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FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Biología Celular

CENTRO CATECOLAMINÉRGICOS EN EL SISTEMA NERVIOSO CENTRAL DE ANFIBIOS: ESTUDIO HODOLÓGICO Y NEUROQUÍMICO

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Cristina Sánchez – Camacho Blázquez

Bajo la dirección del doctor

Agustín González Gallegos

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Trabajo de Investigación que presenta

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Fdo. Dña. Cristina Sánchez-Camacho Blázquez

Dirigido por el Doctor

Agustín González Gallegos

Profesor Titular del Departamento de Biología Celular

de la Facultad de Ciencias Biológicas de la

Universidad Complutense de Madrid

Fdo. D. Agustín González Gallegos

D. Agustín González Gallegos, Profesor Titular del Departamento de Biología Celular de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid,

HACE CONSTAR: Que Doña Cristina Sánchez-Camacho Blázquez ha realizado bajo mi dirección el trabajo de su Tesis Doctoral "Centros catecolaminérgicos en el Sistema Nervioso Central de anfibios. Estudio hodológico e inmunohistoquímico", que ha terminado con el mayor aprovechamiento.

Revisado el presente trabajo, quedo conforme con su presentación para ser juzgado.

Y para que conste y surta los efectos oportunos, lo firmo en Madrid a dieciocho de septiembre de dos mil dos.

Fdo. D. Agustín González Gallegos

A mi padre
A mi madre
A Jorge

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Desde la demostración de su presencia en el sistema nervioso central (SNC) en los años cincuenta, las catecolaminas han recibido gran atención, particularmente debido a su implicación en determinados procesos patológicos y desórdenes neurológicos como la enfermedad de Parkinson, estados de ansiedad y depresión o la esquizofrenia. Asimismo, se han acumulado un gran número de datos acerca de la organización de los sistemas catecolaminérgicos (CA) en el SNC de los vertebrados amniotas (mamíferos, aves y reptiles) y anamniotas (anfibios y peces). La distribución de este grupo de neurotransmisores parece altamente conservada a lo largo de la evolución y constituye posiblemente uno de los sistemas neuroquímicos filogenéticamente más antiguos del encéfalo de vertebrados (Parent, 1984). Sin embargo, existen pocos datos acerca de la conectividad de sus grupos celulares que permitan establecer homologías claras no sólo en función de su topografía sino también en base a su hodología y ontogenia.

Síntesis y Metabolismo de las Catecolaminas

Las catecolaminas (CA) son un grupo de compuestos orgánicos formados por un anillo de benceno con dos grupos hidroxilo adyacentes (grupo catecol) y una cadena amina en el lado opuesto. Las principales CA usadas por el sistema nervioso son la dopamina (DA), la noradrenalina (NA) y la adrenalina (A), derivadas del aminoácido L-tirosina. La primera enzima en la ruta de síntesis de las catecolaminas es la tirosina hidroxilasa (TH) que convierte la L-tirosina derivada de la dieta en la primera catecolamina de la ruta, la L-DOPA (L-dihidroxifenilalanina), tras la adición de un grupo hidroxilo al anillo catecol (Fig. 1). Ésta es la enzima limitante de la reacción y está presente en todas las neuronas catecolaminérgicas. A continuación, la DOPA es transformada en DA mediante la descarboxilación del grupo amino en una reacción catalizada por la enzima DOPA descarboxilasa (también conocida como L-aminoácido aromático descarboxilasa, AACD). En las neuronas noradrenérgicas, la DA actúa como precursor en la síntesis de la NA (o norepinefrina) tras la adición de un grupo hidroxilo a la cadena lateral por la enzima dopamina β-hidroxilasa (DBH). Finalmente, la NA es utilizada como precursor del producto final de la ruta metabólica de las catecolaminas, la adrenalina (o epinefrina). Esta última es sintetizada por la enzima feniletanolamina N-metiltransferasa (PNMT), tras la metilación del grupo amino de la cadena lateral de la NA (Reiner, 1994).

En las células dopaminérgicas, la conversión de la tirosina en L-DOPA y de ésta en DA ocurre en el citoplasma neuronal. Por el contrario, en las neuronas noradrenérgicas la DA pasa a las vesículas de almacenamiento donde sufre la β-hidroxilación para dar lugar a la NA. Así, la mayor parte de la enzima DBH está unida a la membrana vesicular en los terminales nerviosos. Finalmente, la NA es transformada en A en el citoplasma de las neuronas adrenérgicas que contienen la enzima PNMT.

Tras su liberación, las CA actúan sobre sus receptores de membrana localizados en las neuronas postsinápticas. Existen dos tipos de receptores para DA, D1 (con los subtipos D1 y D5) y D2 (D2, D3 y D4), mientras que los efectos de la NA y la A están mediados a través de los receptores α1-adrenérgicos (α1A, α1B, α1D), α2-adrenérgicos (α2A, α2B, α2C) o β-adrenérgicos (β1, β2, β3). Por otro lado, tras la liberación de las CA a la hendidura sináptica se produce un proceso de recaptación dependiente de energía mediante un transportador localizado en la membrana externa del terminal. Existe un transportador específico para la NA (NET) que se encuentra sólo en las neuronas noradrenérgicas, y otro para la

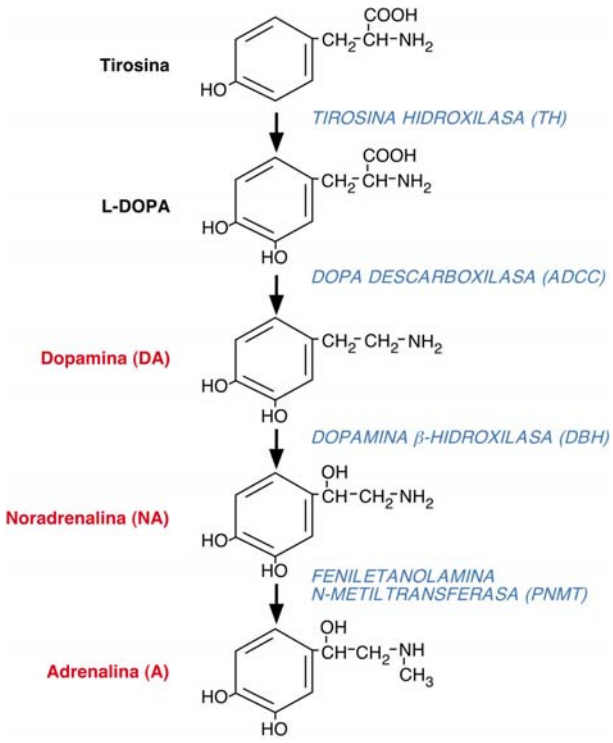


Fig. 1. Ruta de síntesis de las catecolaminas.

recaptación de la DA (DAT). Este proceso permite la reutilización de las CA evitando su degradación extraneuronal, al mismo tiempo que controla su concentración en el espacio extracelular regulando el nivel de activación de los receptores postsinápticos.

Finalmente, en el proceso de inactivación de las CA participan varias enzimas catabólicas, entre las que se incluyen la monoamino oxidasa (con dos isoformas, la MAO-A y MAO-B) y la catecol-O-metiltransfersa (COMT). La MAO se encuentra en neuronas y células gliales y debido a su localización intracelular, juega un papel preponderante en la degradación de las CA que no están almacenadas en vesículas. La COMT está presente virtualmente en casi todas las células unida a la membrana postsináptica, e inactiva las CA que puedan escapar de la recaptación por los terminales sinápticos (Reiner, 1994).

Técnicas de Detección de las Catecolaminas

Las técnicas de histofluorescencia desarrolladas a finales de la década de los sesenta, fueron pioneras en el estudio de la distribución de las catecolaminas en el SNC. En particular, el método de fluorescencia inducida por formaldehído, desarrollado por Falck y cols. (1962), y sus posteriores modificaciones están basadas en las características químicas de las aminas que experimentan con facilidad reacciones de oxido-reducción y condensación. Así, se observó que las catecolaminas y la serotonina daban lugar a productos fluorescentes de color verde o amarillo respectivamente, en presencia de formaldehído. Sin embargo, esta metodología presenta varias limitaciones con respecto a la sensibilidad y especificidad del marcaje de las CA. Así, el principal inconveniente de estas técnicas es que no permiten distinguir entre las diferentes catecolaminas presentes, además de la inestabilidad de los productos formados y su baja sensibilidad.

Con el desarrollo de las técnicas de inmunohistoquímica en los años ochenta, mediante la utilización de anticuerpos contra las catecolaminas o sus enzimas de síntesis, se obtuvo una herramienta más sensible y específica para su localización en el cerebro. Se han desarrollado anticuerpos contra las enzimas TH, AACD, DBH y PNMT, así como anticuerpos frente a la L-DOPA, DA, NA y A, que se han utilizado ampliamente en el estudio de la distribución de las catecolaminas en el SNC y SNP (Smeets y González, 2000). En base a la ruta de síntesis de las CA, los somas neuronales dopaminérgicos se tiñen con anticuerpos frente a DA y la enzima TH, pero no con anticuerpos frente a DBH, NA o PNMT. Del mismo modo, las células noradrenérgicas son inmunopositivas para TH, DBH y NA, mientras que las neuronas adrenérgicas presentan inmunorreactividad para las enzimas TH, DBH y PNMT (Smeets y Steinbusch, 1990; Reiner, 1994). Sin embargo, se ha demostrado la existencia de células que, o bien se tiñen con anticuerpos frente a TH, pero son inmunonegativas para DA, DBH, NA o PNMT, o bien son células que presentan inmunorreactividad frente a DA o NA, pero no frente a las enzimas TH o DBH. En el primer caso, se trata de células que posiblemente acumulan L-DOPA como producto final en la síntesis de CA. En el segundo caso, se trataría de células que acumulan DA o NA, pero que no tienen capacidad de sintetizar dichos neurotransmisores ya que carecen de sus enzimas de síntesis. Estas células están en contacto directo con el líquido cefalorraquídeo del que posiblemente captan el neurotransmisor. Hay que destacar que la falta de inmunorreactividad para una sustancia en particular no demuestra su ausencia, ya que dicha sustancia puede estar presente en niveles muy bajos que no son detectables inmunohistoquímicamente pero que son funcionalmente significativos para la célula.

Organización de los Sistemas CA en el SNC de Mamíferos

El primer estudio de distribución de las catecolaminas en el SNC fue realizado por Dahlström y Fuxe (1964) en el encéfalo de la rata, utilizando el método de histoquímica de fluorescencia. En este trabajo se describen 12 grupos CA diferentes que denominaron de A1 a A12 de caudal a rostral. Estudios posteriores empleando técnicas inmunohistoquímicas, han permitido determinar qué tipo de catecolamina se encuentra presente en cada uno estos grupos, además de identificar nuevos grupos celulares que no se habían descritos con anterioridad mediante las técnicas de histofluorescencia menos sensibles (Hökfelt y cols., 1984). En la actualidad, los grupos dopaminérgicos y noradrenérgicos son en total 17, que se designan como A1-A17, mientras que los grupos adrenérgicos son tres y se designan como C1-C3 (Fig. 2).

Dentro de los grupos catecolaminérgicos localizados en el rombencéfalo caudal se incluyen los denominados *grupo tegmental ventrolateral* (**A1** y **C1**), el *grupo dorsolateral* (**A2** y **C2**), y los grupos **A3** y **C3**. En el rombencéfalo rostral, se localizan los grupos noradrenérgicos **A4**, **A5**, **A6** y **A7**, situados en la formación reticular del puente. El grupo A6 corresponde al *locus coeruleus*, que se subdivide en tres partes: una dorsal (locus coeruleus propiamente dicho), una ventral (locus subcoeruleus) y otra lateral (núcleo de Kölliker-Fuxe). El locus coeruleus es el grupo noradrenérgico más importante, ya que se calcula que contiene más del 40% de las neuronas noradrenérgicas. A pesar de ser la mayor fuente de NA en el cerebro, el locus coeruleus contiene un pequeño número de neuronas. Sin embargo, sus axones forman una red difusa y se calcula que aproximadamente cada célula da lugar a unos 100.000 terminales. Así, una única neurona proyectaría a varias regiones del encéfalo, incluso a zonas muy alejadas entre sí. El grupo A4 constituye una continuación dorsal del complejo A6. El grupo A7 se encuentra localizado en la región ventrolateral de la formación reticular del puente, cerca del *branchium conjunctivum*, mientras que el grupo A5 se continúa rostralmente con el grupo A7 y se localiza dorsal a la oliva superior y ventromedial al tracto del nervio facial.

Los grupos mesencefálicos **A8**, **A9** y **A10**, están constituidos por células dopaminérgicas del *área retrorubral*, la *parte compacta de la sustancia negra* y el *área tegmental ventral*,

Abreviaturas

aob	bulbo olfatorio accesorio
Apl	amígdala, pars lateralis
Apm	amígdala, pars medialis
ATV	área tegmental ventral
C	núcleo talámico central
Cb	cerebelo
cp	comisura posterior
epl	capa plexiforme externa
Dp	palio dorsal
gl	capa glomerular del bulbo olfatorio
Hd	habénula dorsal
Hv	habénula ventral
igl	capa granular interna
Ip	núcleo interpeduncular
Is	núcleo del ístmo
Jc	núcleo yuxtacomisural
Lc	locus coeruleus
LH	hipotálamo lateral
Lp	palio lateral
Lpv	núcleo posteroventral lateral
Ls	septo lateral
m	tegmento mesencefálico
Ma	núcleo mamilar
mes	mesencéfalo
ml	capa celular mitral del bulbo olfatorio
Mp	palio medial
Ms	septo medial
Nsol	núcleo del tracto solitario
ob	bulbo olfatorio
oc	quiasma óptico
POa	área preóptica anterior
Prm	núcleo profundo mesencefálico
PTrG	sustancia gris pretoral
PV	núcleo paraventricular
Ri	núcleo reticular inferior
RM	núcleo retromamilar
Rs	núcleo reticular superior
SC	núcleo supraquiasmático
SM	núcleo supramamilar
sol	tracto solitario
SPr	prosencéfalo secundario
To	techo óptico
TP	tubérculo posterior
TPdm	tubérculo posterior dorsomedial
TPvl	tubérculo posterior ventrolateral
v	ventrículo
VH	hipotálamo ventral
VLd	núcleo talámico ventrolateral, parte dorsal
VLv	núcleo talámico ventrolateral, parte ventral
VM	núcleo talámico ventromedial
Zip	núcleo periventricular de la zona incerta
III	núcleo del nervio oculomotor

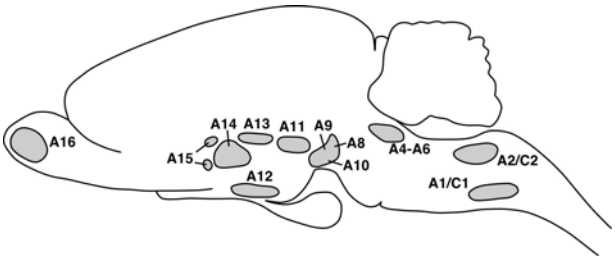


Fig. 2. Distribución de los grupos catecolaminérgicos en el SNC de mamíferos (modificado de Hökfelt y cols., 1984).

respectivamente. Posteriormente, estudios más detallados han permitido subdividir estos grupos celulares. Así, en el grupo A9 se distingue una porción dorsal y otra ventral, mientras que en el grupo A10 se diferencian las regiones dorsolateral, dorsocaudal, ventrorrostral y caudal (Hökfelt y cols., 1984).

En el diencéfalo se localizan los grupos dopaminérgicos **A11-A15.** El grupo A11 se denomina *grupo diencefálico caudal* y se localiza en la sustancia gris periacueductal del tálamo, hipotálamo y mesencéfalo rostral. El grupo A12 de mamíferos denominado *grupo celular tuberal*, se localiza principalmente en el núcleo arcuato, y da lugar al sistema dopaminérgico tuberoinfundibular. El grupo A13 forma parte del *grupo hipotalámico rostral*, con algunas células localizadas en la zona incerta. Finalmente, el grupo A14 se designa como *grupo periventricular rostral*, mientras que el grupo A15 incluye células dopaminérgicas situadas en los *núcleos supraóptico* y *paraventricular*.

El grupo **A16** incluye neuronas dopaminérgicas localizadas en el *bulbo olfatorio*, principalmente situadas en la capa glomerular, aunque también se encuentran algunas células en la capa plexiforme externa. La *retina* de mamíferos contiene también numerosas células dopaminérgicas que constituyen el grupo **A17**. Son células amacrinas que se distribuyen principalmente en la capa nuclear interna, aunque también se encuentran algunas células desplazadas en la capa plexiforme interna y la capa de células ganglionares. Existen evidencias que señalan la existencia de NA y A en la retina de algunas especies de mamíferos (Smeets y Reiner, 1994).

Por último, se ha descrito la presencia de otros grupos catecolaminérgicos no clasificados en diferentes especies de mamíferos. Así, se ha demostrado la presencia de células catecolaminérgicas en la médula espinal, en las regiones pretectal y habenular, en núcleos talámicos de la línea media, en varias regiones hipotalámicas y la zona incerta, en la corteza y en regiones del telencéfalo basal (tubérculo olfatorio, banda diagonal de Broca, y estriado dorsal y ventral) (Smeets y González, 2000).

Organización de los Sistemas CA en el SNC de Anfibios

Existen trabajos previos que han analizado la distribución de los sistemas catecolaminérgicos en anfibios mediante el empleo de técnicas inmunohistoquímicas. Así, mediante la utilización de anticuerpos frente a DA y NA, o el uso de anticuerpos contra sus enzimas de síntesis TH, DBH y PNMT disponemos de información detallada sobre la distribución de las catecolaminas en el SNC de diversas especies pertenecientes a los tres órdenes de Amphibia: Anura (o Salentia), Urodela (o Caudata) y Gymnophiona (o Apoda) (ver Tabla 1). Todos los datos existentes apuntan a la presencia de un patrón básico común dentro de este grupo de vertebrados, aunque con la existencia de algunas caraterísticas particulares en función de la especie.

El desarrollo de los sistemas catecolaminérgicos también se ha estudiado en detalle en diferentes especies de anfibios (González y cols., 1994a,b, 1995), demostrando la presencia de células CA en el SNC desde estadios embrionarios tempranos. En concreto, mediante técnicas de inmunodetección para la DA y la enzima TH, se ha realizado un estudio comparado de su distribución durante el desarrollo ontogenético en dos especies de anfibios anuros, *Rana ridibunda* y *Xenopus laevis* (González y cols., 1994a,b), y en el urodelo *Pleurodeles waltl* (González y cols., 1995). Los resultados de estos trabajos demuestran que existe un patrón común en la secuencia espacio-temporal de aparición de los grupos CA, aunque con la existencia de algunas diferencias interespecíficas. Resulta importante destacar que el estudio ontogenético de estos sistemas es interesante ya que proporciona información acerca de la importancia funcional de las catecolaminas desde estadios tempranos del desarrollo y su implicación en la maduración del SNC.

Orden Anura: *Rana perezi* y *Xenopus laevis*

En el anuro *Rana perezi*, el grupo de células DA más rostral se localiza en el *bulbo olfatorio*, distribuidas en las capas mitral y glomerular (Figs. 3a, 4) (González y Smeets, 1991, 1994a). Además, se ha demostrado la presencia de células TH positivas/DA negativas en la capa granular interna, y se ha propuesto la posibilidad de que estas neuronas acumulen DOPA como producto final en la síntesis de CA. En el telencéfalo de anuros, al igual que en el resto de especies de anfibios analizadas, no se ha detectado la presencia de células CA. Sin embargo, numerosas células inmunopositivas para DA (DAi) y TH (THi) se localizan en el *área preóptica anterior*, distribuidas alrededor del receso preóptico (Figs. 3b, 4). En este grupo se distinguen dos tipos celulares: células bipolares que contactan con el ventrículo, y un segundo tipo de células dispersas que no contactan con el líquido cefalorraquídeo (LCR). Otro grupo formado por células dopaminérgicas se localiza en el *núcleo supraquiasmático*, inmediatamente dorsal al quiasma óptico, y que también contiene algunas células que contactan con el ventrículo (Figs. 3c, 4). En niveles diencefálicos se sitúa el *núcleo periventricular de la zona incerta*, formado por una columna de células dopaminérgicas fuertemente inmunorreactivas y con largos procesos dirigidos lateralmente (Figs. 3d, 4). Este núcleo fue descrito inicialmente como un grupo hipotalámico que se denominó como las "células acompañantes del núcleo del órgano periventricular" (González y Smeets, 1991, 1994a). Sin embargo, un análisis más detallado ha revelado que los cuerpos celulares de este grupo se localizan en el tálamo ventral, justo en el límite con el hipotálamo. En relación con este núcleo, se ha demostrado la presencia de células débilmente marcadas también en la región del tálamo ventral pero localizadas dorsalmente al núcleo periventricular de la zona incerta, que parecen formar un grupo DA separado (Fig. 5) (ver Sánchez-Camacho y cols., 2001). Hay que destacar también la presencia de células TH negativas/DA positivas localizadas en el *núcleo del órgano periventricular*, dentro de la capa subependimal del infundíbulo. Estas células poseen un proceso apical que contacta con el LCR, como posible fuente extracelular de DA en estas neuronas. La región del tubérculo posterior contiene un gran número de células DA en todos los anfibios. En *Rana*, el *tubérculo posterior* está dividido en una porción ventrolateral localizada dorsalmente en el infundíbulo, y una porción dorsomedial que alcanza niveles mesecefálicos (Figs. 3e,f, 4). El grupo dorsomedial está constituido por una columna de células pequeñas situadas en la línea media que se

Tabla 1. Estudios inmunohistoquímicos de los sistemas CA en el SNC de anfibios

Autores	Orden	Especie	Anticuerpo
Yoshida y cols., 1983	Anura	*Rana catesbeiana*	TH
Franzoni y cols., 1986	Urodela	*Triturus cristatus carnifex*	TH
Carr y cols., 1991*	Anura	*Rana catesbeiana*	TH
González y Smeets, 1991	Anura Urodela	*Rana ridibunda (R. perezi)* *Pleurodeles waltl*	TH, DA
Corio y cols., 1992	Urodela	*Triturus alpestris*	TH, DA
González y cols., 1993	Anura	*Xenopus laevis*	TH, DA
González y Smeets, 1993	Anura	*Xenopus laevis*	NA, DBH
González y cols., 1994*	Anura	*Xenopus laevis* *Rana ridibunda (R. perezi)*	TH, DA
González y Smeets, 1994	Gymnophiona	*Typhlonectes compressicauda*	TH
González y cols., 1995*	Urodela	*Pleurodeles waltl*	TH
González y Smeets, 1995	Urodela	*Pleurodeles waltl*	NA, DBH, PNMT
Beltramo y cols., 1998	Urodela	*Ambystoma mexicanum*	TH, DA, AADC
Milán y Puelles, 2000	Anura	*Rana perezi* *Xenopus laevis*	TH
González y Smeets, 1994		Review	
González y cols., 1994*		Review	
Smeets y González, 2000		Review	

(*, trabajos de desarrollo de los sistemas catecolaminérgicos en anfibios)

extiende caudalmente en el tegmento mesencefálico hasta la raíz del nervio oculomotor (Fig. 3f). En base a sus conexiones con el telencéfalo basal, se ha considerado a este grupo como el homólogo de la substantia negra pars compacta y el área tegmental ventral (grupos A9-A10) de amniotas (Marín y cols., 1997, 1998). En la región pretectal, se sitúa el *núcleo yuxtacomisural* formado por células DA, que anteriormente fue descrito como parte del núcleo talámico posterior (Figs. 3d,e, 4). El *locus coeruleus* está formado por células NA y constituye el único grupo celular CA presente en la región ístmica (Figs. 3g, 4). Este grupo de células se localiza medial, ventral y caudal al núcleo del ístmo. El número, localización y morfología de sus células varía de forma notable entre las distintas especies estudiadas. En anuros, el locus coeruleus se extiende a lo largo de todo el segmento ístmico, mientras que en urodelos y gymnophionas, este grupo se sitúa solamente en niveles ístmicos caudales. En todas las especies sin embargo, está formado por neuronas multipolares con largas dendritas dirigidas ventralmente o ventrolateralmente, que se ramifican de forma profusa en la formación reticular. En niveles rombencefálicos, encontramos un grupo de células THi/DAi localizadas a lo largo de la línea media del tegmento rombencefálico, y que se sitúan en capas ependimales y subependimales, muy próximas al ventrículo. En todos los anfibios, el *núcleo del tracto solitario* está formado por una población mixta de células DA, NA y adrenérgicas, localizadas alrededor del tracto en niveles rombencefálicos caudales (Figs. 3h, 4). En *Rana*, este núcleo está formado rostralmente por células grandes, multipolares, situadas ventralmente al tracto solitario, mientras que caudalmente está constituido por células de tamaño medio o pequeño que rodean el tracto. En niveles del óbex, las células de ambos lados se fusionan por encima del ventrículo en el área postrema. Por último, el grupo CA más caudal está formado por células THi/DAi localizadas en la médula espinal. Estas células se sitúan ventrales al canal central, en las capas ependimales y subependimales, y están en contacto directo con el LCR. Forman una columna contínua que se extiende a lo largo de toda la médula espinal hasta el *filum terminale*. Recientemente se ha descrito la presencia de otro grupo de células THi dispersas en la médula, localizadas principalmente en el campo dorsal espinal exclusivamente en niveles cervicales (Sánchez-Camacho y cols., 2001).

En el anuro *Xenopus laevis*, también se ha analizado la distribución de los grupos CA en el SNC (González y cols., 1993; González y Smeets, 1993, 1994a). Aunque se han identificado los mismos grupos descritos en *Rana perezi*, se ha demostrado la existencia de algunas diferencias interespecíficas. En particular, hay que destacar que dentro de la región tuberal no se puede distinguir una porción ventrolateral y otra dorsomedial como sucede en la rana. Sin embargo, existen dos tipos celulares distintos dentro de este grupo: células pequeñas con un soma redondo que ocupan una posición dorsal dentro del grupo, y neuronas de mayor tamaño con el soma en forma de pera localizadas en la parte más ventral, que recuerdan a las subdivisiones dorsomedial y ventrolateral del tubérculo posterior de *Rana*, respectivamente. Además, dentro de la extensión caudal del tubérculo posterior en regiones mesencefálicas, el grupo permanece separado en dos poblaciones distintas próximas a la línea media, pero no llegan a fusionarse como ocurre en *Rana*.

Desarrollo de los Sistemas CA en *Xenopus laevis*. Debido a la fácil disponibilidad de larvas y embriones, *Xenopus laevis* se ha utilizado como especie principal en numerosos estudios de desarrollo en anfibios. La elección de esta especie como modelo se debe a la facilidad con la que se reproducen de forma inducida en el laboratorio, además de la existencia de una tabla de desarrollo muy detallada que facilita la clasificación de los distintos estadios embrionarios y larvarios (Nieuwkoop y Faber, 1967). El desarrollo ontogenético de *Xenopus* se caracteriza por dos etapas distintas: 1) un *período embrionario* (hasta el estadio 45), que comienza con la aparición de un opérculo que cubre las estructuras branquiales externas, y que finaliza con la total reabsorción de las mismas; y 2) un *período larvario* marcado por el comienzo de la alimentación independiente, que se subdivide a su vez en tres fases (Gona y cols., 1982): a) *premetamorfosis* (estadios 45/46 hasta 52/53), en el que la larva sufre un crecimiento general y externamente aparece el esbozo de los miembros posteriores; b) *prometamorfosis* (estadios 52/53 hasta 58/59), caracterizado por la formación gradual de los miembros posteriores y que finaliza con la emergencia de los miembros anteriores; y c) *clímax metamórfico* (estadios 58/59 hasta 66), durante el cual se producen los cambios más pronunciados del desarrollo de la larva, incluyendo la regresión

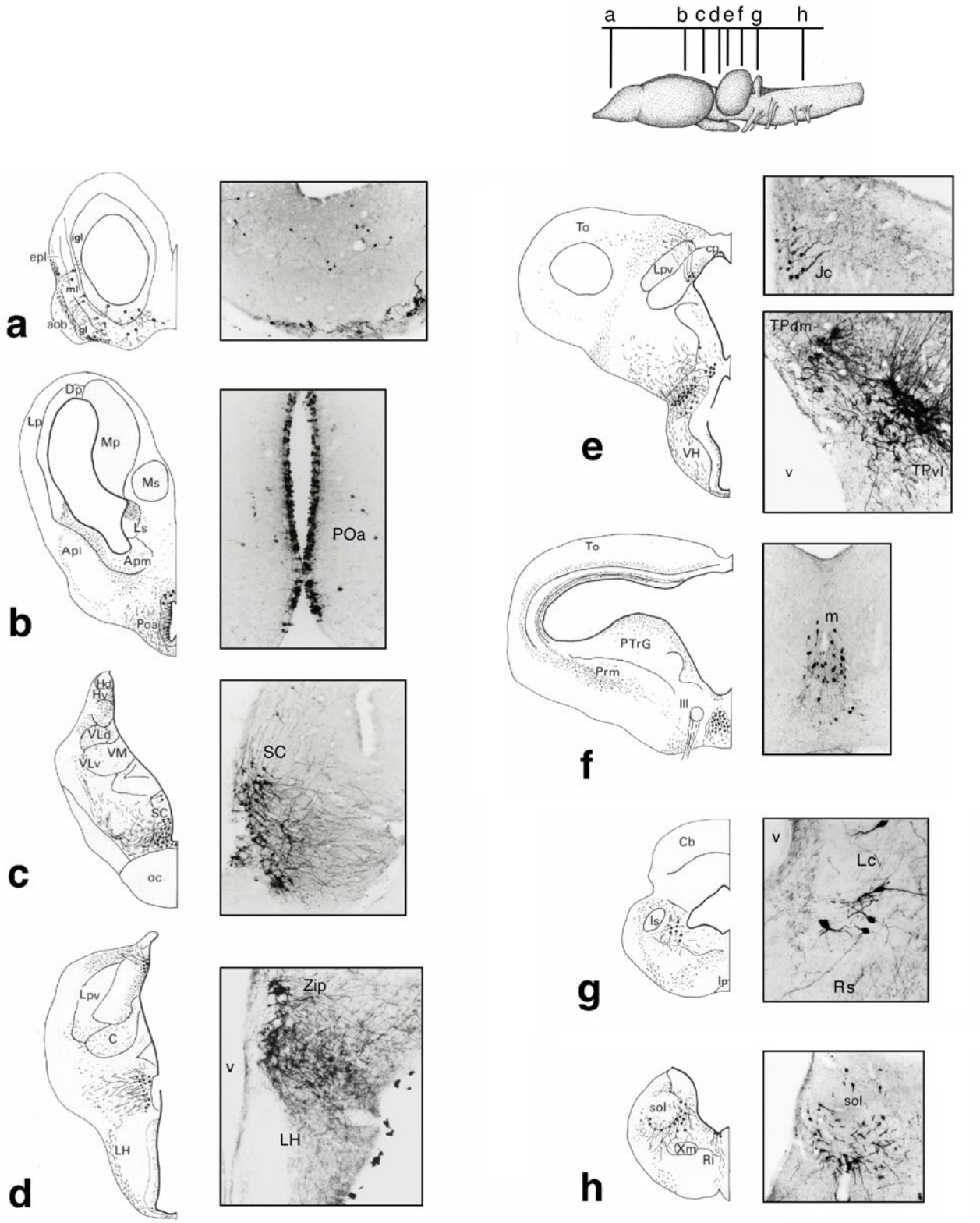


Fig. 3. Distribución de los grupos catecolaminérgicos en el SNC del anuro *Rana perezi* (modificado de González y Smeets, 1991).

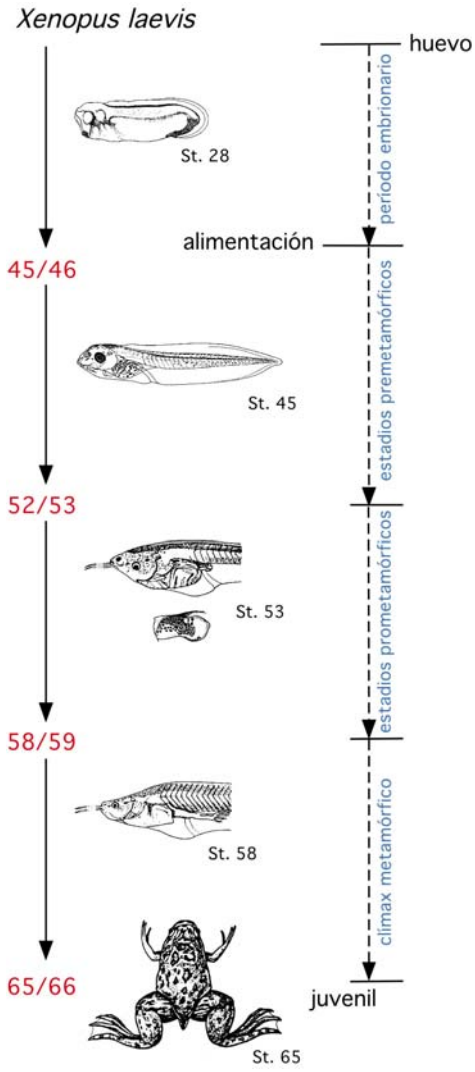


Fig. 4. Tabla del desarrollo embrionario y larvario de *Xenopus laevis* (modificado de Nieuwkoop y Faber, 1967).

total de la cola hasta la aparición de un individuo juvenil de cuatro patas totalmente adaptado a la vida anfibia (Fig. 4).

La presencia de los sistemas CA desde estadios embrionarios tempranos en *Xenopus* sugire la importancia funcional de este grupo de neurotransmisores durante el desarrollo. Así, el trabajo realizado por González y cols. (1994a,b) revela la presencia del primer grupo de células CA desde el estadio 38 en la médula espinal, localizadas ventralmente al canal central. Inmediatamente después, aparecen células CA en el tubérculo posterior (estadio 39), y en los núcleos periventricular de la zona incerta y supraquiasmático (estadios 40/41). Las células NA del locus coeruleus aparecen en el estadio 41 seguidas por la aparición de neuronas THi en el bulbo olfatorio (estadio 42). El período embrionario finaliza con la aparición de neuronas DA en la capa nuclear interna de la retina (estadios 43-45). El período premetamórfico se caracteriza por la maduración progresiva de las células y fibras CA previamente formadas así como la aparición de nuevos grupos celulares. En el estadio 51 aparecen las primeras células CA en el núcleo del tracto solitario, seguidas por las células del núcleo yuxtacomisural (estadio 52/53). Durante la prometamórfosis aparece el grupo CA situado en el tegmento mesencefálico (estadio 54). Finalmente, el desarrollo de los sistemas CA termina con la formación de las células en el área preóptica (estadio 59) (ver Tabla 2).

Orden Urodela: *Pleurodeles waltl*

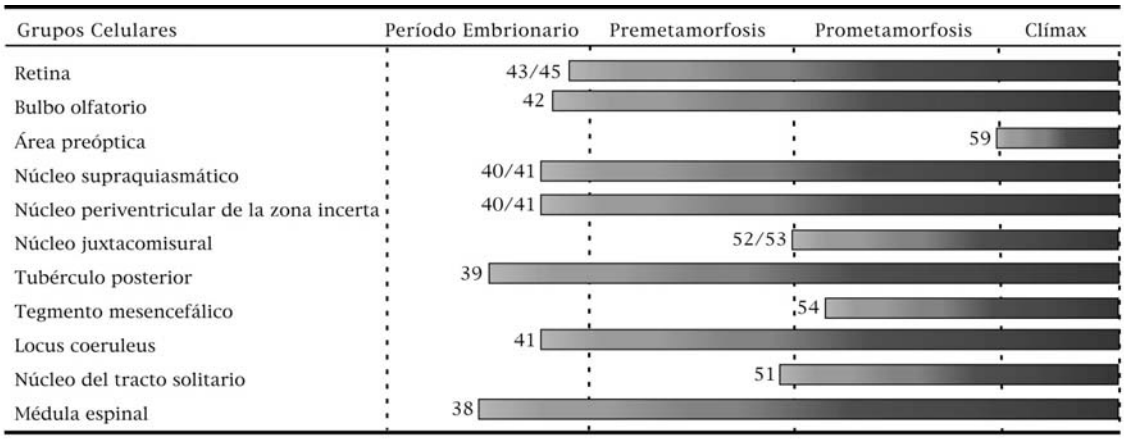
La distribución de los grupos CA en el urodelo *Pleurodeles waltl*, es similar a la descrita en los anfibios anuros, aunque existen algunas diferencias interespecíficas, principalmente en relación a la morfología y número de las neuronas CA (González y Smeets, 1991, 1994a, 1995). En general, las células CA en urodelos son de mayor tamaño y existe un menor grado de migración de sus grupos celulares debido a un proceso conocido como paedomorfosis (Roth y cols., 1993). Este proceso de "simplificación secundaria" del encéfalo, aparece como resultado de una retención de las características juveniles o embrionarias en los individuos adultos. Se caracteriza por una disminución en la complejidad anatómica del cerebro: las neuronas parecen relativamente inmaduras e indiferenciadas, son de gran tamaño, y permanecen próximas al ventrículo debido al escaso grado de migración celular.

Al igual que en *Rana* y *Xenopus*, las células DA más rostrales se sitúan en las capas glomerular y mitral del bulbo olfatorio, con la existencia de un grupo adicional de células TH positivas/DA negativas en la capa granular interna. En el diencéfalo, se encuentran células CA en el área preóptica, los núcleos supraquiasmático y periventricular de la zona incerta, el tubérculo posterior y el núcleo pretectal. Del mismo modo que en la rana, se puede hacer una subdivisión dorsomedial y ventrolateral en el tubérculo posterior. Sin embargo, existe una variación en el grado de fusión del grupo de células DA mesencefálicas. Así, en *Pleurodeles* al igual que sucede en *Xenopus*, este grupo permanece separado en dos poblaciones celulares próximas a la línea media que no llegan a fusionarse totalmente. Por otro lado, el locus coeruleus contiene un pequeño número de células NA que forman un grupo compacto próximo al cuarto ventrículo, situado rostralmente al núcleo motor del nervio trigémino. Finalmente, las células CA del núcleo del tracto solitario son de mayor tamaño que en anuros, aunque no se pueden distinguir distintos tipos neuronales. Estas células forman una columna compacta periventricular situada medialmente al fascículo solitario.

Orden Gymnophiona: *Typhlonectes compressicauda* y *Dermophis mexicanus*

La localización anatómica de los grupos celulares CA se ha analizado en detalle mediante anticuerpos frente a la enzima TH en dos especies de ápodos: *Typhlonectes compressicauda* y *Dermophis mexicanus* (González y Smeets, 1994b; Sánchez-Camacho y cols., 2001). En general, el encéfalo en los ápodos es más parecido en muchos aspectos al de urodelos que al de anuros. También tiene lugar un fenómeno de "simplificación secundaria" o paedomorfosis que hace que el cerebro en estas especies presente una morfología más simple, con un bajo grado de laminación y migración celular en sus grupos. En cuanto a la distribución de las CA en el SNC es bastante similar a la descrita en anuros y urodelos, aunque se ha demostrado la existencia de algunas diferencias interespecíficas. Así, en *Typhlonectes* se ha identificado la presencia de un grupo adicional de células THi en la *formación reticular* y en la *porción prevagal del núcleo del tracto solitario*. Además, estas especies se caracterizan por presentar un gran número de células en el tegmento mesencefálico y el hipotálamo, donde la mayor parte contactan con el LCR. En este sentido, parece que existe una tendencia en las formas acuáticas de anfibios a tener un mayor número de células que están directamente en contacto con el ventrículo, como también se ha observado en otras es-

Tabla 2. Desarrollo ontogenético de los grupos celulares catecolaminérgicos en el SNC de *Xenopus laevis*.



Las barras horizontales indican la presencia y el tiempo de aparición de los grupos celulares que contienen células TH-inmunorreactivas durante el desarrollo embrionario y larvario de *Xenopus* (modificado de González y cols., 1994).

pecies de reptiles y peces (González y Smeets, 1994a,b; Smeets, 1994; Smeets y Reiner, 1994).

El Modelo Neuromérico en el Estudio de la Distribución de los Grupos CA

Numerosos estudios han demostrado que el encéfalo de vertebrados se desarrolla siguiendo un patrón segmental, de manera que este modelo permanece en el cerebro adulto gobernando la distribucion topográfica de sus poblaciones neuronales. Más aún, el número y organización de estos segmentos neurales (neurómeros) es una característica constante en todos los vertebrados. El modelo neuromérico constituye así una herramienta muy útil para estudiar la variación en la organización del cerebro de los vertebrados (Puelles y Rubenstein, 1993; Puelles, 1995).

Recientemente se ha analizado la distribución de los grupos CA en el SNC de peces teleósteos (Anadón y cols., 2002), anfibios (Milán y Puelles, 2000), reptiles (Medina y cols., 1994), aves (Puelles y Medina, 1994) y mamíferos (Puelles y Verney, 1998) siguiendo una aproximación segmental. El análisis de la distribución neuromérica de las catecolaminas facilita el estudio de la variación evolutiva de la organización de este grupo de neurotransmisores en el encéfalo de los vertebrados, permitiendo establecer homologías entre los distintos grupos celulares atendiendo a su localización topográfica. En particular, se ha propuesto recientemente el mapa prosomérico del diencéfalo de anuros (*Rana perezi* y *Xenopus laevis*) analizando la distribución de distintos marcadores incluyendo la enzima TH y demostrando que su distribución segmental es comparable a la descrita en otros vertebrados (Puelles y cols., 1996; Milán y Puelles, 2000).

De acuerdo con la organización segmental, el cerebro anterior se divide en el diencéfalo y el prosencéfalo secundario, cada uno de los cuales está subdivido a su vez en tres prosómeros (p1-p6). Los prosómeros p1-p3 (también llamados sinencéfalo, parencéfalo posterior y parencéfalo anterior respectivamente) contienen el pretecho, el complejo tálamo dorsal/epitálamo, y el tálamo ventral respectivamente, dentro de la placa alar. Por otro lado, los prosómeros p4-p6 del prosencéfalo secundario incluyen el hipotálamo retroquiasmático y se extienden dorsalmente en el telencéfalo. Dentro de la placa alar del prosencéfalo secundario, se localizarían células THi pertenecientes al núcleo epiquiasmático en p6, mientras que el área preóptica (concretamente la porción dorsal, según Milán y Puelles, 2000) y el núcleo supraquiasmático se situarían en p5. El núcleo paraventricular estaría incluído dentro del prosómero 4. Las células que forman parte de este grupo forman una banda paralela a los límites entre p4/p5 y p3/p4. En conjunto, los núcleos epiquiasmático y paraventricular, junto con las porciones rostral y caudal del núcleo supraquiasmático, correponden al denominado núcleo supraquiasmático según la definición clásica del diencefálo de Neary y Northcutt (1983). En la placa alar de p3 se sitúa el núcleo periventricular de la zona incerta, donde también se incluye la población de células DA dorsales a este grupo. La placa alar de p1 contiene núcleo yuxtacomisural dentro de la región pretectal (núcleo subcomisural según Milán y Puelles, 2000). Finalmente, dentro del segmento ístmico se localiza el locus coeruleus, mientras que el núcleo del tracto solitario se sitúa en los rombómeros 7 y 8 (Fig. 5).

Dentro de la placa basal, se localizan los grupos celulares correspondientes a los núcleos superficial mamilar (p4), mamilar (p4) y retromamilar (p3), el tubérculo posterior (p2) y el área tegmental ventral (p1 y mesencéfalo). Los núcleos superficial mamilar y mamilar incluyen el denominado tubérculo posterior ventrolateral. Por otro lado, el núcleo retromamilar y el tubérculo posterior constituyen la porción dorsomedial del tubérculo posterior, mientras que su continuación más caudal incluiría el área tegmental ventral, todo ello constituyendo parte del homólogo de los grupos A9-A10 de amniotas (Fig. 5).

Aspectos Filogenéticos de los Sistemas CA en el SNC de Vertebrados

Durante los últimos veinte años se han acumulado un gran número de datos acerca de la organización de los sistemas catecolaminérgicos en el SNC de las distintas clases de vertebrados (*ciclóstomos*: Wicht y Northcutt, 1994; Pierre y cols., 1997; *condrictios*: Meredith y Smeets, 1987; Stuesse y cols., 1990; Molist y cols., 1993; *osteictios*: Meek, 1994; Adrio y cols., 2002; Anadón y cols., 2002; *anfibios*: González y Smeets, 1991, 1994a; *reptiles*: Smeets y Steinbusch, 1990; Smeets, 1994; *aves*: Moons y cols., 1994; Reiner y cols., 1994; *mamíferos*: Hökfelt y cols., 1984; Kitahama y cols., 1994; Tillet, 1994). Recientemente se han publicado varias revisiones

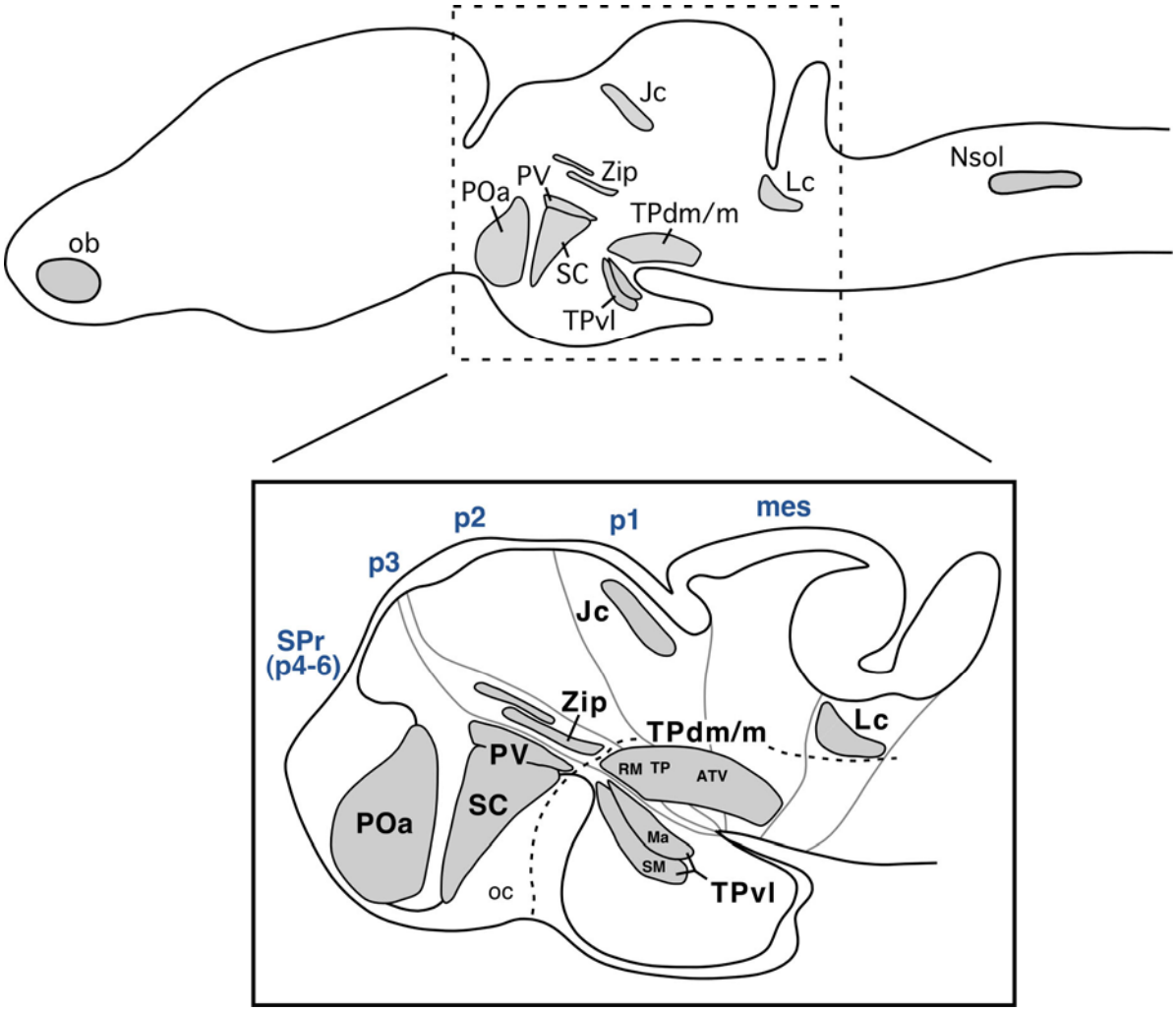


Fig. 5. Distribución de los grupos catecolaminérgicos en el encéfalo de *Rana perezi* según el mapa prosomérico (modificado de Milán y Puelles, 2000).

que han tratado de unificar los numerosos datos existentes para intentar comprender hasta qué punto son comparables los sistemas CA entre vertebrados y si existe una organización básica de estos sistemas (Smeets y Reiner, 1994; Smeets y González, 2000). Así, la distribución de las catecolaminas parece altamente conservada a lo largo de la evolución y constituye posiblemente uno de los sistemas neuroquímicos filogenéticamente más antiguos del encéfalo de vertebrados (Parent, 1984).

En contra de la idea clásica de que la evolución de los sistemas CA está marcada por un incremento en su complejidad desde anamniotas a amniotas, parece evidente ahora que el cerebro de anamniotas contiene grupos CA que en amniotas han perdido la capacidad de producir CA. Sin embargo, a pesar de la variación existente en la morfología y complejidad del cerebro, se pueden identificar seis grupos principales de células CA en el SNC de todos los vertebrados: 1) un grupo rombencefálico caudal que comprende los grupos celulares **A1**-**A3** y **C1**-**C3**; 2) un grupo rombecefálico rostral/ístmico consistente en los grupos **A4**-**A7**; 3) un grupo mesencefálico (**A8**-**A10**); 4) un grupo diencefálico (**A11**-**A15**); 5) un grupo en bulbo olfatorio (**A16**); y 6) un grupo retinal (**A17**). Excepto por el grupo mesencefálico, el resto de los grupos principales están presentes en todas las especies de vertebrados estudiadadas hasta ahora (ver Tabla 3) (Smeets y Reiner, 1994; Smeets y González, 2000).

Objetivos y Metodología

Como acabamos de presentar en la introducción, el estudio de la distribución anatómica y la funcionalidad de las catecolaminas en el SNC ha sido uno de los principales objetos de debate durante décadas. Estos trabajos no se han limitado únicamente al estudio en mamíferos, sino que abarcan un gran número de especies que incluyen tanto vertebrados amniotas como especies anamniotas. Sin embargo, apenas existen trabajos de conectividad, y la mayor parte de ellos se han centrado en el estudio de las rutas mesoestriatal y mesolímbica. De este modo, con el objeto de profundizar en el análisis de la organización de los sistemas CA en los vertebrados anfibios, tratando de aportar un mayor conocimiento en la hodología y ontogenia de sus grupos, hemos realizado este estudio. En este sentido, el principal objetivo que se ha planteado en la presente Tesis Doctoral ha sido el análisis detallado de la inervación y aferencias catecolaminérgicas en tres estructuras diferentes como son la médula espinal, el techo óptico y la región septal en anfibios. Estas tres regiones del SNC se caracterizan por la abundante presencia de fibras y terminales CA que se distribuyen

Tabla 3. Análisis Comparativo de los grupos catecolaminérgicos en el Sistema Nervioso Central de Vertebrados

	ROMBENCÉFALO/ REGIÓN ÍSTMICA										MESENCÉFALO			DIENCÉFALO/TELENCÉFALO						
	C1	**C2**	**C3**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**	**A13**	**A14**	**A15**	**A16**	**A17**
Ciclóstomos	?	?	-	?	?	-	-	-	+	-	-	-	-	-	-	?	?	?	+	+
Condrictios	?	?	-	+	+	-	-	±	+	-	-	±	±	-	-	?	?	?	+	+
Osteictios	+	+	-	+	+	-	-	-	+	-	-	-	±	-	-	-	?	?	+	+
Anfibios	+	+	-	+	+	-	-	±	+	-	-	?	+	-	-	±	+	+	+	+
Reptiles	+	±	-	+	+	-	-	±	+	±	+	+	+	±	-	±	+	+	+	+
Aves	+	+	-	+	+	-	±	+	+	±	+	+	+	+	±	+	+	+	+	+
Mamíferos	+	±	±	+	+	±	±	+	+	±	+	+	+	+	+	+	+	+	+	+

(+, presente en todas las especies estudiadas hasta ahora; -, no encontrado; ±, presente en algunas especies pero no en otras; ?, presente pero no reconocible como un grupo separado. Modificado de Smeets y González, 2000)

de una manera altamente diferencial y específica. Analizar el origen de esta inervación ha sido uno de los objetivos primordiales del presente trabajo. Asimismo, y dado que el uso de las dextranaminas como trazadores axonales ha demostrado ser una herramienta muy útil en el estudio de conexiones, hemos realizado un estudio en paralelo de las vías descendentes a la médula espinal. Así, en el **Capítulo 2** se ha estudiado la organización de las conexiones aferentes a la médula espinal. En este trabajo hemos empleado de técnicas de trazado retrógrado con dextranaminas biotinadas o conjugadas con Texas Red™ que se aplicaron en forma de cristales en distintos niveles medulares. Los experimentos llevados a cabo en este trabajo de investigación se han realizado en cuatro especies representativas de los tres órdenes de anfibios: los anuros *Xenopus laevis* y *Rana perezi*, el urodelo *Pleurodeles waltl* y el ápodo *Dermophis mexicanus*. En la primera parte del **Capítulo 3**, nos planteamos el estudio detallado de la inervación CA en la médula espinal, analizando la distribución de fibras y terminales en todos los niveles medulares mediante el empleo de métodos de inmunodetección para la enzima tirosina hidroxilasa (TH). Además, mediante el uso de técnicas de doble marcaje, combinando el trazado neuronal retrógrado de dextranaminas con inmunohistoquímica para la enzima TH, determinamos el origen y la organización de dicha inervación catecolaminérgica en la médula espinal. Para realizar este estudio hemos utilizado las mismas especies de anfibios empleadas en el Capítulo 2. Con el fin de completar esta parte del estudio acerca de la organización de la médula espinal, en el **Capítulo 4** realizamos el estudio del desarrollo de las proyecciones descendentes espinales, así como la secuencia de aparición de las aferencias catecolaminérgicas a la médula espinal. Estos experimentos se han llevado a cabo utilizando el anuro *Xenopus laevis* como modelo, debido a la disponibilidad de embriones y larvas en nuestro laboratorio. En esta parte del trabajo empleamos técnicas de trazado neuronal *in vitro*, utilizando dextranaminas aplicadas en forma de cristales. El empleo de esta técnica nos ha permitido el análisis detallado de estas conexiones a lo largo del desarrollo, desde estadios embrionarios tardíos hasta el final de la metamorfosis.

En el **Capítulo 5**, hemos realizado un estudio detallado y comparado de la distribución de fibras y terminales en el techo mesencefálico del anuro *Rana perezi* y el urodelo *Pleurodeles waltl*, mediante el empleo de anticuerpos frente a DA y a las enzimas TH y DBH. Además, mediante el uso de técnicas de doble marcaje similares a las utilizadas en los trabajos previos para inviduos adultos, determinamos el origen de dicha inervación presente en el techo óptico. Finalmente, en el **Capítulo 6** estudiamos la organización de la inervación catecolaminérgica en la región septal de *Rana perezi*. Así, analizamos la distribución de fibras y terminales CA en el septo de la rana mediante el empleo de inmunohistoquímica para la DA y las enzimas TH y DBH. Para el análisis del origen de dicha inervación, se emplearon técnicas de trazado retrógrado con dextranaminas aplicadas en forma de cristal o iontoforéticamente, siguiendo una aproximación *in vivo*, similar a la utilizada en trabajos previos para individuos adultos (Capítulos 2, 3 y 5). Asimismo, utilizamos una nueva aproximación *in vitro* parecida a la empleada en estudios de desarrollo (Capítulo 4) pero adaptada para individuos adultos, que resultó ser de gran utilidad para aplicaciones en regiones del cerebro de difícil acceso como puede ser la región septal. Tanto en los experimentos *in vivo* como el los llevados a cabo bajo condiciones in *vitro*, se combinaron con la inmunodetección de la enzima TH para determinar el origen de la inervación CA en el septo.

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Capítulo 2

Conexiones aferentes de la médula espinal

Descending supraspinal pathways in amphibians. I. A dextran amine tracing study of their cells of origin

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Descending Supraspinal Pathways in Amphibians. I. A Dextran Amine Tracing Study of Their Cells of Origin

CRISTINA SÁNCHEZ-CAMACHO[1], OSCAR MARÍN[2], HANS J. TEN DONKELAAR[3] AND AGUSTÍN GONZÁLEZ[1]

[1]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain

[2]Department of Psychiatry and Langley Porter Psychiatric Institute, Nina Ireland Laboratory of Developmental Neurobiology, University of California San Francisco, California

[3]Department of Neurology, University Hospital Nijmegen, The Netherlands

ABSTRACT

The present study is the first of a series on descending supraspinal pathways in amphibians in which hodological and developmental aspects are studied. Representative species of anurans (the green frog, *Rana perezi*, and the clawed toad, *Xenopus laevis*), urodeles (the Iberian ribbed newt, *Pleurodeles waltl*), and gymnophionans (the Mexican caecilian, *Dermophis mexicanus*) have been used. By means of retrograde tracing with dextran amines, previous data in anurans were largely confirmed and extended, but the studies in *P. waltl* and *D. mexicanus* present the first detailed data on descending pathways to the spinal cord in urodeles and gymnophionans. In all three orders, extensive brainstem-spinal pathways were present with only minor representation of spinal projections originating in forebrain regions. In the rhombencephalon spinal projections arise from the reticular formation, several parts of the octavolateral area, the locus coeruleus, the laterodorsal tegmental nucleus, the raphe nucleus, sensory nuclei (trigeminal sensory nuclei and the dorsal column nucleus), and the nucleus of the solitary tract. In all species studied, the cerebellar nucleus and scattered cerebellar cells innervate the spinal cord, predominantly contralaterally. Mesencephalic projections include modest tectospinal projections, torospinal projections, and extensive tegmentospinal projections. The tegmentospinal projections include projections from the nucleus of Edinger-Westphal, the red nucleus, and from anterodorsal, anteroventral and posteroventral tegmental nuclei. In the forebrain, diencephalospinal projections originate in the ventral thalamus, posterior tubercle, the pretectal region and the interstitial nucleus of the fasciculus longitudinalis medialis. The most rostrally located cells of origin of descending spinal pathways were found in the suprachiasmatic nucleus, the preoptic area and a subpallial region in the caudal telencephalic hemisphere, probably belonging to the amygdaloid complex. Our data are discussed in an evolutionary perspective.

Indexing terms: spinal cord input; anurans; urodeles; gymnophionans (apodans); descending pathways; dextran amines; evolution

The organization of descending supraspinal pathways has been the subject of numerous neuroanatomical investigations in vertebrates (for reviews see Kuypers and Martin, 1982; ten Donkelaar, 1982, 2000, 2001; Cruce and Newman, 1984; Nudo and Masterton, 1988). The main goal of these studies was to determine the cell groups in the brain stem, diencephalon and cerebral cortex that give rise to descending spinal projections, and to analyze their role in supraspinal control of motor activity and their modulatory effects on sensory information. Additionally, the comparative analysis of descending spinal pathways in vertebrates proved to be a suitable means to assess evolutionary traits in the central nervous system (Ronan and Northcutt, 1985; Ronan, 1989; Cruce et al., 1999; ten Donkelaar, 2001). During the last three decades our knowledge of the descending spinal pathways in vertebrates increased extensively by the introduction of new tract-tracing techniques. Thus, the classical anterograde and retrograde degeneration techniques were replaced by tract-tracing techniques based on the retrograde transport of macromolecules. In particular, horseradish peroxidase (HRP) backlabeling of the cells of origin of descending supraspinal pathways was widely applied in agnathans (Ronan, 1989), cartilaginous fishes (Smeets and Timerick, 1981; Cruce et al., 1999), bony fishes (Oka et al., 1986; Prasada Rao et al., 1987), lungfishes (Ronan and Northcutt, 1985), amphibians (ten Donkelaar et al., 1981; Naujoks-Manteuffel and Manteuffel, 1988), reptiles (ten Donkelaar et al., 1980; Woodson and Künzle, 1982; Newman et al., 1983), birds (Cabot et al., 1982; Gross and Oppenheim, 1985; Webster et al., 1990), and mammals (see Kuypers, 1981; Nudo and Masterton, 1988) .

Our current understanding of the descending spinal projections in amphibians is primarily based on results obtained in

anurans with anterograde degeneration techniques (Mensah and Thompson, 1978; Corvaja et al., 1973), retrograde HRP-tracing studies (Corvaja and d'Ascanio, 1981; d'Ascanio and Corvaja, 1981; ten Donkelaar et al., 1981; Forehand and Farel, 1982; Will et al., 1985b), and cobalt-labeling studies (Tóth et al., 1985). These studies showed that amphibians share the basic pattern of organization of descending supraspinal control present in terrestrial vertebrates (see ten Donkelaar, 1982, 1990, 2000, 2001) including the general subdivision into lateral and medial descending systems as advocated for mammals (Kuypers, 1981). Interstitiospinal, reticulospinal, and vestibulospinal pathways pass via the ventral funiculus and ventral part of the lateral funiculus and terminate in the mediodorsal parts of the ventral horn and the adjacent parts of the intermediate zone. This *medial* system is functionally related to postural activities and progression and constitutes a basic system by which the brain exerts control over movements. The *lateral* system consists of fibers occupying a lateral position in the lower brain stem and descending into the lateral funiculus of the spinal cord. This system is mainly composed of rubrospinal fibers. The rubrospinal tract terminates in lateral and dorsal parts of the intermediate zone, and is involved in the steering of limb movements. Moreover, evidence is accumulating for the presence of a third, emotional component of the motor system including coeruleospinal and raphespinal pathways in amphibians which, like in other amniotes (Kuypers, 1982; Holstege and Kuypers, 1987; ten Donkelaar, 1990; Holstege, 1991), may be under the control of the limbic system. Distinct noradrenergic coeruleospinal (Marín et al., 1996) and serotonergic (van Mier et al., 1986; Tan and Miletic, 1990) pathways are found in anurans. Unfortunately, much less information is available on descending supraspinal pathways in urodeles, although a number of retrograde tracer studies (Clarke et al., 1988; Naujoks-Manteuffel and Manteuffel, 1988; Will, 1988; Davis et al., 1989) and immunohistochemical data (González and Smeets, 1991, 1994a, 1995; Clairambault et al., 1994; Dicke et al., 1997) have demonstrated that elaborate descending connections, comparable to those of other vertebrates, are present in newts and salamanders despite their apparently poorly differentiated, secondarily simplified brains (see Roth et al., 1993). Moreover, apart from a study aimed to clarify the presence of a rubrospinal tract (Naujoks-Manteuffel et al., 1988) and some immunohistochemical data (Clairambault et al., 1994; González and Smeets, 1994b, 1997), studies on descending pathways to the spinal cord of gymnophionans (caecilians or apodan amphibians) are lacking.

Previous studies on the organization of descending supraspinal pathways in amphibians revealed rather variable results. Thus, controversy still exists about the presence of certain descending projections as, for example, telencephalo-, tecto-, rubro-, or cerebellospinal pathways. Recently, a new generation of tracers has been introduced, viz. the dextran amines (Glover et al., 1986; Veenman et al., 1992; Fritzsch, 1993). These tracers, which are transported anterogradely as well as retrogradely, can be delivered to restricted sites of the central nervous system and are very sensitive in tract-tracing studies in amphibians both *in vivo* (Marín et al., 1997a,b; A. Muñoz et al., 1995, 1997, 1998) and *in vitro* (Luksch et al., 1996; Marín et al., 1997f).

Abbreviations

A	anterior thalamic nucleus
Ad	anterodorsal tegmental nucleus
Am	amygdala
Av	anteroventral tegmental nucleus
Cb	cerebellum
cc	central canal
cll	caudal lateral line nucleus
DCN	dorsal column nucleus
Dp	dorsal pallium
dth	dorsal thalamus
gl	granule cell layer of the cerebellum
Hb	habenula
III	oculomotor nucleus
Ip	interpeduncular nucleus
Is	isthmic nucleus
IV	trochlear nucleus
Jc	juxtacommissural nucleus
Lc	locus coeruleus
LDT	laterodorsal tegmental nucleus
Lp	lateral pallium
Lpd	lateral posterodorsal nucleus
MesV	mesencephalic trigeminal nucleus
MN	Mauthner neuron
Mp	medial pallium
ncb	nucleus cerebelli
Nd	dorsal nucleus of the octavolateral area
Nflm	nucleus of the fasciculus longitudinalis medialis
Ni	intermediate nucleus of the octavolateral area
nIII	oculomotor nerve
NPM	nucleus profundus mesencephali
nPT	nucleus pretectalis
NPv	nucleus of the periventricular organ
Nv	ventral nucleus of the octavolateral area
nVIII	octaval nerve
pc	posterior commissure
PMg	magnocellular preoptic nucleus
POa	anterior preoptic area
POp	posterior preoptic area
Pv	posteroventral tegmental nucleus
Ra	raphe nucleus
Ri	inferior reticular nucleus
Ris	isthmic reticular nucleus
Rm	middle reticular nucleus
Rs	superior reticular nucleus
Rub	nucleus ruber
S	septum
SC	suprachiasmatic nucleus
sol	solitary tract
Str	striatum
Tl	laminar nucleus of the torus semicircularis
tm	mesencephalic tectum
Tor	torus semicircularis
TP	tuberculum posterius
TPdm	dorsomedial part of the tuberculum posterius
Tpr	principal nucleus of the torus semicircularis
TPvl	ventrolateral part of the tuberculum posterius
v	ventricle
VH	ventral hypothalamic nucleus
VIII	octaval nucleus
VIIIc	caudal octaval nucleus
VIIIv	ventral octaval nucleus
VIIIvl	lateral division of the ventral octaval nucleus
VIIIvm	medial division of the ventral octaval nucleus
VM	ventromedial thalamic nucleus
VL	ventrolateral thalamic nucleus
vth	ventral thalamus

The present report is the first of a series of studies on descending supraspinal pathways in amphibians in which hodological and developmental aspects are studied with emphasis on immunohistochemically characterized cell groups. Although the outcome of the present study partly corraborates previous HRP studies, it serves several purposes. First, the use of the very sensitive dextran amines as tracers revealed the full complement of the cells of origin of descending spinal pathways. Second, the comparative analysis in representatives of the three amphibian orders (Anura, Urodela and Gymnophiona) provides detailed information on these systems in amphibians helping to identify common and special features of each group. Third, this study provides a basis for subsequent studies. Thus, in the companion paper (Sánchez-Camacho et al., 2001) we studied the origin, funicular trajectory and site of termination of catecholaminergic input to the spinal cord in the same species, using the data of the present report as a framework. Developmental studies are in progress.

MATERIALS AND METHODS

For the present study, a total of 26 adult green frogs (*Rana perezi*), 23 clawed toads (*Xenopus laevis*), 17 Iberian ribbed newts (*Pleurodeles waltl*) and 6 adult Mexican caecilians (*Dermophis mexicanus*) were used. The animals were obtained from the laboratory stocks of the Department of Cell Biology, University Complutense of Madrid or were commercially acquired (*D. mexicanus*). The original research reported herein was performed under the animal care guidelines established by the Spanish Royal Decree 223/1988.

The animals were deeply anesthetized before surgery by immersion in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz). In all experiments, the tracers biotinylated dextran amine (BDA 10 kD, D-1956; Molecular Probes, Eugene, OR, USA) or Texas Red-conjugated dextran amine (TRDA 10 kD, D-1863; Molecular Probes) were applied unilaterally to different levels of the spinal cord, aimed at brachial, thoracic or lumbar segments. The surgery always followed a dorsal approach and a large opening was made by drilling a hole in a vertebra. Tracers were always applied as crystals with a sharp tungsten needle on the tip of which the tracer had been recrystallized from a saturated solution in distilled water. In those cases of tracer application to the dorsal part of the spinal cord, a pure dorsal approach was made, whereas for intermediate and ventral application sites an oblique trajectory of the needle was preferred. By slightly tilting the spinal cord, the needle entered the spinal cord laterally and, in this way contamination of the dorsal regions could be avoided. In the cases where the full complement of descending projections to the spinal cord was studied, large tracer applications were made unilaterally to the hemisection of the cord at brachial levels (see Figs. 1, 4 and 7). Survival times varied from 7-14 days. Following this period the animals were deeply anesthetized with an overdose of MS222 (0.8%), and perfused transcardially with 50 ml of saline solution followed by 200 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4). The brain and spinal cord were removed, postfixed in the same fixative for 2-3 hours and immersed in a solution of 30% sucrose in PB for 5-8 h at 4ºC. They were then blocked in a solution of 15% gelatin and 30% sucrose in PB, and stored for 5 h at 4ºC in a solution containing 4% formaldehyde and 30% sucrose in PB. Sections were cut on a freezing microtome at 40 μm thickness in the frontal or sagittal plane and collected in cold 0.1 M PB, pH 7.4. The sections were treated with 1% H_2O_2 in PB for 10 min, and rinsed again three times with PB to reduce endogenous peroxidase activity. For visualization of BDA, an avidin biotin complex (Vectastain, ABC Standard kit, Vector Laboratories, Burlingame, CA, USA) was used. Peroxidase activity was visualized with heavy metal intensification of a diaminobenzidine (DAB)-based HRP reaction product (Adams, 1981). Thus, peroxidase activity is visualized as a blue-black reaction product. After 5-10 min, sections were rinsed five times in PB, mounted on glass slides (mounting medium: 0.2% gelatin in PB), and dried overnight. After ethanol dehydration and xylene cleaning, they were coverslipped with Entellan (Merck). Some sections were counterstained with cresyl violet to facilitate the localization of labeled structures. Sections from TRDA experiments were mounted immediately after sectioning (mounting medium as above) and coverslipped with Vectashield (Vector).

The distribution of retrogradely labeled cells and fibers in the brain was charted in representative transverse sections. In BDA cases, drawings were made by means of a camera lucida. Sections of TRDA experiments were analyzed with a Zeiss fluorescence microscope with the appropriate filter combinations. In the latter cases, the distribution of retrogradely labeled cells was charted using a computer-aided X-Y plotting system (Minnesota Datametrics, MD-2 digitizer and software). In the description of the results, when possible, the extent of the projections is mentioned aimed at brachial, thoracic and lumbar spinal segments. In anurans, brachial (segments 3-4), thoracic (segments 5-7) and lumbar (segments 8-9) can be distinguished and the brachial and lumbar enlargements related to the innervation of the extremities can serve as landmarks (ten Donkelaar, 1998b). In the tailed amphibians, the spinal cord extends throughout the whole length of the vertebral canal and distinct brachial and lumbar enlargements are also present (ten Donkelaar, 1998a). In contrast, the limbless gymnophionans do not possess brachial and lumbar spinal enlargements and in the very long spinal cord no distinction can be made into brachial, thoracic or lumbar segments. Therefore, in the present study, only rostral and caudal segments are considered. The nomenclature is largely the same as that used in our previous studies on the basal ganglia, catecholaminergic and cholinergic systems and ascending spinal pathways in amphibians (González and Smeets, 1991, 1994a,b, 1995; A. Muñoz et al., 1995, 1998; M. Muñoz et al., 1996; González et al., 1996; Marín et al., 1997a-e, 1998a-c).

RESULTS

In the following descriptions, for each amphibian order we will present a general scheme of the pattern of distribution of the cells of origin of the descending pathways to the brachial spinal cord. In these representative experiments (Figs. 1, 4 and 7), the left half of the spinal cord was filled with TRDA. Since the tracer applications damaged the spinal white matter at this level, and the tracer is also taken up by passing fibers, we assume that the cells retrogradely labeled represent the full complement of the cells of origin of descending supraspinal pathways to one side of the spinal cord. In the cases with restricted tracer applications, often the funicular trajectory of the descending pathways could be determined. A summary of the descending supraspinal pathways in amphibians is presented in Table I.

The distribution of the cells of origin of pathways descending to the spinal cord of anurans is first described. Subsequently, their distribution in the urodele and the gymnophionan (caecilian or apodan), is dealt with. It should be noted that the description of forebrain regions projecting to the spinal cord follows the segmental approach of the brain as advocated by Puelles and co-workers (Puelles, 1995; Puelles et al., 1996; Milán and Puelles, 2000). In this approach the dien-

cephalon is composed of the synencephalon and the posterior and anterior parencephalon. The alar plates of these three subdivisions give rise to pretectal nuclei, the dorsal thalamus and the ventral thalamus respectively, whereas the basal plates are the origins of the interstitial nucleus of the flm, the tuberculum posterius and the retromamillary nucleus, respectively. The hypothalamus and preoptic region arise from the basal part of the secondary prosencephalon, whereas pallial and subpallial parts arise from the alar plate of the secondary prosencephalon. For convenience and to compare our data with previous studies, the hypothalamospinal and preopticospinal projections will be discussed together with the diencephalospinal projections.

Descending projections to the spinal cord in anurans

A series of experiments were performed in which tracers were applied unilaterally to the brachial, thoracic or lumbar spinal cord of *Rana perezi* and *Xenopus laevis*. The descending projections to the spinal cord of the two anuran species were largely comparable, although some interspecific differences were observed. Thus, for the description and mapping of these projections, *Rana perezi* was chosen as the main species.

Rhombencephalon. In all experiments in which the tracer applications involved the ventral (Fig. 2a) and lateral parts of the spinal cord, the bulk of retrogradely labeled cells was observed in the inferior and middle reticular nuclei, and the raphe nuclei (Figs. 1j-l, 2b-e). Ipsilateral and contralateral components of these reticulospinal pathways were present. However, the small cells of the inferior reticular nucleus projected more abundantly ipsilaterally, whereas the large cells in the middle reticular nucleus were predominantly found contralateral to the application site in the spinal cord. The ipsilateral raphespinal pathway was always labeled in experiments in which the tracer was applied to the dorsal part of the spinal cord. Raphespinal cells formed a narrow column immediately beneath the median tip of the fourth ventricle. In those experiments in which the tracer applications involved only the ventral part of the spinal cord, raphespinal cells were predominantly seen in the caudal part of this column, whereas after applications to the dorsal spinal cord the rostral part of the raphe in the rhombencephalon was labeled. The most rostral part of the raphe at isthmic levels was never labeled from the spinal cord.

Conspicuous bilateral vestibulospinal pathways were always labeled in experiments with tracer applications that involved the ventral funiculus at any spinal segment (Figs. 1j,k, 2c-e). The vestibular nuclear complex in anurans comprises four nuclei: anterior, lateral, medial and caudal (Matesz, 1979; Nikundiwe and Nieuwenhuys, 1983; Will et al., 1985a,b; ten Donkelaar, 1998b). Spinal projections have been observed from all these four nuclei. At caudal levels of the medulla a major contralateral component was found originating from small cells of the caudal vestibular nucleus. Additionally, contralateral cells were present in the medial vestibular nucleus (Fig. 2c). The lateral, magnocellular vestibular nucleus gives rise to the massive ipsilateral vestibulospinal pathway (Fig. 2d). Moreover, rostral to the octaval nerve roots, cells in the anterior vestibular nucleus also contributed to the ipsilateral vestibulospinal pathway. Additional projections from the octavolateral area were found in *Xenopus laevis* in which the lateral line system is retained in the adult. In *X. laevis*, the lateral line column comprises four nuclei (Will et al., 1985a,b; ten Donkelaar, 1998b): the medial part of the anterior nucleus, the rostral and caudal lateral line nuclei, and an intermediate nucleus, lateral line nucleus. The rostral and caudal lateral line nuclei were found to project ipsilaterally to the spinal cord (Fig. 2e).

Spinal projections were found to arise in somatosensory nuclei (the sensory trigeminal nuclei and the dorsal column nucleus), and in the nucleus of the solitary tract. In the descending and principal sensory trigeminal nuclei, retrogradely labeled cells formed a long column ipsilateral to the application site in the spinal cord. These cells were labeled from brachial spinal segments only if the tracer was applied to the dorsal and dorsolateral part of the cord. The dorsal column nucleus (DCN; see Fig. 1l) was found to be labeled ipsilaterally after dorsal spinal cord applications, particularly when the tracer was applied to the brachial spinal cord. Retrogradely labeled cells in the nucleus of the solitary tract were consistently observed after tracer applications to the dorsal part of the spinal cord, particularly at rostral spinal segments (Fig. 1k, l). Small and medium-sized cells were found predominantly in the contralateral nucleus, ventral or ventrolateral to the solitary tract.

Cerebellum. In all experiments in which the tracer application involved the lateral funiculus, abundant retrogradely labeled cells were found in the cerebellar nucleus (Fig. 1i). The anuran nucleus cerebelli consists of large bipolar cells, laterally embedded in the cerebellar peduncle, and smaller, medially located cells, which are found scattered in the granule cell layer of the cerebellum (Larson-Prior and Cruce, 1992; ten Donkelaar, 1998b). The cerebellospinal pathway originates, mainly contralaterally, from both cell groups (Fig. 2f) with the more caudally projecting cells located in the deep portion of the granule cell layer.

Isthmus. Within the isthmic tegmentum, abundant reticular cells were labeled in almost all experiments in which the tracers were applied to the ventral spinal cord (Fig. 1h,i). The contralateral projections were more abundant. Most cells with spinal projections were found in the superior reticular nucleus, but also ventromedial and ventrolateral to the isthmic nucleus, where the noradrenergic locus coeruleus, and the cholinergic laterodorsal and pedunculopontine tegmental nuclei have been identified (González and Smeets, 1993, 1994a; Marín et al., 1997e). Spinal projections from the locus coeruleus are predominantly ipsilateral, whereas those from the laterodorsal nucleus are mainly contralateral.

Midbrain. Three main systems of descending fibers arise in the midbrain of anurans, i.e. the mesencephalic tegmentum, the torus semicircularis and the tectum mesencephali (Fig. 1d-g). The first two form pathways to the spinal cord that course via the lateral funiculus, whereas tectospinal fibers run in the ventral funiculus, mainly in its medial portion. The tegmental component innervates the entire spinal cord, whereas torospinal fibers reach upper lumbar segments, and tectospinal fibers do not extend beyond the third to fourth spinal segments. Within the mesencephalic tegmentum, retrogradely labeled cells were found bilaterally in the anteroventral tegmental nucleus, and ipsilaterally in the anterodorsal and posteroventral tegmental nuclei. Additionally, large laterally located cells were labeled in the contralateral nucleus profundus mesencephali (Fig. 1f). More rostrally, bilaterally located small cells were labeled just dorsal and lateral to the oculomotor nucleus in the position of the nucleus of Edinger-Westphal. Among the most conspicuously labeled tegmental cell groups found in anurans was the red nucleus (Fig.1e). This cell group was consistently labeled when the tracer applications involved the contralateral laterodorsal funiculus, from any level of the spinal cord (Fig. 3a).

Spinal cord projections from the torus semicircularis were prominent to the brachial and upper thoracic segments. These

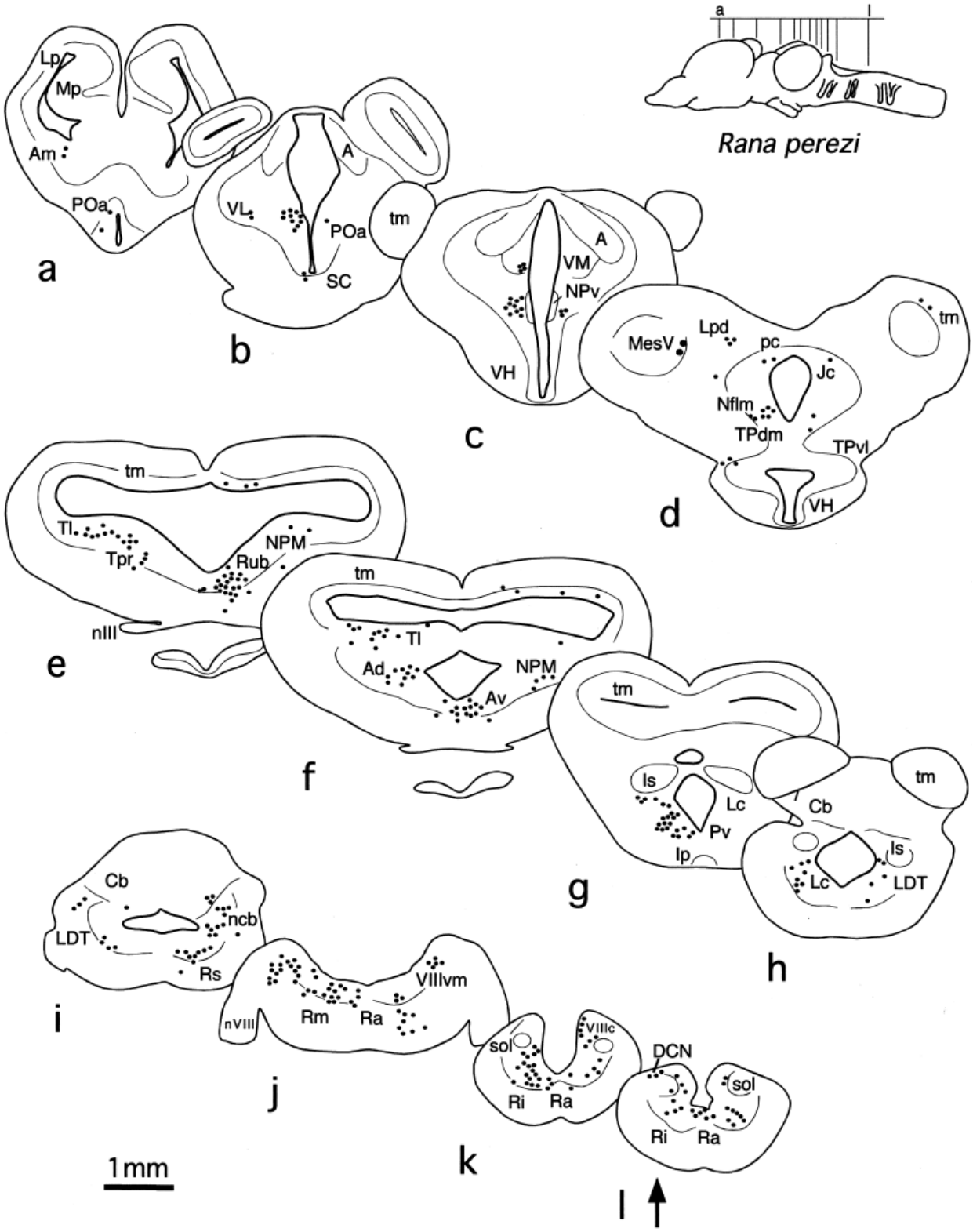

Fig. 1. Schematic drawings of transverse sections through the brain of *Rana perezi* showing the distribution of retrogradely labeled cells (filled dots) after tracer application into the spinal cord. Approximately, a one-to-one correspondence of dots and retrogradely labeled cells is presented. The appropiate levels of the sections are indicated in the upper right scheme. The arrow marks the side of the tracer application in the spinal cord.

TABLE I. Summary of Descending Supraspinal Pathways in Amphibians*

Cell populations	Anura		Urodela		Gymnophiona	
Rhombencephalon						
Raphe nucleus		ipsi		ipsi		ipsi
Inferior reticular nucleus		ipsi		ipsi		ipsi
Middle reticular nucleus		contra		contra		ipsi
Mauthner neuron				contra		
Vestibular nuclei	Caudal nucleus	contra	Ventral nucleus	bilat	Ventral nucleus	bilat
	Ventromedial nucleus	contra				
	Ventrolateral nucleus	ipsi				
	Anterior nucleus	ipsi				
Lateral line nuclei	Rostral and Caudal nuclei (*X. laevis*)	bilat	Intermediate nucleus	bilat	Intermediate nucleus	bilat
Sensory trigeminal nuclei		ipsi		ipsi		ipsi
Dorsal column nucleus		ipsi		ipsi		ipsi (?)
Nucleus of the solitary tract		contra		contra		contra
Cerebellum						
Cebellar nucleus		contra		contra		contra (?)
Isthmus						
Superior reticular nucleus		contra		ipsi		bilat
Locus coeruleus		ipsi		bilat		bilat
Laterodorsal tegmental nucleus		contra		bilat		bilat (?)
Midbrain						
Mesencephalic tegmentum	Av	bilat	Av	bilat		?
	Ad and Pv	ipsi	Ad and Pv	bilat		
	NPM	ipsi				
Edinger-Westphal nucleus		contra		?		?
Red nucleus		contra		contra		contra
Torus semicircularis		ipsi		?		?
Mesencephalic trigeminal nucleus		ipsi		bilat		bilat
Mesencephalic tectum		contra		ipsi		bilat
Diencephalon						
Pretectal Region	JC, PC and Lpd	ipsi		ipsi		ipsi
Interstitial nucleus of the FLM		ipsi		ipsi		ipsi
Posterior tubercle	Dorsomedial division	contra		ipsi		ipsi
	Ventrolateral division	ipsi		ipsi		ipsi
Ventral thalamus	VM and VL	ipsi		ipsi		ipsi
	Zip	ipsi		ipsi		ipsi
Anterior preoptic area		ipsi		ipsi		ipsi
Suprachiasmatic nucleus		ipsi		ipsi		ipsi
Telencephalon						
Ventral/Caudal telencephalon		ipsi		ipsi		ipsi

(?, Not clearly defined cell groups in urodeles or gymnophionans)

(*, Data obtained in the present study)

projections almost exclusively originate from the ipsilateral laminar and principal nuclei of the torus (Figs. 1e,f, 3b). Two components of tectospinal fibers could be distinguished in anurans although it should be noted that they were more prominent in *Rana perezi* than in *Xenopus laevis*. The first component was made up by fibers coursing in the dorsolateral funiculus, and corresponds to the mesencephalic trigeminal descending tract. A set of large ganglionic cells of the mesencephalic trigeminal nucleus was unequivocally labeled after tracer applications that involved the dorsal horn at brachial spinal segments. They were found at the rostral pole of the tectum within deep tectal layers, always ipsilateral to the application side (Figs. 1d, 3c). The second tectospinal pathway is almost exclusively contralateral, and passes via the ventromedial portion of the ventral funiculus. The cells of origin of this pathway were labeled in layer 6 and, less numerous, in layers 2 and 4 (Fig. 1e,f). A small ipsilateral tectospinal component was found to arise from cells in layer 7 in the rostral tectum.

Diencephalon. In the pretectal region, numerous labeled cells were observed to project ipsilaterally to brachial, thoracic and, to a lesser extent, lumbar parts of the spinal cord (Fig. 1d). These pretectal cells were located mainly in the juxtacommissural nucleus with dispersed cells also in the precommissural and the lateral posterodorsal nuclei (subdivision according to Puelles et al., 1996). At the same level, a considerable number of cells in the ventrolateral component of the posterior tubercle also projects ipsilaterally throughout the spinal cord. Occassionally, labeled cells were also found in the dorsomedial division of the posterior tubercle, where contralateral cells were more abundant (Fig. 3d).

At this level, probably the most outstanding cell group that projects to the spinal cord is the interstitial nucleus of the medial longitudinal fasciculus in the basal plate of the synencephalon (see Puelles et al., 1996). This nucleus was always ipsilaterally labeled in experiments in which the tracer applications involved the ventromedial funiculus, and found to innervate the entire spinal cord (Figs. 1d, 3e). The labeled neurons were of two types: small cells located dorsomedially in the nucleus, and large, ventrolaterally located neurons with a huge dendritic arborization directed ventrolaterally.

Within thalamic territories, three cell populations in the anterior parencephalon were found to project as far caudally as the lumbar spinal cord. The majority of thalamospinal cells were observed in the ipsilateral ventromedial thalamic nucleus,

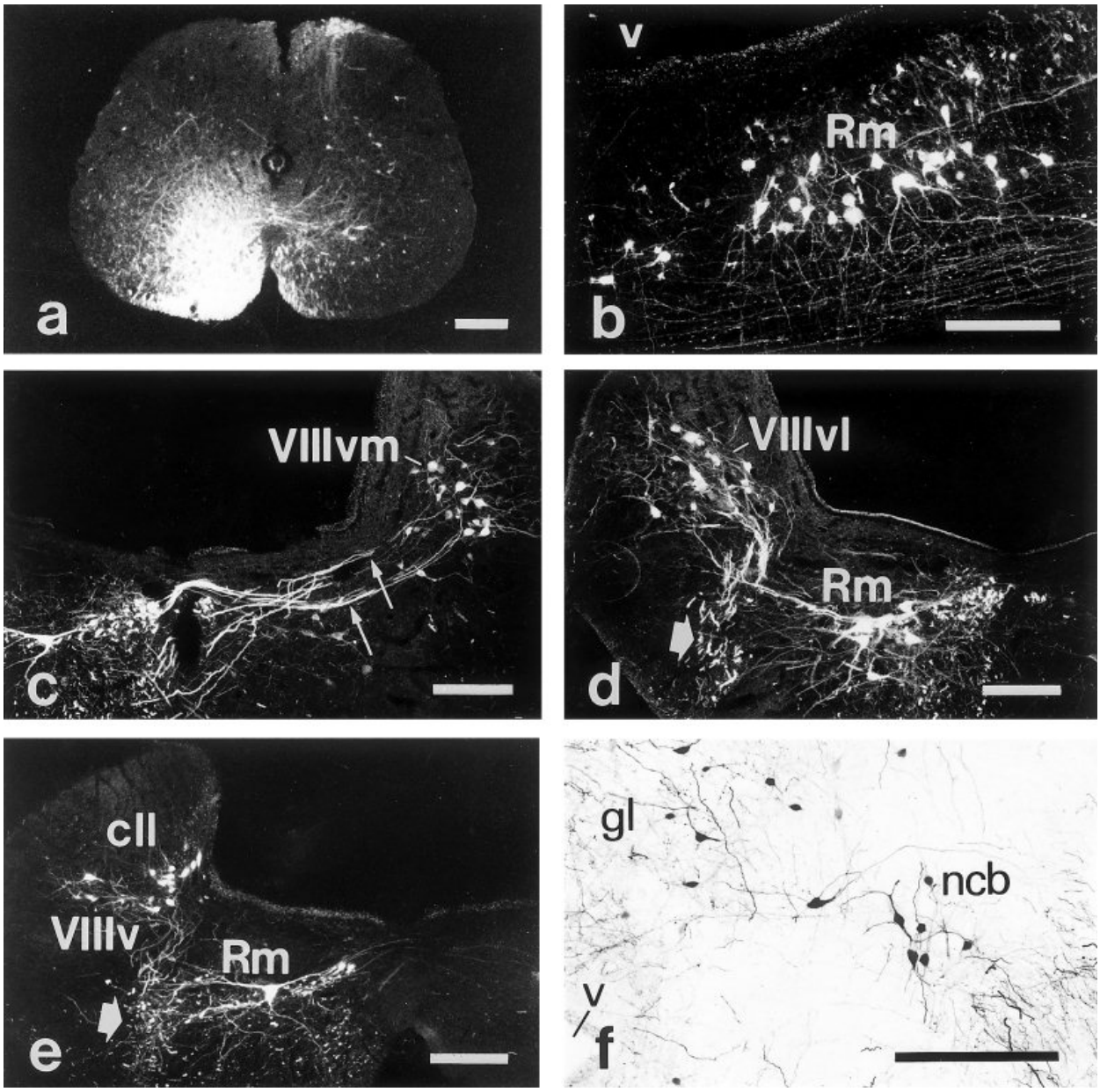

Fig. 2. Photomicrograghs of transverse (a, c-f) and sagittal (b) sections through the brain of *R. perezi* (a-d, f) and *X. laevis* (e) showing the localization of retrogradely labeled cells after spinal tracer application. **a:** Application site of TRDA in the ventromedial part of the spinal cord. **b:** Sagittal section showing the middle reticular population labeled in the ipsilateral medulla after TRDA application. **c:** Contralateral labeled cells in the medial vestibular nucleus. Thin arrows point to vestibular axons coursing to the contralateral side. **d:** Large cells in the ipsilateral middle reticular nucleus and the lateral vestibular nucleus. Note the ipsilateral component of the vestibulospinal pathway (thick arrow). **e:** Large reticular cell and cells in the ventral octaval nucleus in *X. laevis*. Note the ipsilateral vestibular axons descending to the spinal cord (thick arrow). **f:** Lateral group of cells in the contralateral cerebellar nucleus and scattered in the granule layer of the corpus after BDA application. Calibration bars= 200 μm.

mainly in its rostral part (Figs. 1c, 3f). However, a distinct cell group was found labeled caudally in the area between the ventral thalamus and the dorsal hypothalamus just lateral and dorsal to the nucleus of the periventricular organ. At rostral diencephalic levels, ipsilaterally projecting thalamospinal cells were found in the lateral part of the ventrolateral thalamic nucleus (Fig. 1b).

At and rostral to the level of the optic chiasm, already in the secondary prosencephalon, cells projecting to the spinal cord were found in the suprachiasmatic nucleus and in the preoptic region. Only a few cells were found in the suprachiasmatic nucleus at its ventral border lying just lateral to the ventral tip of the third ventricle (Fig. 1b). All along the preoptic region, labeled cells were seen within the magnocellular and parvocellular groups of the preoptic area. Caudally, the cells were compactly arranged in a band-shaped group beneath the ventral thalamus (Fig. 1b). More rostrally, dispersed cells were found in the anterior preoptic area along the wall of the

preoptic recess of the third ventricle (Fig. 1a). The projections to the spinal cord arising in the hypothalamus and ventral thalamus were only found in those experiments in which the dorsal and intermediate grey zones were implicated in the application site, and extended as far caudally as the lumbar spinal cord.

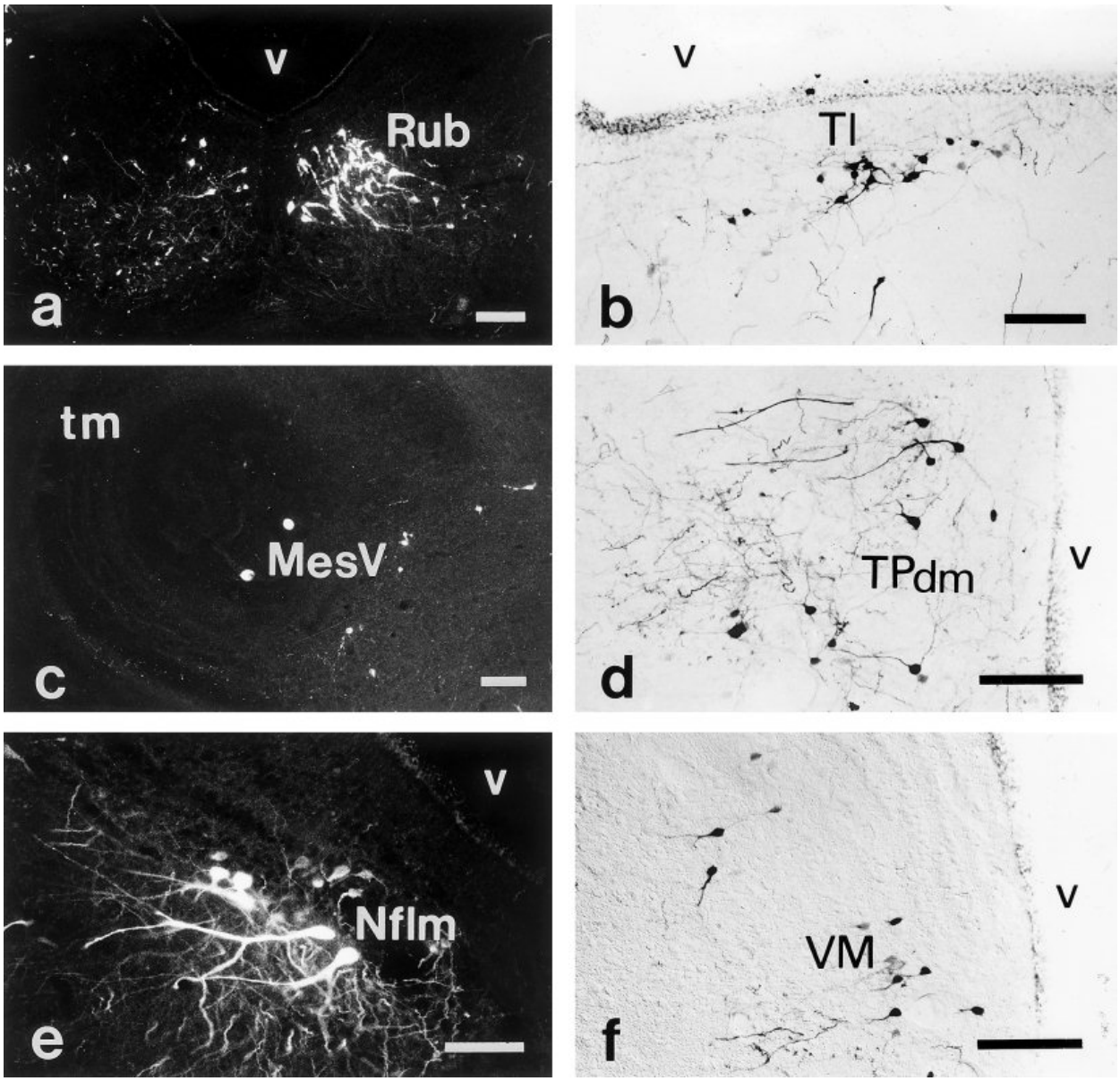

Fig. 3. Photomicrograghs of transverse sections through the brain of *R. perezi* showing the localization of retrogradely labeled cells after spinal tracer application. **a:** Contralaterally projecting cells in the red nucleus. **b:** Laminar nucleus of the torus semicircularis ipsilateral to a BDA application in the spinal cord. **c:** Two large ganglionic cells in the ipsilateral mesencephalic trigeminal nucleus at the rostral pole of the tectum. **d:** BDA labeled cells in the dorsomedial posterior tubercle. **e:** Two types of labeled cells in the ipsilateral interstitial nucleus of the flm: small dorsomedially and large ventrolaterally located neurons. **f:** BDA labeled cells in the ipsilateral ventromedial thalamic nucleus. Calibration bars= 100 μm.

Telencephalon. A small component of descending fibers to the spinal cord was found to arise from cells located at caudal telencephalic levels. These few neurons were labeled ipsilaterally and were located in the ventral part of the hemisphere at the tip of the lateral ventricle, although some cells were observed more laterally (Fig. 1a). This telencephalospinal projection was only observed after large tracer applications at brachial levels.

Descending projections to the spinal cord in urodeles

Like in anurans, small tracer deposits restricted to the dorsal or ventral parts of the spinal cord revealed different patterns of distribution of labeled cells. In Figure 4, the full complement of the cells of origin of descending supraspinal projections to the left third spinal segment is shown. The pattern of retrogradely labeled cells was readily comparable to that obtained in anurans, although differences were observed. The

caudal extent of the various descending supraspinal projections was comparable to that of anurans.

Rhombencephalon. The majority of the supraspinal cells was found in the rhombencephalon (Figs. 4i-m, 5a-c). In the reticular formation, cells projecting ipsilaterally were found mainly close to the midline in the raphe and in the inferior reticular nucleus, whereas cells in the middle reticular nucleus were observed primarily in the contralateral half of the rhombencephalon. Like in anurans, the cells of origin of the raphespinal projections were restricted to the caudal half of the raphe. Subdivisions into distinct reticular nuclei were difficult to establish in urodeles, and small and large cells were labeled along the rhombencephalon. Among the latter, a contralaterally projecting Mauthner cell was labeled at the level of the octaval nerve root (Figs. 4j, 5c).

The spinal projections arising in the octavolateral area were constantly observed after brachial and upper thoracic tracer applications. These projections passed via the ventral funiculus. Our lumbar tracer applications did not include the entire ventral funiculus, and vestibulospinal projections to lumbar levels were only sparsely labeled. In *Pleurodeles waltl*, the octavolateral area is formed by dorsal, intermediate, and ventral zones. Descending pathways to the spinal cord were observed from the intermediate and ventral parts of the octavolateral area (Figs. 4i-k, 5a, 6a). Vestibulospinal projections arise from two parts of the ventral zone: in the caudal rhombencephalon, a predominantly contralateral projection was labeled, whereas more rostrally, mainly ipsilaterally projecting neurons were observed. The cells of origin of this pathway were large, multipolar neurons with laterally and ventrally extended dendrites. In the mechanoreceptive lateral line zone, the neurons innervating the spinal cord were bilaterally distributed and, although their cell bodies were located ventrally, their main dendritic branches extended dorsally into the electroreceptive dorsal zone (Fig. 6a).

Ipsilateral to the application side, small neurons were found at the lateral border of the reticular formation in a position that may include the descending and principal sensory trigeminal nucleus as distinguished by González and Muñoz (1988). Also ipsilaterally, a few cells were labeled dorsomedial to the solitary tract, most likely representing cells of the dorsal column nucleus (A. Muñoz et al., 1998). At mid- and caudal rhombencephalic levels, a population of small cells around the solitary tract was often labeled contralaterally (Fig. 5b).

Cerebellum. Retrogradely labeled cells in the cerebellum were found almost exclusively contralateral to the application site in the spinal cord. The cells of origin of cerebellospinal fibers formed a rather compact group at the lateral margin of the cerebellar plate, whereas scattered cells extended medially within the granule cell layer (Figs. 4h, 5d).

Isthmus. In the reticular formation, ipsilaterally labeled cells were found in the superior reticular nucleus but the most conspicuous cell group was labeled bilaterally in the lateral tegmentum, in the region where the laterodorsal tegmental nucleus and the locus coeruleus (Figs. 4h, 5d) were immunohistochemically identified (González and Smeets, 1994a, 1995; Marín et al., 1997e).

Midbrain. Like in anurans, in *Pleurodeles waltl*, spinal projections from the midbrain include tegmentospinal, torospinal and tectospinal projections. Tegmentospinal pathways arise bilaterally in the anterodorsal, anteroventral and posteroventral tegmental nuclei (Fig. 4e-g). Additionally, a conspicuous red nucleus was found dorsolateral to the oculomotor nucleus in the contralateral tegmentum (Figs. 4e, 5e). Projections from the torus semicircularis were difficult to distinguish and tectospinal pathways were observed only after tracer applications to the ventral part of the brachial spinal cord. Labeled cells in the tectum were mainly found ipsilaterally in layers 6 and 8 (Figs. 4d-g, 6b). Like in anurans, large mesencephalic trigeminal neurons were labeled after tracer applications to dorsal parts of the brachial spinal cord. In urodeles, labeled cells in the mesencephalic trigeminal nucleus were found bilaterally in the deep layers of the tectum (Fig. 4f,g).

Diencephalon. Abundant spinal projections originated from pretectal neurons. These cells were found almost exclusively ipsilateral to the side of the tracer application and formed a band beneath the fibers of the posterior commissure (Fig. 6c). The nucleus interstitialis of the flm was always labeled ipsilaterally after tracer applications to the ventral part the spinal cord (Fig. 4d). Actually, two components were observed: a small-celled group close to the ventricle, and a group of large cells with extensive dendritic branching, located dorsolaterally (Fig. 6d). This situation resembles that found in anurans, although the localization of the small and large cells was reversed. A small population of cells in the posterior tubercle, mainly in its ventrolateral part, was found to project to the dorsal part of the spinal cord. More rostrally, cells were abundantly labeled lateral to the nucleus of the periventricular organ and in the ventral thalamus (Fig. 4b-d).

At the level of the suprachiasmatic nucleus as well as more rostrally, a population of cells in the ipsilateral preoptic region was found to project to the dorsal half of the spinal cord (Fig. 4a,b). These cells included neurons of the suprachiasmatic nucleus (Fig. 6e) and cells of the magnocellular component of the preoptic nucleus (Fig. 6f). The cells located more rostrally in the preoptic area were small and lined the ventricular recess, but did not touch the ventricle.

Telencephalon. Telencephalospinal cells were unequivocally labeled after tracer applications to the brachial spinal cord. These cells were found scattered in the ventral and ventrolateral hemispheric wall, at middle and caudal levels (Fig. 6g,h). The actual location of the cells may correspond with striatal or lateral amygdaloid regions.

Descending projections to the spinal cord in gymnophionans

The cells of origin of descending pathways to the spinal cord of a gymnophionan amphibian (*Dermophis mexicanus*) were investigated in six experiments in which large unilateral tracer applications were made at upper spinal levels only. The limited number of animals available led us to investigate the full complement of descending projections to the spinal cord by these rostral applications. In two cases, the tracer applications involved only the dorsal part of the spinal cord, whereas in one experiment the tracer was restricted to its ventralmost part. The other three experiments were large applications in which almost the complete half of the spinal cord at the level of the operation was filled with the tracer. Figure 7 summarizes the distribution of retrogradely labeled cells revealed in such an experiment. Due to the marked flexure of the brain axis at isthmic-mesencephalic levels, the appearance of "transverse" sections is modified since mesencephalic, isthmic and rhombencephalic portions are cut in the same section (levels f, g and h). This peculiarity makes it difficult to identify structures in certain brain regions. In general, the distribution pattern of the cells of origin of descending pathways to the spinal cord in *Dermophis mexicanus* was largely comparable to that of anurans and urodeles.

Rhombencephalon. The present experiments revealed an extremely well-organized system of descending pathways from the medulla to the spinal cord (Fig. 7h-k). Strikingly

abundant are the reticulospinal pathways. At caudal rhombencephalic levels, labeled cells were found, mainly ipsilaterally in the inferior reticular nucleus. Medium or small-sized cells, with round or elongated perikarya were located medially, whereas

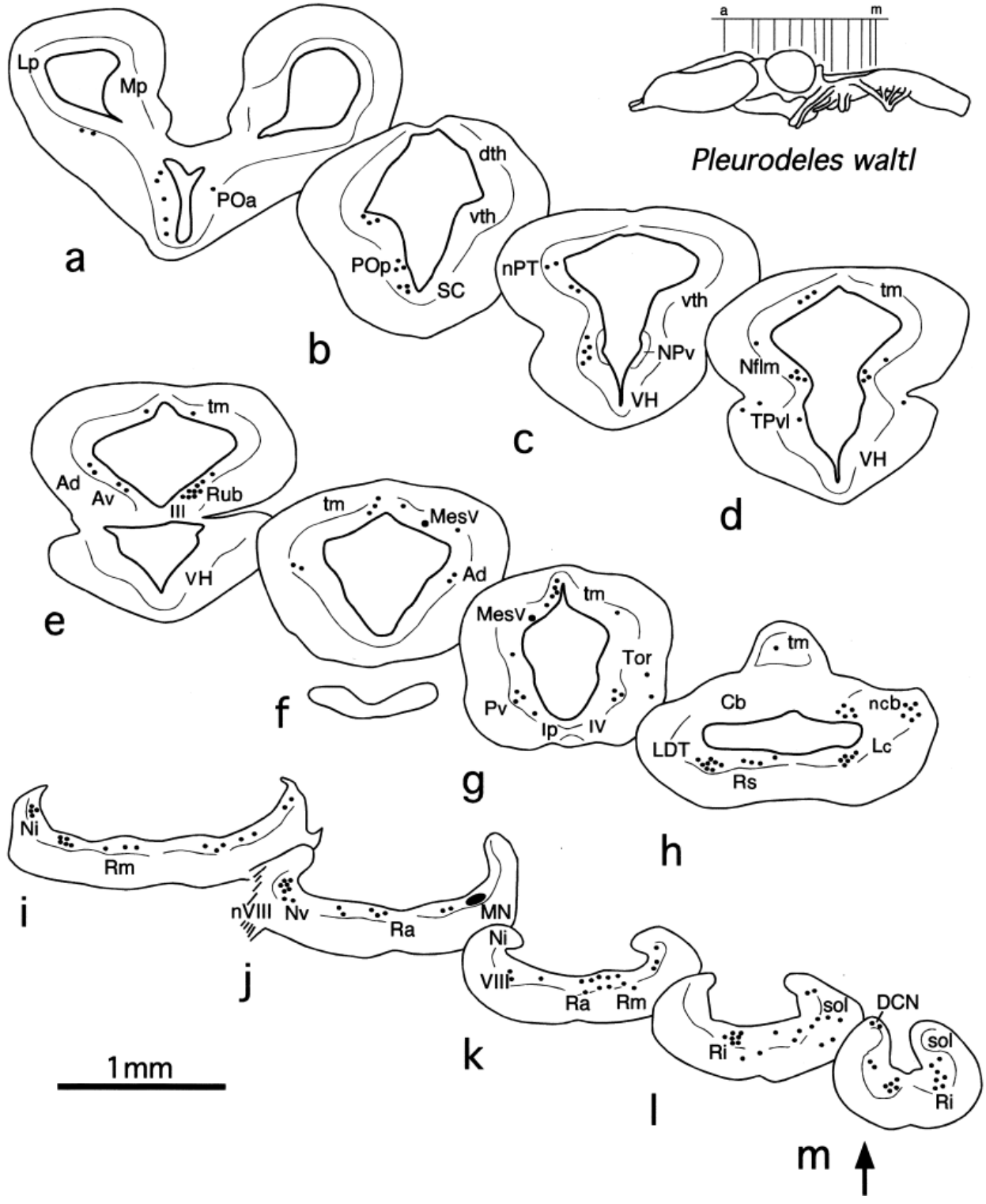

Fig. 4. Schematic drawings of transverse sections through the brain of *Pleurodeles waltl* showing the distribution of retrogradely labeled cells (filled dots) after tracer application into the spinal cord. Approximately, a one-to-one correspondence of dors and retrogradely labeled cells is presented. The appropriate levels of the sections are indicated in the upper right scheme. The arrow marks the side of the tracer application in the spinal cord.

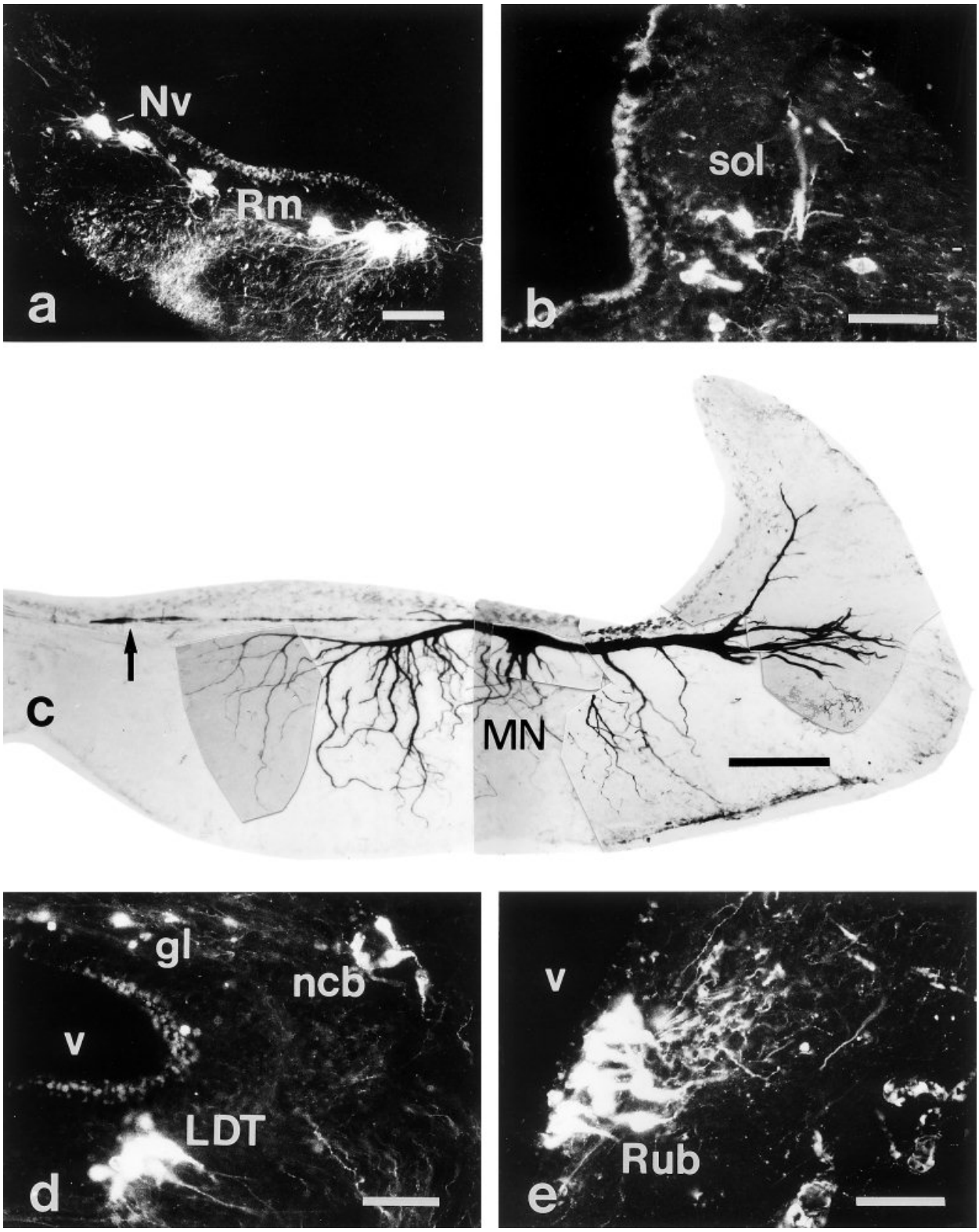

Fig. 5. Photomicrograghs of transverse sections through the brain of *Pleurodeles waltl* showing the localization of retrogradely labeled cells after spinal tracer application. **a:** Reticular and octaval cells of the ventral nucleus in the ipsilateral rhombencephalon. **b:** Contralateral labeled cells in the alar plate around the solitary tract at caudal rhombencephalic levels. **c:** Contralateral Mauthner neuron at the caudal level of the octa-

val nerve root after BDA application into the spinal cord. Note the axon passing along the dorsal aspect of the flm (arrow). **d:** Contralateral reticular cells in the isthmic tegmentum and medial and lateral neurons in the cerebellum. **e:** Contralaterally projecting cells in the red nucleus. Calibration bars= 100 μm.

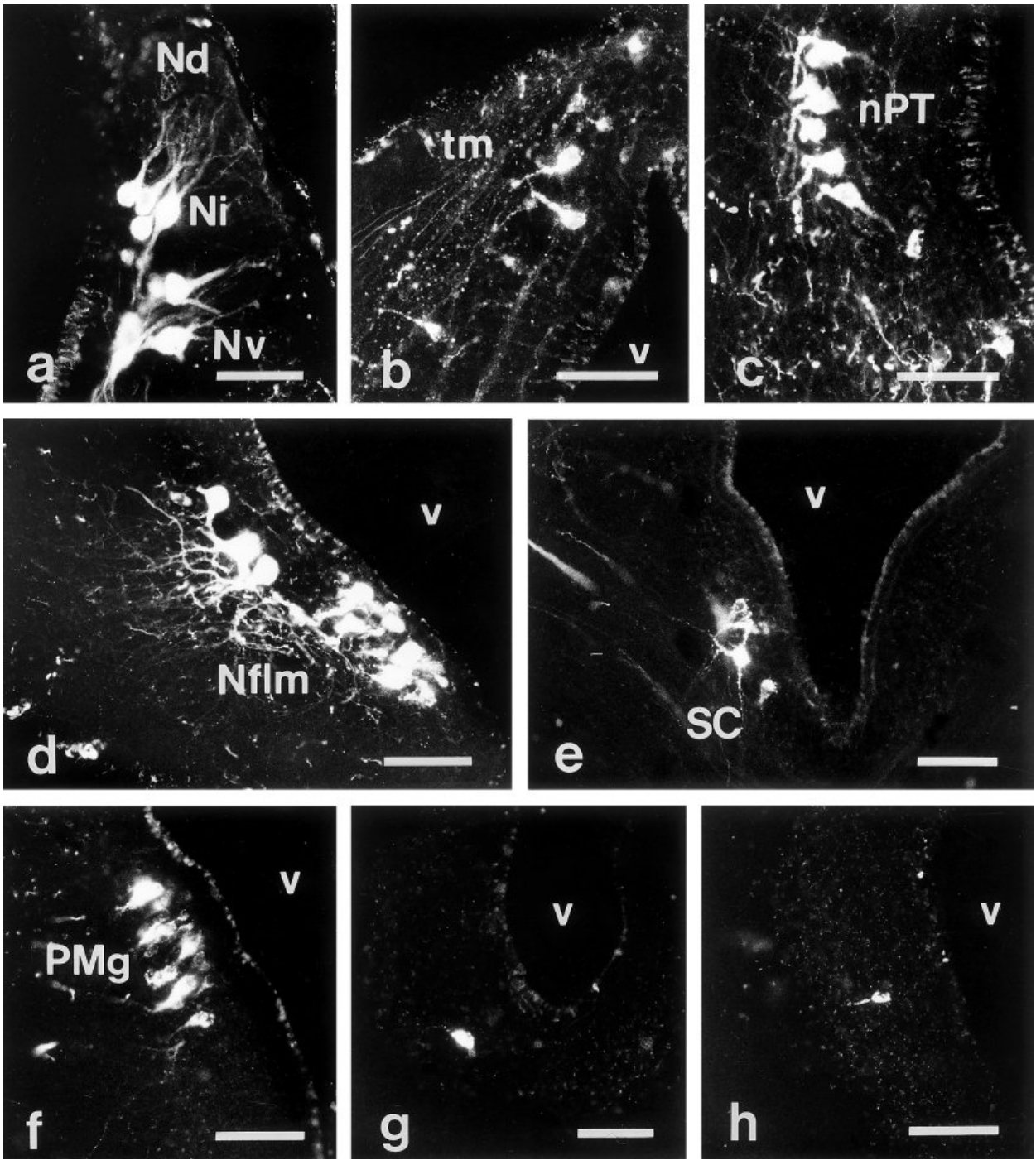

Fig. 6. Photomicrograghs of transverse sections through the brain of *Pleurodeles waltl* showing the localization of retrogradely labeled cells after spinal tracer application. **a:** Retrogradely labeled cells in the contralateral intermediate and ventral nuclei of the octavolateral area. **b:** Ipsilateral labeled neurons in layers 6 and 8 of the mesencephalic tectum. **c:** Ipsilateral spinal projection from pretectal neurons. **d:** Small-celled group and large cells located dorsolaterally in the interstitial nucleus of the flm. **e:** Neurons of the suprachiasmatic nucleus. **f:** Magnocellular neurons in the ipsilateral preoptic region. **g** and **h:** Labeled cells in the ventral telencephalon scattered in the ventral and ventrolateral hemispheric wall, at middle and caudal levels. Calibration bars= 100 μm.

more laterally, medium-sized cells were found with fusiform or bipolar perikarya (Fig. 8a). At mid-rhombencephalic levels, small or medium-sized labeled cells were observed, mainly ipsilaterally, in the middle reticular nucleus (Fig. 8c). At these levels, also some ipsilaterally labeled neurons with small round perikarya were found in the raphe nucleus. Raphespinal cells extended from middle to caudal rhombencephalic levels. No Mauthner cells were found in the brain stem.

In the rostral rhombencephalon and continuing into the isthmus region, two differents groups could be distinguished bilaterally. Within the alar plate, a compact group of small or medium-sized neurons with round or oval perikarya was observed in the octavolateral area (Fig. 8b,c). The anatomy of the octavolateral area of *Dermophis* resembles that of gymnophionans with a free larval stage, where a dorsal nucleus, an intermediate nucleus and a ventral zone are present (Will and Fritzsch, 1988). The ventral zone receives VIIIth nerve afferents and contains neurons that project to the spinal cord. These vestibulospinal neurons showed a rich dendritic arborization that included processes directed dorsally into the dorsal part of the alar plate. In the caudal half of the rhombencephalon, most of the vestibulospinal tract neurons were located contralaterally (Fig. 8b), whereas rostrally the labeled cells were found mainly in a tightly packed group projecting ipsilaterally (Fig. 8c). Some more dorsally situated neurons with spinal projections may belong to the mechanoreceptive intermediate nucleus (Figs. 7i, 8b).

Ventral to the octavolateral area, a conspicous group of small and medium-sized cells was found in the trigeminal sensory nucleus from middle to the most rostral rhombencephalic levels (Fig. 7h,i). Labeled cells were also found in the alar region comparable to the DCN as distinguished in *Pleurodeles*. Dorsolateral to the labeled cells in the inferior reticular nucleus, small round cells were located lateroventral to the solitary tract. The number of cells in this region was increased at caudal rhombencephalic regions, just rostral to the obex.

Isthmus. Large, fusiform reticulospinal neurons were observed bilaterally in the superior reticular nucleus (Fig. 8d). Intermingled with these cells, medium-sized neurons with round or elongated perikarya were found in the most rostral part of the reticular formation. In the region where the brain axis is bended dorsally (and the isthmic tegmentum is seen almost horizontally in "transverse" sections), a population of large reticular cells was labeled just lateral to the flm in the isthmic reticular nucleus (Fig. 8e). These neurons showed pear-shaped perikarya with long processes that intermingled with the fibers coursing in the flm. Also within the isthmic tegmentum, in a region just above the rostral lateral recess of the IVth ventricle, two cell groups were retrogradely labeled (Figs. 7g, 8d). These cells were located in a region where the locus coeruleus is located (González and Smeets, 1994b). However, the small cells located laterally in this region may correspond to a cerebellar nucleus related to the indistinct cerebellum present in this species (Fig. 7g).

Midbrain. Spinal projections from the mesencephalic tegmentum were sparse. However, in all experiments in which the tracer was applied to the dorsal part of the spinal cord, a contralateral cell group was labeled in the lateral tegmentum (Figs. 7f, 8f). This cell group was made up by loosely arranged neurons whose axons passed via the contralateral lateral part of the mesencephalic tegmentum and continued caudally through the lateral part of the rhombencephalon. Its exclusive contralateral projection, the course of ITS descending axons and the localization of its cell bodies suggest that this nucleus is comparable to the red nucleus of anurans and urodeles.

Retrogradely labeled cells in the tectum mesencephali were found in particular after tracer applications that involved the ventral part of the spinal cord. Tectospinal cells were observed bilaterally in the dorsal part of the tectum at rostral as well as at caudal levels (Fig. 7e-h). Mainly at caudal levels of the tectum, round and slightly larger tectal cells were labeled that may correspond to mesencephalic trigeminal neurons (Fig. 9a).

Diencephalon. A few labeled cells located beneath the rostral part of the mesencephalic tectum give rise to a pretectospinal projection to the rostral spinal cord. A large cell population was labeled in the interstitial nucleus of the flm (Figs. 7e, 9b). This nucleus was made up by two different cell types, i.e. large dorsally situated cells and small ventrally located cells. A similar pattern of labeling was observed in urodeles, but in *Dermophis*, the population of dorsal large cells was labeled ipsilaterally, and the ventral component bilaterally. Possibly, this ventral part of the interstitial nucleus of the flm may correspond to the anteroventral tegmental nucleus like in the other amphibians studied or form an accessory oculomotor nucleus.

More rostrally, labeled cells were found scattered in areas that included the ventral thalamus, the dorsal hypothalamus and the posterior tubercle. All these cells were located along the band of grey matter that surrounds the third ventricle, where clear landmarks are not discernible. However, two different types of neurons were seen in the ventral thalamus. Mainly ipsilaterally, medium or small-sized cells with round perykarya were found in the ventral thalamus (Fig. 7d). Ventral to this cell group, and just at the border between the thalamus and the hypothalamus, another type of cells was observed, also mainly ipsilaterally. These were medium-sized neurons, with an oval perikaryon and a main dendrite directed laterally. In the rostral hypothalamus, cells retrogradely labeled from the spinal cord were found at the level of the optic nerve. A few small cells were seen ventrally, whereas a conspicuous group of retrogradely labeled neurons were located close to the ventricle in the magnocellular nucleus of the preoptic area (Figs. 7c, 9c). In the anterior preoptic area, scattered small cells were found along the anterior recess of the third ventricle.

Telencephalon. Like in anurans and urodeles, the most rostral projection to the spinal cord in apodans was found to arise in the telencephalon. Dispersed and weakly labeled neurons were observed in the striatum (Fig. 7a). These neurons possessed small and round perikarya and were located either migrated in the lateral fiber zone or in the periventricular cell layer, some of them close to the lateral ventricle (Fig. 9d). In *Dermophis*, where the telencephalic hemisphere is relatively larger than in anurans and urodeles, the telencephalospinal cells were distributed more rostrally along the lateral wall of the telencephalon.

DISCUSSION

The present study shows that in representative species of the three amphibian orders extensive descending pathways from all main brain divisions reach the spinal cord. Our data are summarized in Table I. In *Rana perezi* and *Xenopus laevis*, our data confirm and extend previous studies by Tóth et al. (1985) in *R. esculenta* and ten Donkelaar et al. (1981) in *X. laevis*. Differences found may be due to the sensitivity of the retrograde tracer used and, in addition, to the terminology followed, mainly when the segmental approach to the brain is considered. Moreover, in our study, both the caudal extent and the funicular trajectory of most of the descending projections

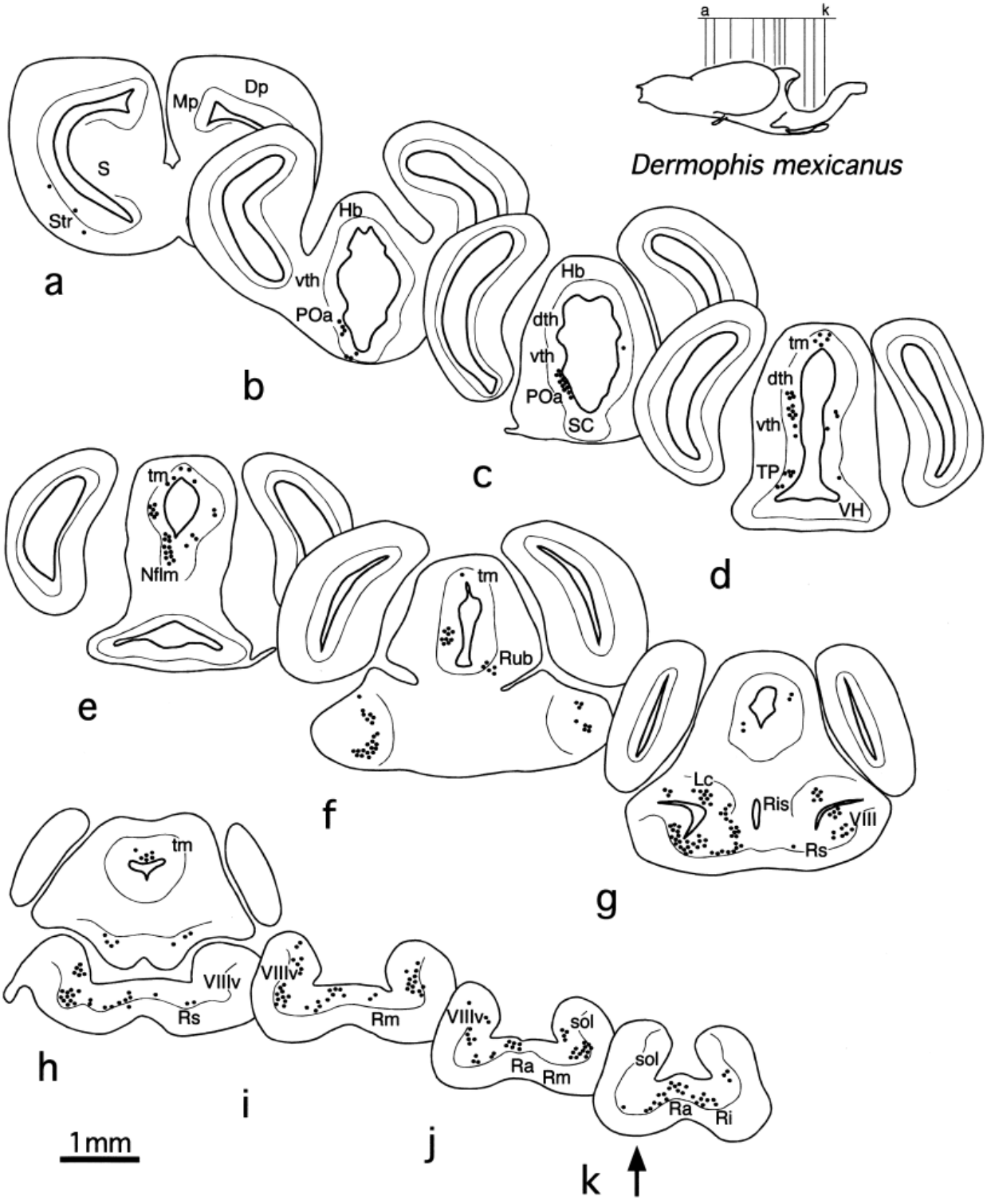

Fig. 7. Schematic drawings of transverse sections through the brain of *Dermophis mexicanus* showing the distribution of retrogradely labeled cells (filled dots) after tracer application into the spinal cord. Approximately, a one-to-one correspondence of dors and retrogradely labeled cells is presented. The appropiate levels of the sections are indicated in the upper right scheme. The arrow marks the side of the tracer application in the spinal cord.

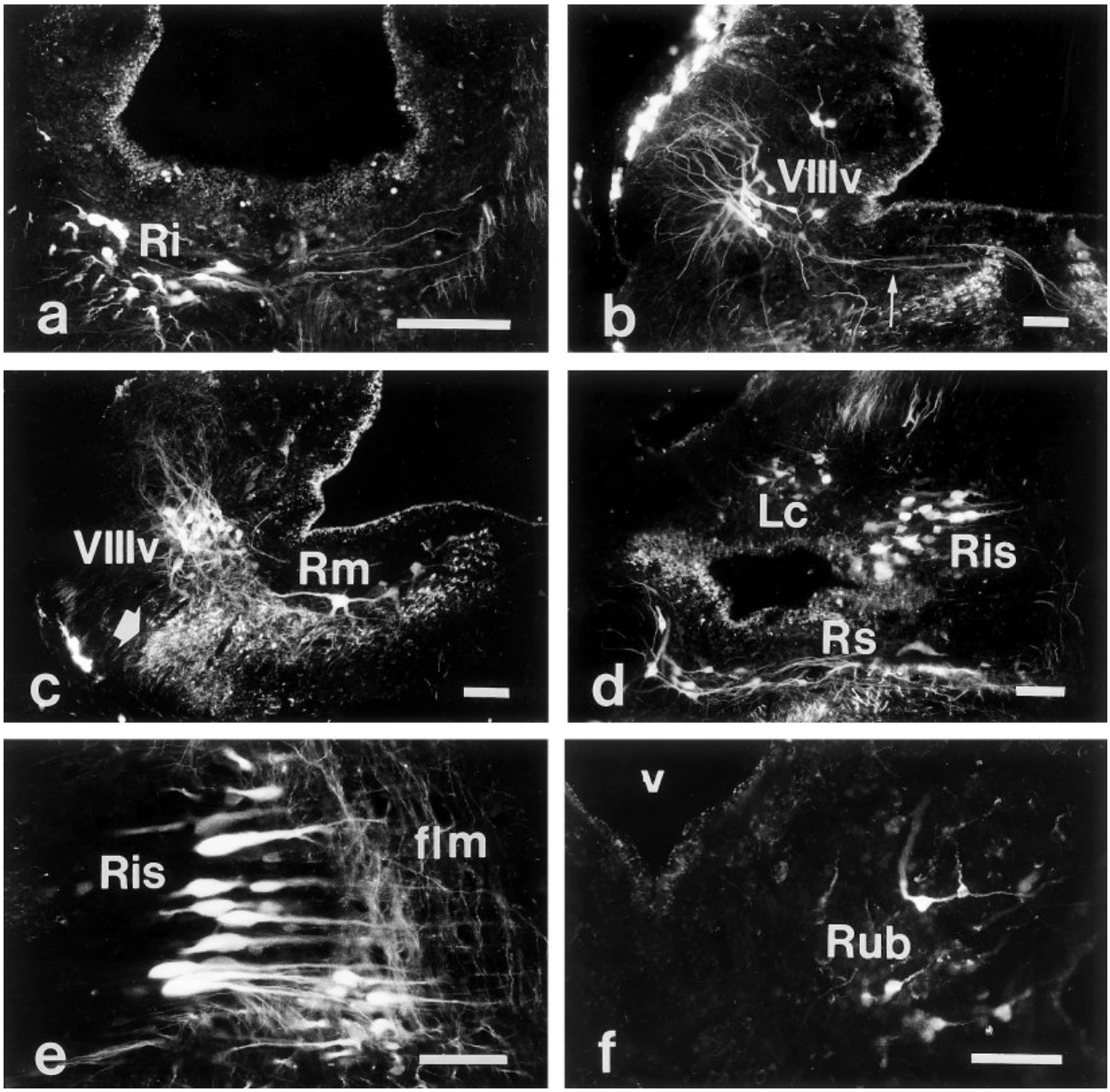

Fig. 8. Photomicrograghs of transverse sections through the brain of *Dermophis mexicanus* showing the localization of retrogradely labeled cells after spinal tracer application. **a:** Contralateral reticular cells in the caudal rhombencephalon. **b:** Octaval cells with axons running to the contralateral side (thin arrow). **c:** Ipsilateral ventral octaval nucleus with axons runnig to the ipsilateral spinal cord (thick arrow) and large reticular cells. **d:** Ipsilateral labeled cells in the zone of the isthmic and superior reticular nuclei and locus coeruleus. **e:** Detail of large cells in the isthmic reticular nucleus shown in Fig. 8d that intermingled with the fibers in the flm. **f:** The cotralateral red nucleus. Calibration bars= 100 μm.

could be determined. The tracer application technique used made it possible to restrict the tracers to small parts of the spinal cord, dorsal, intermediate or ventral, or to particular funiculi. In urodeles, so far only data on the cells of origin of descending pathways to the level of the obex were available (Naujoks-Manteuffel and Manteuffel, 1988). The present study shows their extent into the spinal cord, but moreover their funicular trajectories. The data in the Mexican caecilian, *Dermophis mexicanus*, form the first report on descending pathways to the spinal cord in the order Gymnophiona. In all three amphibian orders, telencephalospinal, diencephalospinal, and extensive brainstem-spinal pathways are present. Most descending pathways appear to be quite conservative and are present throughout vertebrates. In the following discussion the similarities but in particular the differences between amphibians and other vertebrates will be emphasized. The major sources of descending pathwys in vertebrates are summarized in Table II.

The most prominent amphibian descending pathways were found to arise in the vestibular part of the octavolateral area and the rhombencephalic reticular formation. In amphibians which retain their lateral line system such as *Xenopus laevis*, also spinal projections from lateral line nuclei were observed. In *Pleurodeles waltl*, Mauthner cells contralaterally innervate the spinal cord. Although Mauthner cells maintain their spinal projections in adult frogs (Will, 1986, 1991; Davis and Farel, 1990), we found no evidence for such a projection in *Rana perezi* or in *Xenopus laevis*, in line with previous studies in

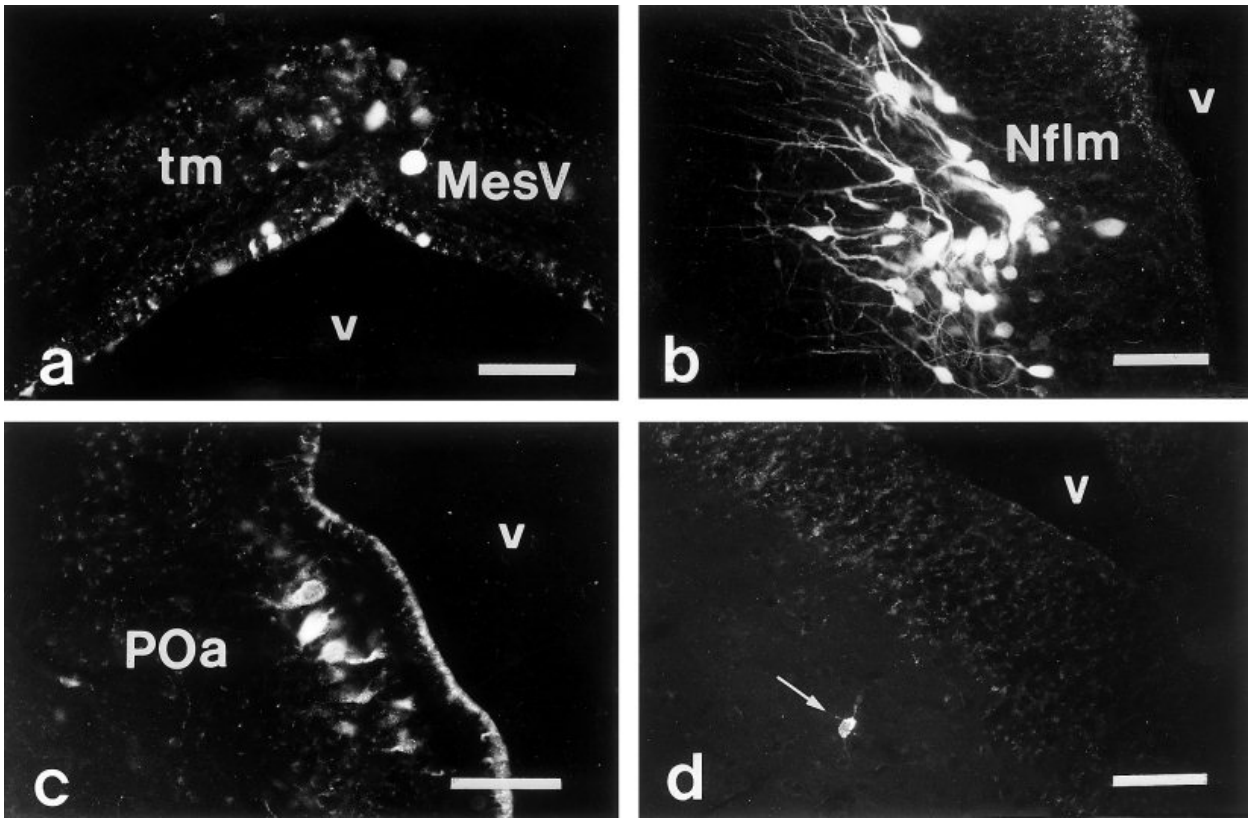

Fig. 9. Photomicrograghs of transverse sections through the brain of *Dermophis mexicanus* illustrating the localization of retrogradely labeled cells after spinal tracer application. **a:** Labeled cells in the mesencephalic tectum. Big and round mesencephalic trigeminal neurons are observed. **b:** Detail of the neurons in the interstitial nucleus of the flm. Note the two different cell types: large dorsally situated cells and small ventrally located cells. **c:** Ipsilateral magnocellular neurons in the preoptic area. **d:** Labeled cell in the striatum (arrow). Calibration bars= 100 μm.

anurans (ten Donkelaar et al., 1981; Tóth et al., 1985). In addition, Mauthner neurons with axons extending into the spinal cord were not observed in gymnophionans (Naujoks-Manteuffel and Manteuffel, 1988; present study). In the four species studied at least two *vestibulospinal pathways* were found, an ipsilateral one from the large-celled lateral vestibular nucleus, and a contralateral projection arising in the medial vestibular nucleus and, if present, in the caudal vestibular nucleus. In anurans, we also found a sparse ipsilateral projection from the anterior vestibular nucleus. These data are in line with previous data on vestibulospinal projections in amphibians (ten Donkelaar et al., 1981; Tóth et al., 1985; Will et al., 1985b; Will, 1988; Naujoks-Manteuffel and Manteuffel, 1988). Vestibulospinal (or octavomotor) projections are found in all vertebrates (see Table II). In agnathans, the intermediate octavomotor nucleus ipsilaterally innervates the spinal cord, and the posterior octavomotor nucleus the contralateral spinal cord (Ronan, 1989). In cartilaginous fishes, the magnocellular vestibular nucleus ipsilaterally innervates the spinal cord, and the caudal vestibular nucleus the contralateral cord (Smeets and Timerick, 1981; Cruce et al., 1999). Comparable observations were made in teleosts (Oka et al., 1986; Prasada Rao et al., 1987). The vestibulospinal projections in reptiles (ten Donkelaar et al., 1980; Woodson and Künzle, 1982), birds (Cabot et al., 1982) and mammals (see Nudo and Masterton, 1988) are more extensive but are *basically* composed of an ipsilateral pathway from the lateral vestibular nucleus and a contralateral pathway, passing via the flm, from the medial and inferior vestibular nuclei.

Reticulospinal projections arise throughout the brainstem reticular formation. In a previous study (ten Donkelaar et al., 1980) evidence was found for two main reticulospinal pathways, one arising in the inferior reticular nucleus and passing, joined by the raphespinal projection, via the lateral funiculus, and another arising from more rostral reticular levels including the interstitial nucleus of the flm in the mesencephalon, passing via the ventral funiculus. The present data are in line with this view, but the reticulospinal and in particular the raphespinal projections can now be more clearly defined. The application of antibodies against serotonin in *Rana catesbeiana* (Yoshida et al., 1983; Ueda et al., 1984), in *R. pipiens* (Adli et al., 1999; Tan and Miletic, 1990), in *Xenopus laevis* (van Mier et al., 1986), in urodeles (Clairambault et al., 1994; Dicke et al., 1997), and in a gymnophionan, *Typhlonectes compressicauda* (Clairambault et al., 1994) clearly suggested bulbospinal serotonergic pathways in amphibians. Moreover, by combining retrograde fluorescent tracing with serotonin immunohistochemistry, Tan and Miletic (1990) showed that the rostral part of the raphe nucleus innervates the dorsal horn, the intermediate zone and the ventral horn, whereas its caudal part only innervates the intermediate zone and the ventral horn. In the amphibian species studied, we observed a similar subdivision reminiscent of the raphe magnus and raphe pallidus projections found in amniotes (See Björklund and Skagerberg, 1982). The most rostral component of the raphe nucleus at the

TABLE II. Major Sources of Descending Supraspinal Pathways in Vertebrates

Class	DCN	Raphe complex	Reticular Formation	Vestibular Nuclear Complex	Locus coeruleus	Cerebellar nuclei	Red nucleus	Interstitial nucleus of the FLM	Pretectum	Hypothalamus	Subpallium	Pallium
Anamniotes												
Agnathans	-	+	+	+	+	-	-	+	-	?	-	-
Cartilaginous fishes	+	+	+	+	+	-	+/-	+	-	+	-	?
Bony fishes	-	+	+	+	+	-	+/-	+	-	+	-	-
Amphibians	+	+	+	+	+	+	+/-	+	+	+	+	-
Amniotes												
Reptiles	+	+	+	+	+	+	+/-	+	-	+	+	-
Birds	+	+	+	+	+	+	+	+	-	+	?	+/-
Mammals	+	+	+	+	+	+	+	+	-	+	+/-	+

(+, present; -, absent; +/-, present in certain species, absent in others; ?, questionable)

(*Agnathans*: Ronan, 1989; *Cartilagionus fishes*: Smeets and Timerick, 1981; Cruce et al., 1999; *Bony fishes*: Oka et al., 1986; Prasada Rao et al., 1987; *Amphibians*: ten Donkelaar et al., 1981; Naujoks-Manteuffel and Manteuffel, 1988; the present study; *Reptiles*: ten Donkelaar et al., 1980; Woodson and Künzle, 1982; Newman et al., 1983; *Birds*: Cabot et al., 1982; Gross and Oppenheim, 1985; Webster et al., 1990; *Mammals*: Kuypers, 1981; Nudo and Masterton, 1988)

isthmic level gives rise to ascending projections to the forebrain (Northcutt and Ronan, 1992; Marín et al., 1997a).

The spinal projections of the principal and descending nuclei of the trigeminal nerve demonstrated are in line with previous studies in amphibians (e.g., ten Donkelaar et al., 1981; Tóth et al., 1985). Likewise, the spinal projections of the dorsal column nucleus confirm previous studies by A. Muñoz et al. (1995, 1998). A rather extensive solitariospinal projection reaching the lumbar cord was found in the anurans studied in line with previous studies in *Xenopus laevis* (ten Donkelaar et al., 1981) and in *Rana esculenta* (Tóth et al., 1985). Its catecholaminergic projection will be discussed in the companion paper (Sánchez-Camacho et al., 2001).

In all amphibian species studied, a mainly contralateral *cerebellospinal projection* has been demonstrated (ten Donkelaar et al., 1981; Tóth et al., 1985; Naujoks-Manteuffel and Manteuffel, 1988; Larson-Prior and Cruce, 1992; present study). The bulk of this projection arises in a cerebellar nucleus located laterally in the cerebellar peduncle. In line with data of Larson-Prior and Cruce (1992) we showed that in anurans and urodeles lateral and medial cell populations of cerebellospinal projecting cells are found, and that the medial group is located within the granule cell layer of the corpus cerebelli. Spinal projections from cerebellar nuclei are absent in agnathans, cartilaginous and bony fishes, and can be viewed as a *tetrapod augmentation* (Nudo and Masterton, 1988) in which across phylogeny the cerebellar nuclei undergo a huge expansion due to the increasing importance of the cerebellar cortex.

At the level of the isthmus, spinal projections arise from the region where scattered noradrenergic cells (locus coeruleus) have been localized in amphibians in close relationship with the laterodorsal tegmental nucleus (González and Smeets, 1994a; Marín et al., 1997e). With NADPH-diaphorase histochemistry and nitric oxide synthase (NOS) immunohistochemistry, a conspicuous cell population was found in the isthmic-pretrigeminal region of *Rana perezi* (M. Muñoz et al., 1996) and *Pleurodeles waltl* (González et al., 1996). This cell population resembles the mesopontine NADPH-diaphorase, cholinergic cells of amniotes (see Vincent and Kimura, 1992). In *R. perezi* and *X. laevis*, Marín et al. (1997e) found extensive staining of choline acetyltransferase (ChAT)-immunoreactive cells in the laterodorsal nucleus and the pedunculopontine nucleus which extends far rostrally into the mesencephalic tegmentum. In *P. waltl*, ChAT-immunoreactive cells are restricted to the laterodorsal nucleus (Marín et al., 1997e). Spinal projections from identified cholinergic or nitrergic cells in these cell groups still await investigation. Preliminary results in our laboratory suggest that only a small number of cholinergic cells in the laterodorsal tegmental nucleus do project to the spinal cord, whereas the pedunculopontine nucleus does not project. Noradrenergic *coeruleospinal projections* were demonstrated in amphibians by means of retrograde tracing combined with immunohistochemistry (Marín et al., 1996). On the basis of the distribution of labeled fibers using antibodies against noradrenaline, spinal projections from homologous groups to the locus coeruleus have been suggested in lampreys (Pierre et al., 1994), cartilaginous fishes (Stuesse and Cruce, 1992; Cruce et al., 1999), bony fishes (Meek, 1994), reptiles (Smeets, 1994), birds (Reiner et al., 1994; Puelles and Medina, 1994), and mammals (Kitahama et al., 1994).

Spinal projections from the mesencephalon include predominantly contralateral *tectospinal projections* to the brachial cord in line with previous data in amphibians (Naujoks-Manteuffel and Manteuffel, 1988; Roth et al., 1990; Dicke and Roth, 1994; Dicke, 1999a,b). A small, contralateral tectospinal projection extending not beyond the cervical spinal cord appears to be the rule in vertebrates. Tectospinal projections were also demonstrated in elasmobranchs (Smeets and Timerick, 1981), lungfishes (Ronan and Northcutt, 1985), reptiles (Woodson and Künzle, 1982), and in mammals (Nudo and Masterton, 1989). However, no tectospinal neurons could be identified in retrograde tracing studies in some elasmobranchs (Cruce et al., 1999), in bony fishes (Oka et al., 1986; Prasada Rao et al., 1987), in *Xenopus laevis* (ten Donkelaar et al., 1981), in some reptiles (ten Donkelaar et al., 1980), and in birds (Cabot et al., 1982; Gross and Oppenheim, 1985; Webster et al., 1990). Presumably, in these species the tectospinal pathway does not extend beyond the caudal medulla oblongata. The lack of sensitivity of the tracers used in these studies, however, may also account for the negative findings. It seems likely that tectospinal connections are a general feature of amphibians for the control of the neck muscles, and the control of the direction and the amplitude of saccadic eye movements (Naujoks-Manteuffel and Manteuffel, 1990). In a similar way, our study with dextran amines as tracers has demonstrated spinal projections from *mesencephalic trigeminal neurons* in the three amphibian orders. Contradictory data have been published on the presence of a spinal projection from the mesencephalic nucleus of the trigeminal nerve. Thus, in anurans HRP studies failed to demonstrate mesencephalic trigeminal descending branches into the spinal cord (ten

Donkelaar et al., 1981; M. Muñoz et al., 1993). This pathway seems to be more prominent in urodeles where it has been constantly described (Naujoks-Manteuffel et al., 1988; Roth et al., 1990). Trigeminal mesencephalic projections to the spinal cord seem to be a shared characteristic in anamniote vertebrates (Smeets and Timerick, 1981; Ronan and Northcutt, 1985; Pombal et al., 1997). In addition, this projection appears to exist also in amniotes (ten Donkelaar et al., 1980; Ebbesson, 1981; Woodson and Künzle, 1982).

The present study has unmistakably demonstrated spinal projections from the *torus semicircularis*. Previous studies in urodeles suggested that descending projections from the torus terminate within the medulla (Naujoks-Manteuffel et al., 1988), but several experimental studies in anurans could not demonstrate these projections (Feng and Lin, 1991; Matesz and Kulik, 1996). However, it seems that in various anuran species, the torus semicircularis gives rise to spinal projections mainly from its laminar nucleus (ten Donkelaar et al., 1981; Tóth et al., 1985; the present study). Similar projections from the laminar nucleus of the torus semicircularis were demonstrated in reptiles (ten Donkelaar et al., 1980; Butler and Bruce, 1981; Woodson and Künzle, 1982). At least part of this projection may be part of what is known in mammals as the periaqueductal grey. The periaqueductal grey is involved in head turning movements, vocalization, locomotion and pain modulation (Holstege, 1991). In mammals, it sparsely innervates the cervical spinal cord (see Holstege, 1991).

In anurans, dorsolateral to the oculomotor nucleus an homologue of the nucleus of Edinger-Westphal has been identified (Matesz and Székely, 1977). Spinal projections from this cell group have now been demonstrated in *Rana perezi* and *Xenopus laevis*, but could not be readily identified in *Pleurodeles waltl* and *Dermophis mexicanus*. Possibly, this cell group is included in the nucleus of the flm. Spinal projections from the nucleus of Edinger-Westphal were also found in reptiles (ten Donkelaar et al., 1980; Woodson and Künzle, 1982), birds (Cabot et al., 1982; Gross and Oppenheim, 1985) and mammals (Nudo and Masterton, 1988).

Rather extensive *tegmentospinal projections* arise in the anterodorsal, anteroventral and posteroventral tegmental groups as distinguished by Potter (1965). These cell populations may be lumped together as the mesencephalic reticular formation. The mesencephalic reticular formation also innervates the spinal cord in agnathans (Ronan, 1989), cartilaginous fishes (Smeets and Timerick, 1981; Cruce et al., 1999), bony fishes (Behrendt and Donicht, 1990), lungfishes (Ronan and Northcutt, 1985), reptiles (ten Donkelaar et al., 1980; Woodson and Künzle, 1982; Newman et al., 1983), birds (Cabot et al., 1982; Gross and Oppenheim, 1985), and mammals (See Nudo and Masterton, 1988). Presumably, at least part of these projections arise in cell groups comparable to the mammalian nucleus cuneiformis (Newman, 1985).

In the four species studied, a modest (*Dermophis mexicanus*) to distinct *rubrospinal tract* was demonstrated. Ten Donkelaar (1988) postulated that the presence of a rubrospinal pathway is related to the presence of limbs or limb-like structures. Smeets and Timerick (1981) recognized a contralateral rubrospinal projection in the thornback ray, *Raja clavata* and in the stingray, *Dasyatis sabina*, but not in a shark, the spotted dogfish, *Scyliorhinus canicula*. In these rays locomotion is achieved by undulating movements of the enlarged pectoral fins, whereas the dogfish swims by way of axial body movements. Cruce et al. (1999) identified a rubrospinal projection in a shark and in the guitarfish. In teleosts, variable data were presented. In the landlocked red salmon, *Onchorhynchus nerka* (Oka et al., 1986) and in the zebrafish, *Danio rerio* (Becker et al., 1997), ipsilateral rubrospinal projections were found, but in the goldfish, *Carassius auratus* (Prasada Rao et al., 1987) and in electric fish (Behrendt and Donicht, 1990) no rubrospinal projections were identified. In lungfishes, a small rubrospinal tract was found (Ronan and Northcutt, 1985). In amphibians, ten Donkelaar et al. (1981) identified the cells of origin of a contralateral rubrospinal projection in *Xenopus laevis*. Naujoks-Manteuffel et al. (1988) presented evidence for a rubrospinal tract in *Salamandra salamandra*, but could not demonstrate such a projection in a limbless amphibian, the caecilian *Ichthyophis kohtaoensis*. The present study now clearly demonstates the presence of a contralaterally projecting red nucleus in another apodan. In most reptiles, a distinct rubrospinal tract is found (ten Donkelaar et al., 1980; ten Donkelaar, 1982; Woodson and Künzle, 1982), but not in boid snakes (ten Donkelaar, 1982; ten Donkelaar and Bangma, 1983). In a colubrid snake, the watersnake *Nerodia*, after HRP injections into the spinal cord a tight contralateral cluster of small cells was observed in the tegmentum mesencephali (Cruce et al., 1983) reminiscent of the red nucleus. Rubrospinal pathways are also found in birds (Wild et al., 1979; Cabot et al., 1982; Gross and Oppenheim, 1985; Webster et al., 1990) and in mammals (see Nudo and Masterton, 1988), but apparently not in man (Nathan and Smith, 1982). Some caution on the identification of a rubrospinal tract in anamniotes may be appropriate. Given the rather extensive tegmentospinal projections, to be certain that the nucleus in question indeed is the red nucleus, studies on its cerebellar connections such as those by González et al. (1984), Fiebig (1988) and Larson-Prior and Cruce (1992) would be helpful.

Diencephalospinal projections in amphibians arise in the anterior parencephalic (ventral thalamus) and synencephalic (pretectal region) alar plates, whereas projections from the diencephalic basal plate originate in the posterior tubercle and in the interstitial nucleus of the flm (terminology after Puelles et al., 1996). In addition, spinal projections are found from neurons in the anterior preoptic area, the magnocellular preoptic nucleus and the suprachiasmatic nucleus. It should be kept in mind that the preoptic area and the suprachiasmatic region are territories derived from the alar plate of the secondary prosencephalon. However, for convenience and to compare our data with previous studies in which they were included in the diencephalon (see Neary and Northcutt, 1983), we will discuss their spinal projections together with those of actual diencephalic origin.

The amphibian diencephalospinal projections are largely ipsilateral and extend as far caudally as the lumbar spinal cord. In *Rana perezi*, *Xenopus laevis*, *Pleurodeles waltl*, and *Typhlonectes*, the magnocellular preoptic and the suprachiasmatic nuclei contain the cells of origin of vasotocinergic and mesotocinergic projections (González and Smeets, 1992a,b, 1997). These two peptides are believed to function similarly to the mammalian vasopressin and oxytocin (Sherwood and Parker, 1990). *Hypothalamospinal projections* were found in most vertebrate classes but, apart from those arising in the posterior tubercle, are apparently missing in agnathans (Ronan, 1989), cartilaginous fishes (Smeets and Timerick, 1981; Cruce et al., 1999) and lungfishes (Ronan and Northcutt, 1985). In the goldfish, the magnocellular part of the preoptic nucleus innervates the spinal cord (Prasada Rao et al., 1987), whereas in the zebrafish spinal projections arise also in the parvocellular preoptic nucleus (Becker et al., 1997). In amniotes, the predominant hypothalamospinal projection arises in the paraventricular nucleus (ten Donkelaar et al., 1980; Woodson and Künzle, 1982; Gross and Oppenheim, 1985; Nudo and Masterton, 1988; Webster et al., 1990) that contains vasopressin and oxytocin (Swanson and Sawchenko, 1983; Smeets et al., 1990). Spinal projections from the *poste-*

rior tubercle were demonstrated in lampreys (Ronan, 1989), elasmobranchs (Smeets and Timerick, 1981; Cruce et al., 1999), amphibians (ten Donkelaar et al., 1981; Tóth et al., 1985; Naujoks-Manteuffel and Manteuffel, 1988; the present study), and in the zebrafish (Becker et al., 1997), but not in agnathans (Ronan, 1989).

Spinal projections from the synencephalon include pretectospinal and interstitiospinal projections. Extensive *pretectospinal projections* are found in amphibians. In anurans, they arise from the posterior nucleus and from the posterodorsal division of the lateral nucleus. Nowadays, both nuclei, previously considered part of the dorsal thalamus, are included in the pretectum (Puelles et al., 1996). In particular, the anuran posterior nucleus is actually a complex tripartite pretectal structure of the alar synencephalon, and it seems that its juxtacommissural portion is the main source of pretectospinal projections. In *Pleurodeles waltl*, pretectospinal neurons were found as a band of cells beneath the fibers of the posterior commissure. In *Salamandra salamandra*, pretectospinal cells were observed in the nucleus pretectalis profundus and in a nucleus rostrodorsal to the nucleus of the FLM named nucleus of Darkschewitsch (Naujoks-Manteuffel and Manteuffel, 1988). The amphibian pretectum plays an important role in visuomotor behavior (see Ewert, 1987; Roth, 1987). Pretectospinal projections are sparse in lungfishes (Ronan and Northcutt, 1985), and are absent in amniotes (reptiles: ten Donkelaar et al., 1980; Cruce and Newman, 1981; birds: Webster et al., 1990; mammals: Nudo and Masterton, 1988). *Interstitiospinal projections* from the interstitial nucleus of the fasciculus longitudinalis medialis are present in all vertebrates, and are among the first descending pathways to develop (ten Donkelaar, 2000). In cartilaginous fishes (Cruce et al., 1999), amphibians (ten Donkelaar et al., 1981; Larson-Prior and Cruce, 1992; the present study), and reptiles (ten Donkelaar et al., 1980; ten Donkelaar, 1982; Woodson and Künzle, 1982), apart from the large-celled, interstitial component smaller neurons were observed described as the so-called nucleus of the flm (Woodson and Künzle, 1982; Cruce et al., 1999). In mammals, spinal projections arising in the rostral mesencephalic reticular formation include the field of Forel and the interstitial nucleus of Cajal (Nudo and Masterton, 1988; Holstege and Cowie, 1989).

In amphibians, *telencephalospinal projections* were found in *Xenopus laevis* (ten Donkelaar et al., 1981; the present study), in *Rana perezi* (the present study), in *Pleurodeles waltl* (the present study), and in *Dermophis mexicanus* (the present study). In all species, ipsilateral projections were found from cells in the ventrocaudal part of the lateral subpallium to the cervical spinal cord. Misinterpretation of the caudal boundaries of the anuran striatum attributed these cells to the ventral striatum. A recent reinterpretation of the anuran basal ganglia based on extensive tract-tracing and immunohistochemical data (Marín et al., 1997a, 1998a-c) makes it likely that the anuran telencephalospinal projections arise in the central amygdala. Possibly, the subpallial spinal projections found in *Pleurodeles waltl* and in *Dermophis mexicanus* also form part of the amygdala, but the necessary tract-tracing and immunohistochemical data are largely missing. Amygdalospinal projections were found in reptiles (Follett, 1989, *Tupinambis teguixin*; Siemen and Künzle, 1994, *Pseudemys scripta elegans*), and in mammals (e.g., Hopkins and Holstege, 1978; Nudo and Masterton, 1988). In birds, the so-called occipitomesencephalic tract extends from the sensorimotor part of the archistriatum into the rostral spinal cord (Dubbeldam et al., 1997). It represents the telencephalic output channel for the trigeminal feeding circuit as well as for the vocalization circuit found in songbirds, and resembles the corticobulbar tract found in mammals (Dubbeldam, 1991). The caudal, "amygdalar" component of the archistriatum may participate in the occipitomesencephalic tract (Dubbeldam et al., 1997). In prehensile birds (a cockatoo, *Cacatua galerita*, and a rosella, *Platycerus eximius*), however, Webster et al. (1990) failed to find evidence for telencephalospinal projections with retrograde tracing techniques. Recently, in passerine birds Wild and Williams (2000) found an avian “pyramidal tract” arising from the hyperstriatum accessorium of the rostral Wulst with extensive brainstem projections, and spinal projections to the cervical spinal cord. Minor telencephalospinal projections, possibly from the central nucleus, were found in the nurse shark, *Ginglymostoma cirratum* (Ebbesson and Schroeder, 1971). In two other elasmobranch fishes, the thornback guitarfish, *Platyrhynoidis triseriata*, and the hornshark, *Heterodontus francisci*, however, Cruce et al. (1999) did not find labeled cells in the telencephalon after upper spinal cord HRP or Fluoro-Gold injections.

In summary, the amphibian species studied have several descending pathways in common with other anamniotes and amniotes including vestibulospinal, reticulospinal and interstitiospinal projections (see Table II). If the lateral line system persists, spinal projections from mechanoreceptive lateral line nuclei remain. The main differences between amphibians and other anamniotes concern the presence of extensive pretectospinal projections. These projections are absent in amniotes. In amphibians they play an important role in visuomotor behavior.

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Capítulo 3

Inervación catecolaminérgica de la médula espinal

Descending supraspinal pathways in amphibians. II. Distribution and origin of the catecholaminergic innervation of the spinal cord

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Descending Supraspinal Pathways in Amphibians. II. Distribution and Origin of the Catecholaminergic Innervation of the Spinal Cord

CRISTINA SÁNCHEZ-CAMACHO[1], OSCAR MARÍN[2], WILHELMUS J.A.J. SMEETS[3], HANS J. TEN DONKELAAR[4] AND AGUSTÍN GONZÁLEZ[1]
[1]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain
[2]Department of Psychiatry and Langley Porter Psychiatric Institute, Nina Ireland Laboratory of Developmental Neurobiology, University of California San Francisco, California
[3]Department of Anatomy and Embryology, Vrije Universiteit, Amsterdam, The Netherlands
[4]Department of Neurology, University Hospital Nijmegen, The Netherlands

ABSTRACT

Immunohistochemical studies with antibodies against tyrosine hydroxylase, dopamine and noradrenaline have revealed that the spinal cord of anuran, urodele and gymnophionan (apodan) amphibians is abundantly innervated by catecholaminergic (CA) fibers and terminals. Since in all three orders of amphibians CA intraspinal cells occur, it is unclear to what extent the CA innervation of the spinal cord is of supraspinal origin. In a previous study we showed that many cell groups throughout the forebrain and brainstem project to the spinal cord of two anurans (the green frog, *Rana perezi*, and the clawed toad, *Xenopus laevis*), a urodele (the Iberian ribbed newt, *Pleurodeles waltl*), and a gymnophionan (the Mexican caecilian, *Dermophis mexicanus*). To determine the exact site of origin of the supraspinal CA innervation of the amphibian spinal cord, retrograde tracing techniques were combined with immunohistochemistry for tyrosine hydroxylase in the same sections. The double labeling experiments demonstrated that four brain centers provide CA innervation to the amphibian spinal cord: 1) the ventrolateral component of the posterior tubercle in the mammillary region; 2) the periventricular nucleus of the zona incerta in the ventral thalamus; 3) the locus coeruleus, and 4) the nucleus of the solitary tract. This pattern holds for all three orders of amphibians, except for the CA projection from the nucleus of the solitary tract in gymnophionans. There are differences in the strength of the projections (based on the number of double labeled cells), but in general, spinal functions in amphibians are controlled by CA innervation from brain centers that can easily be compared with their counterparts in amniotes. The organization of the CA input to the spinal cord of amphibians is largely similar to that described for mammals. Nevertheless, using a segmental approach of the CNS a remarkable difference was observed with respect to the diencephalic CA projections.

Indexing terms: **posterior tubercle; periventricular nucleus of the zona incerta; locus coeruleus; nucleus of the solitary tract; retrograde tracing; tyrosine hydroxylase; catecholamines**

The presence of a rich catecholaminergic (CA) innervation of the spinal cord is a feature shared by all vertebrates (for review see: Smeets and Reiner, 1994; Smeets and González, 2000). The spinal cord of mammals is strongly innervated by dopaminergic (DA), noradrenergic (NA) and, to a lesser extent, adrenergic fibers, all of which are known to play a role in nociception (Jensen and Yaksh, 1984; Jensen, 1986; Barasi et al., 1987; Fleetwood-Walker et al., 1988), autonomic functions (Nicholas et al., 1996; Smith et al., 1996; van Dijken et al., 1996; Rosin et al., 1996), and motor control (Commissiong, 1981; Holstege and Kuypers, 1987; Chan et al., 1986; Barbeau and Rossignol, 1991). Immunohistochemical studies with antibodies against dopamine (DA) showed that *dopaminergic fibers* are predominantly confined to the deep layers of the dorsal horn (laminae III-V) and to lamina X of Rexed (1952). Additionally, a dense plexus of DA immunoreactive (DAi) fibers innervates the intermediolateral cell column and moderate innervation was also reported in the ventral horn (Shirouzu et al., 1990; Mouchet et al., 1992; Holstege et al., 1996).

Noradrenergic fibers innervate densely lamina I and the outer part of lamina II of the dorsal horn, whereas a less dense labeling is found in the inner part of lamina II (Mouchet et al., 1992). Furthermore, dense plexuses of noradrenaline (NA) immunoreactive fibers are present in lamina X throughout the spinal cord and in the intermediolateral cell column at thoracic levels. In the ventral horn, NA fibers are predominantly confined to lamina IX. Compared to DA and NA fibers, a*drenergic fibers* have a much more restricted distribution (Hökfelt et al., 1984; Ross et al., 1984; Carlton et al., 1991). The densest spinal innervation by adrenergic fibers, as shown by

phenylethanolamine-*N*-methyltransferase (PNMT) immunohistochemistry, occurs at thoracic levels within the intermediolateral cell column. A less dense adrenergic plexus is present in a region surrounding the central canal at all segmental levels, whereas only a sparse number of PNMT-immunoreactive fibers is found in the superficial part of the dorsal horn (lamina I) and the substantia gelatinosa (Carlton et al., 1991).

Data on the catecholaminergic innervation of the spinal cord in nonmammalian species are sparse. In reptiles, Smeets (1994) showed that dense plexuses of DAi fibers are mainly located in the dorsal horn of the spinal gray matter, preferentially in layers I and II as distinguished by Cruce (1979) and Kusuma et al. (1979), in the medial part of the dorsal horn and in the dorsal part of layer X. A few DA fibers, but considerably more NA fibers were found in the ventral horn. In chickens, elaborate plexuses of tyrosine hydroxylase-immunoreactive (THi) fibers were found in both the dorsal and ventral horns (Okado et al., 1991; Reiner et al., 1994). The densest plexus was found in Terni's column (the avian sympathetic preganglionic neurons), lamina X and the medial part of layers V-VII of the cervical and the thoracic spinal cord. In amphibians, our knowledge of the origin, course and site of termination of CA fibers is limited. So far, immunohistochemical studies included only rostral spinal segments, where numerous TH-, DA- and NA-immunoreactive fibers were found in the dorsal and, to a lesser extent, ventral part of the spinal gray matter (González and Smeets, 1994a,b, 1995). Therefore, one of the aims of the present study is to investigate how the CA innervation of the spinal cord of amphibians is organized at various spinal cord levels.

A second aim of the present study concerns the supraspinal centers that provide the CA innervation to the amphibian spinal cord. Double labeling techniques combining retrograde tracing with immunohistochemistry for catecholamines have elucidated the connectivity of CA cell groups in mammals, particularly in rats. Double labeling studies showed that the *dopaminergic fibers* in the spinal cord arise from the A11 cell group (Björklund and Lindvall, 1984; Skagerberg and Lindvall, 1985; Takada et al., 1988; Shirouzu et al., 1990). The sites of origin of the *noradrenergic fibers* in the spinal cord of mammals are also well established. The locus coeruleus (A6) as well as the related A5 and A7 cell groups project to the spinal cord (Westlund et al., 1983, 1984; Lyons et al., 1989; Clark et al., 1991; Kitahama et al., 1994). The supraspinal origin of *adrenergic fibers* to the spinal cord is located within the ventrolateral medulla, i.e. the C1 and the C3 cell groups (Ross et al., 1984; Carlton et al., 1991; Guyenet et al., 1994).

Double labeling studies of descending CA pathways to the spinal cord of nonmammalian vertebrates are sparse. Nevertheless, on the basis of retrograde tracing studies and immunohistochemical data, it is conceivable that the supraspinal CA inputs to the spinal cord in reptiles and birds are comparable to those in mammals. Preliminary results in birds support this notion (Chikasawa et al., 1983). The same may hold for amphibians, where recently a noradrenergic projection from the locus coeruleus to the spinal cord was demonstrated by double labeling techniques (Marín et al., 1996). However, due to the relative abundance of CA cells within the spinal cord of anamniotes, it was suggested that in these vertebrates a substantial intraspinal CA innervation might prevail over supraspinal CA projections (see Smeets and González, 2000).

In a companion paper (Sánchez-Camacho et al., 2001), we studied the descending projections to the spinal cord of amphibians and found that the distribution of supraspinal neurons projecting to the spinal cord is much more widespread than previously described. A comparison of the brain regions that project to the spinal cord with those that contain CA cell bodies revealed that there are many candidates that may contribute to the CA innervation of the spinal cord in amphibians. In the present study we examined: 1) the distribution of tyrosine hydroxylase-immunoreactivity in the spinal cord in two anurans (the green frog, *Rana perezi*, and the clawed toad,

Abbreviations

A1-A16	catecholaminergic neuronal groups A1-A16
A5c	caudal component of A5
A5r	rostral component of A5
A11m	mesencephalic portion of A11
Ap	area postrema
AP	alar plate
BP	basal plate
C1-C3	adrenergic neuronal groups C1-C3
Cb	cerebellum
cc	central canal
DF	dorsal funiculus
DLF	dorsolateral funiculus
dth	dorsal thalamus
e	epichiasmatic nucleus
Hb	habenula
HL	lateral hypothalamic cell group
III	oculomotor nucleus
Is	isthmic nucleus
Ist	isthmic segment
Jc	juxtacommissural nucleus
Lc	locus coeruleus
LH	lateral hypothalamic nucleus
Lpd	lateral posterodorsal nucleus
Lpv	lateral posteroventral nucleus
lsc	locus subcoeruleus
LT	Lissauer´s tract
Ma	mammillary nucleus
mes	mesencephalon
nII	optic nerve
nPT	nucleus pretectalis
NPv	nucleus of the periventricular organ
Nsol	nucleus of the solitary tract
nVIII	octaval nerve
oc	optic chiasm
p1-p6	prosomeres 1-6
Pc	precommissural nucleus
POa	anterior preoptic area
ppv	preoptic periventricular nucleus
PV	paraventricular nucleus
Ra	raphe nucleus
Ris	isthmic reticular nucleus
r2-r8	rhombomeres 2-8
rm	retromammillary nucleus
Rm	middle reticular nucleus
Rs	superior reticular nucleus
SC	suprachiasmatic nucleus
sol	solitary tract
SPr	secondary prosencephalon
tm	mesencephalic tectum
TP	tuberculum posterius
TPdm	dorsomedial part of the tuberculum posterius
TPvl	ventrolateral part of the tuberculum posterius
v	ventricle
VF	ventral funiculus
VH	ventral hypothalamic nucleus
VIIIv	ventral octaval nucleus
VLF	ventrolateral funiculus
VM	ventromedial thalamic nucleus
vth	ventral thalamus
XII	hypoglossal nucleus
zi	zona incerta
Zip	periventricular nucleus of the zona incerta

Xenopus laevis), a urodele (the Iberian ribbed newt, *Pleurodeles waltl*), and a gymnophionan (the Mexican caecilian, *Dermophis mexicanus*); and 2) the cells of origin of supraspinal CA projections.

MATERIALS AND METHODS

A total of 16 adult green frogs (*Rana perezi*, Amphibia: Anura), 18 clawed toads (*Xenopus laevis*, Amphibia: Anura), 12 Iberian ribbed newts (*Pleurodeles waltl*, Amphibia: Urodela) and 6 adult specimens of Mexican caecilian (*Dermophis mexicanus*, Amphibia: Gymnophiona) were used. The animals were obtained from the laboratory stocks of the Department of Cell Biology, University Complutense of Madrid and from a pet supplier (*Dermophis mexicanus*). In all experiments the animals were deeply anesthetized by immersion in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz). Immunohistochemistry for tyrosine hydroxylase (TH), the synthetic enzyme of catecholamines (CA), was used to reveal catecholaminergic neurons and fibers. To investigate the sources of CA innervation of the spinal cord, retrograde tracing of dextran amines was combined with TH immunohistochemistry on the same brain sections. The original research reported herein was performed under animal care guidelines establish by the Spanish Royal Decree 223/1988.

TH immunohistochemistry

In a first set of experiments, animals were perfused transcardially with 50 ml of saline followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After two hours of postfixation, the brains and spinal cords were immersed in PB containing 30% sucrose for 3-5 h at 4ºC, embedded in a solution of 15% gelatin with 30% sucrose in PB, and then stored for 5 h in a 4% formaldehyde solution at 4ºC. The brains were cut in the frontal or sagittal plane at 40 μm thickness on a freezing microtome, and the sections were collected in PB. They were then rinsed twice in PB, treated with 1% H_2O_2 in PB for 15 minutes to reduce endogenous peroxidase activity, and rinsed again three times in PB. The sections were then processed for TH immunohistochemistry as described before (González and Smeets, 1991; González et al., 1993). Briefly, the sections were first incubated in a mouse anti-TH serum (Incstar, USA), diluted 1:1000 in PB, for 48 h at 4°C. Subsequently, the sections were rinsed in PB and incubated for 90 min in goat anti-mouse serum (DAKO A/S, Denmark) diluted 1:100 at room temperature. After rinsing again, the sections were incubated for 90 minutes in mouse PAP (1:600, Chemicon, USA). The sections were stained in 0.5 mg/ml 3,3´-diaminobenzidine (DAB) with 0.01% H_2O_2 and 25 mg/ml nickel ammonium sulfate (Merck). Finally, the sections were rinsed twice in PB, mounted (mounting medium: 0.25% gelatin in Tris buffer, pH 7.6) and, after drying overnight, coverslipped. Some sections were counterstained with cresyl violet to facilitate the analysis of the results. For details about the specificity of the TH antibodies, the reader is referred to previous works (González and Smeets, 1991; González et al., 1993).

Double-labeling experiments

In a second series of experiments, the tracers 10 kD or 3 kD biotinylated dextran amine (BDA; Molecular Probes, Oregon, USA) and 10 kD or 3 kD Texas Red-conjugated dextran amine (TRDA; Molecular Probes), recrystallized from distilled water onto sharp tungsten needles, were applied unilaterally into the rostral spinal cord. In all experiments, the tracer was delivered into the spinal region between the obex and the brachial enlargement, through a dorsal approach. Prior to tracer application, a hemisection was made in the spinal cord that subsequently received the tracer. This procedure enhanced the tracer uptake and the bulk of descending projections to the spinal cord was observed. Survival times varied from 7 to 14 days. Following this period, the animals were deeply anesthetized and perfused transcardially with 50 ml saline followed by 200 ml fixative (4% paraformaldehyde in PB). The brains were removed, blocked in gelatin and cut in the frontal or sagittal plane at 40 μm thickness on a freezing microtome as described above. Subsequently, brain sections were processed for TH-immunohistochemistry according to the indirect immunofluorescence method. Briefly, they were first incubated for 48 hours at 4°C with a mouse anti-TH antibody (Incstar), diluted 1:1000 as described above. They were then incubated with a FITC-conjugated mouse-IgG complex (Incstar) diluted 1:150 for 90 min at room temperature. BDA was visualized by incubation with a Texas Red-conjugated streptavidin complex (Vector Labs., diluted 1:200) together with the secondary antibody. The sections were then mounted on glass slides and coverslipped with Vectashield (Vector Labs., Burlingame, CA, USA). Alternating the appropriate filter combinations in a Zeiss fluorescence microscope allowed the identification of TRDA retrogradely labeled cells and TH-immunoreactive (THi) cells. The distribution of retrogradely labeled, THi or double labeled neurons in the brains of *Rana perezi*, *Xenopus laevis*, *Pleurodeles waltl* and *Dermophis mexicanus* was charted in representative transverse sections by means of a camera lucida. Finally, some sections that had been plotted were counterstained with cresyl violet to determine cytoarchitectonic boundaries. The nomenclature is the same as that used in our companion paper (Sánchez-Camacho et al., 2001).

RESULTS

In the following description the distribution of catecholaminergic (CA) structures in the spinal cord of each amphibian order is detailed first. Subsequently, the origin of supraspinal CA fibers will be described, as observed in a series of experiments with double labeling techniques.

TH immunoreactivity in the spinal cord of amphibians

The distribution of CA cells and fibers in the spinal cord has been studied at brachial, thoracic and lumbar levels of the spinal cord of anurans and urodeles. In gymnophionans, the lack of limbs results in the absence of brachial and lumbar enlargements. For this reason, and given the remarkable length of the spinal cord, in the case of *Dermophis mexicanus* we have chosen rostral (close to the obex) and caudal (mid-caudal body levels) sections of the spinal cord to compare the distribution of CA elements between these levels. In the amphibian species studied, a wide distribution of THi fibers was found in all spinal segments, and the presence of CA cells ventral to the central canal was observed throughout the spinal cord (Fig. 1). Notable differences in the number and morphology of these fibers and cells occur between the species studied.

Anurans. The green frog, *Rana perezi*, was chosen as the main species for the description and mapping of the CA structures in the spinal cord of anurans (see Fig. 1). At brachial levels, THi fibers were predominantly distributed in the lateral portion of the dorsal and dorsolateral funiculi and gave off thin and long varicose branches that provided abundant innervation of neurons in the dorsal horn and intermediate gray (Fig. 2a). In the ventrolateral funiculus, less abundant thick THi fibers were observed, and only sparse fibers were present in the ventral funiculus. A characteristic feature is the presence of a dense plexus of thick, THi fibers along the border of the lateral funiculus, extending throughout the spinal cord (Figs. 1, 2a). In the gray matter, numerous THi fibers were observed mainly in the dorsal and lateral fields of Ebbesson (1976), and also in

the central field dorsal to the central canal (Fig. 2b). Only a few fibers were observed in the ventrolateral and ventromedial motor fields. In general, the distribution of CA fibers displays a similar pattern throughout the spinal cord (Fig. 1), although the number of THi longitudinal fibers decreases caudalwards. Remarkably, the innervation of the central field above the central canal is more conspicuous at the thoracic level than at brachial and lumbar levels (Figs. 1, 2c). A small terminal field was found dorsolateral to the central canal throughout the spinal cord (Fig. 2c).

At all spinal segments, THi cells were observed ventral to the central canal, forming a longitudinal column (Figs. 1, 2c). These cells are CSF-contacting neurons located in the ependymal and subependymal layers. Additionally, isolated CA neurons were found in the dorsolateral gray field but only at brachial levels (Fig. 2a,d,e). These cells are generally bipolar with long processes that intermingle with the longitudinally oriented descending fibers. These scattered cells seem to be a caudal continuation of the THi neurons of the nucleus of the solitary tract/area postrema complex (Fig. 2f), although occasionally some isolated cells were observed at caudal brachial levels.

Urodeles. As in anurans, the lateral portion of the dorsal and dorsolateral funiculi is most densely innervated at brachial levels (Figs. 1, 3a). However, these long, varicose fibers are thicker, but less abundant than their counterparts in anurans. Moreover, whereas in anurans these THi fibers extensively innervate neurons in the dorsal horn and intermediate gray matter, in urodeles they remain mainly superficial to the gray matter (Fig. 1). On the other hand, throughout the spinal cord, but particularly at thoracic levels, a distinct plexus of THi fibers was found in Lissauer's tract within the dorsolateral funiculus (Figs. 1, 3a,b). At thoracic and lumbar segments, the number of fibers in the lateral funiculus increased, and labeled fibers were also observed in the ventral and ventrolateral funiculi (Fig. 1). Along the spinal cord, a plexus of weakly THi fibers was found just dorsal to the central canal as in anurans. However, a peculiar feature of *Pleurodeles* is the presence of thin, varicose fibers that outline the profiles of large neurons in the ventral horn primarily at thoracic and lumbar levels (Fig. 3b).

In urodeles, only CSF-contacting neurons ventral to the central canal were found to be TH-immunoreactive. These cells form a column throughout the spinal cord including the tail segments.

Gymnophionans. As in anurans and urodeles, an abundant CA innervation was found in the spinal cord of *Dermophis mexicanus* (Fig. 1). This species lacks brachial and lumbar enlargements because of the lack of limbs and the spinal cord is of about similar diameter throughout its length due to the powerful trunk musculature that is the basis of the animal's locomotion (Fig. 3c,d). A strongly immunoreactive fiber plexus was observed in the dorsolateral funiculus close to the dorsal horn. The distribution of THi fibers in the gray matter was more abundant than in urodeles, but less than in anurans. The most conspicuously labeled THi fibers were observed in Lissauer's tract in the dorsolateral funiculus at all spinal segments (Figs. 1, 3c,d). A remarkable feature of *Dermophis* is the innervation of the ventral horn by THi fibers, which is much more extensive than in urodeles and anurans. As in urodeles, a strong innervation of large neurons in the lateral part of the ventral horn was observed at rostral and caudal levels. A few thin, THi fibers occur just dorsal to the central canal. The distribution of CA fibers does not differ substantially between rostral and caudal levels of the spinal cord, although a decrease in the number of THi fibers from rostral to caudal levels is obvious (Figs. 1, 3c,d). In gymnophionans, spinal CA cells are restricted to a column of CSF-contacting cells located ventral to the central canal, but they are more numerous than in anurans or urodeles.

Origin of descending catecholaminergic projections to the spinal cord

In all experiments showing retrogradely labeled cells and THi neurons in the spinal cord, none or only a few cells were labeled ventral to the central canal. This suggested that most of the CA innervation of the spinal cord in amphibians must be of supraspinal origin. A comparison of the cell masses that project to the spinal cord demonstrated in our companion paper (Sánchez-Camacho et al., 2001) with CA cell groups in the brain of amphibians (González and Smeets, 1994a,b, 1995) reveals several candidates for the CA input to the spinal cord of amphibians. However, the present study has verified experimentally that only four of these centers contribute to the supraspinal CA innervation of the amphibian spinal cord. These centers are the posterior tubercle, the periventricular nucleus of the zona incerta, the locus coeruleus and the nucleus of the solitary tract (Figs. 4-8).

Because the tracers were applied unilaterally to the rostral spinal cord, the full complement of descending fibers to the cord was obtained in these experiments. No attempt was made in this study to observe the origin of CA projections to different spinal segments.

Posterior tubercle. The posterior tubercle region of all amphibians studied contains a large population of dopaminergic neurons (González and Smeets, 1991, 1994a; González et al., 1993). In anurans, particularly in *Rana perezi*, two separate populations of CA cell bodies can be distinguished within the nucleus of the posterior tubercle, i.e. a dorsomedial and a ventrolateral group (Fig. 4a). On the basis of its connections with the basal forebrain, the dorsomedial group has been considered the homologue of the substantia nigra pars compacta and ventral tegmental area (A9-A10 groups) of amniotes (Marín et al., 1997b, 1998), which contains retromammillary, posterior tubercle and prerubral regions (terminology of Puelles et al., 1996). The ventrolateral portion of the posterior tubercle, caps the infundibulum, and is located in an area recently assimilated with at least part of the mammillary region (Puelles et al., 1996).

After applications of retrograde tracers in the spinal cord of *Rana perezi*, labeled cells were found almost exclusively in the ventrolateral component of the posterior tubercle (Fig. 5b). Some of these cells, which are characterized by a pear-shaped soma, were also TH-immunoreactive, and could be found throughout the rostrocaudal extent of the posterior tubercle (Fig. 9a-c).

In *Xenopus laevis*, separate dorsomedial and ventrolateral parts of the posterior tubercle could not be distinguished (González et al., 1993). However, within the single tubercular cell group, small, round cells occupied a dorsal position, whereas large pear-shaped neurons were located in the ventrolateral part (Fig. 4b). After tracer applications into the spinal cord of *Xenopus*, numerous retrogradely labeled neurons were found throughout the entire extent of the posterior tubercle. A large number of retrograde labeled cells were also THi (Fig. 6b). This primarily ipsilateral CA spinal projection arises mostly from medium-sized neurons with oval somata (Fig. 9d-f), but some smaller, round cells also contribute.

As in the green frog, dorsomedial and ventrolateral subdivisions of dopaminergic cells could be identified in the posterior tubercle of the ribbed newt, *Pleurodeles waltl* (Fig. 4c). After tracer applications into the spinal cord of *Pleurodeles*, retrogradely labeled neurons were found in the dorsal hypothalamus, primarily ventral to the CA cells at rostral levels. Double labeled cells were found in the ventrolateral part of the posterior tubercle. The majority of the cells was located in the

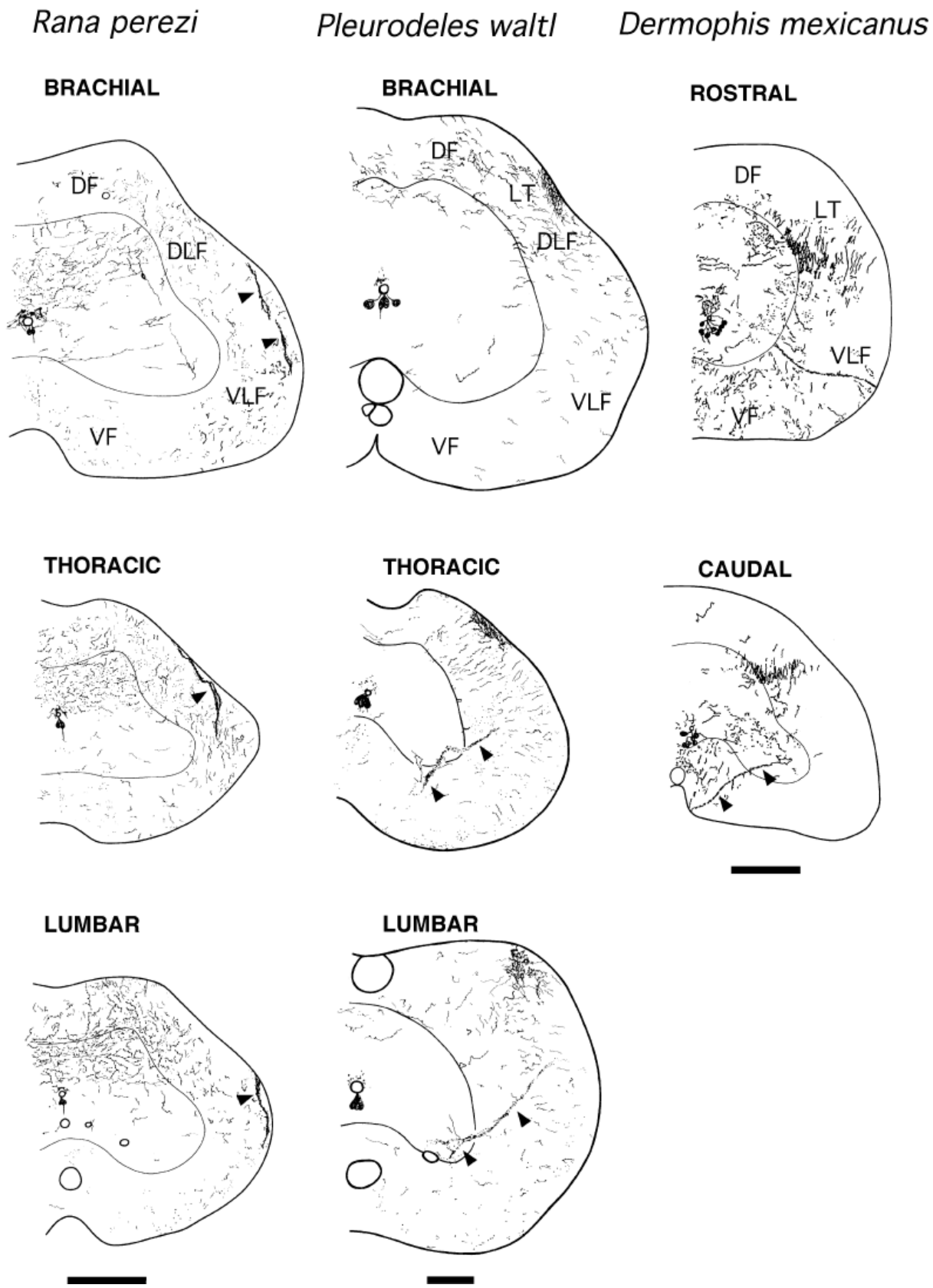

Fig. 1. Diagrams of transverse hemisections through the spinal cord of *Rana perezi*, *Pleurodeles waltl* and *Dermophis mexicanus* showing the distribution of TH immunoreactive cells and fibers at different spinal levels. Arrowheads in the spinal cord of *R. perezi* point to the dense peripheral CA plexus, whereas in *P. waltl* and *D. mexicanus* sections point to somatic profiles densely innervated by CA terminals. Calibration bars= 200 μm (*R. perezi*) and 100 μm (*P. waltl* and *D. mexicanus*).

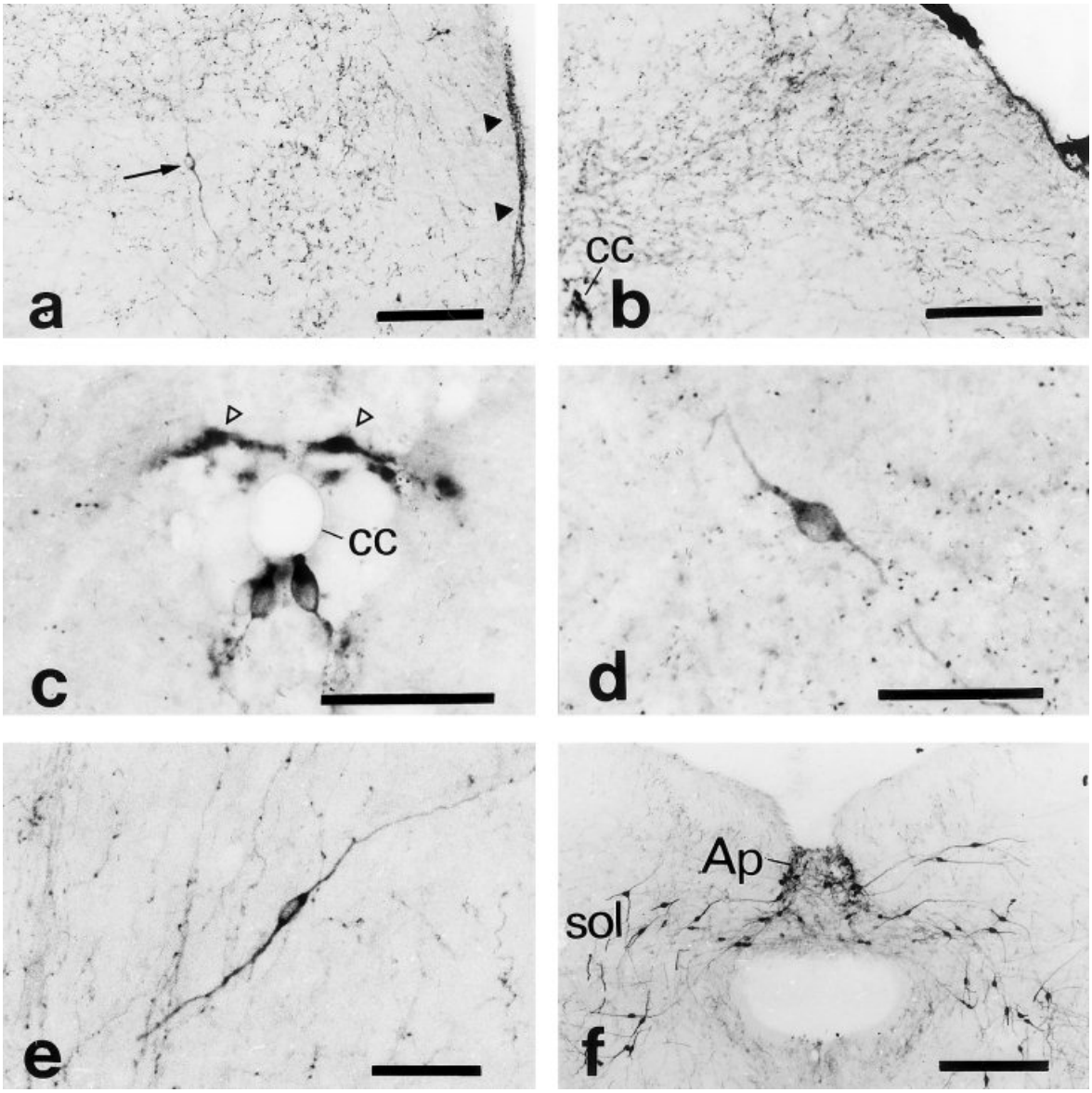

Fig. 2. Photomicrographs of transverse sections through the spinal cord of anurans showing TH immunoreactive cells and fibers. **a**, Dorsolateral field at brachial levels in the spinal cord of *Rana* (arrowheads point to the marginal plexus, whereas arrow indicates an isolated THi cell). **b**, Dorsolateral field at lumbar segments of the spinal cord of *Rana*. **c**, Cells beneath the central canal and plexuses above it (empty arrowheads) in the brachial spinal cord of *Rana*. **d**, Isolated cell in the dorsolateral aspect of the dorsal horn at caudal brachial segment in *Rana*. **e**, Fusiform cell in the dorsal horn of the brachial spinal cord in *Xenopus* as seen in a horizontal section. **f**, Transverse section through the obex-area postrema region in *Xenopus*. Calibration bars= 100 μm (a-d), 50 μm (e-g), and 200 μm (h).

ipsilateral group of large neurons at rostral levels (Fig. 7a), but a few double labeled cells were present at caudal levels of the posterior tubercle (Fig. 7b).

In the gymnophionan brain, dorsomedial and ventrolateral divisions of the posterior tubercle could also be recognized. In the ventrolateral portion, THi cells form a mixed population of large, pear-shaped neurons with long, laterally directed dendrites in the rostral part of the posterior tubercle, and small, round cells extending more caudally along the hypothalamus (Fig. 10a,b). The latter cells were located mainly in the ventral part of the posterior tubercle and some possessed CSF-contacting processes. Only a few retrogradely labeled neurons were found in the dorsal hypothalamus after tracer applications into the spinal cord of *Dermophis* (Fig. 8). Double labeled cells are large and occur ipsilaterally in the ventrolateral division of the posterior tubercle (Fig. 10a-c).

Periventricular nucleus of the zona incerta. The periventricular nucleus of the zona incerta is present in all amphibian species studied. This nucleus was previously described as an hypothalamic group of THi cells lying lateral to the nucleus of the periventricular organ and referred to as “the accompanying cells of the periventricular organ” (González and Smeets, 1991, 1994a). However, a detailed analysis has revealed that the THi cell bodies of this group are located in the ventral thalamus, just at the border with the dorsal hypothalamus (Milán and Puelles, 2000; Smeets and González, 2000).

In *Rana*, the periventricular nucleus of the zona incerta constitutes a column extending from the rostral diencephalon to mid-diencephalic levels. Rostrally, the CA neurons of this

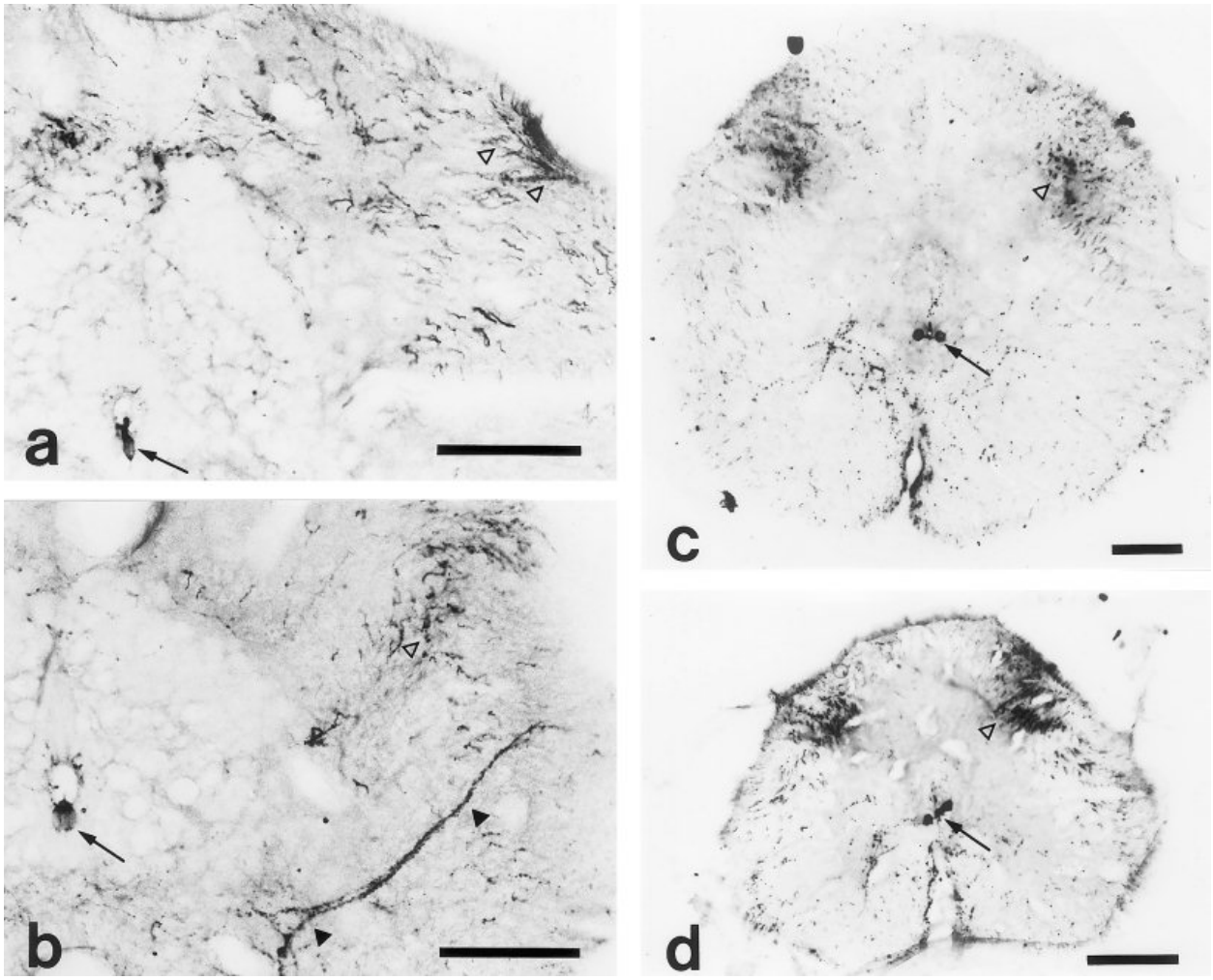

Fig. 3. Photomicrographs of transverse sections through the spinal cord of *Pleurodeles* (**a, b**) and *Dermophis* (**c, d**) showing TH immunoreactive cells and fibers. **a** and **c** are brachial segments whereas **b** and **d** are lumbar (caudal) spinal segments. Arrows point to TH immunoreactive cells beneath the central canal. Arrowheads in b point to a somatic profile densely innervated by CA terminals. Empty arrowheads point to Lissauer´s tract. Calibration bars= 100 μm.

nucleus were disposed in a laminar organization in the ventral thalamus, close to the third ventricle (Fig. 11a,d). At most caudal levels, THi neurons of the periventricular nucleus of the zona incerta were located dorsal to the rostral portion of the dorsomedial posterior tubercle (Figs. 4d, 5a). In close relation to the periventricular zona incerta, also within the ventral thalamus, weakly labeled cells located dorsally seem to form a separate catecholaminergic group (Figs. 5a, 11a).

Spinal applications of retrograde tracers in *Rana*, resulted in labeling of small, round projection neurons that are scattered in the ventral thalamus, mainly ipsilaterally, located dorsal or ventral to the CA neurons in the most external part of the nucleus (Fig. 5a). A different type of retrogradely labeled cells, larger in size and pear-shaped, was found to be THi (Fig. 11a-f). Similar observations were made in *Xenopus* (Fig. 6a). It should be noted that the dorsally located cells within the ventral thalamus were never doubly labeled from the spinal cord (Fig. 5a).

In *Pleurodeles* as in *Dermophis*, the periventricular nucleus of the zona incerta is formed by a small, compact periventricular group of large neurons (Fig. 12). However, in both species, the extent of this nucleus is shorter. As in anurans, a small ipsilateral spinal projection arises from neurons in the dorsal part of the ventral thalamus (Figs. 7a, 8a, 12a-e). Only a few double labeled cells were found more ventrally in the periventricular nucleus of the zona incerta.

Locus coeruleus. The amphibian locus coeruleus is formed by noradrenergic cells that constitute the only CA cell group in the isthmic region (González and Smeets, 1993, 1994a, 1995). The number, location and morphology of the locus coeruleus cells vary notably between the species studied. Whereas in anurans, the locus coeruleus extends along the entire isthmic segment, in urodeles and gymnophionans, this CA cell group is located at caudal isthmic levels only. In all species, however, the long processes of the multipolar neurons in the locus are mainly directed ventrally or ventrolaterally, where they branch profusely in the reticular formation.

After TRDA or BDA applications into the spinal cord of anurans, numerous retrogradely labeled cells were observed in the isthmic region (Figs. 5c, 6c). The double labeling procedure revealed that numerous cells in the superior reticular formation that are retrogradely labeled from the spinal cord intermingle with the THi cell bodies of the locus coeruleus. Remarkably, after spinal cord tracer applications only a few double labeled neurons were found in the rostral part of this catecholaminergic cell group (Fig. 13a,b).

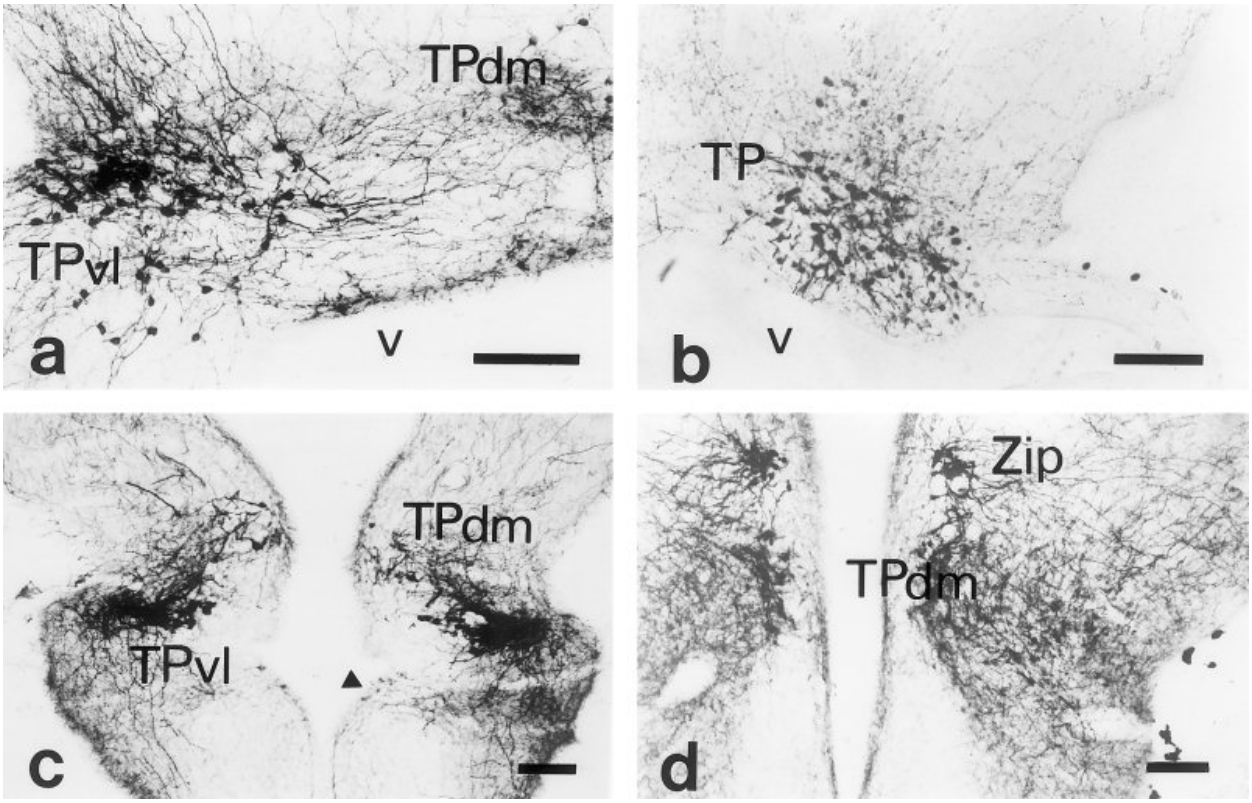

Fig. 4. Photomicrographs of transverse sections through the brain of amphibians at selected levels illustrating examples of the catecholaminergic cell groups that project to the spinal cord, as seen with TH immunohistochemistry. **a**, Posterior tubercle region in *Rana*. **b**, Posterior tubercle region in *Xenopus*. **c**, Posterior tubercle region in *Pleurodeles*. **d**, Periventricular nucleus of the zona incerta and rostral posterior tubercle in *Rana*. Calibration bars= 100 μm.

In the urodele and gymnophionan brains, the locus coeruleus forms a compact group close to the fourth ventricle rostrolateral to the trigeminal motor nucleus. Applications of dextran amines to the spinal cord of *Pleurodeles* resulted in retrogradely labeled cells in the isthmic region and, more abundantly, in the reticular formation. These spinal projection cells form a compact group which lies just ventral to the CA cell group. In each experiment, a few double labeled neurons were observed in the caudal part of the locus coeruleus, at the level of the cerebellum (Fig. 7c) or immediately rostral to the motor trigeminal nucleus, both ipsilateral and contralateral to the injection side of the tracer. After tracer applications into the rostral spinal cord of *Dermophis mexicanus*, numerous retrogradely labeled cells were observed in the reticular formation, close to the CA neurons of the locus coeruleus. Double labeled cells in the gymnophionan locus coeruleus were found both at the ipsilateral and the contralateral side (Fig. 13c,d).

Nucleus of the solitary tract. In all amphibians studied, a mixed population of dopaminergic, noradrenergic and adrenergic cell bodies lies in the nucleus of the solitary tract (González and Smeets, 1993, 1994a, 1995). The cells are large and multipolar, and are mainly located medial and ventral to the tract. At the level of the obex, the cells of both sides fuse above the ventricle.

In anurans, the nucleus of the solitary tract is formed rostrally by large, multipolar THi neurons mainly located ventral to the solitary tract, but more caudally, medium-sized and small THi neurons surround the tract. Following tracer applications into the spinal cord, numerous retrogradely labeled cells were observed in the nucleus of the solitary tract, particularly contralaterally. These projection cells intermingle with the THi neurons of the nucleus. Numerous double labeled cells were present bilaterally from rostral levels of the nucleus of the solitary tract to levels immediately rostral to the obex (Figs. 5d,e; 6d,e). The cells in the nucleus of the solitary tract that project to the spinal cord are located primarily at rostral levels, and are large, multipolar THi neurons located ventral to the tract (Fig. 14a,b). More caudally, a few double labeled cells were located around the tract.

In the urodele brain, the CA neurons in the nucleus of the solitary tract are larger than those in anurans and different cell types could not be distinguished. After tracer applications into the spinal cord, numerous retrogradely labeled neurons were present in the entire extent of the nucleus where they intermingle with the THi cells ventral to the tract. The majority of these cells was located contralaterally, although the ipsilateral component was also prominent. Double labeled cells were observed mainly in the rostral portion of the nucleus, although some double labeled neurons were found at caudal levels as well, particularly on the contralateral side (Fig. 14c-f).

As in urodeles, in *Dermophis* the nucleus of the solitary tract is constituted by a compact periventricular column of cells that lies adjacent to the medial boundary of the solitary tract. The morphology and location of these CA neurons resembles those of urodeles. In contrast with the results obtained in anurans and urodeles, no double labeled neurons could be identified in the nucleus of the solitary tract after tracer applications into the gymnophionan spinal cord. Nevertheless, a few retrogradely labeled cells were observed around the solitary tract at caudal rhombencephalic levels.

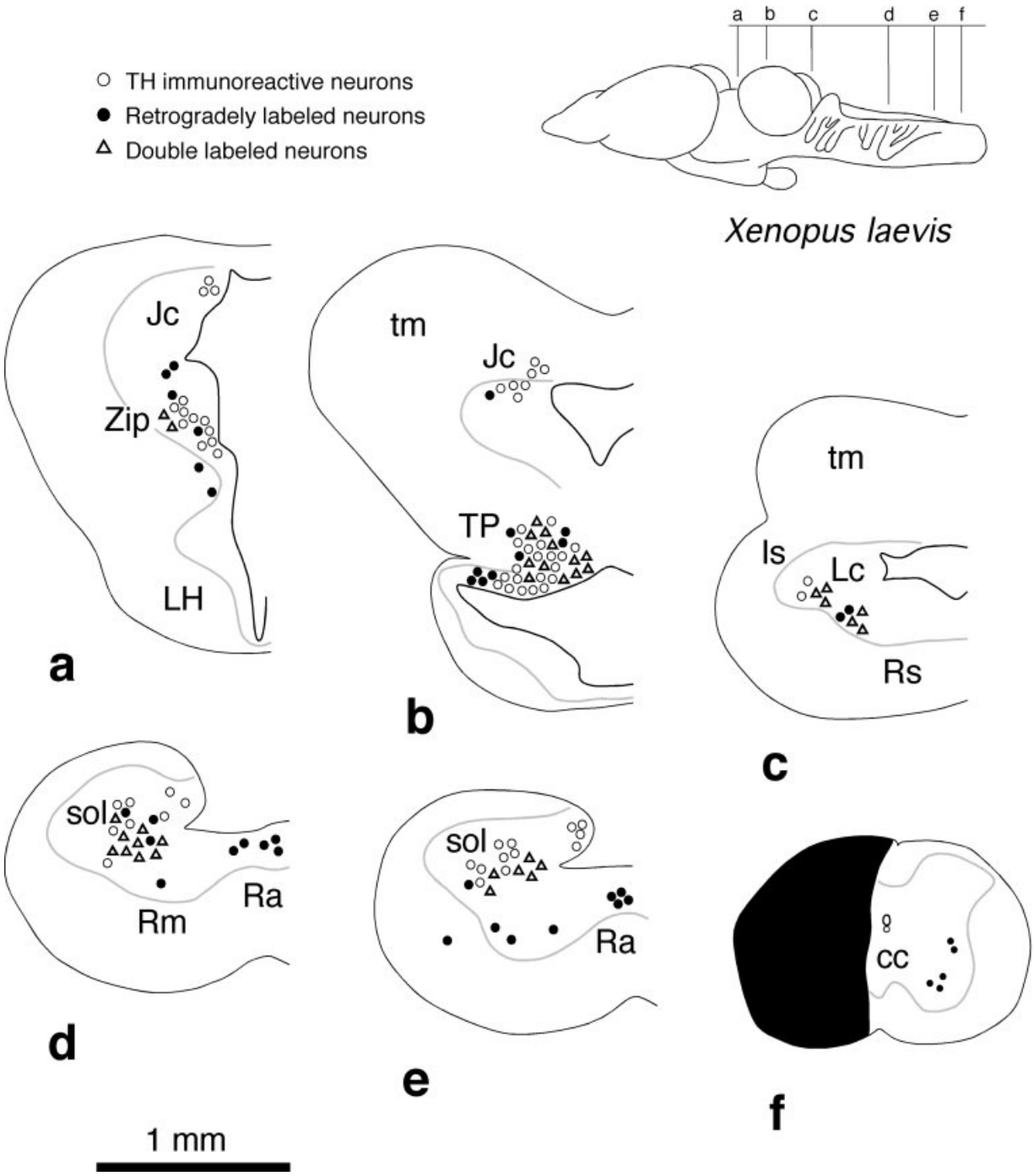

Fig. 5. Schematic drawings of transverse sections through the brain of *Xenopus laevis*, illustrating the distribution of retrogradely labeled cells after tracer applications into the spinal cord (black area). The localization of catecholaminergic cells, as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropiate levels of the sections are indicated in the upper right scheme.

DISCUSSION

Technical considerations

By means of retrograde tracing techniques in combination with TH immunohistochemistry, the present study has revealed the sites of origin of the catecholaminergic innervation of the spinal cord of amphibians. Dextran amines can be transported retrogradely as well as anterogradely depending on the method of application. Thus, tracers applied as crystals on the tip of a sharp tungsten needle yield the best retrograde transport (Marín et al., 1997a). In the present study, relative large application sites were obtained when dextran amine crystals were placed into selected spinal segments. This resulted in highly successful retrograde transport, both with low (3 kD) and high (10 kD) molecular weight dextran amines. The advantage of 3 kD dextran amines is that they are transported faster than the larger ones (see Fritzsch, 1993). However, the crystals of these 3 kD dextran amines dissolve quickly, which

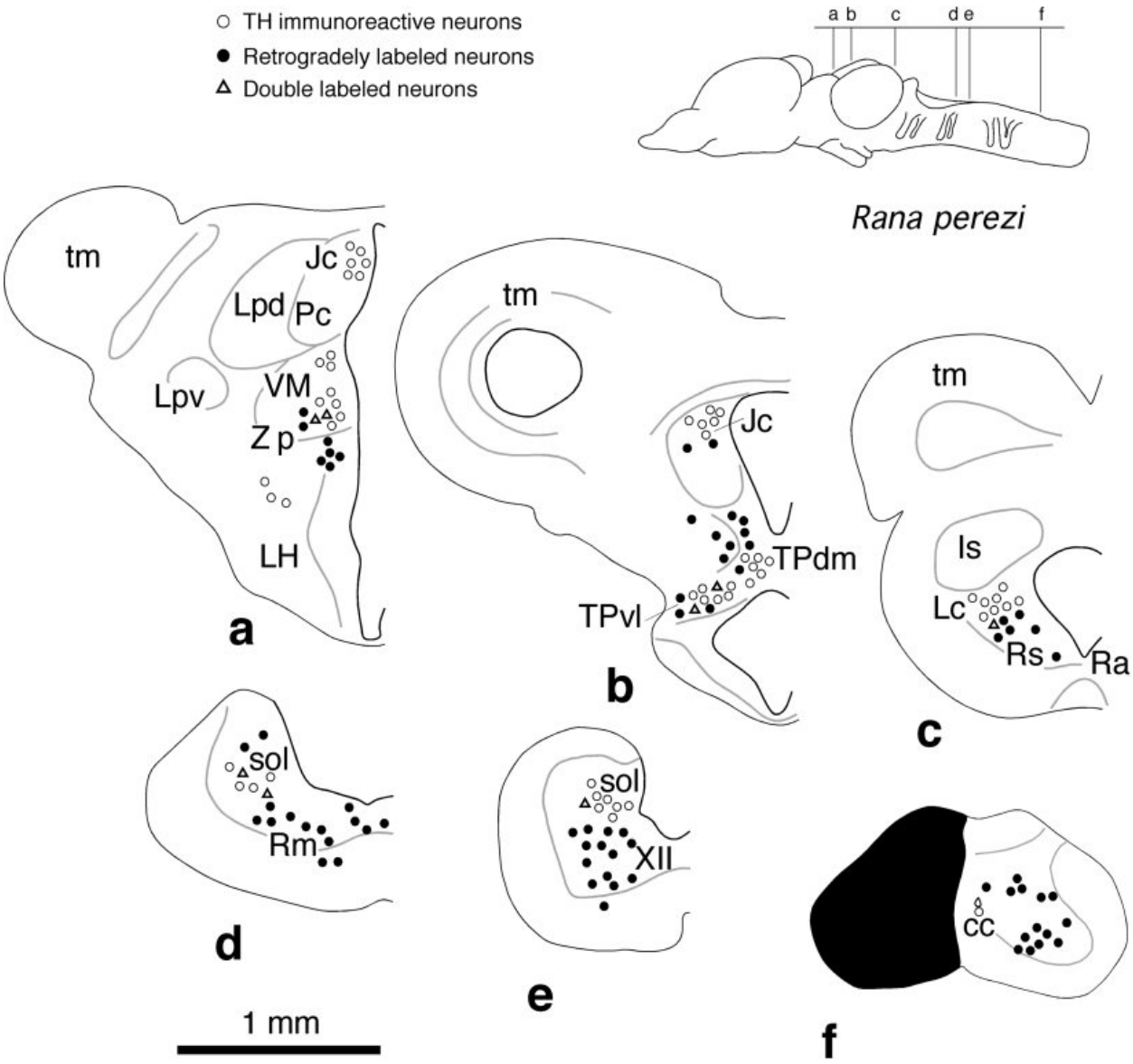

Fig. 6. Schematic drawings of transverse sections through the brain of *Rana perezi*, illustrating the distribution of retrogradely labeled cells after tracer applications into the spinal cord (black area). The localization of catecholaminergic cells, as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropiate levels of the sections are indicated in the upper right scheme.

has a tendency to complicate the application procedure. Biotinylated dextran amines have the advantage that they result in Golgi-like staining of retrogradely labeled cells showing clearly the morphology of the soma and the dendrites. With Texas Red-conjugated dextran amines, on the other hand, the morphology of the labeled cells is less clearly observed, but the detectability of the tracer is very high.

For double labeling experiments, TRDA is favored since it has the advantage of being already fluorescent and the tissue, after cutting, can be immediately processed for TH immunofluorescence. By means of TH immunohistochemistry, no distinction can be made between DA, NA and adrenergic cells. However, since DA and NA cell bodies constitute largely separate cell populations within the brain (González and Smeets, 1994a), their true nature is easily inferred except for the nucleus of the solitary tract, where DA, NA and adrenergic cells intermingle to a large extent (González and Smeets, 1993, 1994a, 1995; González et al., 1993).

CA innervation of the amphibian spinal cord

Our results have demonstrated that catecholaminergic (CA) fibers and terminals abundantly innervate the spinal cord of amphibians. This innervation is present throughout the length of the cord. The distribution of CA fibers is particularly dense in the ventral part of the dorsal horn and in the region above the central canal with only sparsely distributed fibers in ventral horn territories, as was previously observed at brachial levels (Soller, 1977; González and Smeets, 1991, 1994a).

The abundant and wide distribution of CA fibers and terminals displays a similar pattern throughout the spinal cord, although their number decreases caudalwards in all species studied. However, notable differences in the number and morphology of immunoreactive fibers occur among the species

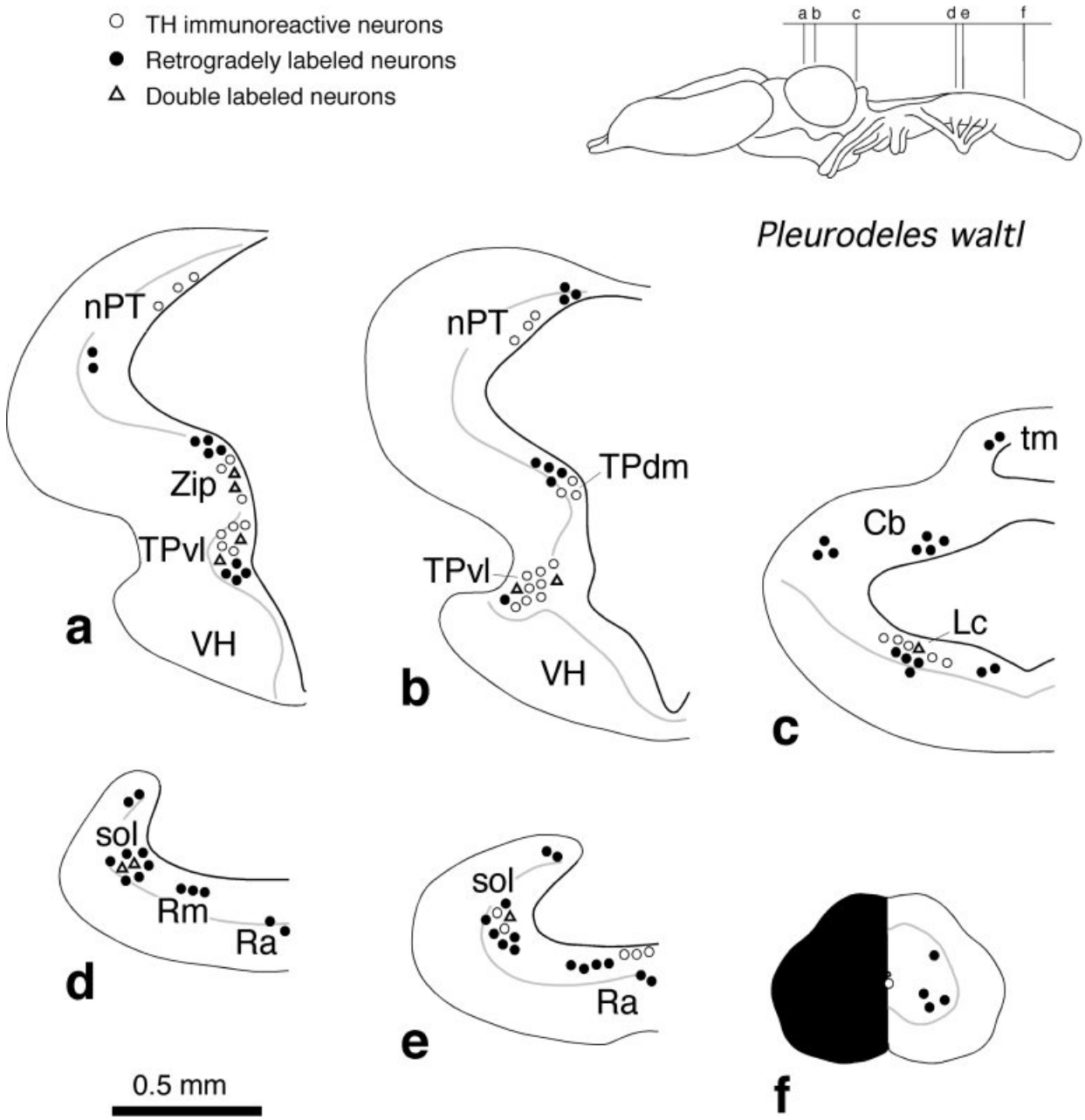

Fig. 7. Schematic drawings of transverse sections through the brain of *Pleurodeles waltl*, illustrating the distribution of retrogradely labeled cells after tracer applications into the spinal cord (black area). The localization of catecholaminergic cells, as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropiate levels of the sections are indicated in the upper right scheme.

studied. Thus, THi fibers in anurans are longer and thinner than those of urodeles and apodans, with small varicosities. Moreover, in the spinal cord of anurans, these fibers are more numerous and have a more extensive distribution.

Whereas in anurans THi fibers extensively distribute among the neurons in the dorsal horn and intermediate gray, in apodans but particularly in urodeles, they remain mostly superficial to the gray matter. In the ventral horn of anurans only scattered CA fibers were found. In contrast, a strong innervation of large neurons in the ventral horn, mainly at thoracic and lumbar cord, is present in urodeles and apodans. Finally, along the spinal cord a plexus of THi fibers was found in the central field dorsal to the central canal in all species studied.

Comparison with other vertebrates

When the distribution of CA fibers in the spinal cord of amphibians is compared with the pattern observed in mammals, the following general conclusions can be made. Mammals and amphibians share a strong CA innervation of the deep dorsal gray matter and the area above the central canal, whereas the innervation of the ventral horn is only weak to moderate (Pindzola et al., 1988; Yoshida and Tanaka, 1988; Shirouzu et al., 1990; Mouchet et al., 1992; Ridet et al., 1992; Weil-Fugazza and Godefroy, 1993; Holstege et al., 1996). Moreover, in mammals dopamine is involved in the sensory transmission via cells in the dorsal horn that project to the dorsal column nucleus (DCN), but not via cells that give rise

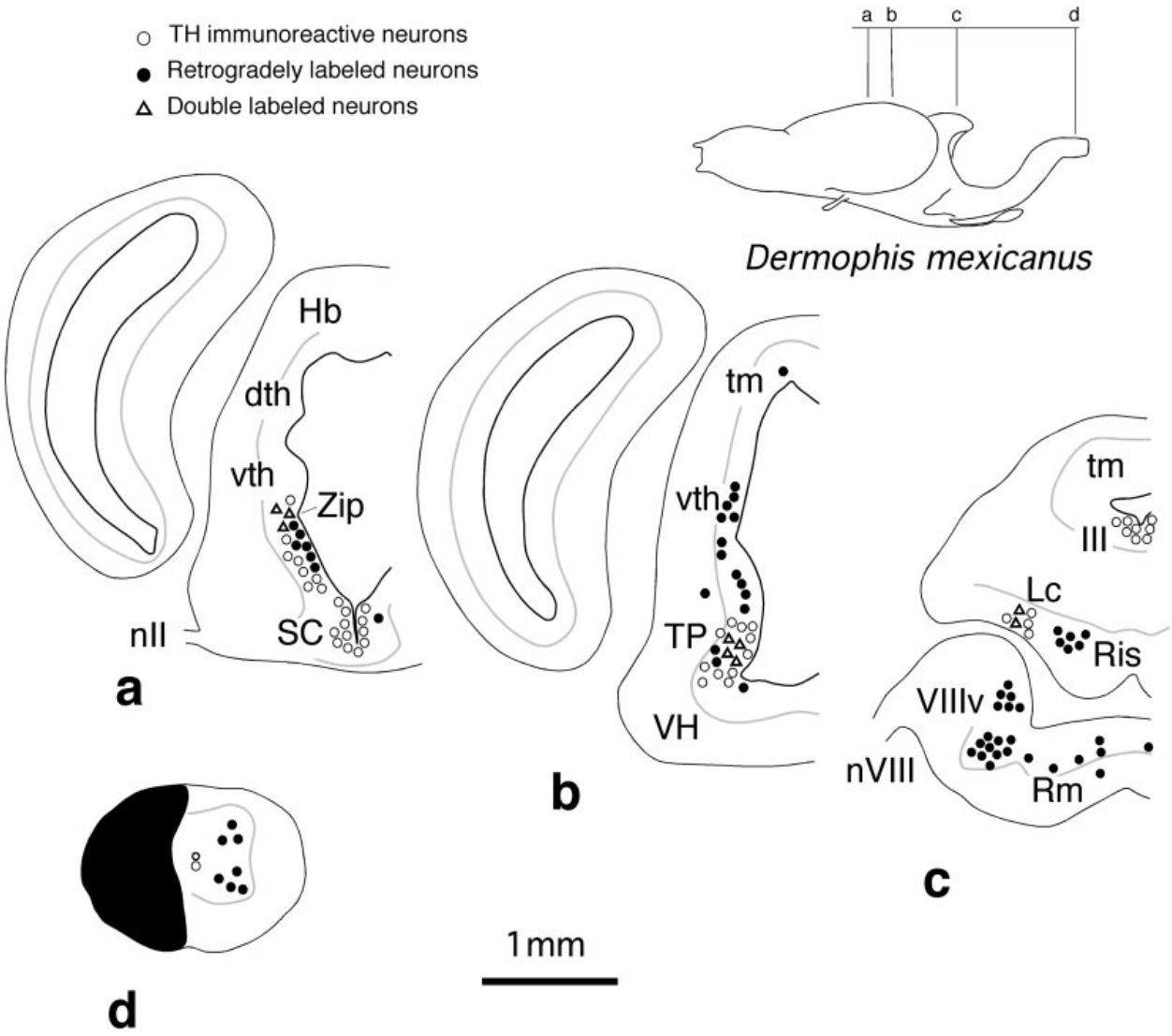

Fig. 8. Schematic drawings of transverse sections through the brain of *Dermophis mexicanus*, illustrating the distribution of retrogradely labeled cells after tracer applications into the spinal cord (black area). The localization of catecholaminergic cells, as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropiate levels of the sections are indicated in the upper right scheme.

to the spinocervical tract (Doyle and Maxwell, 1993; Doyle, 1994). A similar condition may exist in amphibians, although double labeling studies are needed to distinguish between the CA innervation of cells that project to the DCN and those constituting the origin of the spinocervical tract (Muñoz et al., 1995, 1996, 1997).

In amphibians, particularly in anurans, well-organized intermediolateral and intercalated groups of cholinergic cells have been recently characterized in the thoracic spinal cord (Muñoz et al., 2000). These correspond to the column of sympathetic preganglionic neurons studied in anurans with tracing techniques (Robertson, 1987; Horn and Stofer 1988; Peruzzi and Forehand 1994). In addition, the parasympathetic nucleus in the intermediate gray at sacral spinal levels has been described in anurans (Campbell et al. 1994; Muñoz et al., 2000). The comparison of the distribution of CA fibers in spinal segments where the autonomic cells are located suggest the innervation of these neurons. However, in contrast to mammals, the amphibian spinal cord does not show a specific condensation of THi fibers among preganglionic cells.

In amphibians, CA innervation was observed in the ventral gray matter, including the fields of the motoneuron pools. Noteworthy, in the urodele and gymnophionan spinal cord we have observed the peculiar feature of perisomatic terminal-like structures on large neurons of the ventral horn. This situation prompted us to consider a specific innervation of distinct motoneurons of the ventral horn. However, ongoing experiments in our laboratory have showed that the cells surrounded by TH terminals are ChAT immunonegative and, therefore, would represent a type of interneurons in the ventral gray matter. On the contrary, in two different studies with antibodies against TH and DA respectively, Pindzola et al. (1988) in the North American opossum (*Didelphis virginiana*) and Yoshida and Tanaka (1988) in the rat, showed the presence of CA fibers concentrated around motoneurons in laminae IX, particularly at middle to lower thoracic and upper lumbar levels in the opossum.

Data on the nature of the catecholaminergic innervation of the spinal cord in non-mammalian species are sparse. In *lampreys*, THi and DAi fibers are present in the dorsal half of the

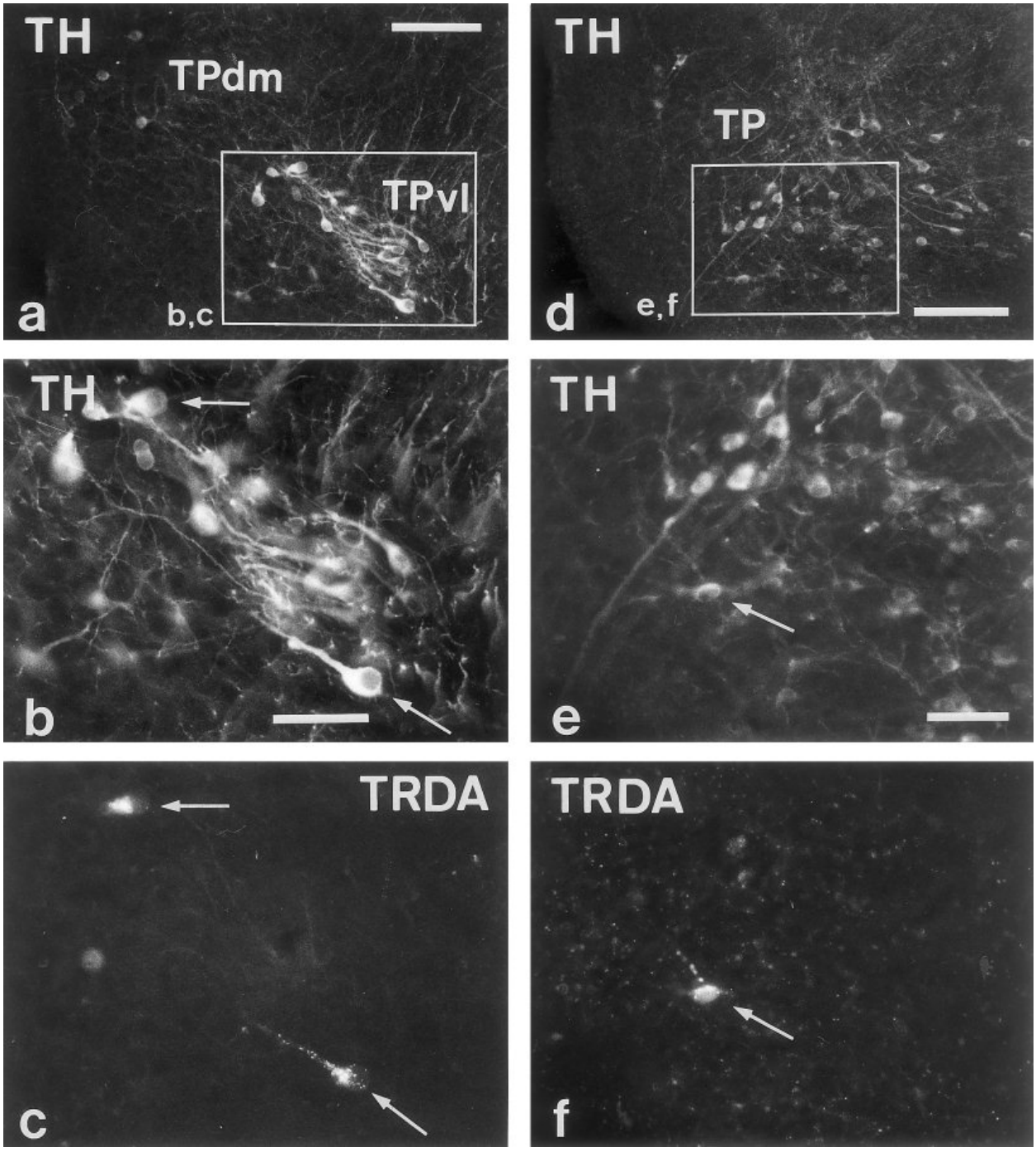

Fig. 9. Transverse sections through the brain of *Rana* (**a-c**) and *Xenopus* (**d-f**) showing the localization of THi cells (**a,b** and **d,e**) and retrograde labeled cells in the posterior tubercle (**c, f**) after tracer application in the spinal cord. Arrows point to double labeled cells. Calibration bars= 100 μm (a,d) and 50 μm (b,c,e,f).

rostralmost part of the spinal cord, but more caudally they decrease rapidly in number (Schotland et al., 1996; Pombal et al., 1997). On the other hand, DAi fibers in the ventromedial column are present throughout the rostrocaudal extent of the spinal cord. In a cartilaginous fish, i.e. the skate *Raja radiata*, DA immunohistochemistry revealed the existence of immunoreactive fibers that are primarily located around the central canal (Roberts and Meredith, 1987). However, small numbers of fibers extend into the dorsal horn, the ventral horn and the ventral funiculus. Similar observations were made in a teleost (Roberts et al., 1989). Studies in the lizard *Gekko gecko* with antibodies against DA and NA (Smeets, 1994) have shown that rather dense plexuses of DAi and NAi fibers are mainly located in the dorsal horn of the gray matter, preferentially in the presumed laminae I and II, in the medial part of the dorsal horn and in the dorsal part of lamina X. A few DAi fibers, but considerably more DBHi/NAi fibers are found in the ventral horn. Elaborate plexuses of THi fibers have been reported for the spinal cord of birds, both in the dorsal and ventral horn (Okado et al., 1991; Reiner et al., 1994). The densest plexus is

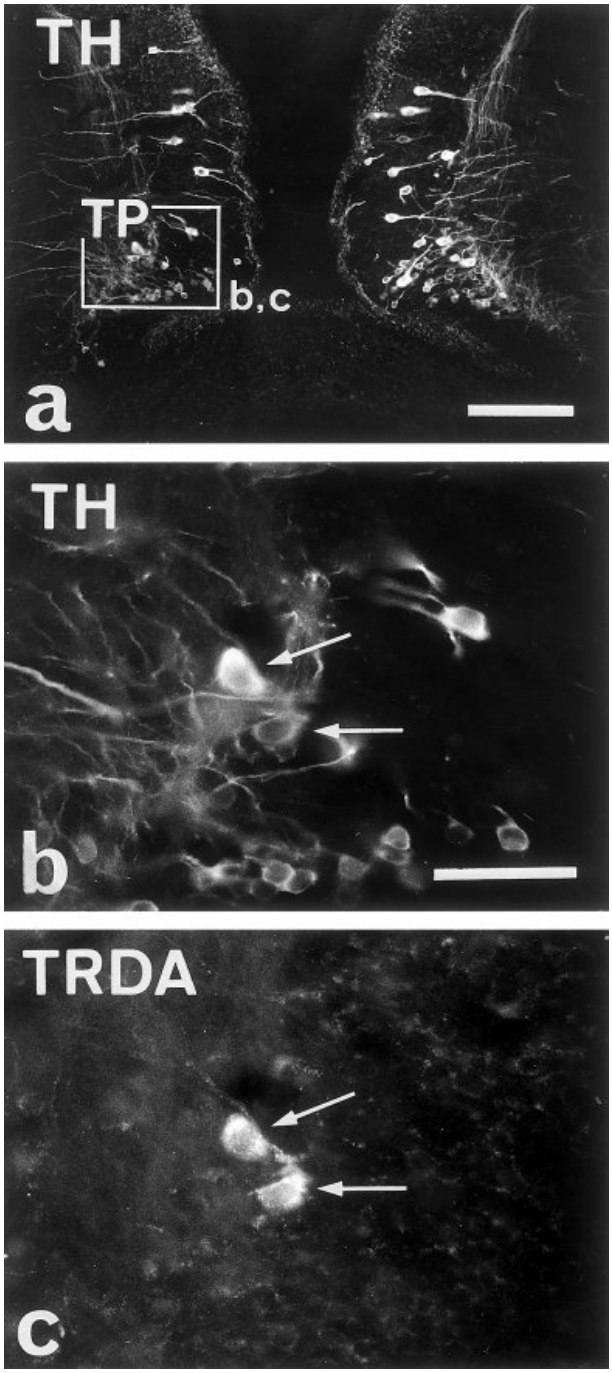

Fig. 10. Transverse sections through the brain of *Dermophis* showing the localization of THi cells (**a,b**) and retrograde labeled cells in the posterior tubercle (**c**) after tracer application in the spinal cord. Arrows point to double labeled cells. Calibration bars= 200 μm (a) and 100 μm (b,c).

found in Terni´s column, in lamina X and in the medial part of laminae V-VII of the cervical and thoracic spinal cord, but the remaining laminae also contain many immunoreactive fibers (Reiner et al. 1994). In particular, there seems to be a selective innervation of the preganglionic neurons of the thoracic cord (Coote, 1985). Obviously, more specific information about the dopaminergic, noradrenergic and adrenergic innervation throughout the spinal cord of non-mammalian vertebrates is greatly needed.

Cells of origin of supraspinal CA fibers

Although a minor proportion of the CA fibers found in the spinal cord of amphibians may be of intraspinal origin, it seems that THi fibers of supraspinal origin form the bulk of the CA innervation of the spinal cord. A similar situation is likely to be present in all vertebrates. In amphibians four centers in the forebrain and brain stem were found to contribute to the CA innervation of the spinal cord. These CA cell groups are, from rostral to caudal, the posterior tubercle, the periventricular nucleus of the zona incerta, the locus coeruleus and the nucleus of the solitary tract.

Numerous studies in mammals have attempted to clarify the origin of the CA innervation in the spinal cord. The adrenergic bulbospinal system in mammals arises in the ventrolateral medulla within the C1 and, to a lesser extent, C3 cell groups (Ross et al., 1984; Carlton et al., 1991; Guyenet et al., 1994). The adrenergic cells of the C1 group are intermingled with a population of noradrenergic cells (the A1 group), whose rostral portion projects also to the spinal cord (Blessing et al., 1981; Fleetwood-Walker and Coote, 1981; Fleetwood-Walker et al., 1983; Maisky and Doroshenko, 1991). A similar situation is found in birds where the termination of this spinal projection arising in the C1/A1 group selectively innervates the autonomic nuclei of the thoracic cord (Coote, 1985).

In amphibians, the nucleus of the solitary tract has been described as an ill-defined cell population that surrounds, mainly laterally and ventrally, the tract. However, this region seems to be highly heterogeneous in terms of chemoarchitecture. In particular, dopaminergic, noradrenergic and adrenergic cells have been found in this nucleus (González and Smeets, 1991, 1993, 1994a, 1995; González et al., 1993). Due to the lack of ventrolaterally migrated cells that could account for the corresponding groups in mammals, the nucleus of the solitary tract of amphibians may be regarded as a CA complex equivalent to the C1/A1-C3/A3 groups of amniotes. In the present study, since only TH immunohistochemistry was used in combination with the retrograde tracer, the CA implicated in the solitariospinal pathway could not be determined. However, considering the position and density of the NA cells in the region of the nucleus of the solitary tract (González and Smeets, 1993, 1995) it seems that most probably this projection is primarily noradrenergic, as in mammals.

The majority of supraspinal NA input to the spinal cord of mammals arises from the A5-A7 CA cell groups located at pontine levels. Detailed information exists for rats about the contribution of each group to this innervation, the trajectory of the descending fibers and their terminal sites (for review, see Smeets and Gonzalez, 2000). Only a single CA cell population has been identified in the isthmic region of amphibians (Dubé and Parent, 1982; Yoshida et al., 1983; Franzoni et al., 1986; González and Smeets, 1994a). This group has been considered the amphibian homologue of the locus coeruleus of mammals primarily on the basis of its position and NA content (González and Smeets, 1993, 1995). More recently, this notion was corroborated by connectional data, showing projections to both the telencephalon and spinal cord in anurans and urodeles (Marín et al., 1996). Whether the CA cell group in the isthmic region contains also cells comparable to the A5 and A7 cell groups will be discussed in the following section.

Double labeling techniques similar to those used in our study have revealed that the dopaminergic fibers in the spinal cord in mammals arise in the "periventricular nuclei of the hypothalamus" (Björklund and Skagerberg, 1979; Martin et al., 1982; Skagerberg et al., 1982; Lindvall et al., 1983; Björklund and Lindvall, 1984; Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Takada et al., 1988; Shirouzu et al., 1990). According to the classification of Hökfelt et al., (1984),

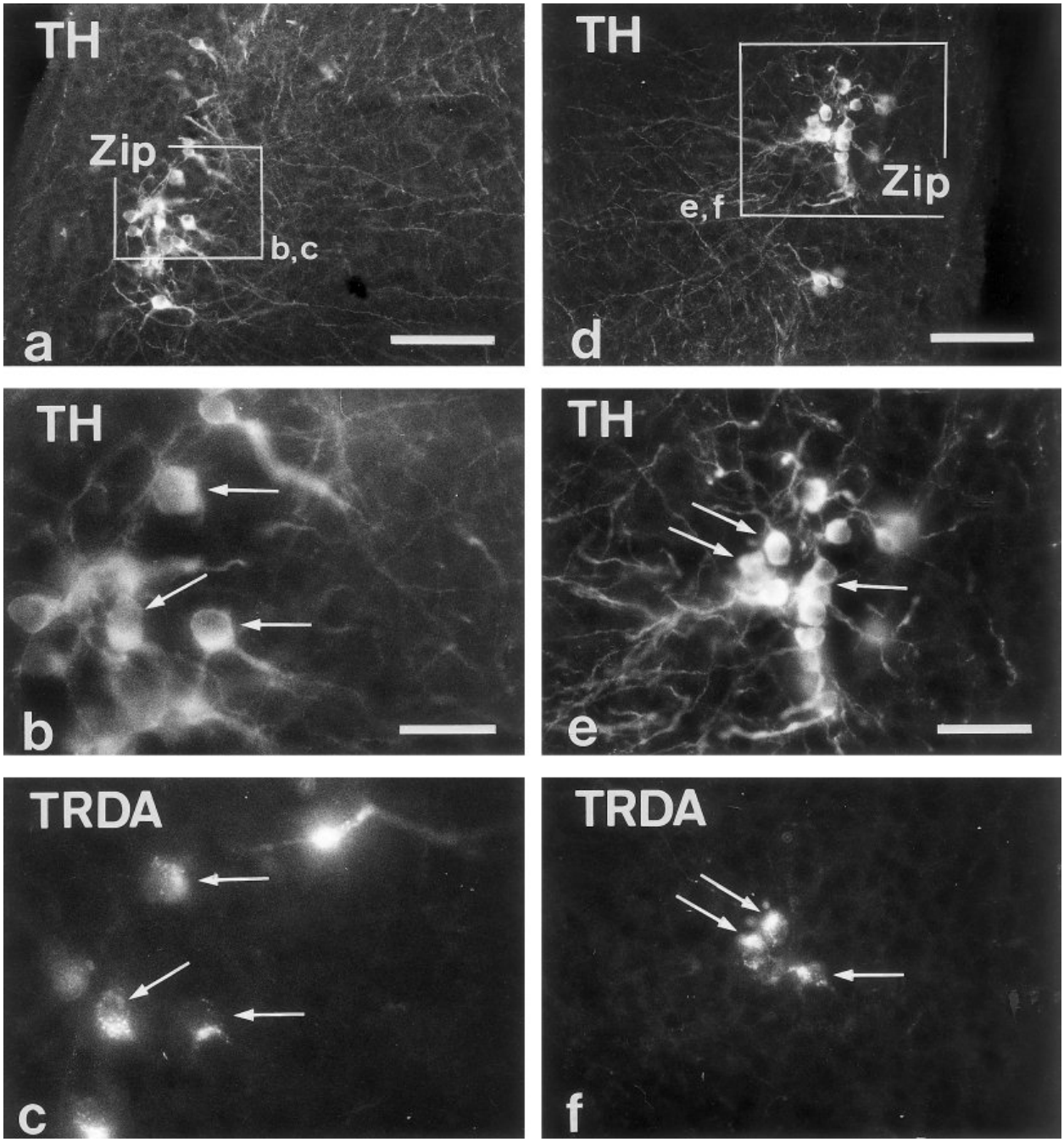

Fig. 11. Transverse sections through the brain of *Rana* (**a-c**) and *Xenopus* (**d-f**) showing the localization of THi cells (**a,b** and **d,e**) and retrograde labeled cells in periventricular nucleus of the zona incerta (**c, f**) after tracer application in the spinal cord. Arrows point to double labeled cells. Calibration bars= 100 μm (a,d) and 50 μm (b,c,e,f).

the A11 cell group is the principal, and perhaps exclusive, source of the supraspinal DA innervation. Although the A11 cell group was described within the hypothalamic territories more recent analysis have located this group in the caudal thalamus. In particular, the cells in the subparafascicular thalamic nucleus (a part of the A11 group) has been demonstrated to project abundantly to the spinal cord and the same neurons also project to the neocortex (Takada et al., 1988; Takada, 1993).

In amphibians, a "diencephalospinal dopaminergic system" has been demonstrated to arise in the posterior tubercle and in a newly described zone, i.e. the periventricular nucleus of the zona incerta (Puelles et al., 1996; Milán and Puelles, 2000). The comparison of this region with the A11 group of mammals is complicated due to the different topographical locations of the DA cells (see below).

Except for a few studies, double labeling experiments are lacking for non-mammalian vertebrates. Nevertheless, on the basis of retrograde tracing studies and immunohistochemical data, it is conceivable that the supraspinal CA inputs to the spinal cord in reptiles and birds are essentially the same. Preliminary results in birds support this notion (Chikasawa et al.,

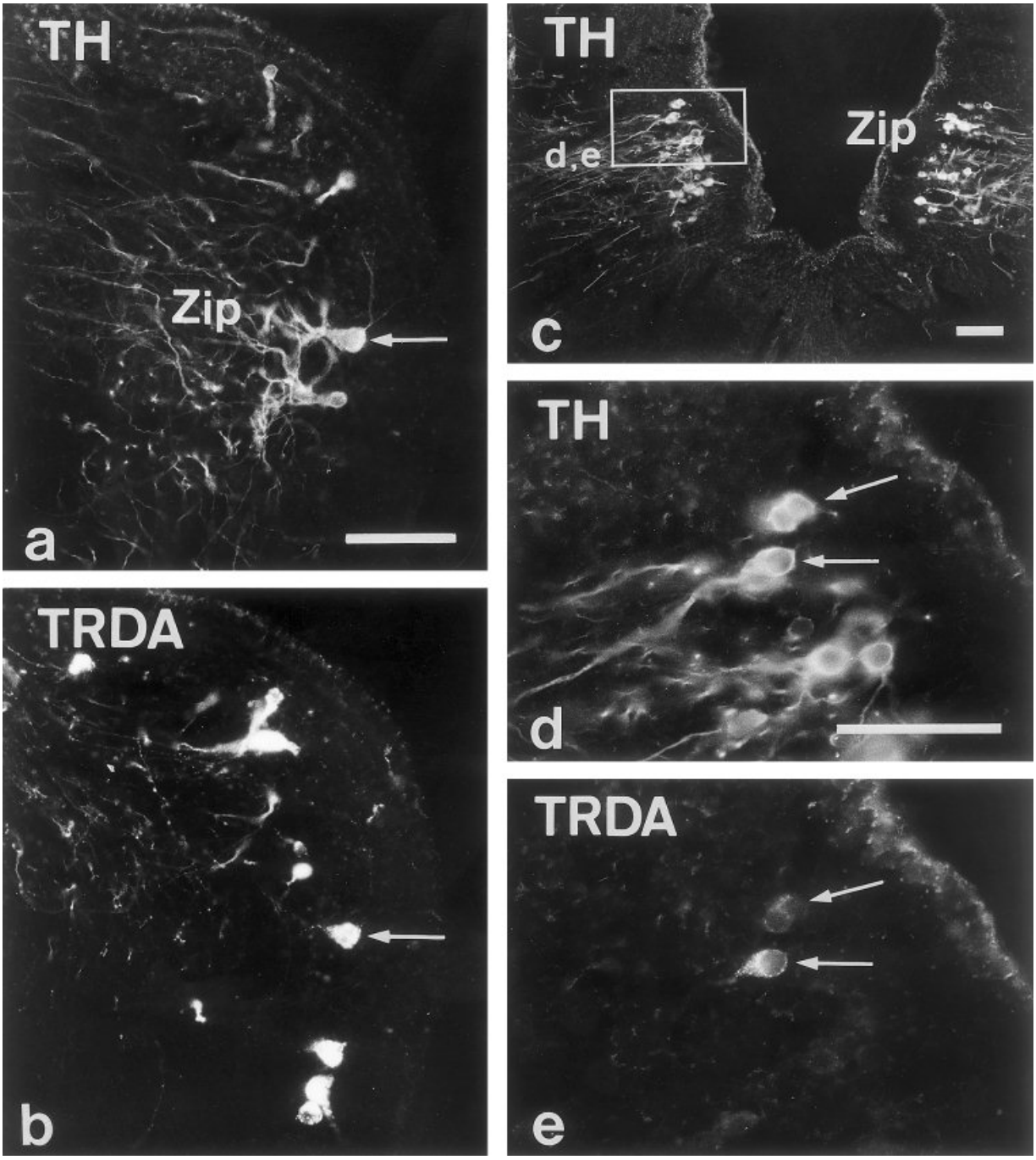

Fig. 12. Transverse sections through the brain of *Pleurodeles* (**a**,**b**) and *Dermophis* (**c**-**e**) showing the localization of THi cells (**a** and **c**,**d**) and retrograde labeled cells (**b, e**) in periventricular nucleus of the zona incerta after tracer application in the spinal cord. Arrows point to double labeled cells. Calibration bars= 100 μm.

1983; Coote, 1985). Furthermore, the distribution of catecholaminergic cell bodies in cartilaginous fish (Meredith and Smeets, 1987; Stuesse et al., 1994) and cells projecting to the spinal cord (Smeets and Timerick, 1981; Timerick et al., 1992) point in the same direction. A different condition may be present in cyclostomes, where descending catecholaminergic fibers can be traced only to rostral spinal cord levels within its dorsal half (Schotland et al., 1996; Pombal et al., 1997).

Segmental organization of the CA cell groups projecting to the spinal cord

Recently, an effort has been made to compare the CA cell groups of vertebrates by using a segmental approach (Smeets and González, 2000). In Figure 15, the localization of the CA cell groups and those giving rise to the descending spinal projections are represented for mammals and anurans within a segmental framework (after Puelles et al., 1996; Puelles and Verney, 1998).

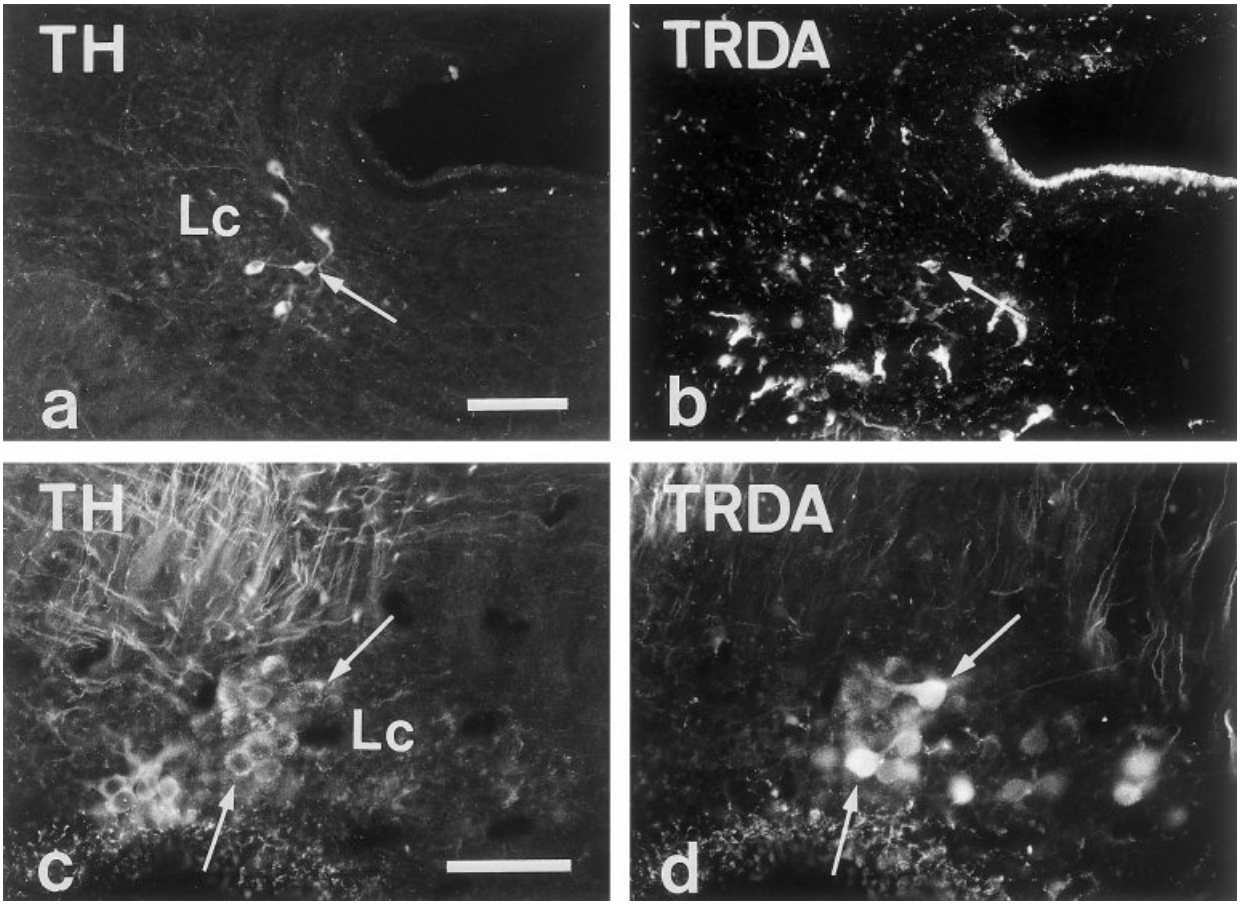

Fig. 13. Transverse sections through the brain of *Rana* (**a,b**) and *Dermophis* (**c,d**) showing the localization of THi cells (**a,c**) and retrograde labeled cells in the locus coeruleus (**b,d**) after tracer application in the spinal cord. Arrows point to double labeled cells. Calibration bars= 100 μm.

The spinal projection arising in A1 in mammals is readily comparable to the projection from the nucleus of the solitary tract of amphibians. The C3 group of mammals is formed in the floor plate in median or paramedian locations in relation with the raphe neurons of rhombomere r5 (Zecevic and Verney, 1995; Puelles and Verney, 1998). Because no CA cells are found in a comparable segmental domain in amphibians, this group seems to be lacking.

Similarly, the basal plate-derived CA cell groups A5 and A7 of mammals are not recognizable in amphibians. The rostral part of the A5 group arises in r2-r3, its caudal part in r5-r6, whereas the A7 group develops in the isthmic segment. In non-mammalian vertebrates, CA cell groups in these rhombomeres have not been consistently reported, but the cells observed in “prevagal locations” in gymnophionan amphibians, turtles and snakes could easily be assimilated to A5c (Smeets, 1994). The gap between A5r and A5c corresponds to neuromere r4 where no CA cells seem to originate. The presence of a pretrigeminal CA cell group (A7) in the basal plate of the isthmic neuromere r1, has been described in rats (König et al., 1988), birds (Puelles and Medina, 1994), and snakes (Smeets and Reiner, 1994), but not in man (Puelles and Verney, 1998). In our experiments in *Dermophis mexicanus* CA cells in prevagal location were not double labeled what accounts for a total lack of the A5 spinal projection in amphibians. This fact can be correlated with the lack of a particularly dense innervation of the autonomic IML in amphibians, which in mammals arises primarily in the A5 group (Stevens et al., 1985; Clark and Proudfit, 1993).

The locus coeruleus proper (A6) develops in the caudal portion of the isthmic segment (r1) and is clearly observed in all vertebrates in this location. However, the A4 group, which extends laterally even into cerebellar regions, has been found in some mammalian species (rat, dog, sheep, cat), but not in others (rabbit, primate). Some larger cells, which are continuous with the locus coeruleus cell group, extend caudally into r2-r3 and represent the locus subcoeruleus. In amphibians, the NA cell bodies in the isthmic region occupy portions of rhombomeres r1 and r2, and they show some morphological differences. Thus, the spinal projection arising in the so-called locus coeruleus in amphibians might represent together the projection from the locus coeruleus (A6 group) and locus subcoeruleus of mammals.

The segmental analysis of the forebrain has revealed that most of the "hypothalamic" regions do not belong to the classic diencephalon but should be included into the so-called secondary prosencephalon (see Puelles and Rubenstein, 1993). Thus, the consideration of the segments and the basal versus alar plates localization of the different CA cell masses in mammals has revealed that the formerly described hypothalamic A11 does not belong to the hypothalamus. Taken together the diencephalic segments (prosomeres p1, p2 and p3, from caudal to rostral), their alar plate develops CA cell groups in all vertebrates studied. In the ventral aspect of this plate, a continuous group of DA cells (A11) is found in p1 and

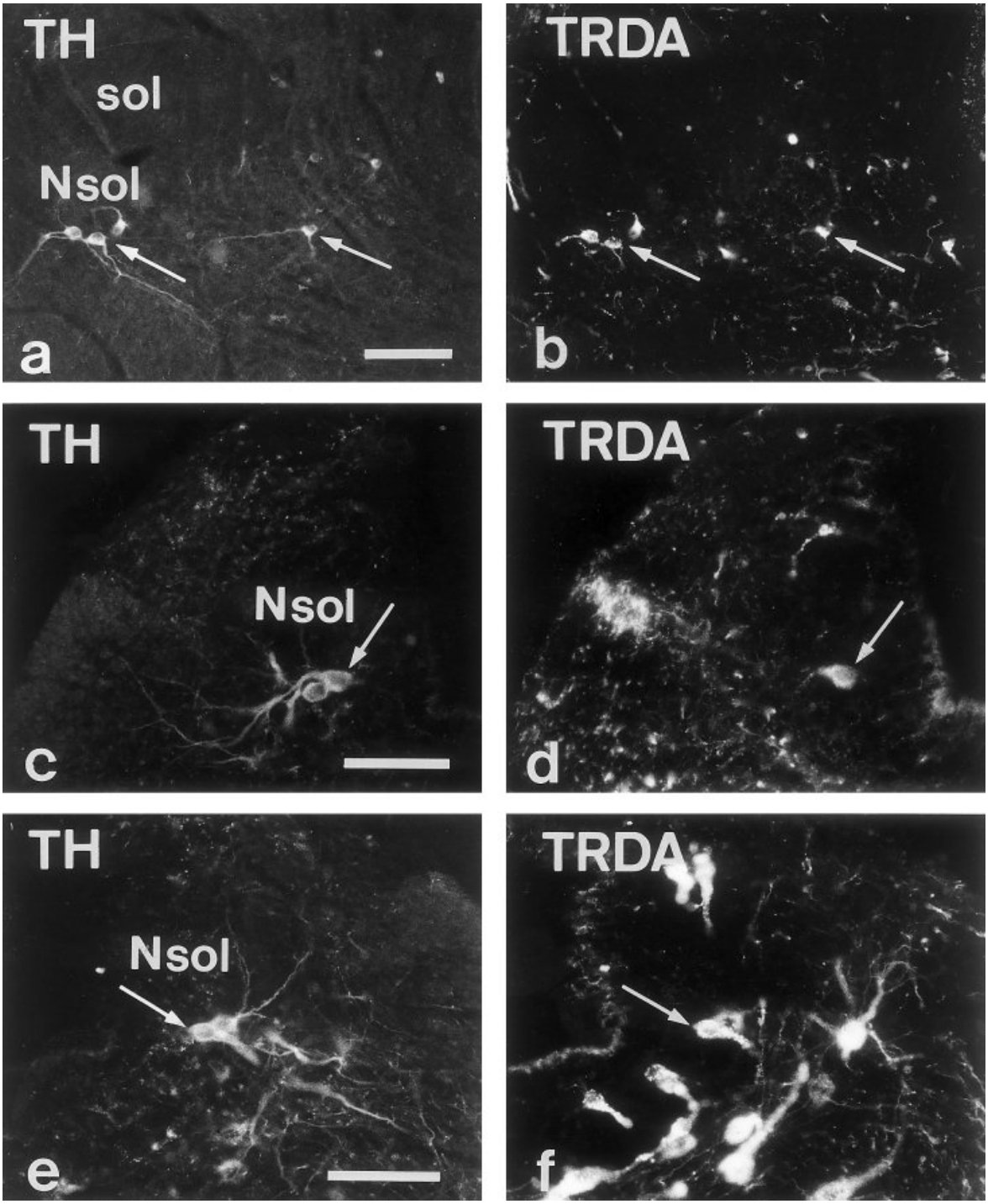

Fig. 14. Transverse sections through the brain of *Rana* (**a,b**) and *Pleurodeles* (**c-f**) showing the localization of THi cells (**a,c** and **e**) and retrograde labeled cells in the nucleus of the solitary tract (**b,d** and **f**) after tracer application in the spinal cord. Photomicrographs **c** and **d** are caudal rhombencephalic levels, whereas **e** and **f** are intermediate rhombencephalic levels. Arrows point to double labeled cells. Calibration bars= 100 μm.

p2 of mammals, forming a rostral continuation of the mesencephalic periaqueductal cells. A separate A13 group (zona incerta) develops in the alar plate of p3. The A13-A11 column seems to be the origin of diencephalospinal projections. Dopaminergic cell groups homologous to the A11 and A13 of mammals have been recognized in birds and reptiles (Medina et al., 1994; Puelles and Medina, 1994). The periventricular cell groups in the ventral alar plate in p1 and p2 (A11) seem to be absent in anamniotes.

In amphibians, two bands of CA cells have been found in the alar plate of prosomere p3 (Fig. 15). The more ventrally (rostrally, according to the longitudinal axis of the brain) is formed by cells with the characteristic extensive dendritic trees directed laterally and described as the periventricular

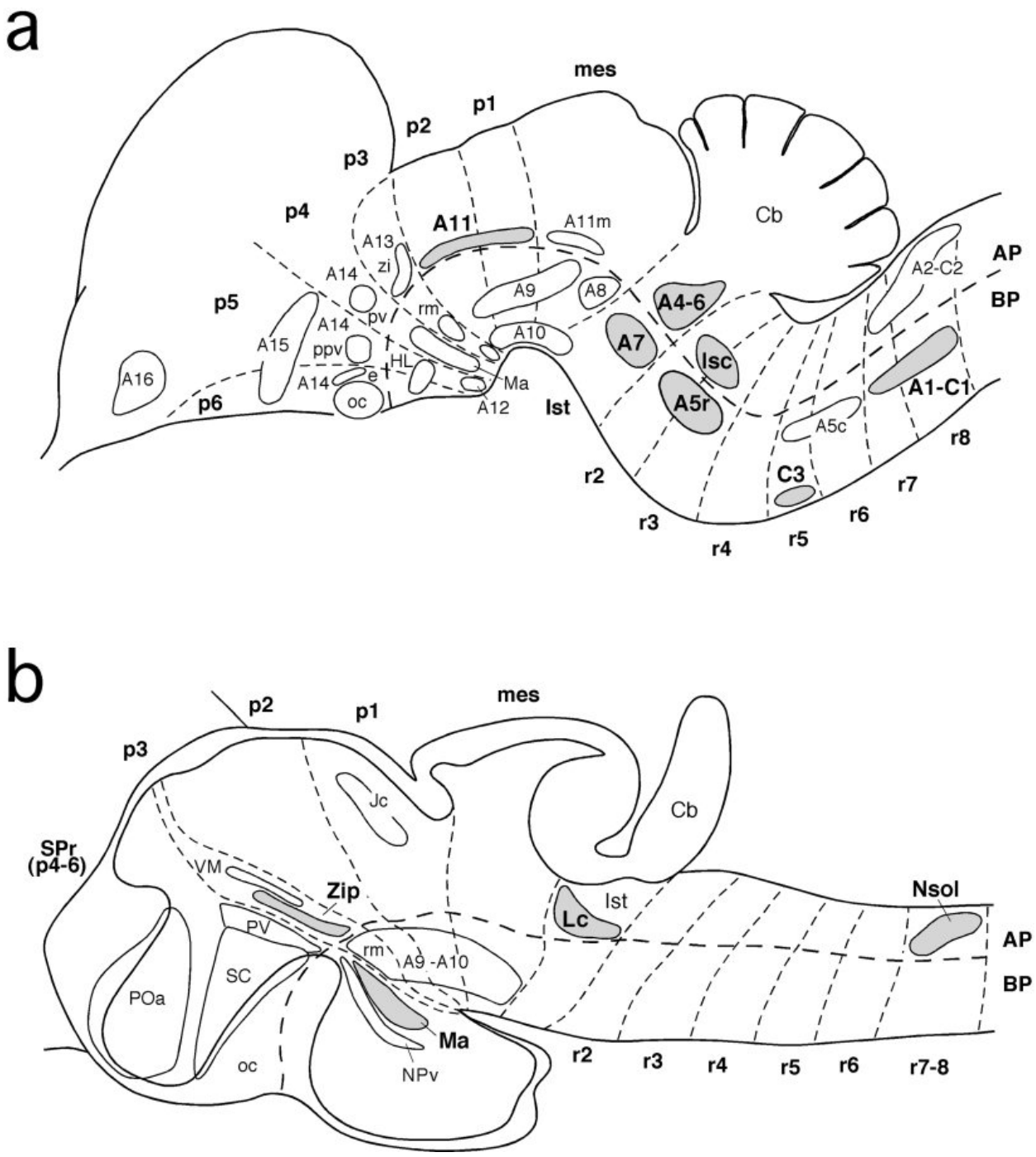

Fig. 15. Schematic diagrams of a midsagittal section through the brain of an hypothetic mammal (**a**) and an anuran (**b**) illustrating, in projection, the localization of the catecholaminergic cell groups. Within this neuromeric model, the catecholaminergic centers that project to the spinal cord are illustrated (shaded nuclei). (Fig.15b reproduced with permission of S. Karger AG, Basel from Puelles et al., 1996).

nucleus of the zona incerta (Milán and Puelles, 2000). The present study showed that these cells are the sole diencephalic source of spinal DA fibers in amphibians. Considering this projection, a comparison with the A11 group of mammals can be made, although in this case the prosomere localization clearly differs. In mammals, spinal DA fibers arising in the A13 group were postulated in the rabbit (Blessing and Chalmers, 1979) but subsequent studies in the rat have demonstrated that the efferent projections from the region of the medial zona incerta containing the A13 dopaminergic cells do not reach the spinal cord (Wagner et al., 1995). Therefore, DA spinal projections arising in p3 are lacking in mammals. The peculiar arrangement found in amphibians points to a more rostral origin of the DA projections to the spinal cord than in mammals. Although a point to point comparison between cell groups in mammals and amphibians seems not possible it is worth mentioning that several interesting clues are available in the literature. For instance in the rat, DA cells in the A11 group that project to the spinal cord contain calcitonin gene-related peptide (CGRP) immunoreactivity (Orazzo et al.,

1993), whereas cells in the A13 group coexpress DA and somatostatin (Meister et al., 1987). Curiously, the region in the periventricular nucleus of the zona incerta, that has been shown to project to the spinal cord (present study), also possesses CGRP-containing cells (Petkó and Sánta, 1992) and the DA cells of the band in the ventromedial nucleus co-express TH and somatostatin (unpublished observations; Inagaki et al., 1981; Petkó and Orosz, 1996).

Finally, an additional DA projection was found to originate from CA cells in the secondary prosencephalon. Following previous descriptions, this projection arises in the ventrolateral portion of the posterior tubercle. However, the segmental topography of these cells (according to Milán and Puelles, 2000) would correspond to the superficial mammillary and mammillary nuclei of the basal part of prosomere p4. Although this situation seems to be different in mammals, it should not be ruled out that when applying a similar segmental analysis to the descending CA projections to the spinal cord in mammals, part of the widely described "hypothalamospinal" system would be comparable to what we have found in amphibians. Clearly, similar studies need to be made in representatives of different vertebrate classes before establishing common patterns or significant differences between the descending CA spinal systems in vertebrates.

CONCLUDING REMARKS

The present study has provided evidence that the CA innervation of the spinal cord in amphibians shares many features with its counterpart in the brain of amniotes. Nevertheless, using a segmental approach a remarkable difference has been observed with respect to the diencephalospinal projection. On the other hand, when the supraspinal CA projections in mammals are reinvestigated, by using a similar approach, it may turn out that even this projection is less different than it now seems to be.

Sparse information is available about the functional significance of the CA innervation of the spinal cord in amphibians. The distribution of CA fibers in the spinal cord and their origin in the brain strongly suggest similar functions as in amniotes. These would include a role in nociception, autonomic functions and motor control (Smeets and González, 2000). Moreover, direct investigations in amphibians have demonstrated that CA in the spinal cord inhibit sympathetic reflexes and produce elevation of pain thresholds (Undesser et al., 1981; Stevens and Brenner, 1996).

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Capítulo 4

Desarrollo de las conexiones aferentes y catecolaminérgicas de la médula espinal

Descending supraspinal pathways in amphibians. III. Development of descending projections to the spinal cord in *Xenopus laevis* with emphasis on the catecholaminergic inputs

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Descending Supraspinal Pathways in Amphibians. III. Development of Descending Projections to the Spinal Cord in *Xenopus laevis* With Emphasis on the Catecholaminergic Inputs

CRISTINA SÁNCHEZ-CAMACHO[1], OSCAR MARÍN[2], HANS J. TEN DONKELAAR[3] AND AGUSTÍN GONZÁLEZ[1]

[1]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain
[2]Department of Psychiatry and Langley Porter Psychiatric Institute, Nina Ireland Laboratory of Developmental Neurobiology, University of California at San Francisco, San Francisco, California
[3]Department of Neurology, University Medical Center Nijmegen, The Netherlands

ABSTRACT

In developmental stages of the clawed toad, *Xenopus laevis*, we describe the ontogeny of descending supraspinal connections, catecholaminergic projections in particular, by means of retrograde tracing techniques with dextran amines. Already at *embryonic stages* (stage 40), spinal projections from the reticular formation, raphe nuclei, Mauthner neurons, vestibular nuclei, the locus coeruleus, the interstitial nucleus of the medial longitudinal fasciculus, the posterior tubercle and the periventricular nucleus of the zona incerta are well developed. At the beginning of the *premetamorphic period* (stage 46), spinal projections arise from the suprachiasmatic nucleus, the torus semicircularis, the pretectal region and the ventral telencephalon. After stage 48, tectospinal and cerebellospinal projections develop, with spinal projections from the preoptic area following at stage 51. Rubrospinal projections are present at stage 50. During the *prometamorphic period*, spinal projections arise in the nucleus of the solitary tract, the lateral line nucleus and the mesencephalic trigeminal nucleus. With *in vitro* double labeling methods, based on retrograde tracing of dextran amines in combination with tyrosine hydroxylase (TH) immunohistochemistry, we show that at stage 40/41, catecholaminergic (CA) neurons in the posterior tubercle are the first to project to the spinal cord. Subsequently, at stage 43, new projections arise in the periventricular nucleus of the zona incerta and the locus coeruleus. The last CA projection to the spinal cord originates from neurons in the nucleus of the solitary tract at the beginning of prometamorphosis (stage 53). Our data show a temporal, rostrocaudal sequence in the development of the CA cell groups projecting to the spinal cord. Moreover, the early appearance of CA fibers, preterminals and terminal-like structures in dorsal, intermediate and ventral zones of the embryonic spinal cord suggests an important role for catecholamines during development in nociception, autonomic functions and motor control at the spinal level.

Indexing terms: posterior tubercle; periventricular nucleus of the zona incerta; locus coruleus; nucleusof the solitary tract; retrograde tracing; tyrosine hydroxylase; catecholamines; ontogeny

In a recent study, we showed that, in adult specimens of the three amphibian orders (Anura, Urodela and Gymnophiona), extensive descending projections from all main brain divisions reach the spinal cord (Sánchez-Camacho et al., 2001a). These data, based on retrograde dextran amine tracing, greatly extended previous observations on the organization of descending supraspinal pathways in amphibians (ten Donkelaar et al., 1981; Tóth et al., 1985; Naujoks-Manteuffel and Manteuffel, 1988). Moreover, the combination of fluorescent dextran amine labeling with immunohistofluorescence against tyrosine hydroxylase (TH) allowed the identification of those neurons that provide the catecholaminergic (CA) innervation to the spinal cord (Sánchez-Camacho et al., 2001b). The results of this study revealed that only four CA cell groups project to the spinal cord of amphibians, i.e. the ventrolateral component of the posterior tubercle in the mamillary region, the periventricular nucleus of the zona incerta in the ventral thalamus, the locus coeruleus and the nucleus of the solitary tract. Comparison of the organization of descending CA projections to the spinal cord of amphibians with that of amniotes showed that many features are shared by all tetrapods (Smeets and González, 2000; Sánchez-Camacho et al., 2001b).

An important aspect of the organization of the descending pathways to the spinal cord in vertebrates concerns their de-

velopment and temporal sequence of appearance. Previous studies in amphibians dealt with the ontogeny of supraspinal input to the spinal cord of *Xenopus laevis* (ten Donkelaar and de Boer-van Huizen, 1982; van Mier and ten Donkelaar, 1984; Nordlander et al., 1985; Roberts and Alford, 1986; Hartenstein, 1993). A number of descending pathways, arising mainly in the brainstem, develop early in the embryo, even before hatching. Tracing studies in species of other vertebrate classes showed that, as in amphibians, reticulospinal and interstitiospinal fibers reach the spinal cord first, followed by vestibulospinal fibers and, much later, by rubrospinal and, if present, corticospinal projections (fish: Kimmel et al, 1982; Mendelson, 1986a,b; chick: Okado and Oppenheim, 1985; Glover and Petursdottir, 1991; Chédotal et al., 1995; mammals: Martin et al., 1978, 1991, 1993; Cabana and Martin, 1982, 1984; Auclair et al., 1993, 1999; Kudo et al., 1993; de Boer-van Huizen and ten Donkelaar, 1999). All these data suggest a phylogenetic constancy of descending supraspinal pathways in vertebrates, and that, at least in the descending input from the brainstem to the spinal cord, a comparable pattern of development exists (ten Donkelaar, 2000).

Catecholaminergic fibers of supraspinal origin innervate the spinal cord in all vertebrates, from early stages of development on (Smeets and González, 2000; ten Donkelaar, 2000). In particular, both anuran and urodele amphibians possess CA fibers from late embryonic stages onwards (González et al., 1994a,b, 1995). As in other brain regions, the early presence of a CA innervation may play an important role in organizing the development of the spinal cord, and may directly influence the maturation of spinal neurons (Tennyson et al., 1973; Specht et al., 1981; Voorn et al., 1988).

The present study is part of a research program on the organization of the spinal cord in amphibians to evaluate: 1) the similarities and differences between amphibian orders, and 2) to what extent the pattern of connectivity in amphibians is comparable to that of amniotes. We describe the temporal sequence of appearance of descending supraspinal pathways in amphibians, with emphasis on the CA inputs, and compare the development of descending spinal connections of amphibians with the data available for amniotes. To reach these goals, the South African clawed toad, *Xenopus laevis*, is used since an accurate timetable of its development (Nieuwkoop and Faber, 1967) is available, and moreover, its spinal cord connections were studied in adults as well as in developmental stages (ten Donkelaar and de Boer-van Huizen, 1982; van Mier and ten Donkelaar, 1984; Nordlander et al., 1985; Roberts and Alford, 1986; Hartenstein, 1993; Muñoz et al., 1997; Sánchez-Camacho et al., 2001a,b). In *in vitro* preparations of developmental stages of *X. laevis*, we applied low-weight (3kD) dextran amines for retrograde tracing known to give good results when studying the formation of neuronal circuitry in the developing brain of amphibians (Luksch et al., 1996, Muñoz et al., 1996). This technique can easily be combined with immunohistochemistry, giving a powerful tool to characterize the neurotransmitters involved in pathways within the central nervous system (Marín et al., 1997).

Abbreviations

Am	amygdala
cc	central canal
dh	dorsal horn
DLF	dorsolateral funiculus
Lc	locus coeruleus
ll	lateral line nucleus
MN	Mauthner neuron
Nflm	nucleus of the fasciculus longitudinalis medialis
nPT	nucleus pretectalis
Nsol	nucleus of the solitary tract
POa	anterior preoptic area
Ra	raphe nucleus
Ri	inferior reticular nucleus
Rm	middle reticular nucleus
Rs	superior reticular nucleus
Rub	nucleus ruber
sol	solitary tract
tm	mesencephalic tectum
TP	posterior tubercle
VF	ventral funiculus
VH	ventral hypothalamic nucleus
VIII	octaval nuclear complex
vth	ventral thalamus
Zip	periventricular nucleus of the zona incerta

MATERIALS AND METHODS

For the present study, a total of 63 *Xenopus laevis* embryos and larvae, ranging from developmental stages 40 to 65 (Nieuwkoop and Faber, 1967), were used (Table I). The animals were obtained by Pregnyl-induced (Organon) breeding and maintained in tap water at 20ºC throughout their development. In all experiments, tadpoles were deeply anesthetized by immersion in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz) in distilled water. Their stage was identified under the microscope before the tracing experiments. The tadpoles were processed under *in vitro* conditions, as previously described (Luksch et al., 1996; Muñoz et al., 1996; Marín et al., 1997). Briefly, under anesthesia the animals were cooled to a body temperature of 4ºC and perfused transcardially with iced Ringer's solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose; Merck), which was oxygenated with carbogen (95% O_2, 5% CO_2) to a pH of 7.3 (Straka and Dieringer, 1993). Subsequently, the brain and spinal cord were rapidly isolated and, after removal of the dura mater and the choroid plexuses, transferred to fresh iced Ringer's solution.

The retrogradely transported tracers 3 kD biotinylated dextran amine (BDA; Molecular Probes, Oregon) or 3 kD Texas Red-conjugated dextran amine (TRDA; Molecular Probes), were applied into the developing spinal cord. On the tip of a tungsten needle the tracer was recrystallized from a saturated solution in distilled water. Due to the small diameter of the spinal cord, it was not always possible to restrict the tracer applications to one side of the spinal cord, particularly in early stages of development. The tracers were always applied into the rostral cord to label the bulk of descending supraspinal projections. The crystal was left for 2-5 min and then was washed away as the brains were immersed and maintained for 15-24 hours at 15ºC in continuously oxygenated Ringer's solution. They were then fixed for 3-5 hours in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), blocked in a solution of 15% gelatin and 30% sucrose in PB, and stored for 5 hours in a solution containing 4% formaldehyde and 30% sucrose in PB at 4ºC. The brains were cut on a freezing microtome at 30 μm thickness in the frontal, horizontal or sagittal plane and collected in cold PB.

In a first series of experiments, BDA was visualized with an avidin biotin complex (Vectastain, ABC Standard kit, Vector Labs., Burlingame, CA) and peroxidase activity with DAB-nickel as chromogen (see Sánchez-Camacho et al., 2001a for details). The sections were mounted on glass slides

(mounting medium: 0.2% gelatin in Tris buffer, pH 7.6), and dried over-

TABLE 1. Number of Animals Investigated at Different Stages of Development With Tracer Applications into the Spinal Cord and TH Immunohistochemistry[1]

Developmental Stage[2]																		
Embryonic						Premetamorphic							Prometamorphic			Metamorphic climax		
40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	57	59	65	n
3	1	2	5	2	6	9	8	6	3	6	2	1	3	2	1	1	2	63

[1]TH, tyrosine hydroxylase.

[2]Staging of the embryos and larvae according to Nieuwkoop and Faber (1967).

night. After ethanol dehydration and xylene cleaning, they were coverslipped with Entellan (Merck). Some sections were counterstained with cresyl violet to facilitate the localization of labeled structures. Sections from TRDA experiments were mounted immediately after sectioning (mounting medium as above) and coverslipped with Vectashield (Vector Labs., Burlingame, CA).

In a second set of experiments, we combined visualization of BDA or TRDA with indirect immunofluorescence for tyrosine hydroxylase (TH). Briefly, brain sections were first incubated for 48 hours at 4ºC with a mouse anti-TH antibody (Incstar), diluted 1:1000. They were then incubated with a FITC-conjugated mouse-IgG complex (Incstar) diluted 1:150 for 90 minutes at room temperature. BDA was visualized by incubation with a Texas Red-conjugated streptavidin complex (Vector Labs., diluted 1:200) together with the secondary antibody. The sections were then mounted on glass slides and coverslipped with Vectashield. Alternating the appropriate filter combinations in a Zeiss fluorescence microscope allowed the identification of BDA or TRDA retrogradely labeled cells and TH-immunoreactive (THi) cells.

The distribution of labeled cells in the brain of *X. laevis* tadpoles was charted in representative transverse sections by means of a camera lucida or a computer-aided X-Y plotting system (Minnesota Datametrics, MD-2 digitizer and software, Minnesota). The nomenclature is largely the same as that used in previous studies on the amphibian brain (e.g., González et al., 1994a,b; Marín et al., 1997; Muñoz et al., 1997; Sánchez-Camacho et al., 2001a,b). The original research reported herein was performed under animal care guidelines established by the Spanish Royal Decree 223/1988.

RESULTS

In the present study, retrograde tracers were applied to the developing spinal cord of *Xenopus laevis* tadpoles from late embryonic to juvenile stages. Thus, the progressive development of supraspinal descending pathways can be analyzed. Additionally, in combination with TH-immunohistochemistry, the gradual maturation of catecholaminergic projections to the spinal cord is demonstrated. Examples of the labeling obtained are shown inmuno Figures 2 and 3. Broadly, we define the *embryonic period* as the first, rather long period of development that ends with the total resorption of the external gills and the beginning of independent feeding (stage 45). In other studies on the development of the anuran brain frequently hatching (stage 35/38 in *X. laevis*) is used to mark the end of the embryonic period (Manelli and Margoritora, 1961; Fox, 1984; van Mier and ten Donkelaar, 1984). In these studies, for the period between hatching and independent feeding the loose term initial larval period is used. In the present study, like in other studies on the development of anuran catecholamine systems (e.g., González et al., 1994a,b; Marín et al., 1997), we divide the larval period, marked by independent feeding, into three sets of stages following the subdivision in ranid frogs by Gona et al. (1982): 1) *premetamorphic stages* (stages 45/46 until 52/53), in which the tadpole grows in size and the hindlimb buds appear on the lateral side of the body; 2) *prometamorphic stages* (stages 52/53 until 58/59), characterized by the gradual formation of the hindlimbs, and ending with the emergence of the forelimbs, and 3) *metamorphic climax* (stages 58/59 until 66), the period of the more drastic changes of metamorphosis when the transformation of the tailed larval form into the tailless, four-legged juvenile occurs. In Figure 1 the development of descending supraspinal pathways to the spinal cord as well as the cells of origin of supraspinal CA input is shown in three representative developmental stages of *X. laevis*. In the following sections, we will describe: 1) the development of the pathways descending to the spinal cord (summarized in Fig. 2), 2) the distribution of TH-immunoreactivity in the spinal cord, and 3) the developmental sequence of the supraspinal catecholaminergic input (summarized in Table II).

Development of descending supraspinal pathways to the spinal cord

Late embryonic stages. The earliest tadpoles of *Xenopus* studied are of developmental stages 40-42, i.e. late embryonic stages, just following hatching and prior to independent feeding. Tracer applications into the spinal cord show that several descending brainstem and diencephalic projections are already present in these embryonic stages (Fig. 1A). The labeled cells found, located mainly in the marginal zone, are round to oval in shape with hardly any labeled dendrites and, when present, with only a main process directed ventrally. Large vacuoles are often present in the cytoplasm of these neurons. At this stage of development, the spinal cord already receives input from the hypothalamus, the ventral thalamus, the interstitial nucleus of the flm, the mesencephalic tegmentum and particularly from the rhombencephalon. The rhombencephalic reticular formation projects extensively to the spinal cord, and the Mauthner cell and the vestibular nuclear complex also innervate the spinal cord from early stages and these projections are very conscupicuous throughout development (Figs. 3a-c). The more medially located reticular neurons may correspond to the raphespinal projection at these stages. Oval-shaped cells medial to the CA-positive neurons of the locus coeruleus also project to the spinal cord (Fig. 1A-d).

At the end of the embryonic period (from stages 43 to 45), a number of new cell groups projects to the spinal cord, and the number of labeled neurons in the cell groups already mentioned increases. The spinal projection neurons of these cell

groups are more mature, with numerous thin and long dendritic ramifications. The most extensive projection to the spi-

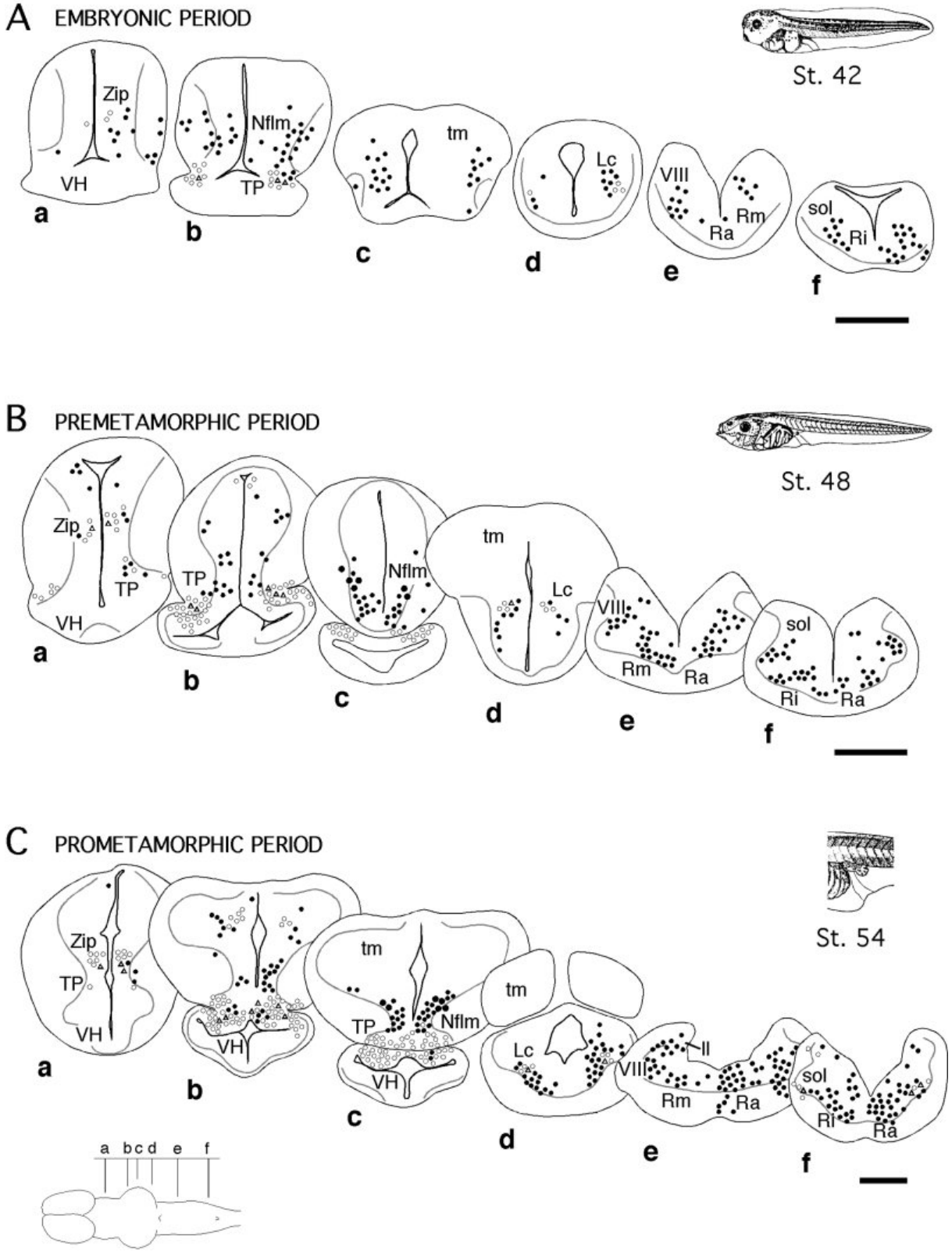

Fig. 1. Schematic drawings of transverse sections through the brain of *Xenopus laevis* at different periods through development (A, stage 42; B, stage 48; C, stage 54) illustrating: the distribution of retrogradely labeled cells after tracer applications into the spinal cord (black dots), the localization of catecholaminergic cells (open dots) as revealed by TH-immunohistochemistry, and double labeled cells (triangles). The approximate levels of the sections are indicated in a scheme of the brain in the lower left. Calibration bars = 20 μm.

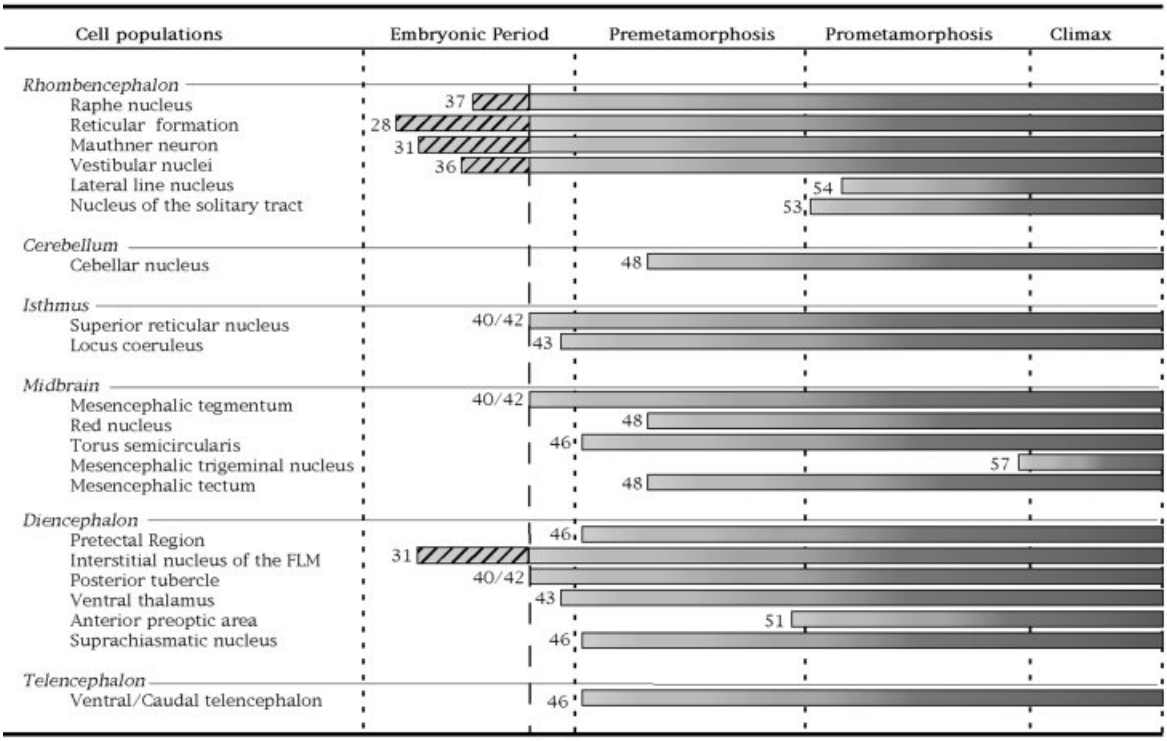

Fig. 2. Summary of the time of onset of descending supraspinal pathways in *Xenopus laevis*.

TABLE 2. Time of appearance of TH immunoreactivity and Catecholaminegic Projections to the Spinal Cord[1]

Catecholaminergic nuclei	Developmental stage[4]	
	TH immunoreactivity[2]	Spinal Cord projection[3]
Posterior Tubercle	39	40
Periventricular nucleus of the Zona Incerta	40-41	43
Locus coeruleus	41	43
Nucleus of the solitary tract	51	53

[1]TH, tyrosine hydroxylase.

[2]González et al., 1994; González and Smeets, 1994.

[3]Data obtained in the present study.

[4]Staging of the embryos and larvae according to Nieuwkoop and Faber (1967).

nal cord at this stage of development arises from the reticular formation throughout the entire rhombencephalon. Two different reticular groups can be distinguished at middle hindbrain levels: 1) large multipolar cells with a profuse dendritic arborization located in the ventral part of the reticular formation, and 2) dorsal to the first group, smaller monopolar round or oval cells with a main process directed ventrally. At rostral rhombencephalic levels the reticulospinal projection neurons are of the second cell type. At caudal levels, small round cells close to the midline represent the raphespinal connection. Numerous retrogradely labeled cells are found in the vestibular nuclear complex, occupying a dorsolateral position in the marginal zone of the rhombencephalic alar plate (Fig. 3b,c). The pair of Mauthner cells stands out at the level of the nucleus reticularis medius, lateral to this nucleus and ventral to the octaval nuclear complex (Fig. 3b). The Mauthner cells have a main dorsolateral dendritic branch extending into the octavolateral area with a profuse arborization, and a second branch directed ventromedially into the reticular formation (Fig. 3c). More rostrally, in the isthmic tegmentum, many cells in the superior reticular nucleus and some neurons in the dorsomedial part of the locus coeruleus project to the spinal cord (Fig. 3d). In the midbrain, numerous spinal projections arise from the mesencephalic tegmentum, and at caudal diencephalic levels from the distinctly labeled interstitial nucleus of the medial longitudinal fasciculus (Figs. 3a, 4a,b). Two cell types projecting to the spinal cord can be distinguished: 1) large neurons with a wide, ventrolaterally oriented, dendritic arborization located in the dorsal and lateral parts of the interstitial nucleus, and 2) small oval cells located ventrally and medially with only a main dendritic process. At this stage of development, the most rostrally located cells, giving rise to spinal projections, are found in the ventral thalamus and in the entire hypothalamus (Fig. 3e). In particular, neurons in the dorsal hypothalamus, presumably in the developing posterior tubercle, and also in the region of the periventricular nucleus of the zona incerta in the ventral thalamus project to the spinal cord.

Larval stages

Premetamorphic stages. During this first period of larval development, the ontogeny of descending supraspinal projections is characterized not only by a progressive maturation of already existing connections, but also by the appearance of

several new cell groups innervating the spinal cord. Moreover, the number of projection neurons increases, particularly in the

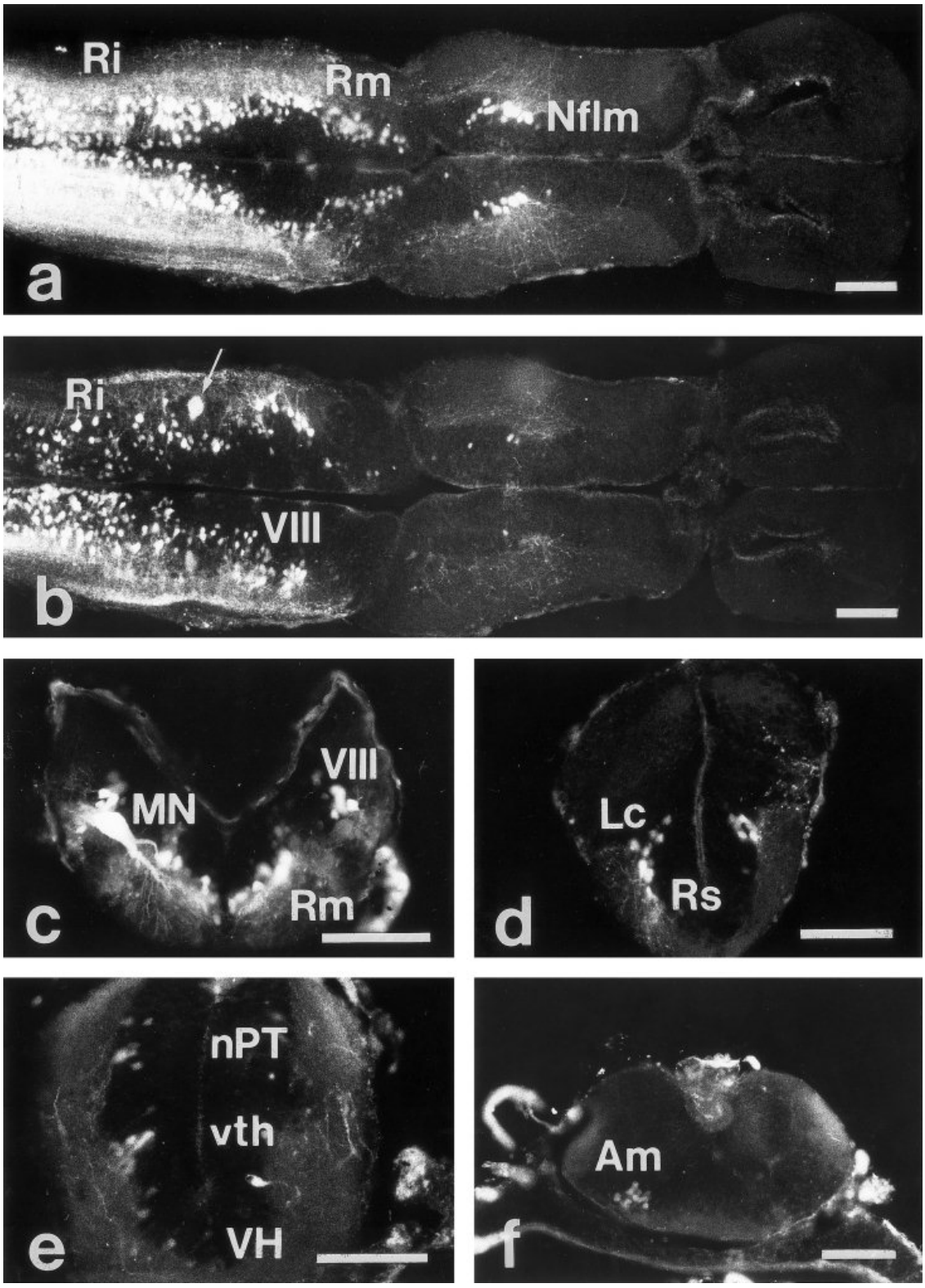

Fig. 3. Photomicrographs of horizontal (a,b) and transverse sections (c-f) through the brain of *Xenopus laevis* at different developmental stages during the embryonic and premetamorphic periods showing the distribution of retrogradely labeled cells after tracer applications into the spinal cord. **a**, Large labeled neurons in the middle and inferior reticular nuclei and the interstitial nucleus of the flm at stage 49 as seen in a horizontal section. **b**, Inferior reticular nucleus and octaval nuclear complex in a more dorsal horizontal section at stage 49. Arrow points to the Mauthner cell. **c**, Mauthner cell, vestibular nuclear complex and middle reticular nucleus at stage 48. **d**, Superior reticular nucleus and locus coeruleus at stage 47. **e**, Labeled neurons in the pretectal nucleus and the ventral thalamus at stage 46. **f**, Retrogradely labeled cells in the ventrocaudal telencephalon at stage 46. Calibration bars = 100 μm.

dorsal hypothalamus and the ventral thalamus (Fig. 1B). The retrogradely labeled cells are more mature: they are smaller in size, without vacuoles in their cytoplasm, round to oval in shape and have a profuse dendritic arborization.

At the beginning of premetamorphosis (stage 46), spinal projections from many neurons along the entire rostrocaudal extent of the dorsal hypothalamus and from a compact cell group in the ventrocaudal part of the telencephalon appear (Fig. 3f). The telencephalospinal projection arises in the region of the future amygdala, lateral to the preoptic area. At this stage of development, retrogradely labeled neurons spinal projections also arise in the suprachiasmatic nucleus, the torus semicircularis and the pretectal region (Fig. 4c). In the region of the locus coeruleus, the labeled cells can be better distinguished from the adjacent mesencephalic tegmentum and rhombencephalic reticular formation than in previous stages. At stage 48, cerebellospinal and tectospinal projections arise (Fig. 4d). Compared to late embryonic stages, the number of cells projecting to the spinal cord from the rostral part of the mesencephalic tegmentum, immediately caudal to the interstitial nucleus of the flm, is increased. Some of these contralaterally labeled cells may correspond to the nucleus ruber, clearly identifable in subsequent stages (Fig. 4e). Finally, at the end of the premetamorphic period, by stage 51, neurons scattered in the preoptic region project to the spinal cord.

Prometamorphic stages. The organization of descending pathways at the beginning of the prometamorphic period (stage 54), is almost similar to that of the adult brain, with easily discernable, separate and well-migrated cell groups (Fig. 1C). Retrogradely labeled neurons are present in the cerebellum, contralateral to the side of the tracer application. More caudally, two new cell groups, i.e. the lateral line nucleus and neurons around the solitary tract project to the spinal cord. At stage 57, the round, large cells of the mesencephalic trigeminal nucleus in the rostral pole of the mesencephalic tectum project to the spinal cord ipsilaterally. At the end of prometamorphosis, two separate cell populations in the octavolateral area project to the spinal cord: 1) a rostral group, consisting of small round cells located medially, in the lateral line nucleus, and 2) more caudally located cells, larger in size and with fusiform soma, primarily found ventrolaterally in the rhombencephalic alar plate.

Metamorphic climax. In this period of development no new cell groups project to the spinal cord. This period is characterized by an increase in the number of retrogradely labeled cells and the maturation of the descending projections. In the mesencephalic tectum, retrogradely labeled neurons are now found along its whole rostrocaudal extent, and the projection from the torus semicircularis is more abundant. By stage 59, the larger size of the spinal cord makes it possible to make unilateral tracer applications. The contralateral projection from the nucleus ruber is well developed, and clearly distinct from other retrogradely labeled neurons in the mesencephalic tegmentum (Fig. 4e). In the rhombencephalon, spinal projections from the entire octavolateral area arise from a compact ventrolateral cell group with fusiform large somata and extensive dendritic arborizations into the dorsal alar plate (Fig. 4f).

Development of TH immunoreactivity in the spinal cord

The distribution of catecholaminergic (CA) fiber systems was studied with TH-immunohistochemistry at rostral levels of the spinal cord during the development of *Xenopus*. The ontogenesis of the CA spinal innervation is characterized by a progressive maturation of THi fibers and terminal-like structures that increase in number and have a wider distribution in the spinal cord during progressive development. THi fibers are present in the spinal cord by late embryonic stages, and occupy primarily the laterodorsal aspect of the marginal zone (Fig. 5a). At the same time, a scattered population of THi cells is found ventral to the central canal (Fig. 5a).

The development of the TH-immunoreactivity in the spinal cord in the first part of the *premetamorphic period* (stages 46 to 48) is characterized by an increase in the number of the labeled fibers, and by a wider distribution in the marginal zone (Fig. 5a). The dorsolateral funiculus contains a strongly immunoreactive plexus of fibers, and scattered fibers and terminals are also distributed in the ventral white matter. At the end of premetamorphosis (stages 49-52) the spinal gray matter contains a few thin long fibers, mainly in its ventral field. THi fibers are thin with small varicosities and, leaving the funiculi, they progress into the spinal gray, mainly into the dorsal horn and the ventrolateral part of the ventral horn (Fig. 5b).

The *prometamorphic period* is marked by an increase of the CA innervation of the spinal gray matter, where it is possible to distinguish THi fibers in the dorsal, the intermediate and the ventral spinal fields. Numerous immunoreactive fibers occur in the white matter, particularly in the ventrolateral and the dorsolateral funiculi. During the *metamorphic climax,* the pattern of CA organization in the spinal cord is similar to that present in the adult. The spinal cord increases in size and complexity, and the CA innervation of the spinal gray matter achieves its final development. In juvenile stages (stage 65), a plexus of THi fibers can be distinguished along the border of the spinal cord almost identical to that observed in the adult cord. Abundant CA fibers are also distributed into the central field dorsal to the central canal.

Development of the descending catecholaminergic projections to the spinal cord

With a double-labeling procedure, the temporal sequence of the supraspinal CA innervation to the spinal cord is described and compared with the onset of the CA cell groups during development in *Xenopus* (González et al., 1994a,b; see Fig. 1, and Table II). In line with a previous study in the adult brain (Sánchez-Camacho et al., 2001b), during development four brain centers contain double labeled neurons: the posterior tubercle in the mamillary region (Figs. 6, 7, 8a), the periventricular nucleus of the zona incerta in the ventral thalamus (Fig. 8b), the locus coeruleus (Fig. 8c) and the nucleus of the solitary tract (Fig. 8d).

After tracer applications into the spinal cord at *late embryonic stages*, scattered retrogradely labeled cells are present in the dorsal hypothalamus. At these stages, the dopaminergic cells of the posterior tubercle are located close to the dorsal infundibulum. At stage 40/41, the combination of tracing with TH immunohistochemistry reveals the first double labeled neurons in the posterior tubercle (Fig. 6a,b). At about the same time, neurons projecting to the spinal cord are also present in the ventral thalamus and the region of the locus coeruleus. However, these cell groups contain no double labeled cells, although weakly THi neurons are already present in these nuclei as early as stage 41. Slightly later, at stage 43, the periventricular nucleus of the zona incerta (Fig. 8b) and the locus coeruleus (Fig. 8c) contain a few double labeled neurons. Noradrenergic cells in the locus coeruleus are always only weakly TH-immunoreactive and, therefore, double labeled cells are difficult to distinguish.

During *premetamorphosis*, in the previously described CA cell groups the number of THi cells increases. From the beginning of this period, double labeled neurons are found along the

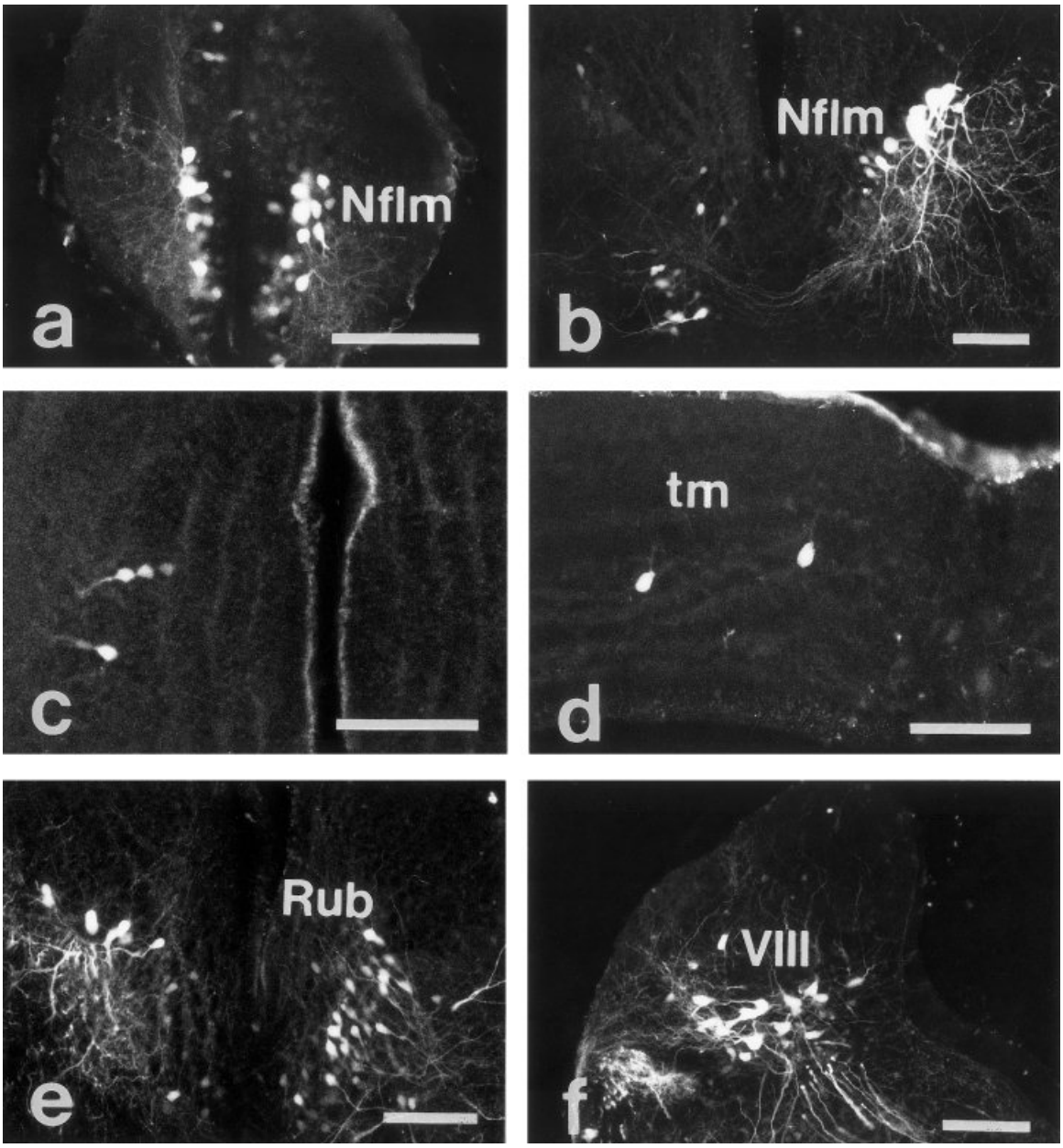

Fig. 4. Photomicrographs of transverse sections through the brain of *Xenopus laevis* at different developmental stages illustrating the labeling after tracer applications into the spinal cord. **a**, Interstitial nucleus of the flm during premetamorphosis (stage 48). **b**, At the beginning of the metamorphic climax (stage 59), two types of labeled cells can be clearly distinguished in the interstitial nucleus of the flm: small medially found and large laterally located neurons. **c**, Labeled neurons in the pretectal region at stage 54 corresponding to level a in Figure 1C. **d**, Retrogradely labeled cells in the mesencephalic tectum at stage 59. **e**, Neurons of the red nucleus projecting to the spinal cord at stage 59. **f**, During the metamorphic climax, the organization of the vestibular nuclear complex in the alar plate is similar to that found in the adult brain (stage 65). Calibration bars = 100 μm.

entire rostrocaudal extent of the posterior tubercle (Fig. 6c,d). At the end of the premetamorphic period (stage 51), TH-immunohistochemistry reveals CA cells in the nucleus of the solitary tract. However, double labeled neurons do not appear before stage 53, i.e. at the beginning of prometamorphosis (Fig. 8d).

During *prometamorphic stages*, the pattern of catecholaminergic projections to the spinal cord observed is already identical to that found in the adult brain (Sánchez-Camacho et al., 2001b; see Fig. 7). The number of retrogradely labeled neurons observed in the ventral thalamus increases. They are located lateral to the THi cells of the periventricular nucleus of the zona incerta (stage 54).

DISCUSSION

In the present study, the development of descending supraspinal pathways, of catecholaminergic (CA) input in particular, is described in the anuran *Xenopus laevis* from late embryonic stages up to the juvenile (stages 40 to 65).

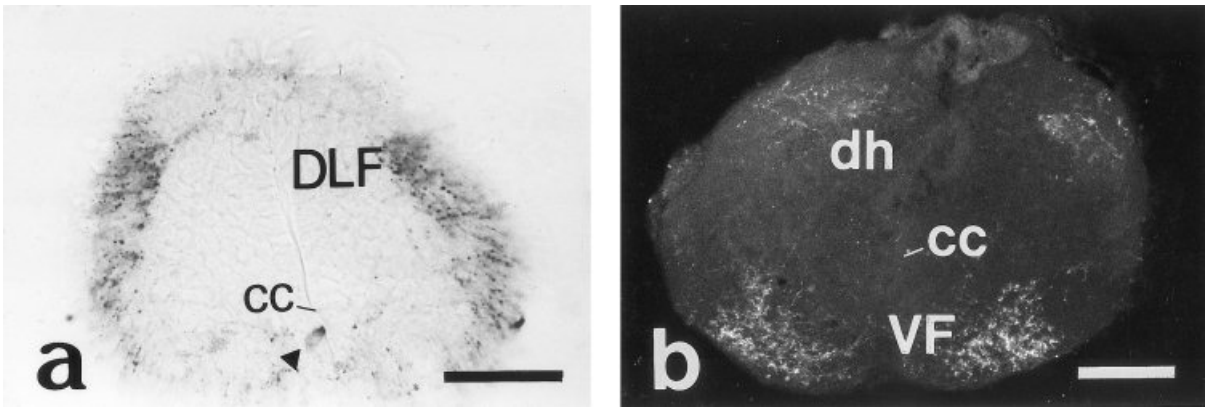

Fig. 5. Photomicrographs of transverse sections through the rostral spinal cord of *Xenopus laevis* showing TH immunoreactive cells and fibers during premetamorphosis at stages 46 (**a**) and 51 (**b**). The arrowhead in **a** points to cells beneath the central canal. Calibration bars = 50 μm (a) and 100 μm (b).

Development of descending supraspinal pathways to the spinal cord

The observed developmental sequence of descending pathways corroborates previous studies but, given the sensitivity of the used dextran amines as retrograde tracers, spinal projections from rostral areas such as the hypothalamus, the preoptic area and the caudal telencephalic hemisphere are described. Van Mier and ten Donkelaar (1984) suggested that, in general, the developmental organization of the supraspinal input to the spinal cord in *Xenopus* is characterized by a temporal, caudorostral sequence. Our data show that although this may be true for the brainstem, the reticular formation in particular, projections from the hypothalamus and the ventral thalamus preceed cerebellospinal and tectospinal projections. Rather than a caudorostral temporal sequence of development, we observe a ventral to dorsal (or basal to alar) pattern in the time of appearance of cells projecting to the spinal cord within each main brain subdivision. Thus, for example in the rhombencephalon, axons from basal groups such as the reticular formation or raphe nuclei reach the spinal cord before those arising from cells in the alar plate as the vestibular nuclei or the nucleus of the solitary tract. Such a ventrodorsal sequence of emergence of descending projection neurons is also apparent when one compares reticulospinal and vestibulospinal projections in zebrafish (Mendelson, 1986a,b), goldfish (Sharma and Berthoud, 1992), chick (Okado and Oppenheim, 1985), opossums (Cabana and Martin, 1982, 1984; Wang et al., 1992; Martin et al., 1993), and rats (Auclair et al., 1993; Kudo et al., 1993).

In zebrafish, a segmental organization of reticulospinal and vestibulospinal projections is evident (Kimmel et al., 1982; Mendelson, 1986a,b; Suwa et al., 1996). Although in *Xenopus* embryos it is easy to recognize the neuromeric limits in the hindbrain (Hartenstein, 1993), the appearance of reticulospinal neurons is not restricted to a certain segmental domain. Instead, a rather compact group continuous across several rhombomeres is found (Roberts and Clarke, 1982; van Mier and ten Donkelaar, 1984; Nordlander et al., 1985; Straka et al., 2001a,b; present study). Vestibulomotor neurons, however, are segmentally arranged, with the major clusters of vestibulospinal neurons located in the ipsilateral rhombomere 4 and the contralateral rhombomere 5 (Straka et al., 2001a,b). Similar observations were made in chickens (Díaz et al., 1998; Díaz and Glover, 2001).

Serotonergic cells in the raphe nucleus develop from stage 25 onwards along a rostrocaudal sequence, and raphespinal fibers reach the rostral spinal cord at stage 32 (van Mier et al., 1986). In our study, the cells of origin of raphespinal projections are restricted to the caudal part of the raphe nucleus, in line with tracer data in adult ranid frogs (Tan and Miletic, 1990).

In *Xenopus*, no labeled cells were found in the diencephalon after HRP applications to the spinal cord of premetamorphic tadpoles. Only at stages just before metamorphic climax (57/58), occasionally labeled cells were observed in the ventral thalamus and the periventricular hypothalamic nucleus (ten Donkelaar and de Boer van Huizen, 1982). With dextran amines we show that diencephalic projections to the spinal cord arise in late embryonic stages. The differences observed in the presence and time of origin of diencephalospinal pathways between our and previous studies are most likely due to the sensitivity of the tracer techniques used. Thus, the *in vitro* retrograde transport of dextran amines is more sensitive than the *in vivo* transport of HRP as we previously demonstrated (Muñoz et al., 1996; Marín et al., 1997). The early presence of hypothalamospinal pathways preceeds changes in locomotor pattern and, therefore, these projections may, apart from a role in the control of autonomic functions, be involved in the transformation of locomotion from the embryonic to the juvenile pattern.

The telencephalospinal projection is rather conspicuous during larval development, and is apparently reduced during metamorphosis, since in adults only a few scattered cells in the ventrolateral part of the hemisphere project to the spinal cord (Sánchez-Camacho et al., 2001a). This suggests the presence of transient projections to the spinal cord during development. In developing chicken embryo, Okado and Oppenheim (1985) showed the existence of transient projections to the spinal cord arising in suprachiasmatic regions and in the lateral hypothalamus. Both of these cell groups do not project to the spinal cord in newly hatched chickens.

In general, descending supraspinal projections in *Xenopus* embryos and larvae develop according to a pattern common to a wide variety of vertebrates ranging from fish to mammals (for review see ten Donkelaar, 2000). In all species studied, descending supraspinal projections are present throughout the spinal cord at early stages. The observation that descending supraspinal input in vertebrates occurs at stages when target cells in the spinal cord are relatively immature raises the pos-

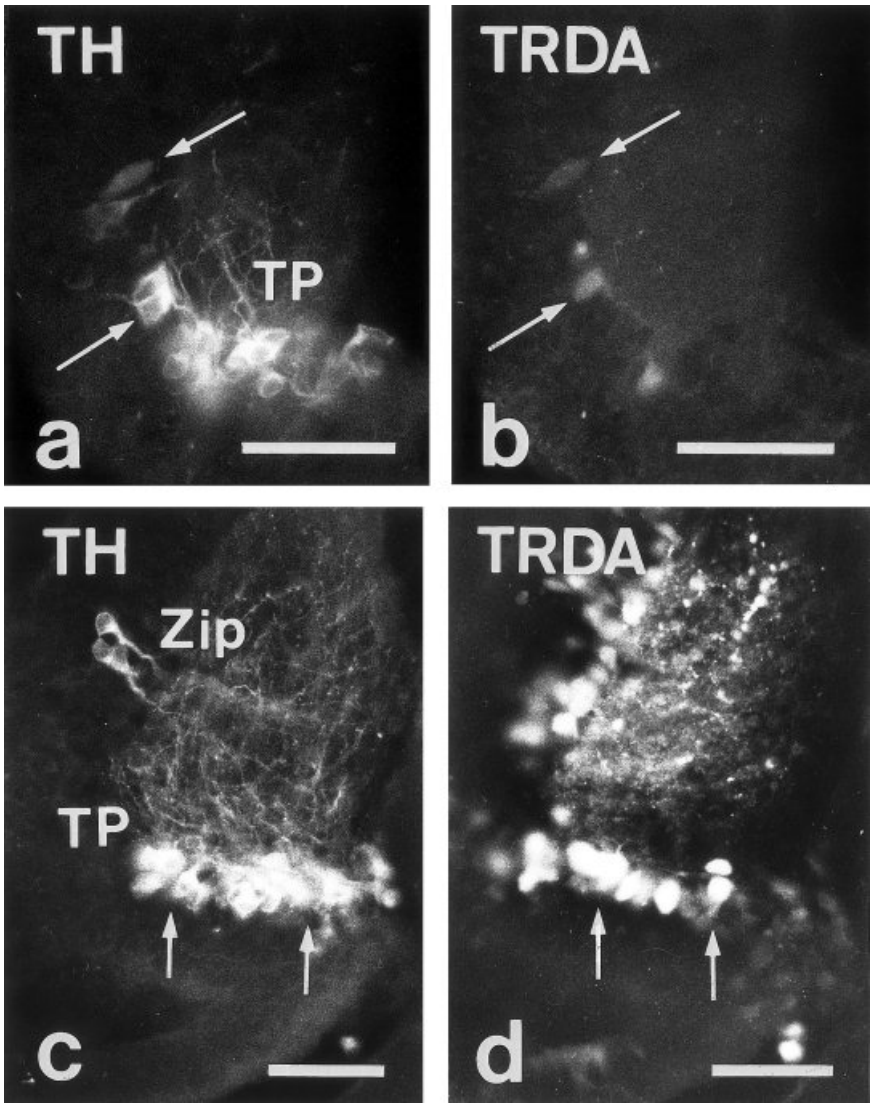

Fig. 6. Photomicrographs of transverse sections through the brain of *Xenopus laevis* showing the localization of THi cells (**a,c**) and retrograde labeled cells (**b,d**) in the posterior tubercle after tracer applications in the spinal cord at the end of the embryonic period (stage 44, a,b) and the beginning of the premetamorphic period (stage 48, c,d). Arrows indicate double labeled cells. Calibration bars = 50 μm.

sibility that these projections may mediate important cellular interactions involved in spinal cord neurogenesis as well as mediate early functional interactions involving synaptic transmission between brain and spinal cord (Okado and Oppenheim, 1985).

Development of TH immunoreactivity in the spinal cord

The presence of an abundant CA innervation throughout the spinal cord in adult amphibians is a common feature shared by all vertebrates (Smeets and González, 2000; Sánchez-Camacho et al., 2001b). The pattern of CA innervation of the spinal cord during development is characterized first by the gradual distribution of THi fibers in the marginal zone within the white matter which is followed by a progressive invasion of the spinal gray matter.

It should be taken into account that throughout late embryonic and larval stages a population of THi cells is located just ventral to the central canal of the spinal cord (González et al., 1994a,b; Heathcote and Chen, 1993, 1994; the present study). However, the pattern of development of cell processes from the somata beneath the central canal strongly supports the notion that most of the TH-immunoreactivity observed in the spinal cord is of supraspinal origin, as it is in the adult (Heathcote and Chen, 1994; Sánchez-Camacho et al., 2001b). In line with our data in amphibians, a catecholaminergic innervation is also present very early in development in the spinal cord of other vertebrate classes (Singer et al., 1980; Commissiong, 1983a,b; Pindzola et al., 1990; Ekström et al., 1992;

Rajaofetra et al., 1992; Medina et al., 1994; Smeets and González, 2000).

Although little is known about the functional significance of catecholamines in the amphibian spinal cord, the localization of THi fibers in the dorsal horn, intermediate gray and ventral horn suggests an involvement in nociception, autonomic functions and motor control (see Smeets and González, 2000; Sánchez-Camacho et al., 2001b). The early appearance of CA fibers in the spinal cord indicates that catecholamines

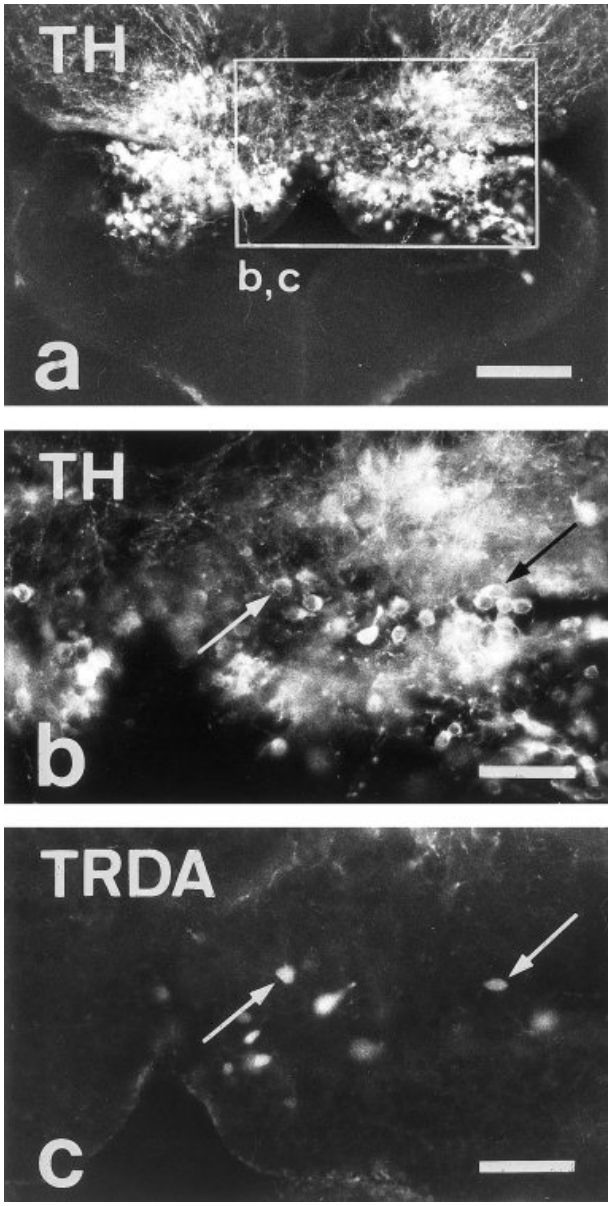

Fig. 7. Transverse sections through the brain of *Xenopus laevis* showing the localization of THi cells (**a,b**) and retrograde labeled cells (**c**) in the posterior tubercle after spinal tracer applications at the beginning of the prometamorphic period (stage 54). Arrows point to double labeled cells. Calibration bars = 100 μm (a) and 50 μm (b,c).

may also play a significant role during development, at least in early locomotor behavior or, as it has been hypothesized for monoamines in mammals (Haydon et al., 1984, 1985; König et al., 1986), as trophic factors implicated in the neurogenesis, differentiation, migration and maturation of spinal neurons.

Development of the descending CA projections to the spinal cord

Recently, we showed that only four brain centers contribute the bulk of the supraspinal CA innervation of the adult spinal cord in amphibians: the posterior tubercle in the mamillary region, the periventricular nucleus of the zona incerta in the ventral thalamus, the locus coeruleus, and the nucleus of the solitary tract (Sánchez-Camacho et al., 2001b). The onset of the CA descending projections in *Xenopus* embryos and larvae follows a rostrocaudal sequence. CA projections from the posterior tubercle, the periventricular nucleus of the zona incerta and the locus coeruleus, reach the spinal cord by the end of the embryonic period, whereas spinal projections from the nucleus of the solitary tract do not arise before the beginning of the prometamorphic period.

Immunohistochemical studies in anurans and urodeles showed a similar sequence in the appearance of THi cell groups through species (González et al., 1994a,b; 1995). In *Xenopus*, cells in the posterior tubercle are TH/DA immunoreactive at embryonic stage 39, soon followed by the "accompanying cell group of the periventricular organ", which is now regarded as the periventricular nucleus of the zona incerta (stage 40/41) and the locus coeruleus (stage 41). CA cells in the nucleus of the solitary tract develop later at the end of the premetamorphic period (stage 51). The comparison of these data with the time when the first CA projections to the spinal cord are detected in our study suggests that TH immunoreactivity develops first in the CA neurons innervating the spinal cord, immediately followed by the outgrowth of descending CA projections to the spinal cord (see Table II). A recent study on the development of the CA input to the basal ganglia in *Xenopus* (Marín et al., 1997) suggests that a rostrocaudal sequence also exists in the time of appearance of the CA innervation of the basal forebrain.

Comparison with mammals. Analysis of the ingrowth of CA fibers into the spinal cord of the North American opossum with TH immunohistochemistry (Pindzola et al., 1990) revealed many similarities with the development of the spinal CA innervation observed in amphibians. At birth, THi axons are present throughout the spinal cord, particularly in the dorsolateral marginal zone, by postnatal day 3 (PD3) in the intermediate zone, and by PD8, they are mostly concentrated in the intermediolateral cell column. However, lamina I and II of the dorsal horn are not innervated until PD15. These results suggest that THi axons grow into the gray matter following a rostral to caudal temporal sequence during development, in line with data by Rajaofetra et al. (1992) on the noradrenergic innervation in the rat spinal cord. As in amphibians, a delay exists between the arrival of supraspinal fibers in the spinal white matter and the penetration of the gray matter.

So far, in amniotes, only the study by Pindzola et al. (1990) in the North American opossum, *Didelphis virginiana*, dealt with the development of CA projections to the lumbosacral spinal cord by means of double labeling techniques. After tracer applications into the spinal cord, they found, among others, double labeled neurons by postnatal day 5 (PD5) in the nucleus paraventricularis hypothalami and the area hypothalamica lateralis, the nucleus coeruleus and the ventrolateral medulla, which also project to the lumbar cord in adult opossum (Pindzola et al., 1988). It is important to note that, in this study, all nuclei mentioned above project to the lumbar spinal cord on the same day and, therefore, projections to rostral

spinal levels are expected to develop much earlier and differences may be found in the time of appearance of the projections from the various nuclei. Comparison with our data in amphibians suggests that the CA projections found in opossums are comparable to those found from the posterior tubercle in the hypothalamus, the locus coeruleus and the nucleus of the solitary tract (caudal medulla) in anurans, respectively. CA projections from the ventral thalamus, comparable to that from the periventricular nucleus of the zona incerta demonstrated in amphibians are not observed in the opossum.

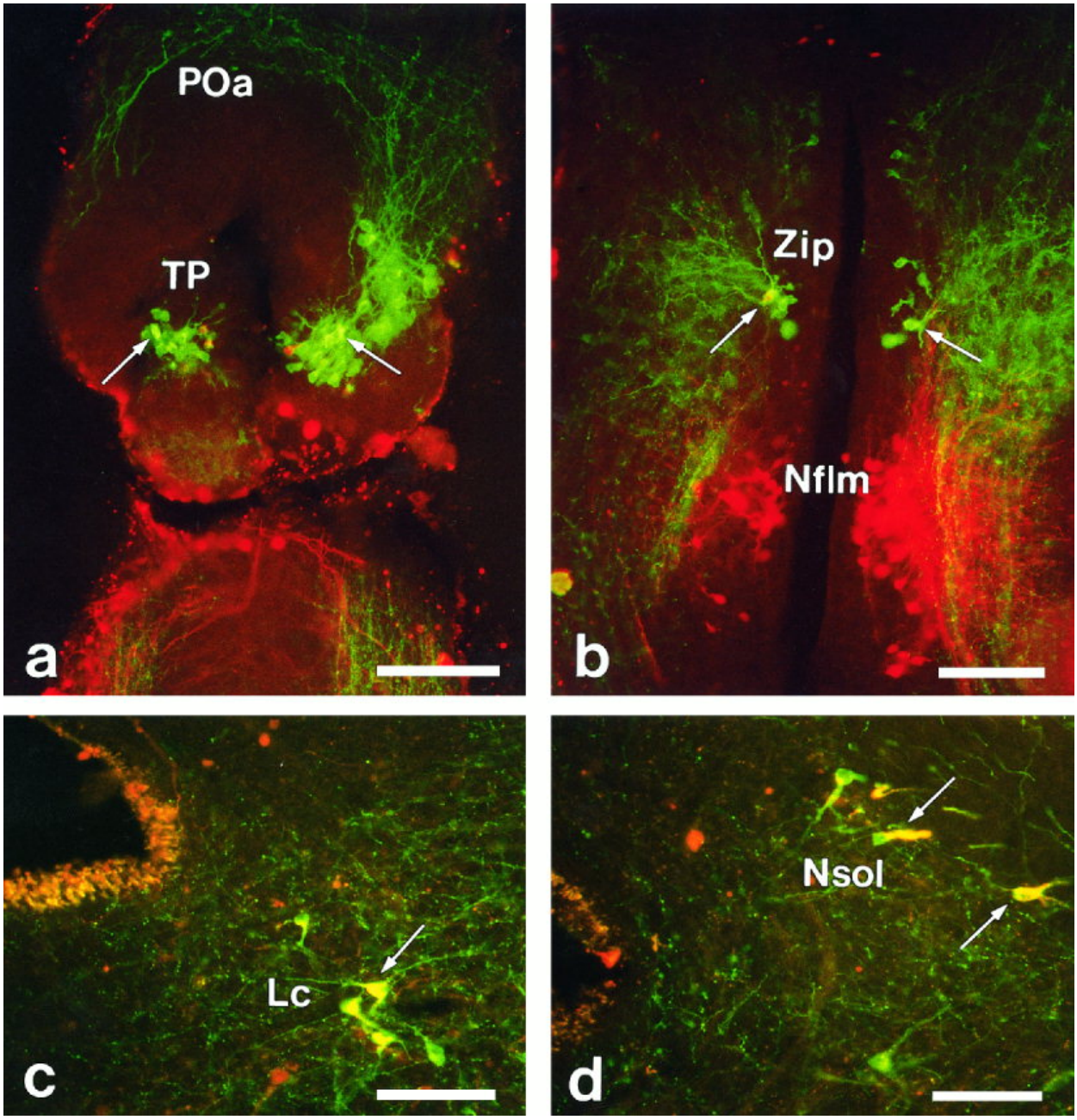

Fig. 8.- Horizontal (a,b) and transverse (c,d) sections through the brain of *Xenopus laevis* showing the localization of THi cells (green) and retrograde labeled cells (red) in the posterior tubercle (**a**) and the periventricular nucleus of the zona incerta (**b**) at stage 51 after tracer application in the spinal cord. **c** and **d** show double labeled neurons in the locus coeruleus (**c**) and in the nucleus of the solitary tract (**d**) close to the metamorphic climax (stages 65/66). Arrows point to double labeled cells. Calibration bars = 100 μm.

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Origin and development of descending catecholaminergic pathways to the spinal cord in amphibians

C. Sánchez-Camacho[1], O. Marín[2], J. M. López[1], N. Moreno[1], W.J.A.J. Smeets[3], H.J. ten Donkelaar[4] and A. González[1]

[1]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense of Madrid, Spain; [2]Department of Psychiatry and Langley Porter Psychiatric Institute, Nina Ireland Laboratory of Developmental Neurobiology, University of California San Francisco, USA; [3]Department of Anatomy and Embryology, Vrije Universiteit, Amsterdam, The Netherlands; and [4]Department of Neurology, University Medical Center Nijmegen, The Netherlands

ABSTRACT: The origin and development of the supraspinal catecholaminergic (CA) innervation of the spinal cord was studied in representative species of the three amphibian orders (Anura: *Xenopus laevis* and *Rana perezi*; Urodela: *Pleurodeles waltl*; Gymnophiona: *Dermophis mexicanus*). Using retrograde dextran amine tracing in combination with tyrosine hydroxylase (TH)-immunohistochemistry, we showed that only four brain centers contribute to the CA innervation of the adult spinal cord: 1) the ventrolateral component of the posterior tubercle; 2) the periventricular nucleus of the zona incerta; 3) the locus coeruleus, and 4) the nucleus of the solitary tract (except for gymnophionans). The pattern observed is largely similar in all amphibian species studied. The development of the CA innervation of the spinal cord was studied with *in vitro* double labeling methods in *Xenopus laevis* tadpoles. At stage 40/41, the first CA neurons projecting to the spinal cord were found to arise in the posterior tubercle. At stage 43, spinal projections were found from the periventricular nucleus of the zona incerta and the locus coeruleus, whereas spinal projections from the nucleus of the solitary tract were not observed before stage 53. These results demonstrate a temporal sequence in the appearance of the CA cell groups projecting to the anuran spinal cord, organized along a rostrocaudal gradient.

KEY WORDS: Posterior tubercle, Zona incerta, Locus coeruleus, Nucleus of the solitary tract, Retrograde tracing, Tyrosine hydroxylase

INTRODUCTION

In amphibians, the spinal cord is abundantly innervated by catecholaminergic (CA) fibers and terminals. However, because of the presence of CA cells within the spinal cord, it is unclear to what extent this intraspinal CA innervation prevails over that of supraspinal origin. Using highly sensitive retrograde dextran amine tracing in combination with immunohistofluorescence against tyrosine hydroxylase (TH), we identified the neuron populations that provide the CA innervation to the spinal cord [17]. Only four CA cell groups appear to project to the spinal cord of amphibians, i. e. the ventrolateral component of the posterior tubercle in the mamillary region, the periventricular nucleus of the zona incerta in the ventral thalamus, the locus coeruleus and the nucleus of the solitary tract. In anuran as well as in urodele amphibians CA fibers innervate the spinal cord from embryonic stages onwards [4,5]. The early presence of CA innervation may play an important role in organizing the development of the spinal cord and may directly influence the maturation of spinal neurons [18]. However, in amphibians, the origin and subsequent organization of this CA innervation during embryonic and larval stages is largely unknown, more in particular since data on the formation of these connections are not avaliable. Therefore, we studied the development of the supraspinal centers that provide the CA innervation of the amphibian spinal cord in the South African clawed toad, *Xenopus laevis*.

We will discuss the following data: 1) the distribution of tyrosine hydroxylase-immunoreactivity in the adult spinal cord in two anurans (the clawed toad, *Xenopus laevis* and the green frog, *Rana perezi*), a urodele (the Iberian ribbed newt, *Pleurodeles waltl*), and a gymnophionan (the Mexican caecilian, *Dermophis mexicanus*); 2) the cells of origin of supraspinal CA projections in these species, and 3) the development of TH immunoreactivity in the spinal cord and the temporal sequence in the appearance of the cells of origin of supraspinal CA projections in *Xenopus laevis*.

MATERIALS AND METHODS

For the present study, representative adult specimens of the three amphibian orders (Anura: *Xenopus laevis* and *Rana perezi*; Urodela: *Pleurodeles waltl*; Gymnophiona: *Dermophis mexicanus*) were used. In all experiments, the animals were deeply anesthetized by immersion in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz). In double labeling experiments, the tracer 10 kD Texas Red-conjugated dextran amine (TRDA; Molecular Probes, Oregon), recrystallized from distilled water onto sharp tungsten needles, was applied unilaterally into the rostral spinal cord. After survival times of 7-14 days, the animals were reanesthetized and perfused transcardially with saline, followed by a mixture

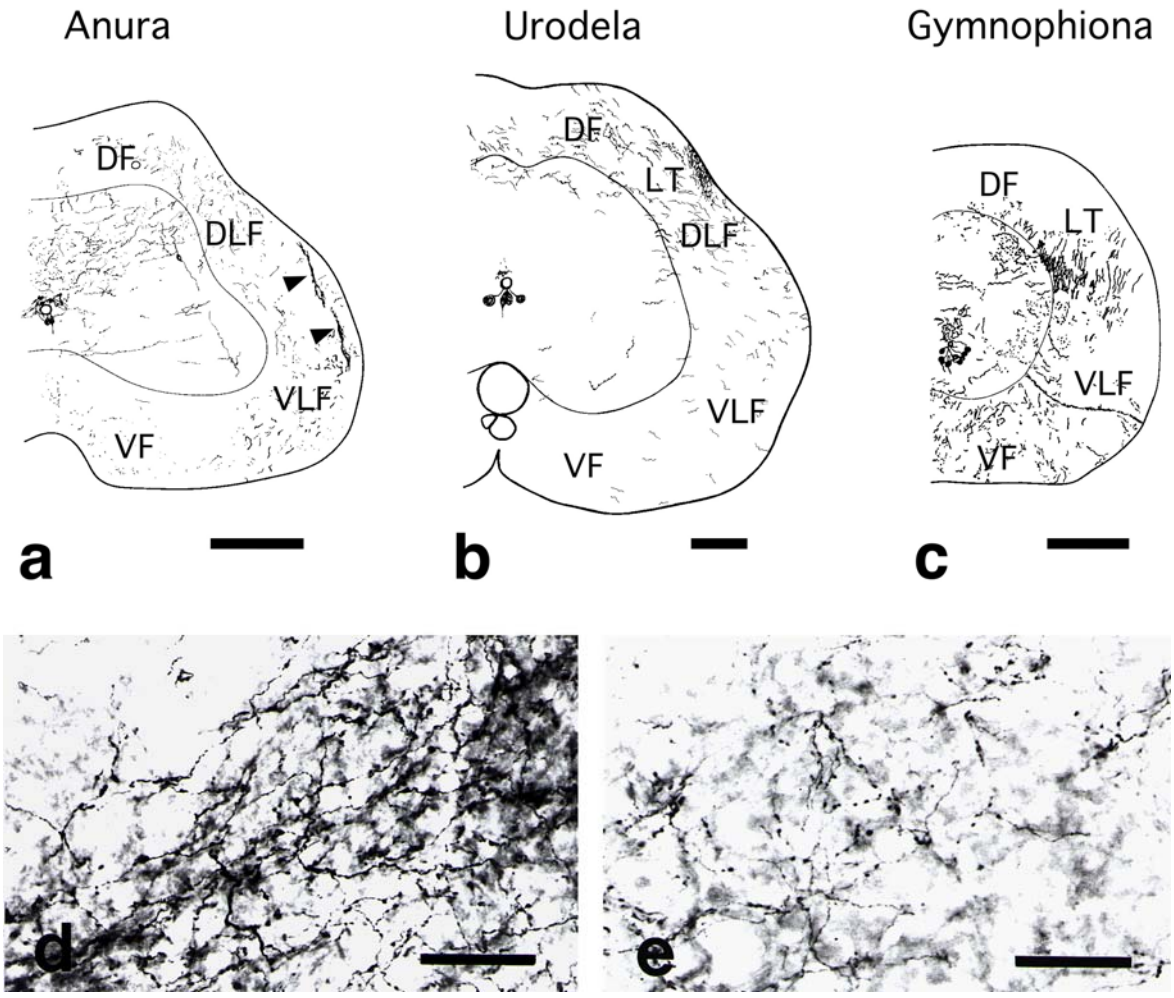

FIG. 1. Diagrams of transverse hemisections through the spinal cord of *Rana perezi* (**a**), *Pleurodeles waltl* (**b**) and *Dermophis mexicanus* (**c**) showing the distribution of TH immunoreactive cells and fibers at rostral spinal levels. Arrowheads in the spinal cord of *R. perezi* point to the dense peripheral CA plexus. Photomicrographs of transverse sections (**d, e**) through the spinal cord of *Rana perezi* showing TH immunoreactive fibers. **d**, Abundant varicose THi fibers in the dorsolateral field at brachial spinal levels. **e**, Thin, varicose THi fibers in the ventral field at brachial spinal levels. Calibration bars= 200 μm (a), 100 μm (b, c) and 50 μm (d, e). Abbreviations: DF, dorsal funiculus; DLF, dorsolateral funiculus; LT, Lissauer´s tract; VF, ventral funiculus; VLF, ventrolateral funiculus.

of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After 2 h of postfixation, the brains and spinal cords were immersed in PB containing 30% sucrose for 3-5 h at 4°C, embedded in a solution of 15% gelatin with 30% sucrose, and stored for 5 h in a 4% formaldehyde solution at 4°C. The brains were cut in the frontal or sagittal plane at 40 μm on a freezing microtome. The sections were first incubated for 48 hours at 4°C with a mouse anti-TH antibody (Incstar) diluted 1:1000 and, subsequently, with a FITC-conjugated mouse-IgG complex (Incstar) diluted 1:150 for 90 min at room temperature. The sections were mounted and coverslipped with Vectashield (Vector Labs., Burlingame).

In a second set of experiments, a total of 63 *Xenopus laevis* embryos and larvae, ranging from developmental stages 40 to 65 [13], were used. In all experiments, animals were processed for tracing experiments by using 3 kD TRDA (Molecular Probes) applications into the developing spinal cord under *in vitro* conditions, as previously described [8,10]. Visualization of TRDA was combined with indirect immunofluorescence for TH as described for the adult brain.

Additionally, some sections were processed for TH immunohistochemistry to reveal the presence of catecholaminergic neurons and fibers in the adult and developing spinal cords following the PAP method. For more details the reader is referred to previous studies [1,4,16,17]. All experiments were carried out under animal care guidelines established by the Spanish Royal Decree 223/1988.

RESULTS

TH-Immunoreactivity in the Spinal Cord of Amphibians

The distribution of CA cells and fibers in the spinal cord was studied at brachial, thoracic and lumbar levels of the spinal cord of anurans and urodeles, and in rostral and caudal sections of gymnophionans. In all species studied an abundant CA innervation was found throughout the spinal cord (Fig. 1). The lateral portion of the dorsal and dorsolateral funiculi was the most densely innervated at brachial levels in all species studied, whereas in the ventrolateral and ventral funiculi less

abundant TH immunoreactive (THi) fibers were observed (Fig. 1a-c). Notable differences were found in the number and morphology of immunoreactive fibers among the species studied. In anurans, THi fibers were longer and thinner than those of urodeles and apodans. Moreover, whereas in anurans THi fibers extensively innervate neurons in the dorsal and intermediate spinal gray fields (Fig. 1d), in urodeles and apodans they remain mainly in the white matter, just superficial to the gray matter. A characteristic feature of anurans is the presence of a dense plexus of thick THi fibers along the border of the lateral funiculus throughout the spinal cord (Fig. 1a). Within the gray matter, numerous THi fibers were observed mainly in the dorsal and lateral fields, and also in the central field dorsal to the central canal (Fig. 1a-c). Only a few fibers were observed in the ventrolateral and ventromedial motor fields in anurans (Fig. 1e). A peculiar feature in *Pleurodeles* and *Dermophis* is the presence of thin, varicose fibers that outline the profiles of large neurons in the ventral horn. Additionally, a distinct plexus of THi fibers was found in the Lissauer's tract within the dorsolateral funiculus at all spinal segments studied in urodeles and apodans (Fig. 1b, c). In all species studied, liquor-contacting THi neurons were observed ventral to the central canal throughout the spinal cord. Additionally, but only in anurans, isolated bipolar CA neurons were found in the dorsolateral gray field at brachial levels.

Origin of Descending Catecholaminergic Projections to the Spinal Cord

Only four brain centers contribute to the supraspinal CA innervation of the spinal cord in amphibians: the posterior tubercle, the periventricular nucleus of the zona incerta, the locus coeruleus and the nucleus of the solitary tract (Fig. 2).

Posterior tubercle. In *Rana* as well as in *Pleurodeles* and *Dermophis*, two separate populations of dopaminergic cell bodies can be distinguished within this nucleus, i.e. a dorsomedial and a ventrolateral group. In *Xenopus*, however, no separate parts of the posterior tubercle could be recognized. Following unilateral applications of dextran amines into the spinal cord of *Rana perezi*, labeled cells were found almost exclusively in the ventrolateral component of the posterior tubercle. Double labeled cells were present throughout the rostrocaudal extent of the posterior tubercle (Fig. 2b). In *Xenopus*, numerous retrogradely labeled neurons were also found throughout the entire extent of the posterior tubercle. A large number of these retrograde labeled cells were also THi. In *Pleurodeles* and *Dermophis*, double labeled cells were found ipsilaterally in the ventrolateral part of the nucleus, mainly at rostral levels.

Periventricular nucleus of the zona incerta. In *Rana* and *Xenopus*, the CA neurons of this nucleus were disposed as layers in the ventral thalamus at rostral levels, whereas more caudally, THi neurons were located dorsal to the rostral portion of the posterior tubercle. In *Pleurodeles* as in *Dermophis*, this nucleus is formed by a small, compact periventricular group of large CA neurons. Unilateral spinal applications of retrograde tracers in *Rana*, resulted in labeling of small, round projection neurons that are scattered in the ventral thalamus, mainly ipsilaterally. A different type of retrogradely labeled cells, larger in size and pear-shaped, was found to be THi in the periventricular nucleus of the zona incerta (Fig. 2a). Similar observations were made in *Xenopus*. As in anurans, a small ipsilateral spinal projection originates from neurons in the dorsal part of the ventral thalamus in *Pleurodeles* and *Dermophis*, but only a few double labeled cells were found more ventrally in this nucleus (Fig. 2e, f).

Locus coeruleus. Whereas in anurans, the locus coeruleus extends along the entire isthmic segment, in urodeles and gymnophionans, this CA cell group is located only at caudal isthmic levels forming a compact group close to the fourth ventricle. After unilateral TRDA applications to the spinal cord of all species studied, numerous labeled cells were found in the isthmic region and, more abundantly, in the reticular formation. Remarkably, in anurans, only a few double labeled neurons were found in the rostral part of this CA cell group (Fig. 2c). In *Pleurodeles*, also a few double labeled neurons were observed bilaterally in the caudal part of the locus coeruleus. In the gymnophionan locus coeruleus, double labeled cells were found bilaterally.

Nucleus of the solitary tract. In anurans, the nucleus of the solitary tract is formed rostrally by large, multipolar THi neurons located ventral to the solitary tract, but more caudally, medium-sized and small THi neurons surround the tract. In the urodele and apodan brains studied, this nucleus is formed by a compact periventricular column of cells adjacent to the medial boundary of the solitary tract, and no different cell types could be distinguished. After spinal tracer applications in anurans, numerous retrogradely labeled cells were observed in the nucleus of the solitary tract, particularly contralaterally. Numerous double labeled cells were present bilaterally from rostral levels of the nucleus to levels immediately rostral to the obex (Fig. 2d). In *Pleurodeles*, numerous retrogradely labeled neurons were present along the entire extent of the nucleus, mainly contralaterally. Double labeled cells were observed mainly in the rostral portion of the nucleus. In *Dermophis*, however, no double labeled neurons could be identified in this nucleus, although a few retrogradely labeled cells were observed around the caudal part of the solitary tract.

Development of the Catecholaminergic Innervation in the Spinal Cord

A progressive maturation of the spinal CA innervation and an increase in the number of THi fibers and terminals were found during development. At *late embryonic stages* 40-41, THi cell bodies were already located ventral to the central canal and scattered short THi fibers with thick varicosities occupied the marginal zone of the spinal cord in the dorsolateral funiculus. During *premetamorphosis* (stages 46-52), the number of the labeled fibers increased with a wider distribution in the marginal zone. A strongly immunoreactive plexus of THi fibers was observed in the dorsolateral funiculus, and by the end of this period, a few scattered thin, long fibers and terminals were distributed into the spinal gray matter, mainly in its ventral field. In this period, THi fibers were more mature, thinner and longer with small varicosities as compared to those in the embryonic stages. The *prometamorphic period* (stages 53-58) was marked by an increase in the CA innervation of the spinal gray matter, where it was possible to distinguish THi fibers in the dorsal and ventral fields. The CA innervation of the spinal cord was complete at the beginning of the *climax of the metamorphosis* (stages 59-66).

Development of the Descending Catecholaminergic Projections to the Spinal Cord

The temporal sequence of the supraspinal CA innervation to the spinal cord was studied in *Xenopus laevis.* After TRDA applications into the spinal cord at *late embryonic stages*, scattered retrogradely labeled cells were present in the dorsal hypothalamus. At these stages, the dopaminergic cells of the

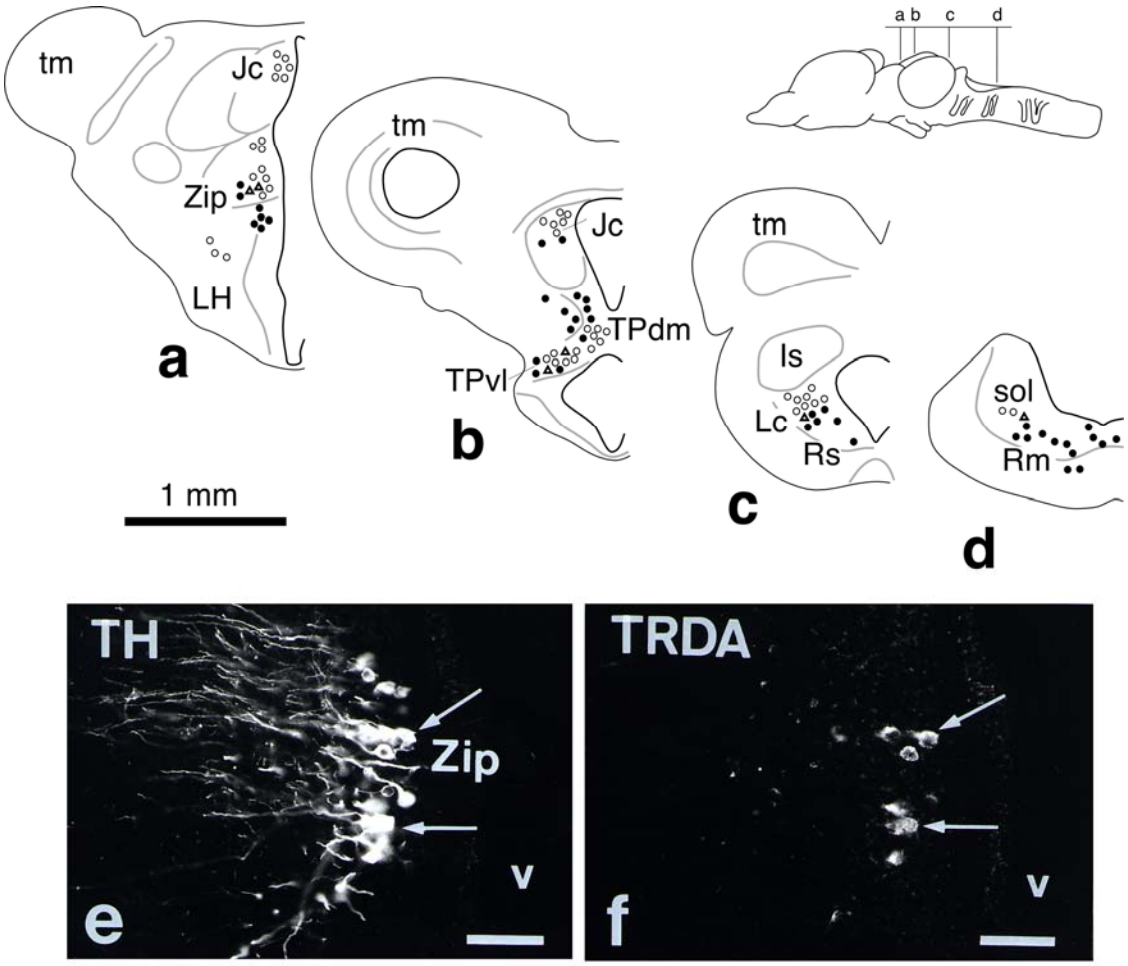

FIG. 2. Schematic drawings of transverse hemisections (**a-d**) through the brain of *Rana perezi*, illustrating the distribution of retrogradely labeled cells (black dots) after unilateral tracer applications into the spinal cord. The localization of CA cells (open dots), as revealed by TH immunohistochemistry, and double labeled neurons (triangles) is also charted. The approximate levels of the sections are indicated in a representative scheme of the brain in the upper right. Photomicrographs of transverse sections (**e, f**) through the brain of *Dermophis mexicanus* showing the localization of THi cells (**e**) and retrograde labeled cells (**f**) in the periventricular nucleus of the zona incerta after tracer applications into the spinal cord. Arrows point to double labeled cells. Calibration bars= 100 μm (e,f). Abbreviations: Is, isthmic nucleus; Jc, juxtacommissural nucleus; Lc, locus coeruleus; LH, lateral hypothalamic nucleus; Rm, middle reticular nucleus; Rs, superior reticular nucleus; sol, solitary tract; TH, tyrosine hydroxylase; tm, mesencephalic tectum; TPdm, dorsomedial part of the posterior tubercle; TPvl, ventrolateral part of the posterior tubercle; TRDA, Texas Red-conjugated dextran amine; v, ventricle; Zip, periventricular nucleus of the zona incerta.

posterior tubercle were located close to the dorsal infundibulum. The first double labeled neurons were already found at stages 40-41 in the posterior tubercle (Fig. 3a, b). At about the same time, spinal projection neurons were also present in the ventral thalamus and the region of the locus coeruleus. In contrast, no double labeled cells were observed although weakly THi neurons were present in these nuclei as early as stage 41. At stage 43, a few double labeled neurons were found in the periventricular nucleus of the zona incerta and the locus coeruleus. During *premetamorphosis*, the previously described catecholaminergic cell groups matured by increasing their number of THi cells. From the beginning of this period, the number of double labeled neurons in the posterior tubercle increased and extended from rostral to caudal levels (Fig. 3c, d). At the end of the premetamorphic period (stage 51), TH immunohistochemistry revealed also catecholaminergic cells in the nucleus of the solitary tract. However, no double labeled neurons were observed until stage 53, at the beginning of the prometamorphosis. During *prometamorphic stages*, the catecholaminergic innervation of the spinal cord was already identical to that found in the adult brain.

DISCUSSION

TH-Immunoreactivity in the Spinal Cord of Amphibians

We demonstrated the presence of an abundant CA innervation throughout the spinal cord of the amphibians studied. The pattern of CA innervation in the spinal cord is similar in all species studied, although interspecific differences in the number and morphology of THi fibers and cells occur. CA innervation is particularly strong in the dorsal horn and the area above the central canal, in line with previous data [1]. Only sparsely distributed fibers were present in the ventral horn of anurans, whereas a strong innervation of large neurons, mainly at thoracic and lumbar cord, was found in urodeles and apodans. Moreover, the distribution of CA fibers

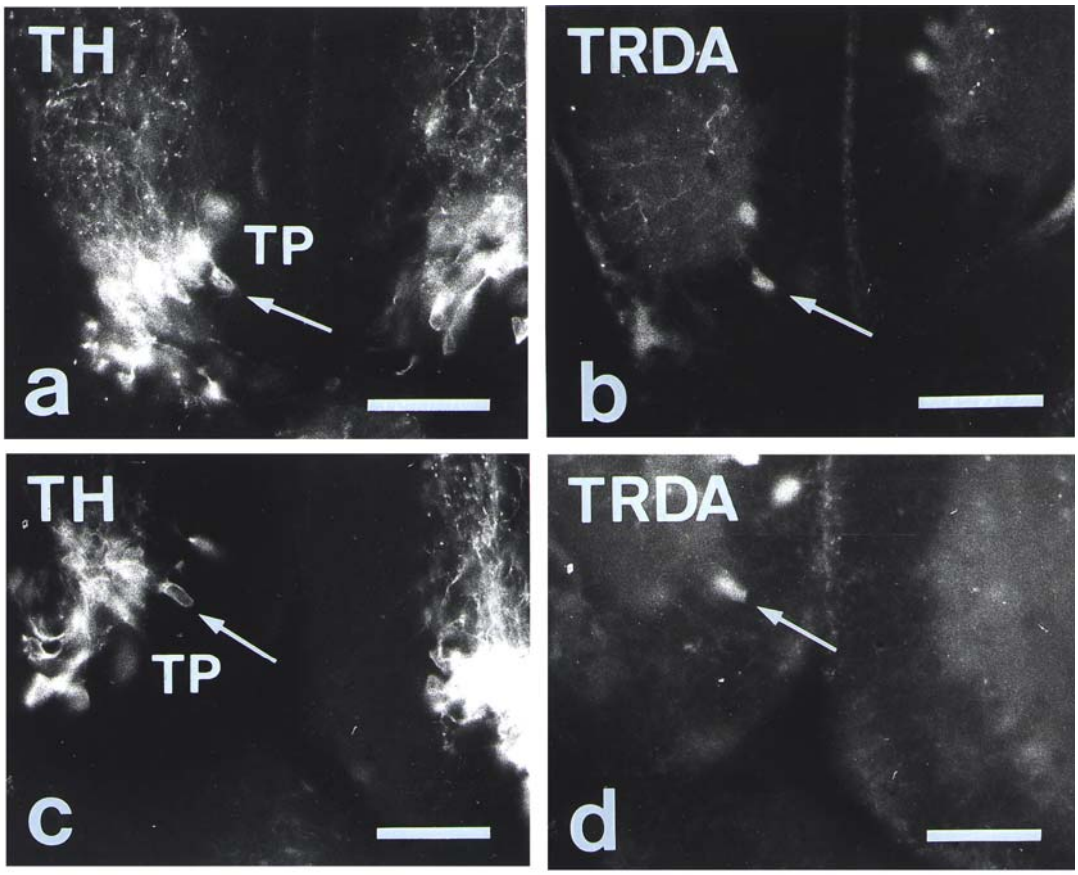

FIG. 3.- Photomicrographs of transverse sections through the brain of *Xenopus laevis* showing the localization of THi cells (**a, c**) and retrograde labeled cells (**b, d**) in the posterior tubercle after tracer applications in the spinal cord at the end of the embryonic period (stage 45, a, b) and the beginning of the premetamorphosis (stage 48, c, d). Arrows point to double labeled cells. Calibration bars = 50 μm. Abbreviations: TH, tyrosine hydroxylase; TP, posterior tubercle; TRDA, Texas Red-conjugated dextran amine.

in intermediolateral field, where the autonomic cells are located [12,14], suggests the innervation of these neurons. The presence of CA cells ventral to the central canal was observed throughout the spinal cord, and only in anurans, scattered THi neurons where also found in the dorsolateral field at rostral spinal levels. These cells seem to be a caudal continuation of the THi neurons of the nucleus of the solitary tract/area postrema complex, although occasionally some isolated cells were observed at caudal brachial levels.

Origin of Descending Catecholaminergic Projections to the Spinal Cord

In the present study, four brain centers were found to contribute to the bulk of the CA innervation of the spinal cord in amphibians: the *posterior tubercle*, the *periventricular nucleus of the zona incerta*, the *locus coeruleus* and the *nucleus of the solitary tract*. This pattern holds for all three orders of amphibians except for the lack of a CA spinal projection from the nucleus of the solitary tract in gymnophionans. Moreover, the organization of the CA input to the spinal cord of amphibians was found to be largely similar to that described for mammals [17]. However, similar studies need to be made for non-mammalian vertebrates, since double labeling experiments dealing with the CA inputs to the spinal cord are lacking.

A dopaminergic (DA) projection was found to originate from CA cells in the secondary prosencephalon, i.e. the *ventrolateral portion of the posterior tubercle*. The segmental topography of these cells [11,15] would correspond to the superficial mammillary and mammillary nuclei of the basal part of prosomere p4. Although this situation seems to be different in mammals, it should not be ruled out that when applying a similar segmental analysis to the descending CA projections to the spinal cord in mammals, part of the widely described "hypothalamospinal" system would be comparable to what we have found in amphibians.

The present study showed that projections from the *periventricular nucleus of the zona incerta*, located in the alar plate of prosomere p3, are the sole diencephalic source of spinal DA fibers in amphibians. The mammalian A11 cell group was described within the hypothalamic territories, but more recent analysis has located this group in the caudal thalamus. Thus, the A13-A11 alar column seems to be the origin of diencephalospinal projections in mammals (prosomeres p1, p2 and p3, from caudal to rostral). Considering this projection, a comparison with the A11 group of mammals can be made, but the prosomere localization clearly differs and the DA spinal projections arising in p3 from the A13 group seem to be lacking in mammals [20]. Thus, the peculiar arrangement found in amphibians points to a more rostral origin of the DA projections to the spinal cord than in mammals.

Only a single CA cell population has been identified in the isthmic region of amphibians, which has been considered

the homologue of the *locus coeruleus* of mammals primarily on the basis of its position, noradrenergic content, and projections to both the telencephalon and spinal cord in anurans and urodeles [3,6,9]. Similarly, following a segmental approach, the spinal projection arising in the so-called locus coeruleus in amphibians may represent together the projections from the locus coeruleus (A6 group) and locus subcoeruleus of mammals [17,18].

In amphibians, dopaminergic, noradrenergic and adrenergic cells were found in the nucleus of the solitary tract [1-3,6]. This nucleus may be regarded as a CA complex equivalent to the C1/A1-C3/A3 groups of amniotes. However, by using a segmental approach, Smeets and González [18] concluded that only the spinal projection arising in the mammalian A1 group is comparable to the projection from the *nucleus of the solitary tract* of amphibians.

Development of the Descending Catecholaminergic Projections to the Spinal Cord

The early appearance of the CA spinal innervation by late embryonic stages suggests an important role for catecholamines in the development of these supraspinal projections. We showed the presence of a developmental sequence in the formation of descending CA pathways to the spinal cord along a rostrocaudal gradient. Projections from the posterior tubercle, the periventricular nucleus of the zona incerta and the locus coeruleus, reach the spinal cord by the end of the embryonic period, whereas spinal projections from the nucleus of the solitary tract do not arise before the beginning of the prometamorphic period.

The spatiotemporal expression of TH immunoreactivity and the appearance of CA cell groups has been shown in a recent work by González et al. [4]. At stage 40/41, THi fibers could already be traced to the marginal zone of the spinal cord, in line with our data. Additionally, dopaminergic cells in the posterior tubercle were detected during the embryonic period (stage 39), soon followed by the "accompanying cell group of the periventricular organ", which is now known as the periventricular nucleus of the zona incerta (stage 40/41), and the locus coeruleus (stage 41). CA cells in the nucleus of the solitary tract develop later at the end of the premetamorphic period (stage 51). TH immunoreactivity of CSF-contacting cells ventral to the central canal was detected as early as stage 28 of embryonic development by Heathcote and Chen [7].

In conclusion, our data suggest that TH immunoreactivity develops first in the CA neurons innervating the spinal cord, immediately followed by the outgrowth of descending CA projections to the spinal cord. Since cells within the dopaminergic posterior tubercle become immunoreactive for TH as early as stage 39 [4], and the spinal cord contains THi fibers already at stage 40, it is not surprising that the first dopaminergic projections from this nucleus reach the spinal cord also at early stage 40. The same may hold for the rest of the CA cell groups projecting to the spinal cord. This situation is in line with data by van Mier et al. [19] on the development of raphespinal connections. They proposed that the formation of neurons in the raphe nuclei is characterized by different phases, first transmitter production and formation of an axonal protusion, and ending with the development of axonal collaterals and more axonal varicosities. A rostrocaudal gradient seems also to be present as well in the generation of serotonergic neurons which project to the spinal cord.

ACKNOWLEDGEMENT

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Capítulo 5

Inervación catecolaminérgica del techo óptico

Distribution and origin of the catecholaminergic innervation in the amphibian mesencephalic tectum

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Distribution and origin of the catecholaminergic innervation in the amphibian mesencephalic tectum

CRISTINA SÁNCHEZ-CAMACHO[1], OSCAR MARÍN[2] AND AGUSTÍN GONZÁLEZ[1]
[1]Departamento de Biología Celular, Facultad de Biología, Universidad Complutense of Madrid, Spain
[2]Department of Psychiatry and Langley Porter Psychiatric Institute, Nina Ireland Laboratory of Developmental Neurobiology, University of California at San Francisco

Abstract

The mesencephalic tectum plays a prominent role in integrating both visual and multimodal sensory information essential for normal behavior in amphibians. Activity in the mesencephalic tectum is though to be modulated by the influence of distinct neurochemical inputs, including the catecholaminergic and the cholinergic systems. In the present study, we have investigated the distribution and the origin of the catecholaminergic innervation of the mesencephalic tectum in two amphibian species, the anuran *Rana perezi* and the urodele *Pleurodeles waltl*. Immunohistochemistry for dopamine and two enzymes required for the synthesis of catecholamines, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) revealed a complex pattern of catecholaminergic (CA) innervation in the anuran and urodele mesencephalic tectum. Dopaminergic fibers were primarily present in deep tectal layers, whereas noradrenergic (DBH immunoreactive) fibers predominated in superficial layers. Catecholaminergic cell bodies were never observed within the tectum. To determine the origin of this innervation, applications of retrograde tracers into the optic tectum were combined with immunohistochemistry for TH. Results from these experiments demonstrate that dopaminergic neurons in the suprachiasmatic and juxtacommissural nuclei (in *Rana*) or in the nucleus pretectalis (in *Pleurodeles*), together with noradrenergic cells of the locus coeruleus, are the sources of CA input to the amphibian mesencephalic tectum. The present results suggest that similar CA modulatory inputs are present in the mesecencephalic tectum of both anurans and urodeles.

Keywords: Locus coeruleus, Retrograde tracing, Dopamine, Catecholamines, Optic tectum

Introduction

In amphibians, the mesencephalic tectum is involved in the integration of both visual and multisensory information (Comer & Grobstein, 1981; Roth, 1987; Ewert, 1989; Roth et al., 1998), and it is also implicated in the generation of different motor behaviors, such as saccadic eye movements, fixation, turning, approach, prey capture, and escape behaviors (see Ewert, 1989).

The existence of abundant catecholaminergic (CA) inputs to the tectum (superior colliculus in mammals) seems to be a feature shared by all vertebrates (for review see Smeets & González, 2000). Among other possible roles, tectal catecholamines appear to have a modulatory effect in the sensory processing of visual system. For example, several studies have provided evidence that dopamine (DA) exerts an inhibitory influence in the tectum and that an increase of DA in the tectum results in suppression of prey-orienting turning behavior in amphibians (Glasgow & Ewert, 1996, 1997; Ewert et al., 1999). In addition, several studies analyzing the effect of noradrenaline (NA) on the mammalian superior colliculus have shown that NA generally suppresses the response of collicular neurons (Sato & Kayama, 1983; Mooney et al., 1990).

Regarding the anatomical distribution of CA fibers and terminals in the tectum, significant differences exist among vertebrates. In teleost fish, for example, immunoreactive fibers are predominantly distributed within deep tectal laminae (Meek et al., 1989; Roberts et al., 1989), whereas in reptiles (Medina & Smeets, 1992), birds (Rodman & Karten, 1995) and mammals (Morrison & Foote, 1986; Mooney et al., 1990) superficial retinorecipient layers are the most densely innervated. In amphibians, immunohistochemical studies have demonstrated that the midbrain tectum is richly innervated by CA fibers and terminals, particularly within deep layers (González & Smeets, 1991, 1993, 1994a; González et al., 1993).

Only a few studies in birds and mammals have dealt with the origin of the CA innervation of the tectum. In mammals, the DA input to the superior colliculus arises from the midbrain DA cell groups (Campbell & Takada, 1989), whereas in birds the pretectal region is the main source of the tectal DA innervation. In contrast, the locus coeruleus is the source of NA projections to the tectum in both birds (Rodman & Karten, 1995) and mammals (Mooney et al., 1990). In amphibians, the

pretectal region has been proposed as the source of DA fibers in the optic tectum (Glasgow & Ewert, 1996; Ewert, 1997; Marín et al., 1999).

The goal of the present study was to provide a detailed description of the pattern of catecholaminergic innervation and the sources of this input to the tectum in anuran (*Rana perezi*) and urodele (*Pleurodeles waltl*) amphibians. These two species were selected because previous research in our group dealt with the organization of the catecholaminergic systems in these species (González and Smeets, 1991, 1994a, 1995) and several aspects of tectal connectivity related to neurotransmitter content were also investigated (Marín et al., 1997c, 1999; Marín & González, 1999). We used immunohistochemistry for dopamine (DA) and the enzymes that are involved in the biosynthesis of catecholamines, particularly tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). In addition, we combined TH immunohistochemistry with retrograde tracing of dextran amines to characterize the cells of origin of the catecholaminergic innervation.

Materials and methods

For the present study, a total of 32 adult green frogs (*Rana perezi*, Amphibia: Anura) and 12 specimens of Iberian ribbed newts (*Pleurodeles waltl*, Amphibia: Urodela) were used. The animals were obtained from the laboratory stocks of the Department of Cell Biology, University Complutense of Madrid. In all experiments the animals were deeply anesthetized by immersion in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz). The original research reported herein was performed under animal care guidelines established by the Spanish Royal Decree 223/1988.

DA immunohistochemistry

Under anesthesia, two animals of each species were perfused transcardially with saline followed by a mixture of 5% glutaraldehyde in 0.05M sodium-cacodylate and 1% $Na_2S_2O_5$ (pH 7.1). The brains were removed and further fixed in the same solution for 1-2 hours at room temperature. They were then immersed in a solution of 30% sucrose with 1% $Na_2S_2O_5$ in 0.1M phosphate buffer (pH 7.1) for 3-5 hours at 4°C, embedded in a solution of 15% gelatin with 30% sucrose, and stored for 5 hours in a 4% formaldehyde solution at room temperature. The brains were cut on a freezing microtome at 40 μm in the frontal plane, and the sections were collected in Tris-NaCl buffer containing 1% $Na_2S_2O_5$ (pH 7.1). The sections were subsequently processed immunohistochemically according to the peroxidase antiperoxidase (PAP) technique (Sternberger, 1979), using a DA antiserum generously provided by Dr. Buijs (Netherlands Institute for Brain Research, Amsterdam). This includes the following steps: (1) incubation with the DA antiserum (raised in rabbit), diluted 1:2,000 in Tris-NaCl buffered saline (TBS, pH 7.6) containing 1% $Na_2S_2O_5$ and 0.5% Triton X-100 for 16 hours at 4°C; (2) rinsing 3 times for 10 minutes in TBS containing 0.5% Triton X-100 (TBS-T); (3) incubation in TBS-T with swine antirabbit antiserum (Nordic), diluted 1:50 for 60 minutes; (4) incubation with rabbit peroxidase antiperoxidase complex (Dakopatts), diluted 1:800; (5) rinsing 3 times in TBS-T and twice in TBS; (6) staining in 0.5 mgr/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in TBS for 10-20 minutes. The sections were then mounted on glass slides (mounting medium: 0.25% gelatin in Tris buffer) and, after drying overnight, coverslipped.

Tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) immunohistochemistry

In this set of experiments the animals (*R. perezi* n=6, *P. waltl* n=6) were perfused transcardially with saline followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After two hours of postfixation, the brains were immersed in PB containing 30% sucrose for 3-5 h at 4ºC, embedded in gelatin and cut in the frontal or sagittal plane at 40 μm thickness on a freezing microtome. The sections were collected in PB. They were then rinsed in PB, and treated with 1% H_2O_2 in PB for 15 minutes to reduce endogenous peroxidase activity. The sections were then processed for TH and DBH immunohistochemistry as described before following the PAP method. Briefly, the sections were first incubated in a mouse anti-TH serum (Incstar, USA), diluted 1:1,000 or a rabbit anti-DBH (Eugene Tech International), diluted 1:300 in PB, for 48 h at 4°C. Subsequently, the sections were rinsed in PB and incubated for 90 minutes in goat anti-mouse serum (1:100; DAKO A/S, Denmark) for TH immunohistochemistry and swine anti-rabbit (1: 50; Nordic) for DBH immunohistochemistry at room temperature, and then processed following the PAP method. After rinsing again, the sections were incubated for 90 minutes in mouse or rabbit PAP (1:600, Chemicon, USA), respectively. The sections were DAB-stained, mounted and coverslipped as described above. For more details the reader is referred to previous works (González and Smeets, 1991, 1995; González et al., 1993).

Double-labeling experiments

Retrograde tracing of dextran amines was combined with TH immunohistochemistry to investigate the sources of CA innervation of the mesencephalic tectum. Both in *R. perezi* (n=9) and *P. waltl* (n=7), the tracers 10 kD or 3 kD biotinylated dextran amine (BDA; Molecular Probes, Oregon, USA) and 10 kD or 3 kD Texas Red-conjugated dextran amine (TRDA; Molecular Probes), recrystallized from distilled water onto sharp tungsten needles, were applied unilaterally into mesencephalic tectum. This approach has been ver successful in the study of afferent connections in the brain of amphibians and clearly superior to iontophoretical injections of dissolved tracers (Marín et al., 1997a; Sánchez-Camacho et al., 2001a,b). Survival times varied from 5 to 10 days. After this period, the animals were deeply anesthetized and perfused transcardially with 50 ml saline followed by 200 ml fixative (4% paraformaldehyde in PB). The brains were removed, blocked in gelatin and cut in the frontal or sagittal plane at 40 μm thickness on a freezing microtome, as described above. Subsequently, brain sections were processed for TH-immunohistochemistry according to the indirect immunofluorescence method. Briefly, they were first incubated for 48 hours at 4°C with a mouse anti-TH antibody (Incstar), diluted 1:1,000 as described above. They were then incubated with a FITC-conjugated mouse-IgG complex (Incstar) diluted 1:150 for 90 minutes at room temperature. BDA was visualized by incubation with a Texas Red-conjugated streptavidin complex (Vector Labs., diluted 1:200) together with the secondary antibody. The sections were then mounted on glass slides and coverslipped with Vectashield (Vector Labs., Burlingame, CA, USA). Alternating the appropriate filter combinations in a Zeiss fluorescence microscope allowed the identification of TRDA retrogradely labeled cells and TH immunopositive cells. The distribution of retrogradely labeled, THir or double labeled neurons was charted in representative, transverse brain sections by means of a camera lucida. The nomenclature is the same as that used in our previous studies on the connections of the CA cell groups in the brain of amphibians (Marín et al.,

1997a; Sánchez-Camacho et al., 2001 b). In addition, for the laminar organization of the tectum, the descriptions of Potter (1969) and Lázár et al. (1983) for anurans, and Roth et al. (1990) for urodeles, are followed.

Results

The overall distribution of catecholaminergic fibers and terminals was revealed by immunohistochemistry for TH, the first and rate-limiting enzyme in catecholamine synthesis (Fig. 1). The comparison with the results obtained for DA immunohistochemistry showed that, as expected, TH immunostaining resulted in the labeling of DA structures but revealed additional fibers that surely corresponded with non-dopaminergic fibers. Immunohistochemistry for DBH, the enzyme that catalyses the synthesis of NA from DA, resulted in labeling of a subpopulation of TH immunoreactive (THir) fibers, distinct from the dopamine immunoreactive (DAir) fibers, that most likely are noradrenergic fibers (Fig. 1). Catecholaminergic cell bodies were never observed within the tectum. In the following sections the detailed distribution of these immunostainings is described in the tectum of *R. perezi* and *P. waltl*.

Distribution of catecholaminergic structures in the tectum

Tyrosine hydroxylase immunoreactivity

In the anuran *Rana perezi*, abundant TH innervation was consistently found throughout the rostrocaudal extent of the mesencephalic tectum. Although almost all tectal layers contained THir fibers and terminals, the densest plexus occupied plexiform layers 3 and 5 (Figs. 1, 2a). Additionally, a significant number of disperse immunoreactive fibers was observed in layer 7, primarily located parallel to the layer. Few scattered fibers were also located tangentially in layer 4 and, more abundantly, within layer 6. Finally, dispersed THir fibers were also labeled in layer 8 and deep sublaminae F and G of the tectal layer 9, whereas in more superficial sublaminae scattered fibers were occasionally observed.

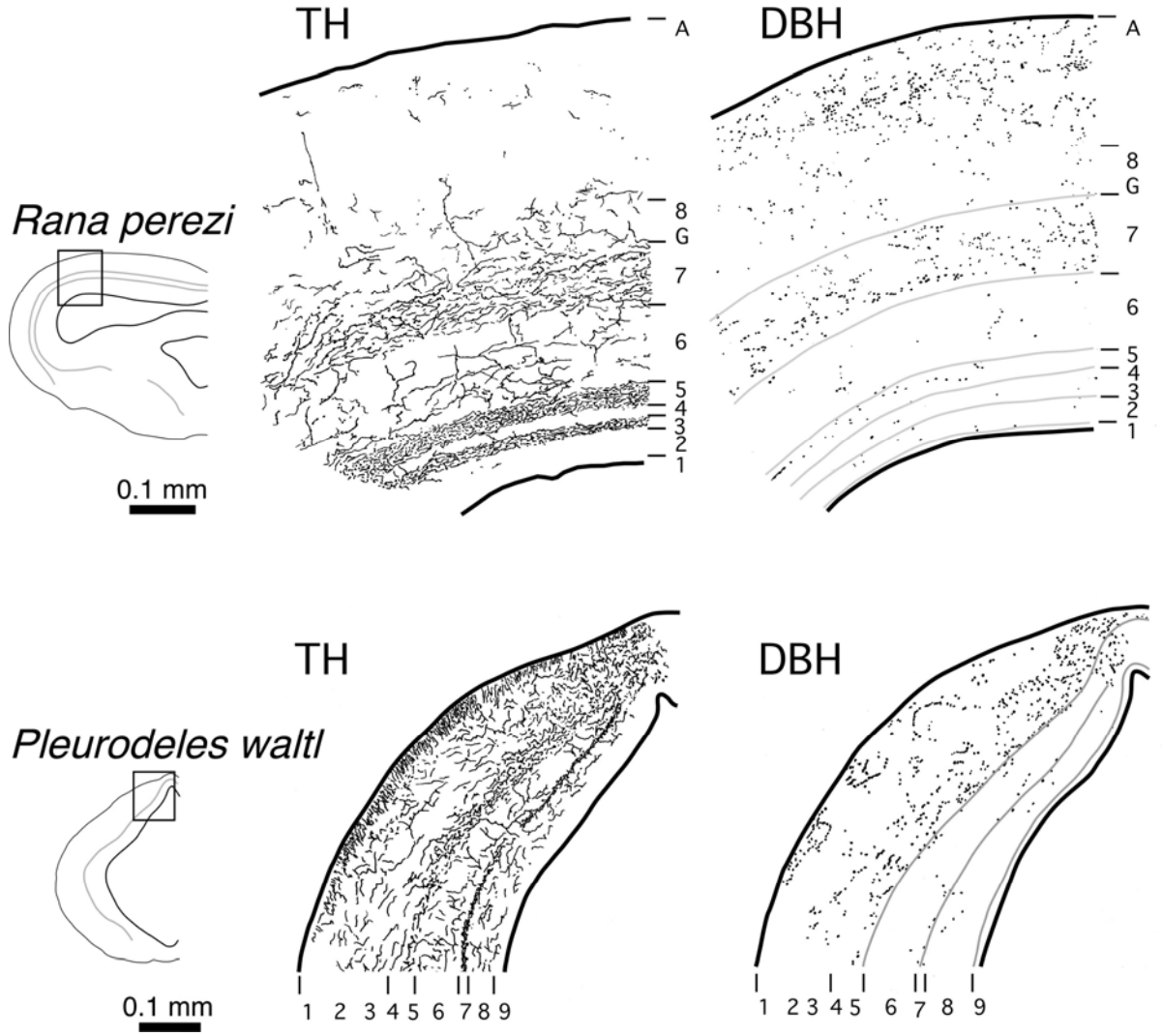

Fig. 1. Camera lucida drawings illustrating the distribution of THir and DBHir fibers and terminals in the tectum of the frog (*Rana perezi*) and the newt (*Pleurodeles waltl*). The localization of the selected tectal regions for each case is indicated by rectangles in the transverse hemisections represented on the left. Letters and numbers refer to tectal layers according to Potter (1969) and Lázár et al. (1983) for *Rana*, and Roth et al. (1990) for *Pleurodeles*.

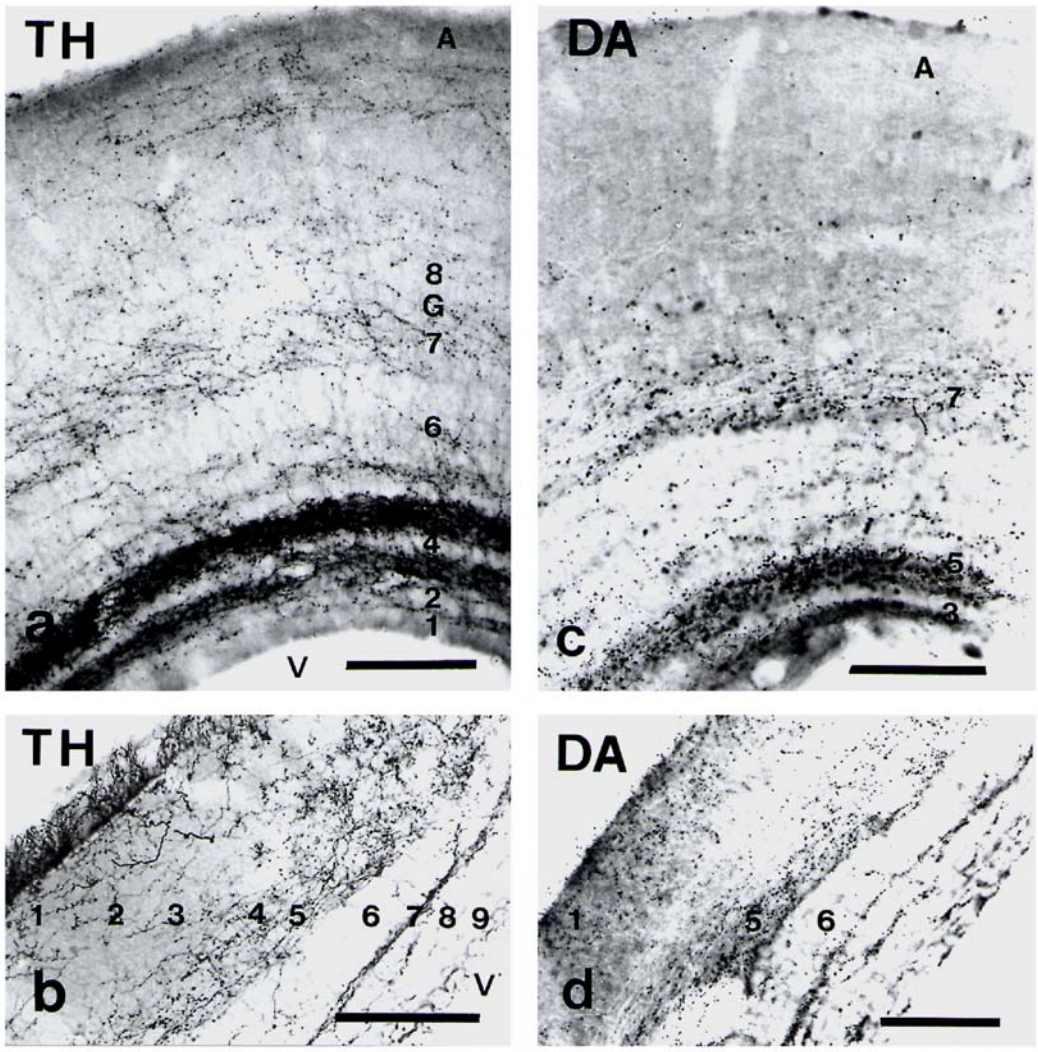

Fig. 2. Photomicrographs of transverse sections through the tectum illustrating THir fibers and terminals (**a** and **b**) in the mesencephalic tectum of *R. perezi* (**a**) and *P. waltl* (**b**). DAir terminals are illustrated in comparable sections (**c** and **d**). Letters and numbers refer to tectal layers according to Potter (1969) and Lázár et al. (1983) for *Rana*, and Roth et al. (1990) for *Pleurodeles*. *v*: ventricle. Calibration bars = 100 μm.

In the optic tectum of the urodele *Pleurodeles waltl*, THir fibers were primarily located within the deep tectal layer 7, which is a very thin plexiform layer (Figs. 1, 2b). In addition, numerous THir fibers coursed in layers 4 and 5. Layers 2 and 3 contained less numerous fibers. Scattered thin, varicose fibers were also disposed in cellular layers 8 and 6, oriented tangentially. An additional feature of the urodele was the presence of a strong innervation in the upper superficial layer (layer 1), with shorter and thicker fibers directed tangentially. Thus, in contrast with the anuran tectum, superficial tectal layers were more densely innervated in the urodele. In general the pattern was consistent from rostral to caudal levels, as well as from medial to lateral sides.

Dopamine immunoreactivity

Immunohistochemistry with antibodies against DA revealed only terminal-like structures and, therefore, the course of dopaminergic fibers in the tectum could not be observed (Fig. 2c, d). In the case of the anuran tectum, most of the DAir structures concentrated in layers 3, 5 and 7 with only scattered terminals among the cell bodies in the cellular layers 4, 6 and 8 (Fig. 2c). Labeling in superficial tectal layers was almost absent in all tectal regions. The urodele tectum showed a dense DA innervation in fiber layers 5 and 7. In addition, DA labeling was also present in superficial layer 1 (Fig. 2d).

Dopamine β-hydroxylase immunoreactivity

The distribution of DBHir fibers within the optic tectum in *Rana* was almost restricted to superficial layers (Fig. 1). Moreover, immunohistochemistry for DBH only revealed varicosities or large, round presumptive boutons as punctate terminal-like labeling. The densest innervation was located in layer 8 and superficial sublaminae of layer 9 (A-E). In addition, layer 7 was also well innervated and only scattered fibers could be found within deep layers 3 and 5.

As in the frog, DBH staining in the urodele mesencephalic tectum was moderate to weak and only the laminar organization of varicosities could be charted (Fig. 1). The pattern of distribution of DBH immunoreactivity showed that

tectal layers 4 and 5 were the most densely innervated, but also

Table 1. Summary of brain centers that project to the tectum *R. perezi* and *P. waltl* in relation to their catecholaminergic content[1]

Cell populations	Projection neurons to the tectum	CA neurons
Forebrain		
Striatum (A)	+	–
Dorsal pallidum (A)	+	–
Central amygdala (A)	+	–
Anterior preoptic area	+	+DA,NA
Suprachiasmatic nucleus	+	+DA
Posterior tubercle, ventrolateral portion	+	+DA
Ventral hypothalamus	+	–
A, C, La (A)	+	–
Dorsal thalamus (U)	+	–
Periventricular nucleus of the zona incerta	+	+DA
VM, VL (A)	+	–
Ventral thalamus (U)	+	–
Juxtacommissural nucleus (A)	+	+DA
Lpd, L, PC (A)	+	–
Nucleus pretectalis (U)	+	+DA
Midbrain		
Mesencephalic tegmentum	+	–
Mesencephalic tectum	+	–
Torus semicircularis	+	–
Isthmus		
Locus coeruleus	+	+NA
Isthmic nucleus	+	–
Hindbrain		
Reticular formation	+	–
Nucleus of the solitary tract	+	+DA,NA, A

[1]Based on González & Smeets, 1991, 1994a, 1995 and the present results.
A = Adrenaline; DA = Dopamine; NA = Noradrenaline
(A) Anuran; (U) Urodele

layers 1 and 2 contained an important amount of DBHir terminal-like structures. Only scattered varicosities could be observed within tectal layer 7.

Tectal afferent projections and sources of the catecholaminergic input

We investigated the afferent connections of the midbrain tectum of *R. perezi* and *P. waltl* by means of retrograde tracing with dextran amines. The localization of the afferent cells to the mesencephalic tectum is summarized in Table 1. Although these results mostly corroborate previous studies on tectal afferents in amphibians, additional afferent centers have been found due to the sensitivity of the technique used (Wilczynski & Northcutt, 1977; Finkenstädt et al., 1983; Rettig, 1988; Hofmann et al., 1990).

In the case of the anuran, in the forebrain, retrograde labeled cells were found particularly in the striatum, central amygdala, dorsal pallidum, anterior preoptic area and suprachiasmatic nucleus. Scattered labeled cells were also observed bilaterally throughout the ventral hypothalamus and the ventrolateral portion of the posterior tubercle. However, the most numerous groups of retrograde labeled neurons were observed in the dorsal and ventral thalamus and in the pretectal region. In particular, an important projection was found from the anterior, central and lateral anterior thalamic nuclei. This projection was mainly ipsilateral and originated from cells with small round perikarya and a main process directed laterally. Less prominent was the projection found from the ventral thalamus, mainly ipsilaterally. Cells were observed in the ventromedial and ventrolateral nuclei, and in the periventricular nucleus of the zona incerta. The pretectotectal projection originated primarily in the ipsilateral juxtacommissural and the lateral posterodorsal nuclei, but a few cells were labeled in the precommissural nucleus and the nucleus lentiformis. Cells located in the upper portion of layer 6, and a smaller population in layer 7 in the contralateral tectum, were the source of a fairly dense tectotectal projection. In addition, conspicuous isthmotectal, tegmentotectal and torotectal projections were found. Cells were also retrogradely labeled in the rhombencephalic reticular formation and in the nucleus of the solitary tract.

As in the frog, afferent projections to the urodele optic tectum were found to arise from the anterior preoptic area, suprachiasmatic nucleus, ventral hypothalamus, ventrolateral posterior tubercle, dorsal and ventral thalamus, pretectal region, isthmic nucleus and nucleus of the solitary tract. However, tectal projections from the striatum or the amygdala could not be demonstrated.

Many different brain regions project to the tectum and a comparison with the distribution of the CA cell groups revealed several candidates for the CA input to the amphibian tectum (see Table 1). The combination of TH immunohistochemistry with retrograde tracing demonstrated that in some of these centers neurons that project to the tectum intermingle with CA cells that do not project to the tectum (Figs. 3, 5). These centers are, therefore, not considered as sources of the CA input to the tectum. In only three centers, however, a subpopulation of THir cells was also found to contain retrogradely transported dextran amines. These are, from rostral to caudal, the dopaminergic suprachiasmatic nucleus, the dopa-

minergic cells in the pretectal region and the noradrenergic locus coeruleus (Figs. 3-6). In the following section we will

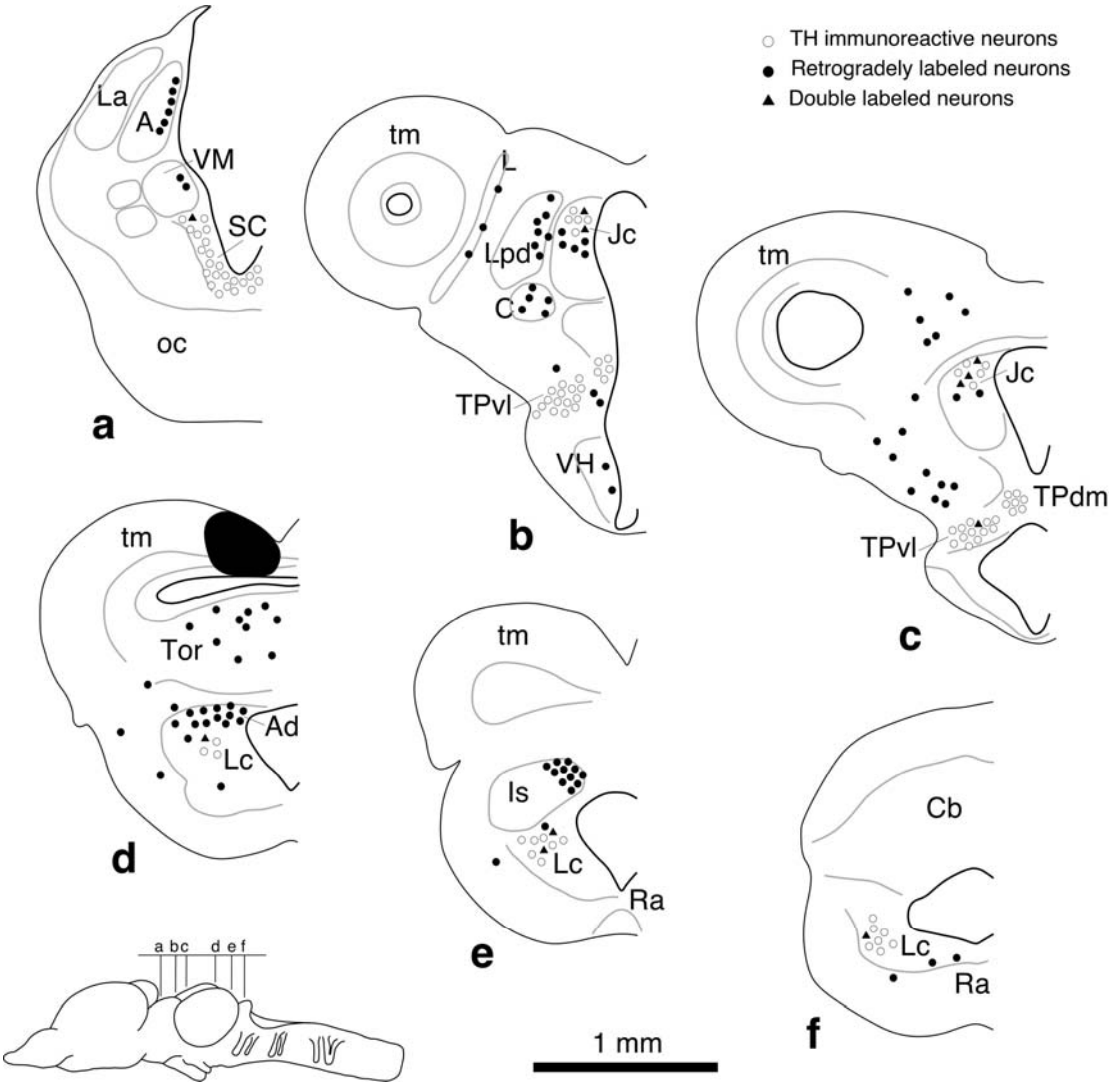

Fig. 3. Schematic drawings of transverse hemisections through the brain of *Rana perezi* summarizing the localization of retrogradely labeled neurons after tracer applications to the mesencephalic tectum (black area). The distribution of catecholaminergic cells as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropriate levels of the sections are indicated in the lower left. *A*: anterior thalamic nucleus; *Ad*: anterodorsal tegmental nucleus; *C*: central thalamic nucleus; *Cb*: cerebellum; *Is*: isthmic nucleus; *Jc*: juxtacommissural nucleus; *L*: nucleus lentiformis; *La*: lateral anterior nucleus; *Lc*: locus coeruleus; *Lpd*: lateral posterodorsal nucleus; *oc*: optic chiasm; *Ra*: raphe nucleus; *SC*: suprachiasmatic nucleus; *tm*: mesencephalic tectum; *Tor*: torus semicircularis; *TPdm*: dorsomedial part of the tuberculum posterius; *TPvl*: ventrolateral part of the tuberculum posterius; *VH*: ventral hypothalamic nucleus; *VM*: ventromedial nucleus.

describe first the brain centers that do provide the catecholaminergic input to the tectum of anuran and urodele amphibians. Subsequently, some comments are given on the centers that contain THir cell bodies, which do not provide catecholaminergic input to the tectum.

Suprachiasmatic nucleus

After tracer applications into the tectum of the frog, a few retrogradely labeled cells were consistently found in the suprachiasmatic nucleus. These cells were located primarily ipsilateral to the application site. Double labeling techniques demonstrated that practically all neurons in the suprachiasmatic nucleus that project to the tectum are THir (Figs. 3a, 4a,b). A similar situation was found in the case of the urodele where only a few retrograde labeled cells were found in the suprachiasmatic nucleus, throughout the rostrocaudal extent of the nucleus (Figs. 5a,b, 6a-c). In contrast with the frog, however, a few double labeled cells were found also in the contralateral side of the tracer application.

Pretectal region

Tracer application into the tectum resulted in abundant labeled neurons within the pretectal region. This population was the most numerous both in the anuran and in the urodele. In the case of the anuran, the bulk of this projection to the optic tectum was found from the ipsilateral juxtacommissural and

the lateral posterodorsal nuclei, but also from the precommissural nucleus and the nucleus lentiformis (Fig. 3b,c). Double

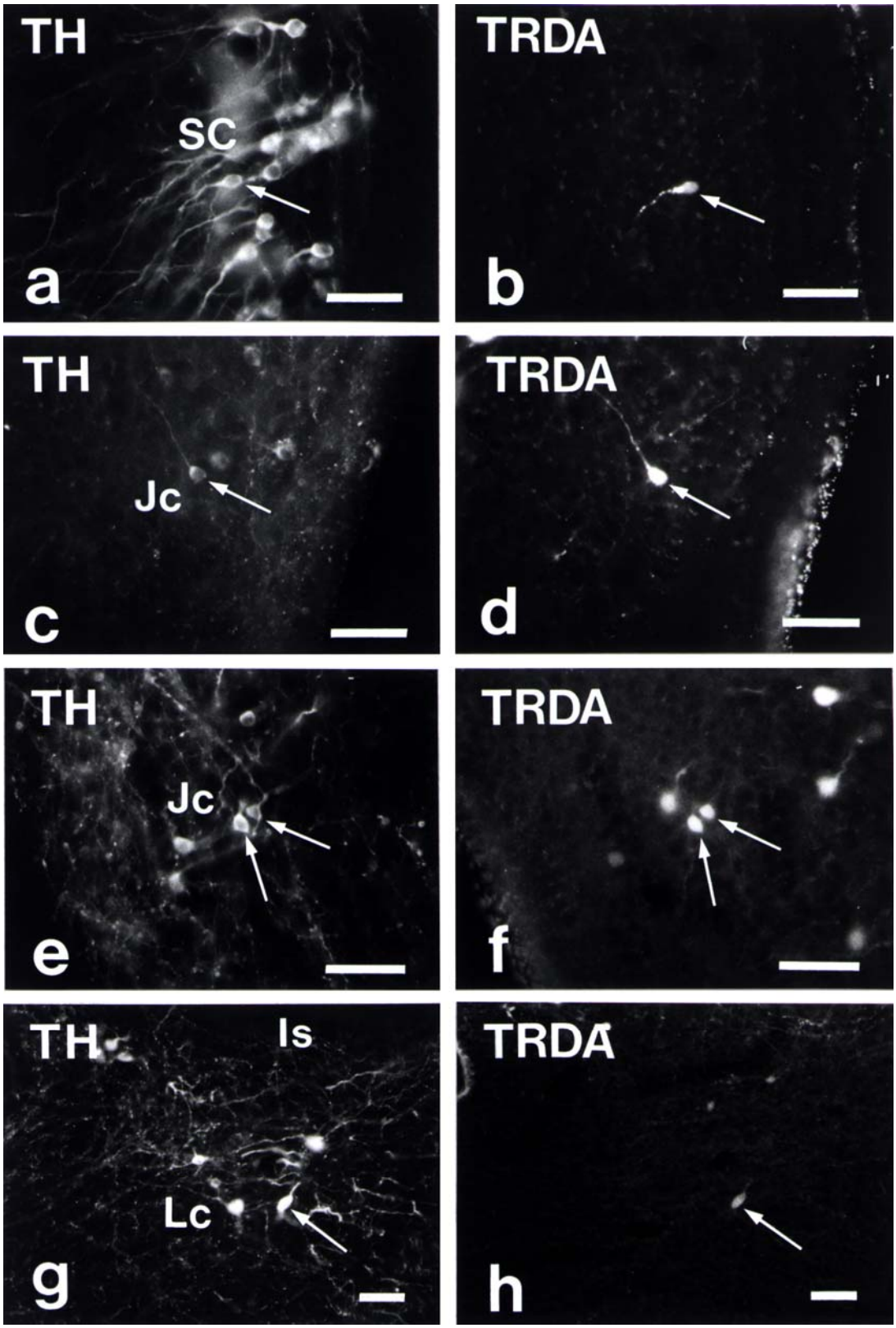

Fig. 4. Photomicrographs of transverse sections through the brain of *Rana perezi* showing the localization of THir cells (**a**,**c**,**e**,**g**) and retrogradely labeled cells after a tectal Texas Red-conjugated dextran amine (TRDA) application (**b**,**d**,**f**,**h**) in the ipsilateral suprachiasmatic (**a**,**b**) and juxtacommissural (**c**,**d**) nuclei, and in the contralateral juxtacommissural nucleus (**e**,**f**) and the locus coeruleus (**g**,**h**). Arrows point to double labeled cells. Calibration bars = 50 μm.

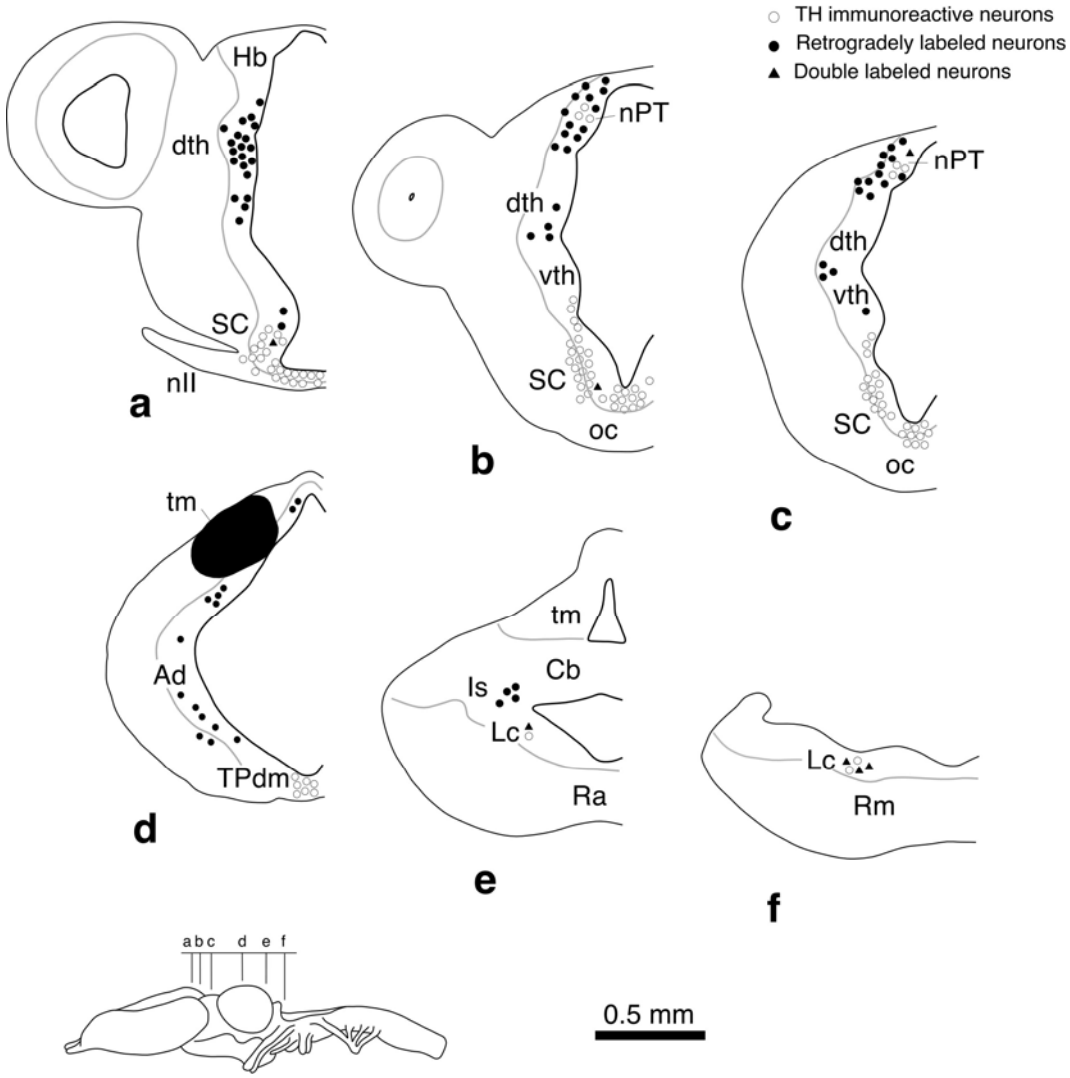

Fig. 5. Schematic drawings of transverse hemisections through the brain of *Pleurodeles waltl* summarizing the localization of retrogradely labeled neurons after tracer applications to the mesencephalic tectum (black area). The distribution of catecholaminergic cells as revealed by TH immunohistochemistry, and double labeled cells is also charted. Contralateral cells are not illustrated. The appropriate levels of the sections are indicated in the lower left. *dth*: dorsal thalamus; *Hb*: habenula; *nII*: optic nerve; *nPT*: nucleus pretectalis; *Rm*: middle reticular nucleus; *vth*: ventral thalamus.

labeling experiments revealed that, in *R. perezi*, THir cells projecting to the tectum were located primarily in the ipsilateral juxtacommissural nucleus, although some contralateral cells were also doubly labeled. They were small, round cells with a main thin process directed dorsolaterally (Fig. 4c-f).

In the urodele, numerous retrograde labeled cells from the tectum were located in the pretectal region (nucleus pretectalis, Fig. 5b, c) in close relation to the THir cells, which are characterized by a large soma located close to the ventricle and a main process directed dorsolaterally. Pretectal neurons projecting to the tectum were generally located lateral to the CA cell group, and only some of these cells were double labeled (Figs. 5c, 6d-f).

Locus coeruleus

A very conspicuous contingent of tectal afferent cells were located in the isthmic region of both amphibians (Figs. 3d-f, 5e,f). In the frog, numerous labeled neurons from the tectum were located in the anterodorsal tegmental nucleus and the isthmic nucleus. Ventrally to these nuclei, double labeled cells were demonstrated mainly in the rostral portion of the locus coeruleus (Figs. 3d-f, 4g,h). This population was mainly ipsilateral although a small contralateral component was also found. Similarly, in *P. waltl* double labeled cells were observed throughout the extent of the locus coeruleus, located ventrally to the isthmic nucleus, particularly in the ipsilateral side (Fig. 5e,f).

Other catecholaminergic cell groups

At several places in the brain of amphibians, catecholaminergic cell bodies intermingle with cells that project to the tectum. The centers that do not provide a catecholaminergic input to the tectum are considered bellow.

With the exception of the dopaminergic cells in the olfactory bulb, the group of cells in the anterior preoptic area is the most rostrally located in the brain of amphibians (González &

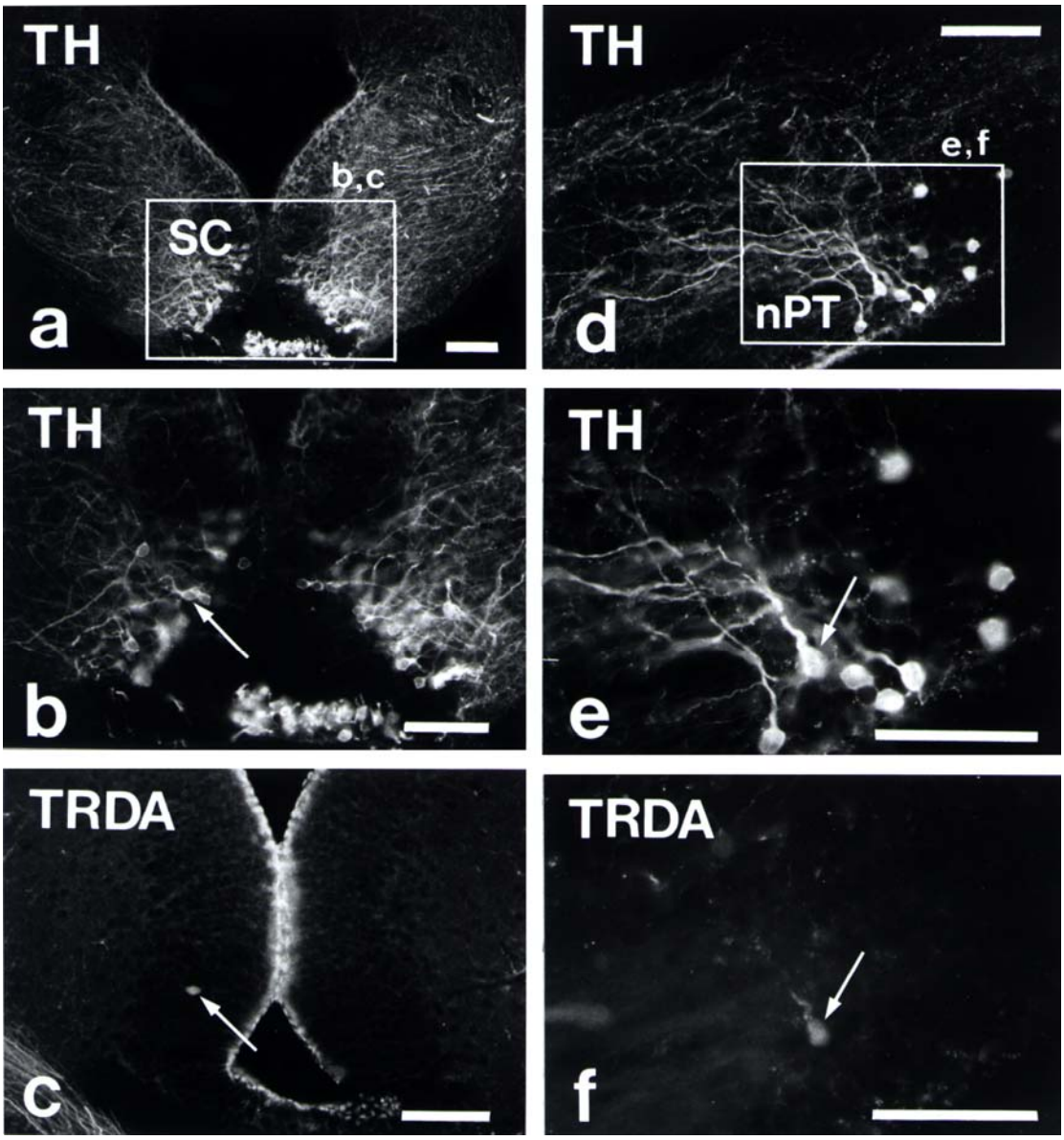

Fig. 6. Photomicrographs of transverse sections through the brain of *Pleurodeles waltl* showing the localization of THir cells (**a**,**b**,**d**,**e**) and retrogradely labeled cells (**c**,**f**) in the ipsilateral suprachiasmatic nucleus (**a-c**) and the nucleus pretectalis (**d-f**) after Texas Red-conjugated dextran amine (TRDA) applications to the mesencephalic tectum. Arrows point to double labeled cells. Calibration bars = 100 μm.

Smeets, 1994a). In experiments with retrograde tracer applications to the tectum, labeled cells were always found in the preoptic area. The morphology of the cells projecting to the tectum resembled that of the catecholaminergic cells and codistribution of both cell groups was observed. However, double labeled cells were never found in the preoptic area.

A contingent of tectal afferent cells was located in the recently described periventricular nucleus of the zona incerta. This nucleus was identified by its localization in the ventral thalamus and by its content of dopaminergic cells (Puelles et al., 1996; Milán & Puelles, 2000). The double labeling experiments of this study did not reveal any catecholaminergic cell in this region that projected to the tectum.

The posterior tubercle of amphibians contains a large population of dopaminergic neurons (González & Smeets, 1991, 1994a). The dopaminergic cells form two separate populations in the dorsomedial and ventrolateral regions of the posterior tubercle (Fig. 3b, c). In the case of *R. perezi*, cells in the posterior tubercle projecting to the tectum were observed in the medial aspect of the ventrolateral component. It should be mentioned that in two cases, an occasional double labeled cell was found in the posterior tubercle (Fig. 3c) and, therefore, a small dopaminergic projection from this region seems to reach the tectum of the frog. A comparable result was not observed in the case of the urodele.

Finally, in anurans and urodeles, a mixed population of dopaminergic, noradrenergic and adrenergic cells are present in the nucleus of the solitary tract. Furthermore, this nucleus seems to be the only place where adrenergic cells are located in the amphibian brain (González & Smeets, 1994a, 1995). Following tracing applications to the tectum retrogradely labeled cells were consistently observed in the ipsilateral nu-

cleus of the solitary tract in the two amphibian species studied. The combined immunohistochemical and tract-tracing techniques did not reveal double stained cells in the nucleus of the solitary tract, although a high degree of codistribution of THir cells and neurons projecting to the tectum was observed.

Discussion

Distribution of the CA innervation in the tectum of amphibians

The complete distribution of CA elements in the tectum of amphibians was analyzed by means of TH immunohistochemistry. In addition, the use of antibodies against DA and DBH revealed the specific distribution of dopaminergic and noradrenergic structures, respectively. DBH immunohistochemistry might also reveal adrenergic structures (see Smeets & González, 2000). However, the lack of immunoreactivity for phenylethanolamine N-methyltransferase, the enzyme that catalyses the synthesis of adrenaline from noradrenaline, in the tectum of amphibians (Yoshida et al., 1983; González & Smeets, 1995), together with the lack of afferences from the adrenergic cells in the nucleus of the solitary tract (present study), support the notion that the DBH staining observed in the tectum is specific for noradrenergic elements.

Differences were found in the morphology of labeled fibers and terminals by using immunohistochemistry for TH, DA, or DBH. Thus, in general, TH immunohistochemistry revealed thin and long varicose fibers with clearly visible intervaricose segments. In contrast, immunoreactive fibers for DA or DBH showed a typical morphology, in which these segments were hardly visible and mostly varicosities were labeled. One explanation for the morphological differences between the TH and DBH stainings could be the different axonal distribution of these enzymes. It is known that the enzyme TH is located in the cytosol (Pickel et al., 1996), whereas DA and DBH are located in the vesicles (Venter et al., 1988; Pickel et al., 1996), which can be specifically accumulated in the varicosities. This situation could explain the presence of TH immunoreactivity in the whole fiber, whereas DA and DBH immunoreactivities appear only in the varicosities.

The present results have shown that the pattern for CA innervation shows a high, selective degree of organization throughout the tectum and that numerous immunoreactive fibers are distributed in a laminar organization. In *Rana*, THir fibers were distributed mainly within deep tectal layers, particularly in layers 3 and 5 and in layer 7, whereas in the newt layers 4, 5 and 7 were the most densely innervated. This pattern in deep tectal layers practically matched that found for DA immunohistochemistry. In contrast, DBHir fibers were more abundantly labeled within superficial tectal layers in both the anuran and the urodele tectum. Due to the position of TH in the synthetic pathway of catecholamines, these superficial fibers were also weakly labeled for TH but, as expected, not for DA immunohistochemistry.

Although strong differences in the laminar structure of the tectum exist between anurans and urodeles, previous studies have shown that, on the basis of their dendritic arborization and pattern of ascending and descending projections, both groups possess very similar functional and morphological types of tectal neurons (Lázár et al., 1983; Roth et al., 1990, 1999; Dicke & Roth, 1996; Dicke, 1999; Sánchez-Camacho et al., 2001a). Thus, periventricular layers 6-9 in urodeles are homologous to layers 1-6 in frogs. The bulk of efferent fibers of the optic tectum in urodeles course in layers 4 and 5 and in frogs in layer 7. Finally, retinal afferents terminate in layers 1-3 in urodeles and in tectal layers 8 and 9 in anurans. The present results support that this homology is also consistent with the pattern of CA innervation and thus, THir fibers located in tectal layers 3 and 5 in *Rana* would be homologous to those in layer 7 in *Pleurodeles*, whereas innervation of layer 7 in the frog would correspond to that in layers 4 and 5 of the newt. Noteworthy, the pattern of distribution of cholinergic fibers in the tectum also corroborated this homology between anuran and urodele lamination (Marín & González, 1999).

The presence of a strong catecholaminergic innervation, particularly with DA, in the layers of the main efferent pathways of the tectum in *Rana* (layer 7) and *Pleurodeles* (layers 4 and 5) suggests that catecholamines would modulate descending tectobulbar and tectospinal output, which mediate different aspects of visual and visuomotor processing. However, because of the wide distribution of DA and NA fibers in almost all tectal layers, catecholamines might be implicated at all tectal levels modulating tectofugal projections and sensory inputs from other brain centers, as well as local intratectal circuits in the processing of visual and nonvisual information.

The laminar distribution of catecholaminergic fibers seems to vary among amphibian species. Thus, in the anuran *Xenopus laevis* all tectal layers contain DA fibers but their density in the superficial and deep tectal zones is higher than that in the intermediate zone (González et al., 1993). The NA fibers, on the contrary, appear to be more numerous in the superficial zone than in the deep tectal zone (González and Smeets, 1993). Thus, whereas in *Rana* THir fibers are predominantly distributed in deep tectal layers, in *Xenopus* and *Pleurodeles* an additional plexus of immunoreactive fibers were found in superficial retinocipient laminae. Of note, in a representative of the gymnophionan order of amphibians, *Typhlonectes compressicauda*, the majority of THir fibers was found in the dorsomedial portion of the superficial tectal zone, in the place where most retinotectal fibers distribute (González & Smeets, 1994b).

The origin of the CA innervation in the tectum of amphibians

Retrograde tracing techniques in combination with TH immunohistochemistry revealed the sources of catecholaminergic inputs to the tectum of amphibians. Catecholaminergic cell bodies in the pretectal region are the major source of the dopaminergic input with a smaller component from the suprachiasmatic nucleus and, in the frog, from the posterior tubercle. The locus coeruleus is the only center that provides the noradrenergic innervation of the tectum.

An interesting result of this study is the identification of an ipsilateral DA projection from the *suprachiasmatic nucleus* to the midbrain tectum. The suprachiasmatic nucleus is involved in the control of the synthesis and release of α-melanocyte-stimulating hormone (αMHS) in the hypophysial pars intermedia of amphibians, and plays a key role in the mechanism of background adaptation (Artero et al., 1994; Tuinhof et al., 1994; Kramer et al., 2001a,b). It has been shown the coexpression of NPY, DA and GABA immunoreactivities within this nucleus, which exert a marked inhibitory effect on αMSH secretion (de Rijk et al., 1992; Tonon et al., 1992; Battaglia et al., 1995). Moreover, a recent study has demonstrated that a region of the suprachiasmatic nucleus receives striatal afferent fibers (Marín et al., 1999), and in turn, projects to the striatum (Allison & Wilczynski, 1994), whereas other portion of the nucleus receives retinal fibers (Tuinhof et al., 1994). As shown in this work, DA projections to the tectum arise from cells in a region of this nucleus that is contacted by striatal fibers but not by retinofugal fibers.

The quantitatively most important DA input to the amphibian tectum arises from the pretectal region, in particular from the juxtacommissural nucleus in the frog and the nucleus pretectalis in the newt. Efferent striatal projections have been demonstrated to terminate in close relation to DA pretectal neurons (Marín et al., 1997b, 1999). Moreover, the striatum influences tectal functions by means of two different indirect routes through the pretectal region: a striato-pallido-pretecto-tectal pathway and a striato-pretecto-tectal pathway (Marín et al., 1998). In the first one, striatal stimulation leads to inhibition of tectofugal neurons, whereas through the striato-pretecto-tectal pathway the final outcome results in disinhibition of tectofugal neurons. Thus, the juxtacommissural nucleus might function as a relay center in these indirect pathways, and possibly also the nucleus pretectalis in urodeles.

The locus coeruleus is the source of NA innervation to the amphibian tectum. Recent studies have demonstrated that this nucleus projects both to the basal ganglia and the spinal cord in amphibians (Marín et al., 1996, 1997a; Sánchez-Camacho et al., 2001a,b). In addition, Marín et al. (1999) suggested the implication of the locus coeruleus in the indirect striatopallidal-tegmento-tectal pathway. The present results reveal an additional connection of this NA cell group with the midbrain tectum not only in the anuran but also in the urodele, and support the idea of the involvement of the locus coeruleus in the processing of visual information in amphibians. Moreover, since no double-labeled neurons have been demonstrated in other NA or adrenergic cell groups as the nucleus of the solitary tract, we conclude that the distribution of DBHir fibers in the tectum are originated exclusively from the NA cells in the locus coeruleus, which project predominantly to superficial, retinocipient tectal layers.

It is known that the basal ganglia in amphibians influence the midbrain tectum via a direct pathway providing a modulatory effect on the tectal responses to visual stimuli resulting in orienting or avoidance behavior (Marín et al., 1997c, 1998). In addition, striatal information influences tectal function by indirect routes through the pretectum and a striatopallidal-tegmento-tectal pathway (Marín et al., 1997c, 1999). Thus, we propose that CA projections found from the dopaminergic juxtacommissural nucleus (in *Rana*) or nucleus pretectalis (in *Pleurodeles*), and the noradrenergic locus coeruleus may mediate part of the striatal input to the tectum, providing an inhibitory effect over the sensory processing in both the anuran and the urodele tectum. In addition, another possible indirect pathway mediated by DA demonstrated in this study would relay striatal information to the tectum by DA cells in the suprachiasmatic nucleus.

Comparative aspects of the CA tectal innervation

The presence of abundant CA fibers and terminals in the midbrain tectum (superior colliculus in mammals) seems to be a common feature of vertebrates, although differences in the laminar pattern of this CA innervation exist. Thus, in teleost fish, there is weak immunoreactivity only in deep and intermediate layers but not in the superficial layers, which receive visual information (Meek et al., 1989; Roberts et al., 1989). In accordance with the present study, CA innervation of the tectum in amphibians is also preferentially distributed within deep layers, although some terminals are also present superficially, particularly DBHir fibers. In contrast with this, the densest CA innervation in birds was found within the superficial retinocipient layers 4, 5 and 7, corresponding roughly to the inferior retinal representation (Rodman & Karten, 1995). In reptiles, abundant CA innervation in the tectum of the lizards *Gekko gecko* and *Gallotia galloti* was found and several differences were noted in the distribution of DAir fibers (Smeets et al., 1986; Medina & Smeets, 1992). Thus, in *Gekko* immunoreactive fibers were present within tectal layers 9 and 11, whereas in *Gallotia* superficial laminae only showed weak DA immunoreactivity. Moreover, overlap between retinal afferences and DAir fibers in layer 11 and particularly in layer 9 appears to exist. The presence of a strong plexus of immunoreactive fibers in superficial retinocipient laminae in the anuran *Xenopus* and the urodele *Pleurodeles* could be comparable to the situation described in reptiles and birds.

In contrast to the tectum of nonmammalian vertebrates, the superior colliculus of mammals is poorly laminated, and laminar specialization of CA innervation is not pronounced. In hamsters, DBH immunohistochemistry revealed the densest innervation within the stratum griseum superficiale, whereas it was very low in the stratum opticum and increased in density in the stratum griseum intermediale and other deep layers (Mooney et al., 1990). In accordance with these data, Morrison & Foote (1986) showed that in primates the superior colliculus is also heavily innervated by DBHir fibers, which are denser and highly arborized in the superficial laminae than in deep laminae. In this study, it was also suggested that functionally related visual regions share common densities of NA innervation and that within the visual system, NA fibers preferentially innervate the regions involved in spatial analysis and visuomotor response, rather than those involved in feature extraction and pattern analysis. Studies on the content of NA in the rabbit superior colliculus have also corroborated that NA concentration is higher in superficial than in deep collicular layers (Wichmann & Starke, 1988).

Only a few studies in birds and mammals have dealt with the sources of CA input to the tectum or superior colliculus by means of double labeling techniques (Campbell & Takada, 1989; Mooney et al., 1990; Rodman & Karten, 1995). In birds, the study by Rodman & Karten (1995) in the pigeon (*Columba livia*) demonstrated double labeled neurons bilaterally in the pontine tegmentum, within the nuclei locus coeruleus and subcoeruleus, and in a portion of the pretectum surrounding the nucleus pretectalis (nucleus pretectalis medialis). Whereas double labeled neurons in the pontine tegmentum do not appear to depend on the site of tectal injection, pretectal cells were mainly labeled after anterior tectal injections. In addition, they found that the majority of the CA input to the optic tectum derives, as in mammals, from the locus coeruleus region, with a small contribution from the pretectum.

Noradrenergic projections to the mammalian superior colliculus appear to derive almost exclusively from the contralateral locus coeruleus (Mooney et al., 1990). Although a pretectal DA cell group seems to be absent in mammals, Campbell & Takada (1989) demonstrated a different source of DA input to the superior colliculus in the rat. By means of double/triple labeling techniques they found that a DA population of substantia nigra pars reticulata cells send axon collaterals to both the ipsilateral striatum and bilateral superior colliculus. This nigrotectal pathway has been suggested to be implicated in the initiation of saccadic responses by cells in the deep layers of the superior colliculus.

In reptiles, DAir and THir cell bodies are present in the pretectal posterodorsal nucleus, which has been proposed to be homologous to the juxtacommissural nucleus in amphibians on basis of its connections (González & Smeets, 1991). Although double labeling studies are lacking, by comparison of hodological data with immunohistochemical observations, Medina & Smeets (1992) proposed the hypothalamic periventricular nucleus, the pretectal posterodorsal nucleus and the substantia nigra as possible candidates for the DA innervation of the midbrain tectum in reptiles. However, double labeling

studies are needed in this group and other vertebrate species in order to assess common features in the origin of the CA projections to the tectum among vertebrates classes.

Functional significance of the CA innervation in the mesencephalic tectum

Catecholamines are known to function as modulatory neurotransmitters in the midbrain tectum, although their precise contribution and mode of action remains essentially unclear. It is known, for example, that the alteration in catecholamine levels can cause profound alterations in attention behavior (Clark et al., 1987) and that damage to the brainstem groups that synthesize NA, or transection of their axons, can also produce deficits in attention and orienting behavior (Carli et al., 1983). In this section we summarize the available data from previous studies on the role of dopamine and noradrenaline in the tectum. In general, both neurotransmitters have been demonstrated to exert an inhibitory effect on neurons of the midbrain tectum.

Dopamine

In several species of vertebrates, systemic administration of the dopamine agonist apomorphine (APO) facilitates oral behaviors (biting, licking) and reduces target oriented motor responses (Blackburn et al., 1992; Glasgow & Ewert, 1997). In amphibians, several studies on the DA modulation of visuomotor functions have provided evidence that dopamine (DA) has an inhibitory influence in the tectum and that an increase of its level results in suppression of prey-orienting turning behavior (Glasgow & Ewert, 1996). Thus, the systemic administration of APO alters prey-catching strategies in a manner that prey-oriented turning movements and locomotion are attenuated, whereas prey snapping is facilitated (Glasgow & Ewert, 1997). In these studies, it was proposed that an APO-induced enhancement of pretecto-tectal inhibitory influences contribute to the reduction of tectal output, thus suppressing prey-oriented turning behavior. However, it should be noted that, as we have shown in this study, the pretectal region is not the only source of DA input to the amphibian tectum. DA projections from the suprachiasmatic nucleus and, to a lesser extent, from the posterior tubercle can also contribute to this tectal inhibition.

Noradrenaline

Several studies have assessed the effects of NA on collicular cells in mammals. Thus, Mooney et al. (1990) demonstrated by means of NA iontophoresis that NA generally suppresses the response of superior colliculus neurons in hamsters and this effect is blocked or attenuated by β-adrenergic antagonists. These results are very similar to those reported for rat (Sato & Kayama, 1983), in which NA also inhibited most collicular neurons, both in the superficial and deep laminae. In addition, in both studies they found that NA excited a small percentage of collicular cells, more commonly in deep laminae.

Recent studies using intracellular recording techniques in hamster have shown that NA acts primarily through α2 and β1 adrenoreceptors suppressing visual responses of collicular neurons in superficial layers and these effects are primarily postsynaptic (Tan et el., 1999; Zhang et al, 1999). However, Arce et al. (1994) suggested that NA may have both pre- and postsynaptic actions in the superficial laminae of the hamster superior colliculus. As shown in a recent pharmacological work, NA released in the rabbit superior colliculus is modulated specifically by presynaptic α2 adrenoreceptors, which act as autoreceptors in a negative feedback on transmitter release (Wichmann & Starke, 1988). Moreover, they did not find evidence for a modulation of NA release through β-adrenoreceptors, but presynaptic inhibition by dopamine D2-receptors, opioid k-receptors, and nicotine and muscarine receptors.

Concluding remarks

The presence of abundant CA fibers and terminals in the midbrain tectum (superior colliculus in mammals) seems to be a common feature of vertebrates. In amphibians, the pattern for CA innervation shows a high, selective degree of organization throughout the tectum, and points to a greater complexity of CA systems in anamniotes than previously thought. Thus, as shown in this study, dopaminergic fibers were primarily present in deep tectal layers, whereas noradrenergic fibers predominated in superficial layers, both in the anuran and the urodele tectum.

It should be also noted that differences appear to exist in the origin of the DA innervation of the tectum among vertebrates. In mammals the DA input to the superior colliculus arises from the midbrain DA cell groups, whereas in birds and amphibians the sources of this DA innervation are located in the pretectal region, and additionally in amphibians in the suprachiasmatic nucleus. In reptiles, it was suggested that both the pretectal region and the midbrain tegmentum could be the sources of this input to the tectum, but double labeling techniques are needed to demonstrate this situation. In contrast, the NA projection to the tectum seems to arise exclusively from the locus coeruleus in all vertebrates.

Finally, functional data support the implication of catecholamines as modulatory neurotransmitters in the midbrain tectum. In general, both dopamine and noradrenaline have been demonstrated to exert an inhibitory effect on tectal neurons. Catecholamines would modulate different aspects of visual and visuomotor processing, such as attention and orienting behavior, prey-oriented turning movements, and oral behaviors.

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Capítulo 6

Inervación catecolaminérgica de la región septal

Catecholaminergic innervation of the septum in the frog: a combined immunohistochemical and tract-tracing study

The Journal of Comparative Neurology (en prensa)

Catecholaminergic Innervation of the Septum in the Frog: a Combined Immunohistochemical and Tract-Tracing Study

CRISTINA SÁNCHEZ-CAMACHO, JUAN J. PEÑA AND AGUSTÍN GONZÁLEZ
Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain

ABSTRACT

In the present study, we have investigated the distribution and the origin of the catecholaminergic innervation of the septal region in the frog *Rana perezi*. Immunohistochemistry for dopamine and two enzymes required for the synthesis of catecholamines, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) revealed a complex pattern of catecholaminergic (CA) innervation in the anuran septum. Dopaminergic fibers were primarily present in the dorsal portion of the lateral septum, whereas noradrenergic (DBH immunoreactive) fibers predominated in the medial septum/diagonal band complex. Catecholaminergic cell bodies were never observed within the septum. To determine the origin of this innervation, applications of dextran amines, both under *in vivo* and *in vitro* conditions, into the septum were combined with immunohistochemistry for TH. Results from these experiments demonstrated that four catecholaminergic cell groups project to the septum: (1) the group related to the zona incerta in the ventral thalamus, (2) the posterior tubercle/mesencephalic group, (3) the locus coeruleus, and (4) the nucleus of the solitary tract. While the two first groups provide dopaminergic innervation to the septum, the locus coeruleus provides the major noradrenergic projection. Noradrenergic fibers most likely arise also in the nucleus of the solitary tract. The results obtained in *Rana perezi* are readily comparable to those in mammals suggesting that the role of catecholamines in the septum is well conserved through phylogeny and that the CA innervation of the amphibian septum may be involved in functional circuits similar to those in mammals.

Indexing terms: dopamine; noradrenaline; catecholamines; locus coeruleus; retrograde tracing; amphibians

The septal area is a subcortical telencephalic structure that occupies a strategic position in the limbic system of all terrestrial vertebrates. Through extensive and reciprocal interconnections with limbic telencephalic and diencephalic areas and, to a lesser extent, with mesencephalic, lower brainstem and spinal cord regions, the septum is believed to have important functions in behavioral, autonomic and endocrine mechanisms (Risold and Swanson, 1997a,b). Catecholamines, dopamine (DA) and noradrenaline (NA) in particular, have been shown to play a key role in such activities. Moreover, the presence of a strong catecholaminergic (CA) innervation is a feature of the septal region of mammals, characterized by pericellular baskets of DA terminals surrounding unstained cell bodies (Lindvall and Stenevi, 1978; Moore, 1978; Gall and Moore, 1984; Onteniente et al., 1984; Gaspar et al., 1985; Jakab and Leranth, 1990). The origin for this abundant distribution of CA fibers and terminals has been located in the incerto-hypothalamic and mesencephalic dopaminergic groups, and in the locus coeruleus and rhombencephalic noradrenergic groups (Lindvall and Stenevi, 1978; Moore, 1978). Furthermore, this specific and highly organized connections, together with the interaction with the cholinergic and peptidergic systems has been shown to be relevant to human physiopathological problems, such a Alzheimer´s or Parkinson´s disease and the pathogenesis of schizophrenia or epilepsy (Berger, 1984; Ferencz et al., 2001).

Studies on the connections and chemical characteristics of the septal region in birds and reptiles have shown that all amniotic vertebrates share many of the features of the mammalian septum (Reiner et al., 1994; Font et al., 1995, 1997, 1998; Wynne and Güntürkün, 1995). Among them, the dense and distinct catecholaminergic innervation stands out as a main feature of the septal organization and comparable functions for catecholamines in the septum of amniotes were proposed (Reiner et al., 1994; Smeets, 1994, Wynne and Güntürkün, 1995; Risold and Swanson, 1997a).

The septal region in *Rana*, located in the medial hemispheric wall, is homologous to the septal region, as a whole, in mammals (Northcutt, 1974). The septum is clearly separated dorsally from the medial pallium and ventrally, with no clear boundary, from the basal ganglia. Within the septal complex of anurans medial and lateral nuclei were traditionally considered (Northcutt and Kicliter, 1980; Northcutt, 1981). In addition, the nucleus of the diagonal band of Broca was considered as a component of the medial septum (Kicliter and Ebbesson, 1976). Other nuclei previously considered as belonging to the

septal complex, such as the pars medialis of the amygdala, are now regarded as distinct entities of the subpallium (Marín et al., 1998a). Available data about the septal connectivity in amphibians are only fragmentary and scarce. Thus, septal projections to pallial regions and hypothalamus have been demonstrated in anurans (Neary, 1990, 1995). Moreover, the homology of the medial septum of anurans with its counterpart in mammals has been recently strengthened by the demonstration of a forerunner of the cholinergic septo-hippocampal pathway in anurans (González and López, 2002). The organization of the septal afferents in amphibians is practically unknown.

Previous mapping studies in our laboratory and others demonstrated that catecholamine immunoreactivity was moderate to high in the septum of amphibians and the labeled structures were heterogeneously distributed in the different septal regions (Yoshida et al., 1983; González and Smeets, 1991, 1993, 1994a). As part of a research program on the organization of the catecholaminergic cell groups in the brain of amphibians, the first purpose of the present study was to provide a detailed description of dopaminergic and noradrenergic innervation of the septal area in *Rana perezi*. Thus, we analyzed the distribution of tyrosine hydroxylase (TH), dopamine (DA) and dopamine β-hydroxylase (DBH) immunoreactivities throughout the septum. Our second goal was to identify the neuronal groups that give rise to the septal catecholaminergic innervation. In these experiments, retrograde tracing with dextran amines under *in vivo* and *in vitro* conditions was combined with TH immunohistochemistry.

MATERIALS AND METHODS

For the present study, a total of 34 adult green frogs, *Rana perezi*, were used. The animals were obtained from the laboratory stocks of the Department of Cell Biology, University Complutense of Madrid. In all experiments the animals were

Abbreviations

Acc	nucleus accumbens
Cb	cerebellum
CeA	central amygdala
DB	nucleus of the diagonal band of Broca
Is	isthmic nucleus
Jc	juxtacommissural nucleus
Lc	locus coeruleus
Lp	lateral pallium
LS	lateral septum
LSd	dorsal part of the lateral septum
LSv	ventral part of the lateral septum
m	mesencephalic tegmentum
MS	medial septum
MS/DB	medial septum/diagonal band complex
Nsol	nucleus of the solitary tract
nIII	oculomotor nerve
ob	olfactory bulb
Pb	parabrachial nucleus
POa	anterior preoptic area
Ra	raphe nucleus
Rs	superior reticular nucleus
Ri	inferior reticular nucleus
Str	striatum
tm	mesencephalic tectum
TPdm	dorsomedial part of the tuberculum posterius
TPvl	ventrolateral part of the tuberculum posterius
v	ventricle
VH	ventral hypothalamic nucleus
VP	ventral pallidum
vth	ventral thalamus
Zip	periventricular nucleus of the zona incerta

deeply anesthetized by immersion in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz). The original research reported herein was performed under animal care guidelines established by the Spanish Royal Decree 223/1988.

DA immunohistochemistry

Under anesthesia, five animals were perfused transcardially with saline followed by a mixture of 5% glutaraldehyde in 0.05M sodium-cacodylate and 1% $Na_2S_2O_5$ (pH 7.1). The brains were removed and further fixed in the same solution for 1-2 hours at room temperature. They were then immersed in a solution of 30% sucrose with 1% $Na_2S_2O_5$ in 0.1M phosphate buffer (pH 7.1) for 3-5 hours at 4°C, embedded in a solution of 15% gelatin with 30% sucrose, and stored for 5 hours in a 4% formaldehyde solution at room temperature. The brains were cut on a freezing microtome at 40 μm in the frontal, sagittal or horizontal plane, and the sections were collected in Tris-NaCl buffer containing 1% $Na_2S_2O_5$ (pH 7.1). The sections were subsequently processed immunohistochemically according to the peroxidase antiperoxidase (PAP) technique (Sternberger, 1979), using a DA antiserum generously provided by Dr. Buijs (Netherlands Institute for Brain Research, Amsterdam). This includes the following steps: (1) incubation with the DA antiserum (raised in rabbit), diluted 1:2,000 in Tris-NaCl buffered saline (TBS, pH 7.6) containing 1% $Na_2S_2O_5$ and 0.5% Triton X-100 for 16 hours at 4°C; (2) rinsing 3 times for 10 minutes in TBS containing 0.5% Triton X-100 (TBS-T); (3) incubation in TBS-T with swine anti-rabbit antiserum (Nordic), diluted 1:50 for 60 minutes; (4) incubation with rabbit peroxidase anti-peroxidase complex (Dakopatts), diluted 1:800; (5) rinsing 3 times in TBS-T and twice in TBS; (6) staining in 0.5 mgr/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in TBS for 10-20 minutes. The sections were then mounted on glass slides (mounting medium: 0.25% gelatin in Tris buffer) and, after drying overnight, coverslipped.

TH and DBH immunohistochemistry

In this set of experiments, six animals were perfused transcardially with saline followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After two hours of postfixation, the brains were immersed in PB containing 30% sucrose for 3-5 h at 4ºC, embedded in gelatin and cut in the frontal, sagittal or horizontal plane at 40 μm thickness on a freezing microtome. The sections were rinsed in PB and treated with 1% H_2O_2 in PB for 15 minutes to reduce endogenous peroxidase activity. The sections were then processed for TH and DBH immunohistochemistry as described before following the PAP method. Briefly, the sections were first incubated in a mouse anti-TH serum (Incstar, USA), diluted 1:1,000 or a rabbit anti-DBH (Eugene Tech International), diluted 1:300 in PB, for 48 h at 4°C. Subsequently, the sections were rinsed in PB and incubated for 90 minutes in goat anti-mouse serum (1:100; DAKO A/S, Denmark) for TH immunohistochemistry and swine anti-rabbit (1: 50; Nordic) for DBH immunohistochemistry at room temperature, and then processed following the PAP method. After rinsing again, the sections were incubated for 90 minutes in mouse or rabbit PAP (1:600, Chemicon, USA), respectively. The sections were DAB-stained, mounted and coverslipped as described above. For more details the reader is referred to previous works (González and Smeets, 1991, 1995; González et al., 1993).

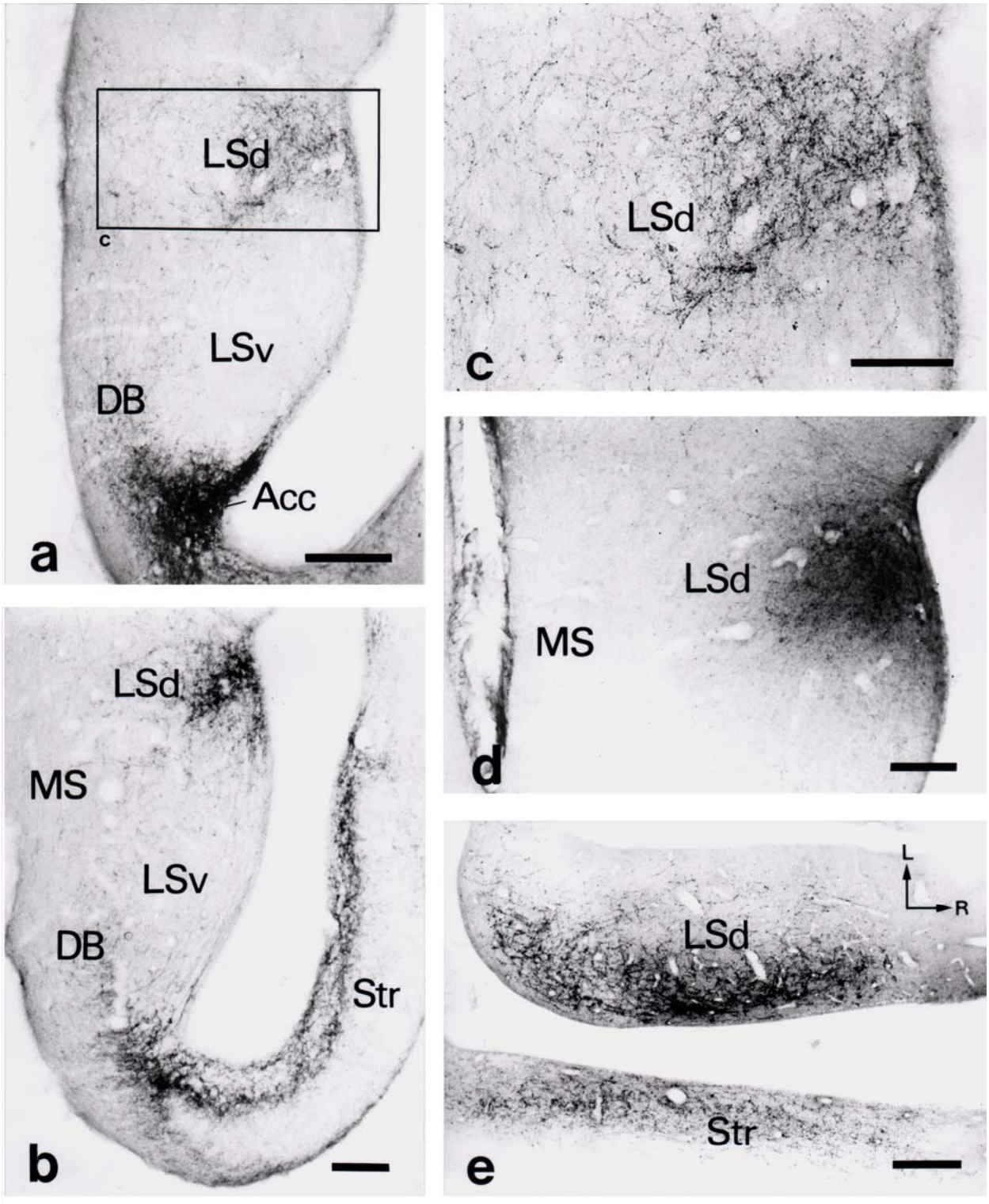

Fig. 1. Photomicrographs of transverse (**a-d**) and horizontal (**e**) sections through the septum of *Rana perezi* illustrating the distribution of THir (**a-c**) and DAir (**d,e**) fibers and terminals. The framed area in **a** corresponds with the photograph shown in **c**. Note that photograph **e** is a horizontal section through the DAir plexus shown in **d**. Calibration bars = 200 μm (a,b), 100 μm (c,d) and 50 μm (e).

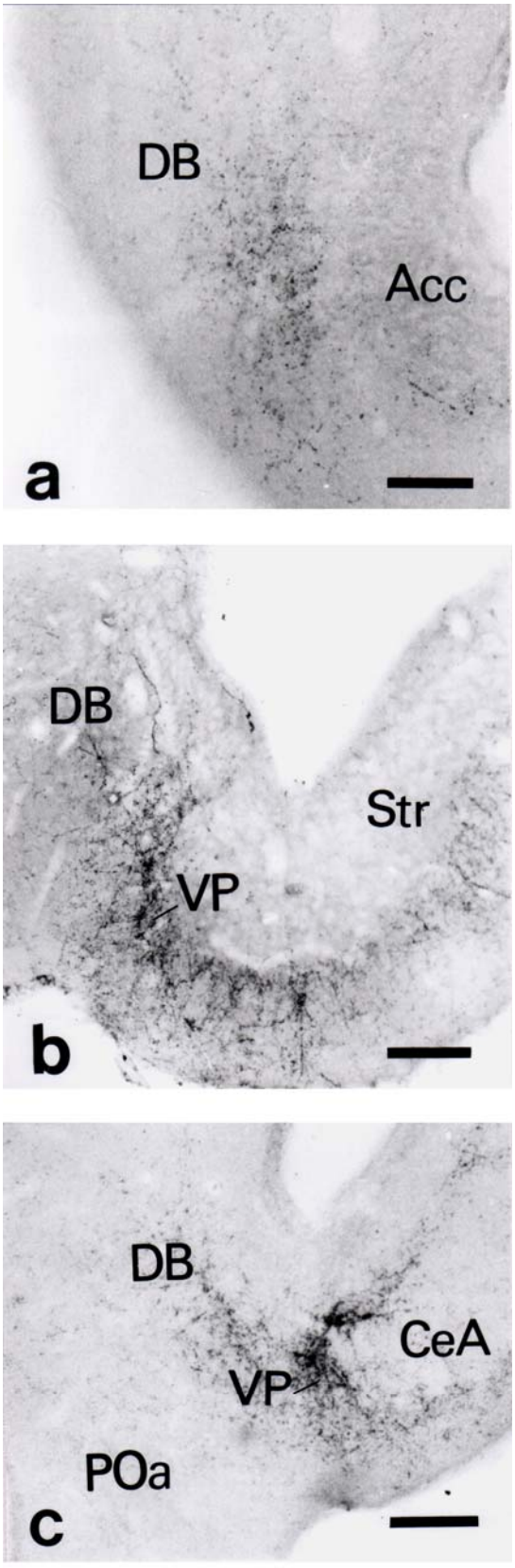

Fig. 2. Photomicrographs of transverse sections through the ventral septum of *Rana perezi* illustrating the distribution of DBHir fibers and terminals at rostral (**a**), intermediate (**b**) and caudal (**c**) levels. Calibration bars = 100 μm (a,b) and 200 μm (c).

Dextran amine tracing combined with TH immunohistochemistry

Retrograde tracing of dextran amines was combined with TH immunohistochemistry to investigate the sources of the CA innervation of the septum. Either *in vivo* or *in vitro* approaches were followed in these experiments.

For the *in vivo* experiments, the tracers biotinylated dextran amine (BDA 3 kD or 10 kD; Molecular Probes) or Texas Red-conjugated dextran amine (TRDA 10 kD; Molecular Probes) were applied unilaterally to different levels of the septal region. The tracers were applied as a solution by means of iontophoretic injections or as crystals on the tip of a needle. Iontophoretic injections were made by applying 4.5-5 μA positive pulsed current (7 s on/7 s off) to the tracer solution (10% BDA 3 kD in 0.9% saline solution) in a glass micropipette (outer tip diameter 10-20 μm) for a period of 10-15 min. Cases with tracer applications as dry crystals were made by impaling the selected brain region with a very sharp tungsten needle on the tip of which the tracer had been recrystalized from a saturated solution in distilled water. In all cases, survival times varied from 5-7 days. Following this period, the animals were deeply anesthetized in 0.3% MS222 and perfused transcardially with saline followed by 150 ml of fixative (4% paraformaldehyde in 0.1 M PB, pH 7.4). The brains were removed and postfixed for two hours in the same fixative. They were then immersed in sucrose, blocked in gelatin and sectioned, as previously described.

A second set of experiments was carried out under *in vitro* whole-brain conditions (modified from Luksch et al., 1996). Only the tracer BDA 3 kD (Molecular Probes) was used in these cases because it is transported faster than those dextran amines of higher molecular weights. Animals were deeply anesthetized in a MS222 solution and transcardially perfused with 50-100 ml iced oxygenated Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose; Merck), which was oxygenated with carbogen (95% O_2, 5% CO_2) to a pH of 7.3 (Straka and Dieringer, 1993). Subsequently, the animals were killed by decapitation and the skin was removed to avoid spread of cutaneous toxins. The brain was rapidly isolated, and after removal of the dura mater and the choroid plexuses to facilitate oxygen diffusion into the tissue, transferred to fresh iced Ringer's solution. The application of the tracer followed immediately. The tracer was always applied as a solution by means of iontophoretic injections as described for the *in vivo* protocol. Then, the brains were immersed and maintained for 15-24 hours at 8ºC in continuously oxygenated Ringer's solution. They were then fixed for 48 hours in 4% paraformaldehyde in 0.1 M PB (pH 7.4), blocked in gelatin and cut on a freezing microtome.

In all cases, tracing experiments were combined with indirect immunofluorescence for TH to reveal catecholaminergic afferent cells to the anuran septal complex. Briefly, brain sections were first incubated for 48 hours at 4ºC with a mouse anti-TH antibody (Incstar) diluted 1:1,000, or a rabbit anti-TH serum (Chemicon) diluted 1:100. They were then incubated with an Alexa™-488-conjugated goat anti-mouse or an Alexa™-594-conjugated goat anti-rabbit serum (Molecular Probes), respectively, both diluted 1:500 for 90 minutes at room temperature. BDA was visualized by incubation with a Texas Red-conjugated streptavidin complex (1:500; Vector Labs.) or Oregon Green streptavidin complex (1:500; Molecular Probes) respectively, together with the secondary antibody. The sections were then mounted on glass slides and coverslipped with Vectashield. Alternation of the appropriate filter combinations in a Zeiss fluorescence microscope allowed the identification of BDA or TRDA retrogradely labeled cells and

TH positive cells. The distribution of labeled cells in the brain of *Rana perezi* was charted in representative transverse sections by means of a camera lucida or a computer-aided X-Y plotting system (Minnesota Datametrics, MD-2 digitizer and software, Minnesota). Photomicrographs were recorded with an Olympus photomicroscope (Olympus, Tokyo, Japan) by using Kodak T-Max 100 professional black-and-white film (Eastman-kodak, Rochester, NY). The nomenclature is the same as that used in our previous studies on the connections of the CA cell groups in the brain of amphibians (Marín et al., 1997b; Sánchez-Camacho et al., 2001b, 2002a,b).

RESULTS

The pattern of distribution of the different catecholamines in the septum was investigated by means of TH, DA and DBH immunohistochemistry. TH, the first and rate-limiting enzyme in catecholamine synthesis is present in all catecholaminergic cells and, thus, the overall distribution of catecholaminergic structures in the septum was revealed by TH immunohistochemistry (Fig. 1a-c). DA immunohistochemistry was used as a specific marker for the dopaminergic system (Fig. 1d,e). Immunohistochemistry for DBH, the enzyme that catalyses the synthesis of NA from DA, resulted in labeling of a subpopulation of TH immunoreactive (THir) fibers, distinct from the domamine immunoreactive (DAir) fibers, that most likely are noradrenergic fibers (Fig. 2). Catecholaminergic cell bodies were never observed within the septum of the frog.

Catecholaminergic structures in the septum

Tyrosine hydroxylase immunoreactivity. Abundant TH innervation was found in restricted septal areas throughout the rostrocaudal extent of the medial telencephalic wall. At rostral levels, the most conspicuous THir fibers and terminals were located in the postolfactory eminence and in the nucleus accumbens. Within the septum proper, a dense THir neuropil was localized in the dorsal portion of the lateral septum (Fig. 1a,c). This neuropil was formed by varicose fibers and terminal-like structures that, in some cases, grouped around unstained cell bodies, although clear pericellular baskets formed by the concentration of these terminals were not identified (Fig. 1c). Sparse THir fibers were also found in the medial septal region, in particular in the diagonal band of Broca. This innervation was observed as a medial concentration of the dense plexus formed in the nucleus accumbens (Fig. 1a).

THir fibers in the dorsal aspect of the lateral septum and in the diagonal band continued caudally up to the level of the anterior commissure but they were most abundant at mid-telencephalic levels (Fig. 1b). Labeled fibers sparsely distributed in the medial septal nucleus and in the ventral lateral septum were also observed, primarily at caudal levels.

Dopamine immunoreactivity. Immunohistochemistry with antibodies against DA revealed only terminal-like structures in a subset of THir regions, as it would be expected given the catecholamine synthetic pathway. In particular, the heavy CA innervation of the dorsal aspect of the lateral septum was demonstrated to contain DA (Fig. 1d,e). The DA staining in this region defined a band throughout the rostrocaudal extent of the septum where the most densely packed DAir structures were localized at mid-telencephalic levels (Fig. 1e). Scattered DA labeling was found in the medial septum but the diagonal band was particularly devoid of DAir structures.

Dopamine β-hydroxylase immunoreactivity. The distribution of DBH immunoreactive (DBHir) fibers within the septum was almost restricted to its ventral portion. Moreover, DBH immunohistochemistry was characterized by labeling only small round varicosities in the septal region, whereas

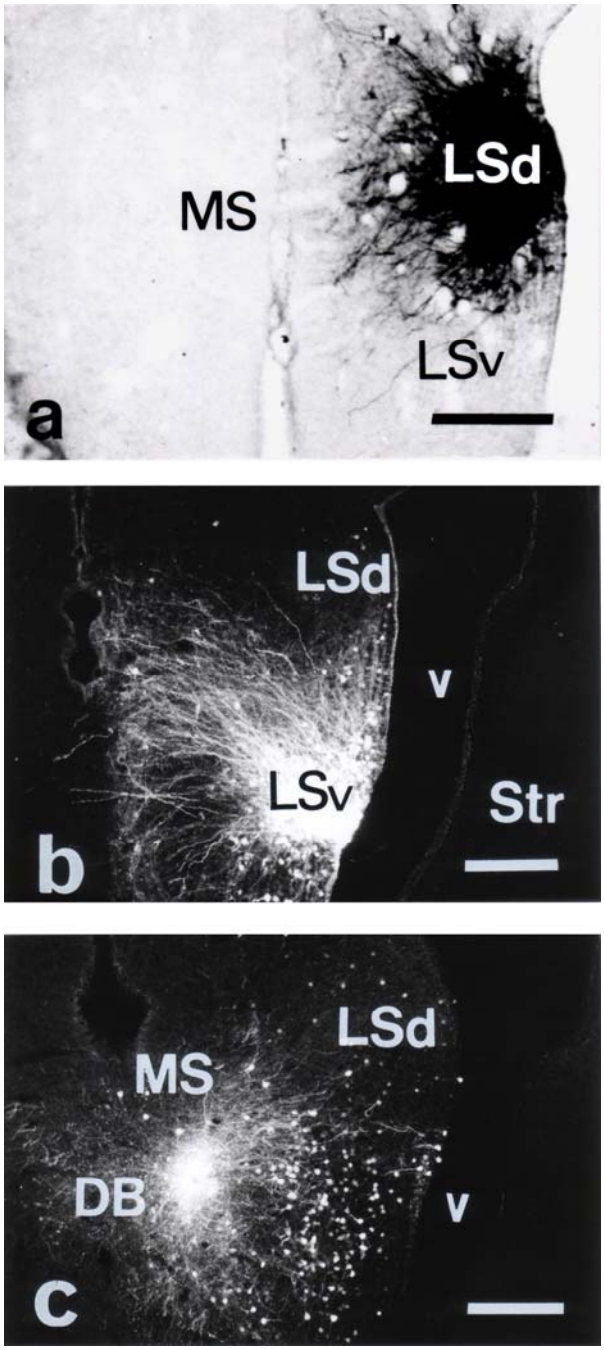

Fig. 3. Photomicrographs of transverse sections through the telencephalon showing representative small injection sites in the dorsolateral (**a**), ventrolateral (**b**) and diagonal band (**c**) nuclei in the septal region of the frog (*Rana perezi*). Calibration bars = 200 μm.

intervaricose segments of immunoreactive fibers were hardly visible. All through the rostrocaudal extent of the septum, the lateral septal region was almost devoid of DBHir structures. Distinct labeling was found exclusively in the nucleus of the diagonal band from rostral to caudal levels (Fig. 2). Starting medially to the nucleus accumbens, which showed almost no DBHir fibers, the nucleus of the diagonal band was densely innervated by DBH fibers that were restricted to its ventral aspect, as a continuation of the conspicuous DBH labeling in the ventral pallidum (Marín et al., 1998a). Only isolated and scattered DBHir terminal-like boutons were found in the dor-

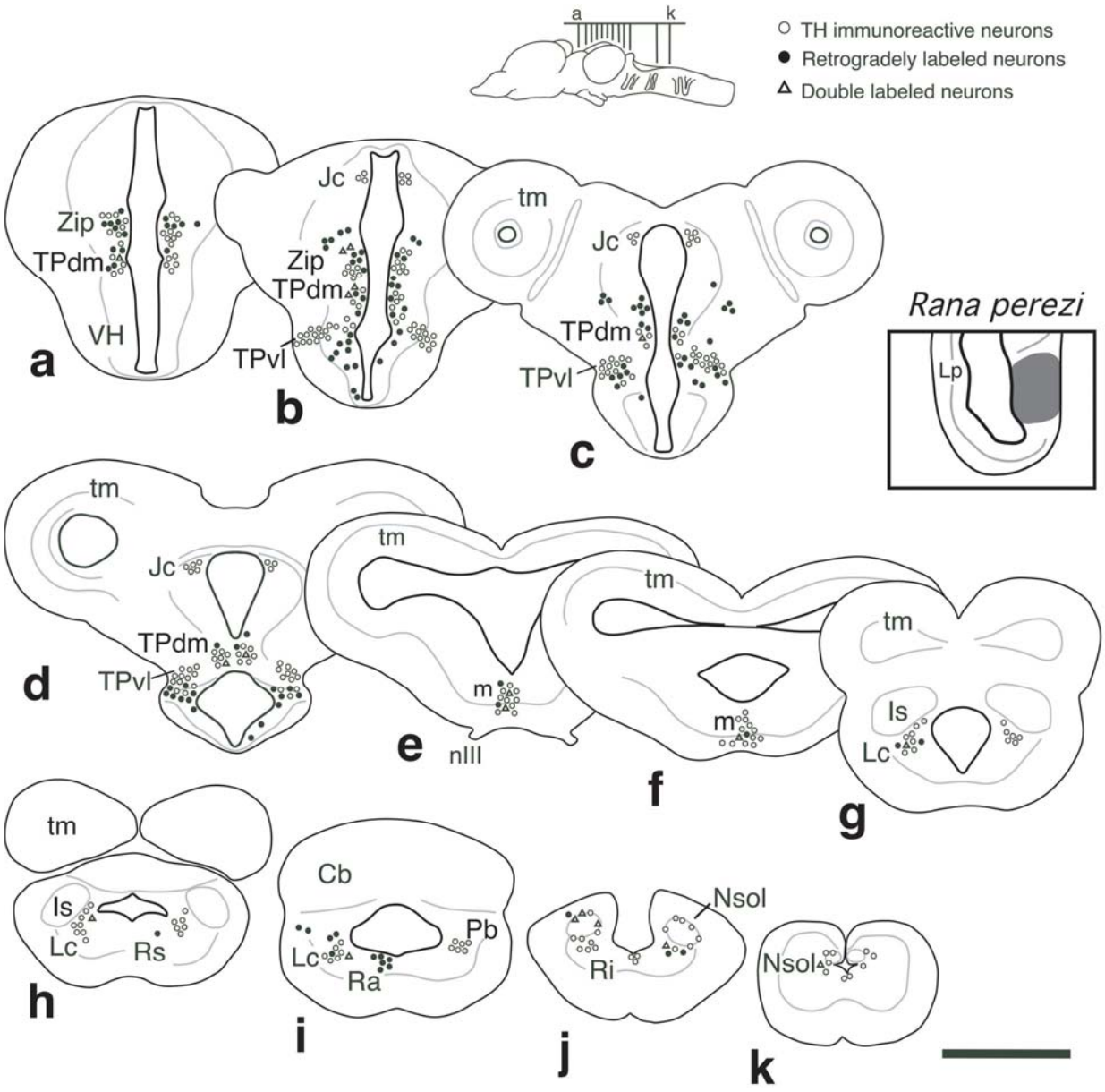

Fig. 4. Schematic drawings of transverse sections through the brain of *Rana perezi* summarizing the localization of retrogradely labeled neurons after tracer applications to the septal region (square in the top). The distribution of catecholaminergic cells as revealed by TH immunohistochemistry, and double labeled cells is also charted. The appropriate levels of the sections are indicated on the scheme of the lateral view of the brain. Calibration bar = 1 mm.

sal aspect of the medial septum and, to a lesser extent, in the ventral lateral septum.

Origin of the catecholaminergic innervation of the septum

Double labeling experiments, both under *in vitro* or *in vivo* conditions, were performed to demonstrate the origin and organization of the catecholaminergic innervation in the anuran septum. Comparable results were obtained by means of both techniques in experiments with injection sites located in similar regions of the septum. The bulk of the CA afferent cells to the septum was revealed in experiments with large injection sites which involved most septal regions. Clues about topography of the CA afferents were achieved following small injections restricted to particular locations in the septum (Fig. 3).

After large tracer applications into the septum, numerous retrogradely labeled neurons were demonstrated in different regions that also contained catecholaminergic cell bodies (Fig. 4). Thus, numerous cells were found in the preoptic area, the suprachiasmatic nucleus, the ventral thalamus, the isthmic tegmentum, and the nucleus of the solitary tract. However, the combination of TH immunohistochemistry with retrograde tracing demonstrated that in some of these centers neurons that project to the septum intermingle with CA cells that do not project to the septum (Fig. 4). These centers are, therefore, not considered as sources of the CA input to the septum. In only four centers a subpopulation of THir cells was also found to

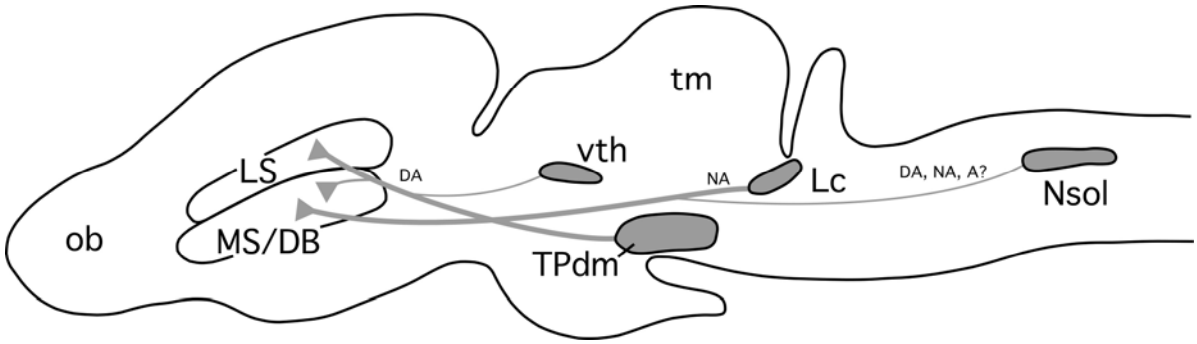

Fig. 5. Schematic diagram summarizing the four CA cell groups that provide innervation to the septum of the frog. The putative CA involved in each projection is indicated. Thick lines correspond to main pathways, whereas thin lines indicate minor projections.

contain retrogradely transported dextran amines (Fig. 5). These are, from rostral to caudal, a small cell population in the ventral thalamus, the dorsomedial part of the posterior tubercle, the locus coeruleus in the isthmic region, and the nucleus of the solitary tract (Figs. 4, 6, 7). All these CA projections were ipsilateral to the injection site, with only a small number of contralateral cells in the nucleus of the solitary tract.

Ventral thalamus. Cells projecting to the septum were located dorsal to the periventricular nucleus of the zona incerta within the ventral thalamus (Puelles et al., 1996). Among these rather numerous cells a small contingent were double labeled with TH immunohistochemistry (Fig. 4b). The CA cells in the ventral thalamus that project to the septum were observed mainly after injections centered in the medial septal nucleus. The CA cell group of the zona incerta and adjacent regions of the ventral thalamus contain dopamine because they stain with TH and DA antibodies but are not revealed with DBH immunohistochemistry (González and Smeets, 1991, 1994a). Therefore, the small number of dopaminergic cells doubly labeled in our experiments in the dorsal portion of the zona incerta region most likely are the origin of the few DAir fibers observed in the medial septal region.

Posterior tubercle. The most important source of septal catecholamines, dopamine in particular, arise from the dorsomedial part of the posterior tubercle and its caudal extent in the mesencephalic tegmentum (Figs. 4a-f, 5, 6). Numerous retrogradely labeled neurons were found bilaterally within this region from rostral to caudal levels, which intermingled with CA cells of the posterior tubercle. However, double labeled neurons were distributed only ipsilaterally from the most rostral part of the nucleus (Fig. 6a,b) to its caudal extent in the mesencephalic tegmentum (Fig. 6c-f). The retrogradely labeled neurons were small, with characteristic round to oval cell bodies. The highest number of double labeled cells concentrated at mesencephalic levels, close to the midline (Figs. 4e,f, 6e,f). This population of CA cells projecting to the septum in the posterior tubercle-mesencephalic tegmental group was always revealed after tracer injections that involved exclusively or partly the dorsolateral aspect of the septum (Fig. 3a). Thus, the bulk of dopamine fibers found in the lateral septum seems to originate in this CA cell group (Fig. 5).

Locus coeruleus. At isthmic levels, retrogradely labeled cells were located in distinct nuclei as the parabrachial or the raphe nuclei. Only a few double labeled cells were found in the ipsilateral locus coeruleus, as the source of noradrenergic fibers in the septal nuclei (Figs. 4g-i, 5, 7a,b). Double labeled neurons in the locus coeruleus distributed from rostral levels ventral to the isthmic nucleus, to caudal levels immediately rostral to de trigeminal motor nucleus. In this location, only noradrenergic cells are located and, therefore, they are assumed to provide all, or most of the DBHir fibers observed in the septum. As a matter of fact, these noradrenergic projection was revealed in those experiments in which the medial septum and, in particular, the nucleus of the diagonal band were totally or partially included in the injection site (Fig. 3c).

Nucleus of the solitary tract. Following tracer applications that involved the caudal portion of the nucleus of the diagonal band (Figs. 3c, 4j,k) numerous retrogradely labeled cells were found around the solitary tract, from mid to caudal rhombencephalic levels. Combination with TH immunohistochemistry demonstrated double labeled neurons within the nucleus of the solitary tract projecting bilaterally to the septum (Figs 4j,k, 7c-f). Because most of these double labeled cells were revealed in experiments with injection sites centered in septal regions with abundant DBHir fiber, it is tempting to assume that noradrenaline is provided to the septum from the nucleus of the solitary tract. However, this nucleus possesses a mixed catecholaminergic cell population where DA, NA and adrenaline containing neurons intermingle (González and Smeets, 1994a) and, therefore, double labeling with TH cannot demonstrate the actual catecholamine involved in the septal projection.

DISCUSSION

Technical considerations

In the present study, TH immunohistochemistry was used to unravel the overall distribution of CA structures in the septum of *Rana perezi*. TH is the first enzyme in catecholamine synthesis and all catecholaminergic cells possess it and, therefore, discrimination between catecholamines (dopamine, noradrenaline or adrenaline) cannot be obtained by this method (Reiner, 1994; Smeets and González, 2000). The use of DA antibodies, however, served to reveal a subset of the TH labeling that accounts for the dopaminergic innervation of the septum. By means of DBH immunohistochemistry, in principle, both noradrenergic and adrenergic structures were revealed (Smeets and Steinbush, 1989). Therefore, in our study, together with the complete pattern of CA innervation of the septum achieved by TH immunohistochemistry, the distinct DA innervation versus the noradrenergic/adrenergic innervation was discriminated in subregions of the septal complex.

Differences were found in the morphology of labeled fibers and terminals by using immunohistochemistry for TH, DA or DBH. Thus, in general, TH immunohistochemistry revealed thin and long varicose fibers with clearly visible intervaricose segments. In contrast, immunoreactive fibers for

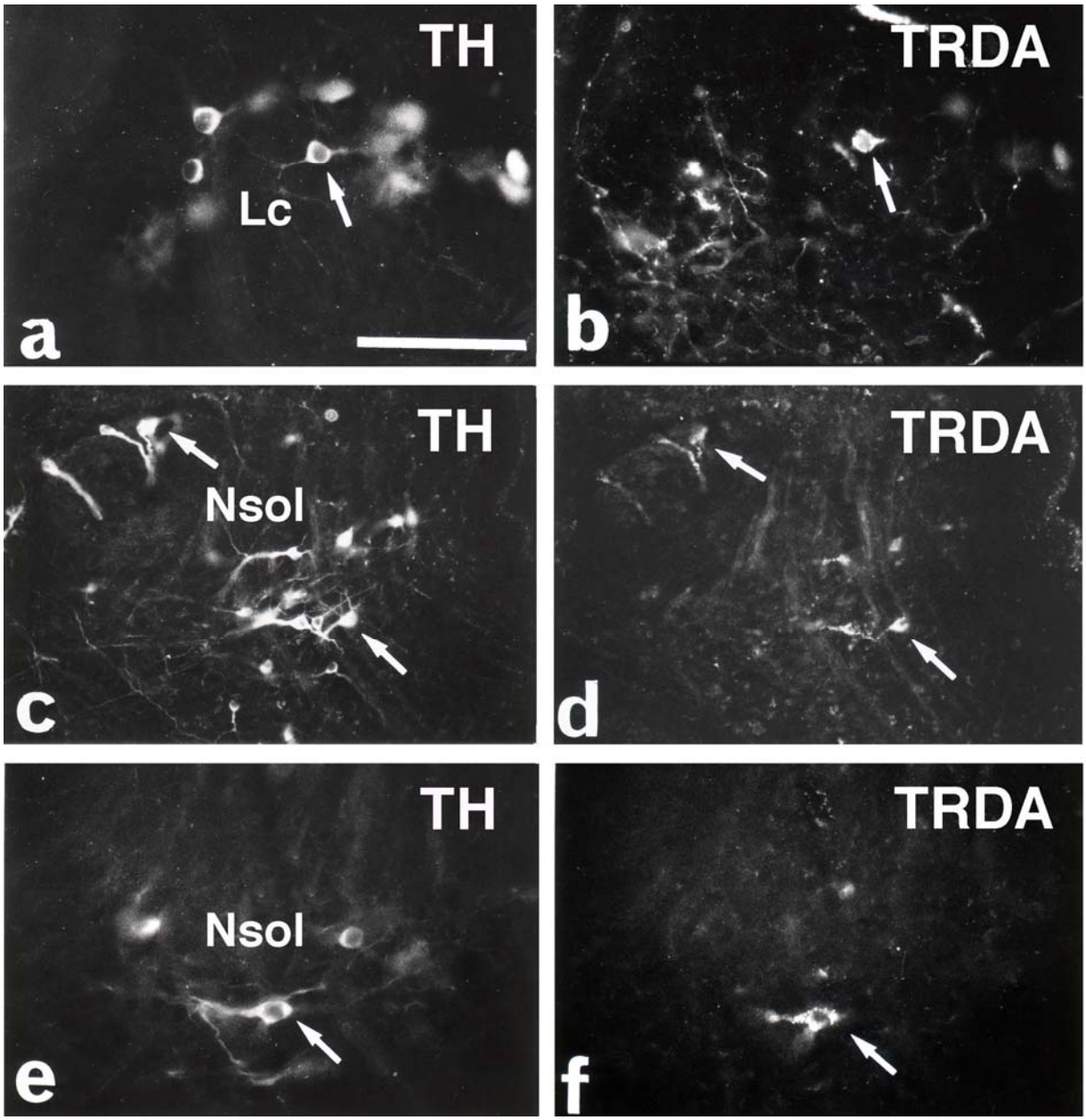

Fig. 6. Photomicrographs of transverse sections through the brain of *Rana perezi* showing the localization of THir cells (**a**,**c**,**e**) and retrogradely labeled cells (**b**,**d**,**f**) after tracer application into the septum in the ipsilateral dorsomedial posterior tubercle (**a**,**b**) and the mesencephalic tegmentum (**c**-**f**). Arrows point to double labeled cells. TH, tyrosine hydroxylase. TRDA, Texas Red-conjugated dextran amine. Calibration bar = 100 μm.

DA or DBH showed a typical morphology, in which these segments were hardly visible and mostly varicosities were labeled. These morphological differences may reflect the different axonal distribution of these substances because TH is located in the cytosol (Pickel et al., 1996), whereas DA and DBH are located in the vesicles which can be specifically accumulated in the varicosities (Venter et al., 1988; Pickel et al., 1996).

For tract-tracing experiments, different approaches were used in our study, both under *in vivo* and *in vitro* conditions. The use of dextran amines *in vivo* was demonstrated to be a powerful tool for axonal tracing in amphibians yielding better results than other tracers (Muñoz et al., 1996; Marín et al., 1997a; Sánchez-Camacho et al., 2001a,b). The use of *in vitro* preparations where dextran amines can be precisely placed in selected brain regions was specially convenient for developmental studies and to deal with regions of the CNS practically inaccessible under *in vivo* conditions (Luksch et al., 1996; Marín et al., 1997c; Sánchez-Camacho et al., 2002a). Dextran amines recrystalized on the tip of sharp needles were used in those studies, whereas in the present study we have obtained successful results by means of iontophoretic injections, applied in the same manner as *in vivo*. The later approach allowed small and precisely located injection sites within the septal region. Importantly, results obtained *in vivo* and *in vitro* were fully comparable, with the only exception of long dis-

tance projections that were generally better traced under *in vivo* conditions. For this reason, the use of low molecular weight dextran amines in the cases of *in vitro* experiments was selected because of their fast axonal diffusion (Fritzsch, 1993).

Comparative aspects of the CA distribution in the septum

The presence of CA fibers and terminals in the septum of amphibians has been corroborated in diverse species (González and Smeets, 1991, 1993, 1994b, 1995). The distinct distribution of DA fibers and DBH fibers in the septum of *Rana perezi* points to a major involvement of DA in laterodorsal septal regions, whereas noradrenergic (or adrenergic) fibers would be primarily related to the medial septum/diagonal band complex. A dense plexus of DA fibers occurs in the lateral septal region of reptiles (Smeets et al., 1986, 1987; Smeets, 1994), birds (Reiner et al., 1994; Wynne and Güntürkün, 1995) and mammals (Moore, 1978; Gall and Moore, 1984; Gaspar et al., 1985), characterized by pericellular baskets of DA terminals surrounding unstained cells. In addition, thinner DA fibers innervate diffusely the lateral septum and , to a lesser extent, the medial septum and do not form baskets. This typical DA innervation in amniotes is differently observed in amphibians where pericellular baskets are not present in the septum of anurans and urodeles but they are found in the large septum of gymnophionans (González and Smeets, 1991, 1994a,b; González et al., 1993). DA innervation of the medial septum is much weaker than in the lateral septum in all amniotes and, among them, lizards seem to possess the strongest innervation in the medial septum/diagonal band complex (Smeets et al., 1986, 1987; Reiner et al., 1994; Smeets, 1994; Wynne and Gütürkün, 1995). The situation observed in *Rana perezi* resembles that of turtles and birds where the medial aspect of the septum was only scantly innervated by DA fibers.

DBH immunoreactive fibers within the septum of amniotes are distinctly distributed in the medial septum/diagonal band complex and, less conspicuously, in the caudoventral lateral septum (Smeets and Steinbusch, 1989; Risold and Swanson, 1997a). The specific use of antibodies against NA and phenylethanolamine-N-methyltransferase (PNMT; the enzyme that catalyzes the conversion of NA to adrenaline) demonstrated in reptiles that both noradrenergic and adrenergic fibers intermingle in the same septal regions (Smeets and Steinbusch, 1989; Smeets and Jonker, 1990). The strong innervation of medial septal regions by noradrenergic/adrenergic fibers seems to be a shared feature of amniotes that, on the basis of our study, can be extended to amphibians.

Four CA cell groups project to the septum

Despite the rich CA innervation of the septum, in all species studied no CA cell bodies were detected within the septum and, therefore, extraseptal origin was assumed for all septal catecholamines. A single exception, however, was reported in the basal forebrain of primates (*Macaca mulatta* and *Macaca fascicularis*) where a distinct subpopulation of THir neurons was observed in the medial septal nucleus and in the diagonal band (Gouras et al., 1992).

Investigations on the origin of the CA innervation of the septum have been performed only in mammals and no data are available for other vertebrate classes. Strikingly, the results of our study are readily comparable to those obtained in mammals. Thus, only four CA cell groups in the brain provide the CA innervation to the septum in both mammals and amphibians.

Incerto-hypothalamic group. Scattered cells within this area (including groups A11, A12 and A13 of Hökfelt et al., 1984) were demonstrated to provide DA innervation to the septum in mammals (Lindvall and Stenevi, 1978). The topographical analysis of the precise location of those cells within the diencephalon points to them as belonging to the ventral thalamus or prosomere 3, according to the segmental criterion (Puelles and Rubenstein, 1993; Smeets and González, 2000). In the present study, we found a small population of THir cells that were retrogradely labeled from the septum and occupied a position between the thalamus and hypothalamus. Following a segmental approach this TH cell group has been compared to the catecholaminergic zona incerta of mammals in prosomere 3 (Puelles et al., 1996; Milán and Puelles, 2000). Since in this location only DA neurons are located in amphibians (González and Smeets, 1994a) the neurons in the region of the zona incerta of *Rana perezi* would provide DA innervation to the septum, as in mammals. This observation strengthens the homology between the amphibian catecholaminergic groups in the ventral thalamus and their counterparts in mammals; as was proposed on the basis of their descending projections (Sánchez-Camacho et al., 2001b).

Mesencephalic tegmental group. The most important source of DA innervation to the septum in mammals arises in the mesencephalic ventral tegmental area (A10 group) as part of the mesolimbic system (Lindvall, 1975; Lindvall and Stenevi, 1978, Swanson, 1982). The A10 cell bodies projecting to different regions in the basal forebrain showed a topographical arrangement in the rat (Fallon and Moore, 1978). In the frog, clear substantia nigra and ventral tegmental area (A9 and A10 groups, respectively) cannot be recognized (González and Smeets, 1994a). However, using the segmental approach and data on their connectivity, the dopaminergic cells in the dorsomedial portion of the posterior tubercle and their caudal continuation in the mesencephalic tegmentum has been proposed as homologous of the A9-A10 groups of amniotes (Marín et al., 1998b). Moreover, within this group a degree of somatotopy was found according to the projections of the dopaminergic neurons to distinct areas of the basal ganglia (Marín et al., 1997b). Thus, the caudal portion of the group was found to project primarily to the nucleus accumbens and, according to the present results, neurons in this region provide also projections to the septum. Therefore, within the rostrocaudal extent of the posterior tubercle-mesencephalic group, the caudal portion would be primarily related with mesolimbic projections and would be compared with the A10 group of mammals.

Locus coeruleus. The majority, if not all, of the NA projections to the septum of mammals was demonstrated to arise in the noradrenergic cell groups of the coeruleus complex, at isthmic levels (Lindvall and Stenevi, 1978; Moore, 1978; Risold and Swanson, 1997a; Senatorov and Renoud, 1999). In our study, a few CA cells in the isthmus were found to project to the septum. Only noradrenergic cells are present in the isthmus and because their catecholaminergic content and connectivity these cells have been considered the amphibian locus coeruleus (González and Smeets, 1993, 1995; Marín et al., 1996). As occurred with their projections to the basal ganglia, the optic tectum or the spinal cord, only few cells in the locus coeruleus seem to provide the septal innervation in amphibians (Marín et al., 1997b; Sánchez-Camacho et al., 2001b, 2002b). This fact points to extensive colateralization of locus coeruleus axons that may reach very different regions in the brain.

Nucleus of the solitary tract. Recent investigations in the rat have demonstrated that a considerable noradrenergic projection to the diagonal band of Broca arises in the nucleus of

the solitary tract (A2 group; Senatorov and Renoud, 1999). In agreement with this result, in *Rana perezi* we found that after tracer injections that involved the diagonal band retrogradely

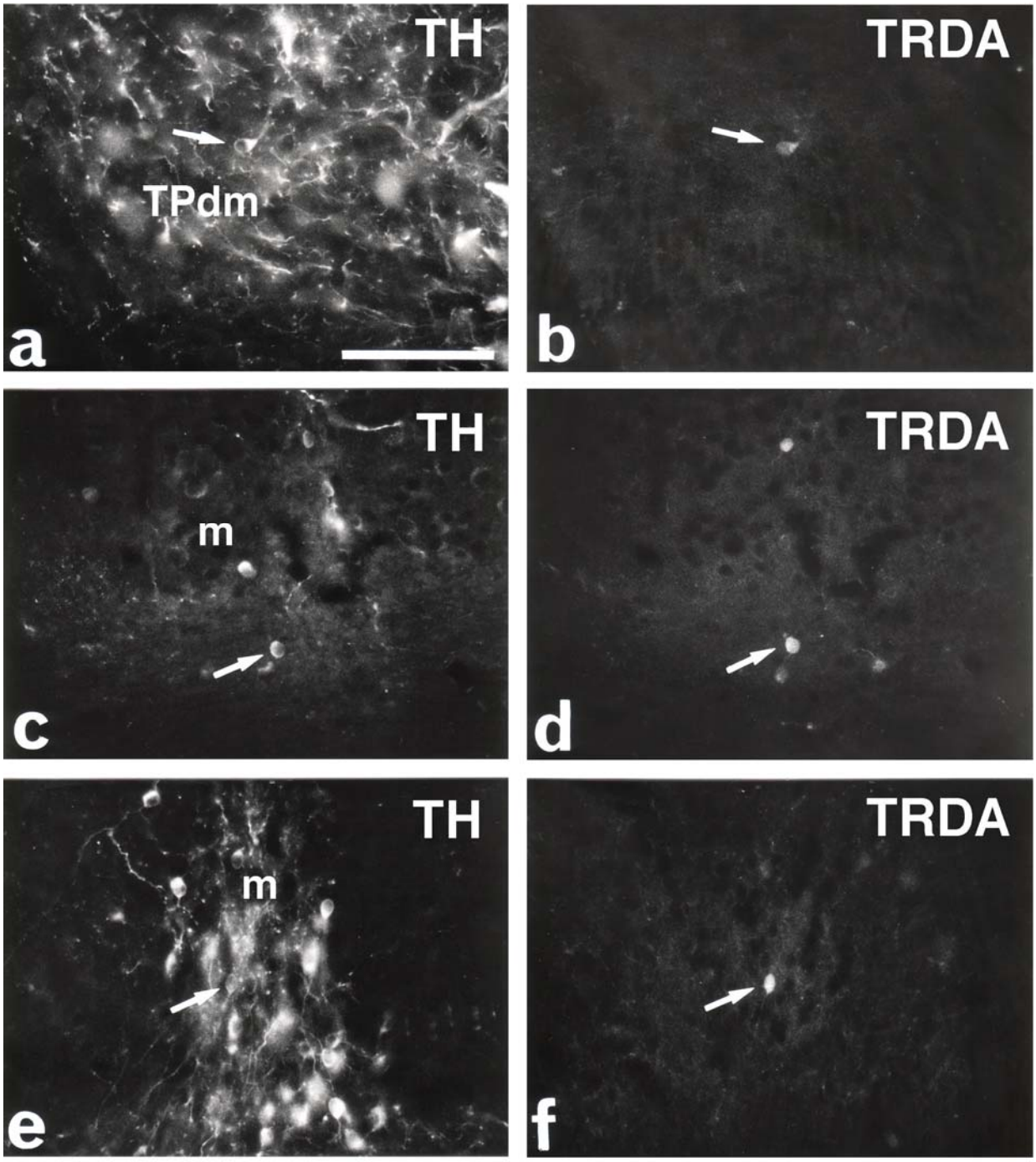

Fig. 7. Photomicrographs of transverse sections through the brain of *Rana perezi* showing the localization of THir cells (**a**,**c**,**e**) and retrogradely labeled cells (**b**,**d**,**f**) after tracer application into the septum in the ipsilateral locus coeruleus (**a**,**b**) and the nucleus of the solitary tract at rostral (**c**,**d**) and caudal levels (**e**,**f**). Arrows point to double labeled cells. TH, tyrosine hydroxylase. TRDA, Texas Red-conjugated dextran amine. Calibration bar = 100 μm.

labeled cells were found in the nucleus of the solitary tract. However, the CA group in the nucleus of the solitary tract of amphibians possesses neurons immunoreactive not only for NA but also for DA and PNMT (González and Smeets, 1994a). Thus, it was not possible to ascertain the actual noradrenergic projection from this nucleus to the septum. However, on the basis of topography and morphology, it seems likely that the bulk of the solitary-septal projection is noradrenergic (González and Smeets, 1993, 1994a).

Based on PNMT immunohistochemistry, the counterparts of the adrenergic groups of the ventrolateral medulla of mammals were proposed to be localized within the nucleus of the solitary tract of amphibians (González and Smeets, 1994a, 1995). The ventrolateral groups in the rat project bilaterally to the diagonal band, whereas the A2 group (nucleus of the solitary tract) projects only ipsilaterally (Senatorov and Renoud, 1999). Thus, the small bilateral projection observed in our study in the nucleus of the solitary tract could be equivalent to the bilateral projection from the ventrolateral medulla of the rat, but specific experiments using PNMT antibodies in combination with retrograde tracing are needed to confirm this projection.

Functional actions of CA in the septum

The functional significance of CA innervation within the septum is largely unknown but numerous physiological studies have dealt with the role of DA and NA within the septal area and their functional interaction with other neurotransmitters.

Dopamine. Contradictory results concerning the physiological influence of the DA system in neurons of the septum have been reported and, therefore, the action of DA may be more complex than a simple excitation or inhibition. Two different DA inputs in the rat lateral septum were proposed: through axodendritic synapses that may cause inhibition, and through asymmetrical axosomatic synapses that may cause excitation (Antonopoulos et al., 1997). In addition, DA has a concentration-dependent modulatory effect on other neurotransmitters, such as Glu and GABA (Chiodo and Berger, 1986). Jakab and Leranth (1990) suggested that high levels of DA release in the septum can result in depression in the activity of lateral septal neurons, while basal levels of DA release can maintain their sensitivity to both excitatory and inhibitory inputs.

DA is involved in the modulation of the activity at all levels within the septal circuitry: in the regulation of the hippocamposeptal pathway by the influence of glutamatergic hippocampal neurons, in the septohippocampal route over cholinergic cells in the medial septum/diagonal band nucleus (Robinson et al., 1979; Costa et al., 1983), and finally influencing GABAergic neurons of the lateral septum (Jakab and Leranth, 1990).

Robinson and coworkers (1979) demonstrated a role of the mesolimbic DA system in the regulation of the turnover rate of acetylcholine in the hippocampus. Thus, DA neurons exert a tonic inhibitory effect on acetylcholine metabolism of septohippocampal pathway. In the lateral septal area, GABAergic spiny neurons receive inputs from CA afferents (Jakab and Leranth, 1990), which. provide a morphological basis of the interaction of glutamate and DA.

The possible role of DA in the septum of anurans may only be inferred by the distribution of the DAir fibers found in our study. Thus, as in mammals, interactions with cholinergic cells in the medial septum (Marín et al. 1997d) and GABAergic cells in the lateral septum (Franzoni and Morino, 1989) seem likely in different anuran species.Additionally, a close relation of DA fibers with cholinergic cells origin of a primitive septohippocampal pathway may also be present in *Rana perezi* (González and López, 2002).

Noradrenaline. In mammals, excitatory and inhibitory effects of NA on GABAergic activity have been previously shown in the lateral septum as well as the medial septum/diagonal band nucleus. Thus, NA via α1-adrenoreceptors, excites medial septum-diagonal band septohippocampal GABAergic neurons and influences both septal and septohippocampal circuitry (Alreja and Liu, 1996). In the lateral septum, NA also modulates the activity of intranuclear GABAergic circuits. Neurons within this region are inhibited or excited directly by NA by activation of α2 and α1 adrenoreceptors and indirectly via a modification of GABA release (Carette, 1999; Carette et al., 2001).

A particular role has been proposed for NA on the diagonal band whose neurons participate in a central baroreceptor-initiated inhibitory modulation of neurohypophysial vasopressin secretion in the rat (Senatorov and Renoud, 1999). This action would be accomplished by regulation of diagonal band cells projecting to vasopressin containing cell groups.

The dense NA innervation of the medial septum/diagonal band complex in the frog, as in mammals, suggests similar functions in both vertebrate groups, but no data are available for amphibians. However the possible implication of NA inputs to the diagonal band on cells that project to vasotocin (vasopressin homologue) cell groups may be guaranteed on the basis of hodology (González and Smeets, 1992; Neary, 1995)

CONCLUDING REMARKS

The organization of the CA input to the amphibian septal area shares many features with that of amniotes. Thus, dopaminergic fibers are preferentially located in the dorsal part of the lateral septum, whereas DBHir fibers distribute mainly at caudal levels in the nucleus of the diagonal band. The origin of the CA innervation of the septum seems to be well conserved across vertebrates because of the extreme similarity found between amphibians and mammals, although further research is needed in other vertebrate classes. The anatomical similarities between amphibians and mammals make it likely that the CA innervation of the amphibian septum may be involved in functional circuits similar to those in mammals.

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Capítulo 7

Resumen de los resultados y Discusión general

Resumen de los Resultados

En el presente trabajo hemos realizado una caracterización hodológica de los sistemas CA en anfibios, tanto en especímenes adultos como durante el desarrollo. En primer lugar, mediante el empleo de técnicas inmunohistoquímicas analizamos en detalle la distribución de fibras y terminales CA en tres estructuras diferentes: la médula espinal, el techo mesencefálico y la región septal de diferentes especies de anfibios. Además, mediante métodos de doble marcaje, determinamos los centros de origen de dichas aferencias CA a estas estructuras encefálicas. Aunque los principales resultados de este estudio se han centrado en analizar la conectividad, ontogenia y distribución de las proyecciones CA a varias regiones del encéfalo, también se ha obtenido información detallada y completa sobre la organización y el desarrollo de las vías descendentes a la médula espinal. Con todos estos datos, intentamos evaluar las similitudes y diferencias en la organización de los grupos CA entre los distintos órdenes de la clase Amphibia y conocer hasta qué punto el patrón de conectividad en los vertebrados anfibios es comparable al existente en amniotas.

En el **Capítulo 2** se describe la organización de los sistemas descendentes a la médula espinal de los anfibios, empleando técnicas de trazado retrógrado con dextranaminas aplicadas en forma de cristal en diferentes niveles medulares. En este apartado describimos en detalle la trayectoria funicular de las aferencias espinales, así como el nivel que alcanzan dichas proyecciones en la médula espinal. Nuestros resultados corroboran datos que se habían publicado con anterioridad en anuros, pero a la vez amplian dicha información demostrando la presencia de conexiones que no se habían descrito hasta ahora. Este hecho se debe fundamentalmente a la mayor eficacia y sensibilidad de las dextranaminas como trazadores frente a otro tipo de compuestos como la peroxidasa de rábano o el complejo cobalto-lisina que se habían empleado en estudios precedentes (ten Donkelaar y cols., 1981; Tóth y cols., 1985). Asimismo, los resultados que presentamos sobre las conexiones espinales en urodelos y ápodos constituyen los primeros datos conocidos hasta el momento en estos órdenes de anfibios. Hay que destacar también, que para este estudio hemos empleado especies representativas de los tres órdenes de la clase Amphibia, concretamente los anuros *Rana perezi* y *Xenopus laevis*, el urodelo *Pleurodeles waltl*, y el ápodo *Dermophis mexicanus*. El estudio llevado a cabo en distintas especies resulta de gran importancia a la hora de comparar los resultados obtenidos y poder inferir diferencias o características comunes en cuanto a la organización de los sistemas descendentes espinales entre los tres órdenes de anfibios en función del modo de vida de la especie. Así, en el caso de *Rana*, se trata de una especie adaptada tanto al medio terrestre como al acuático, que se desplaza mediante saltos, mientras que *Xenopus* está adaptado a una vida exclusivamente acuática. En el caso de *Pleurodeles*, se desenvuelve con mayor dificultad en el medio terrestre, utilizando su cola para nadar. El ápodo *Dermophis* se caracteriza porque pasa la mayor parte de su tiempo enterrado, y presenta una morfología en forma de gusano caracterizada por la ausencia de extremidades, desplazándose mediante movimientos ondulados de su cuerpo.

Los resultados de este trabajo demuestran la presencia de los principales tractos descendentes espinales en las cuatro especies de anfibios estudiadas. En todos ellos, está presente una importante proyección troncoencefálico-espinal, mientras que las conexiones espinales originadas en regiones del prosencéfalo tienen una menor representación (Fig. 1). Así, en el rombencéfalo, las proyecciones a la médula espinal se originan en la formación reticular, el área octavolateral, el locus coeruleus, el núcleo tegmental laterodorsal, el núcleo del rafe, núcleos sensoriales trigeminales, el núcleo de la columna dorsal, y el núcleo del tracto solitario. En todas las especies estudiadas, encontramos células marcadas retrógradamente en el núcleo cerebeloso y algunas células dispersas en el cerebelo, que inervan principalmente la médula espinal contralateral. Las proyecciones mesencefálicas incluyen las aferencias tectoespinales y toroespinales, y una importante proyección tegmentoespinal. Dentro de las conexiones con el tegmento mesencefálico encontramos células de proyección en el núcleo de Edinger-Westphal, el núcleo rojo, y los núcleos tegmentales anterodorsal, anteroventral y posteroventral. Las proyecciones diencéfalo-espinales se originan en el tálamo ventral, el tubérculo posterior, la región pretectal y el núcleo intersticial del fascículo longitudinal medial (flm). Por último, las células localizadas más rostralmente que dan lugar a las vías descendentes espinales se encontraron en el núcleo supraquiasmático, el área preóptica, y una región supalial en el hemisferio telencefálico caudal, que posiblemente pertenece al complejo amigdalino.

En el **Capítulo 3**, analizamos en primer lugar la distribución de la inmunorreactividad frente a la enzima TH en varios niveles espinales de dos especies de anuros (*Rana perezi* y *Xenopus laevis*), un urodelo (*Pleurodeles waltl*), y un ápodo (*Dermophis mexicanus*). El patrón de inervación CA en la médula espinal es similar en todas las especies estudiadas, aunque encontramos diferencias interespecíficas en el número y la morfología de las fibras TH-inmunoreactivas (THi). Así, la inervación CA es particularmente densa en el asta dorsal y la zona dorsal al canal central. Sólo se encontraron algunas fibras dispersas distribuidas en el asta ventral de anuros, mientras que en urodelos y ápodos se demostró la presencia de una fuerte inervación de grandes neuronas localizadas en el campo ventral espinal. Más aún, la distribución de las fibras CA en el campo intermediolateral espinal, sugiere la inervación de las células del sistema autónomo localizadas en esta zona. En cuanto a la presencia de células CA intraespinales, se observó un grupo de neuronas ventrales al canal central a lo largo de la médula espinal que contactan con el líquido cefalorraquídeo. En anuros, encontramos además neuronas THi dispersas en el campo dorsolateral en niveles espinales rostrales. Estas células constituyen la continuación caudal del núcleo del tracto solitario/área postrema, aunque ocasionalmente se observaron algunas células aisladas en niveles braquiales caudales.

Aunque en los tres órdenes de anfibios existen células CA intraespinales, no está claro hasta qué punto la inervación CA de la médula espinal es de origen intra- o supraespinal. Así, el segundo objetivo que planteamos en esta parte del trabajo fue la demostración de los centros encefálicos responsables de las aferencias CA a la médula espinal, empleando técnicas de doble marcaje mediante el uso de trazado retrógrado con dextranaminas combinado con inmunohistoquímica para la enzima TH. Estos experimentos demostraron que cuatro grupos celulares proporcionan la inervación CA a la médula espinal: 1) la porción ventrolateral del tubérculo posterior en la región mamilar; 2) el núcleo periventricular de la zona incerta en el tálamo ventral; 3) el locus coeruleus en la región ístmica; y 4) el núcleo del tracto solitario en niveles rombencefálicos caudales (Fig. 2). Este patrón es constante en todas las especies estudiadas, excepto por la falta de una proyección CA espinal desde el núcleo del tracto solitario en ápodos.

Para completar los resultados obtenidos en los trabajos previos acerca de la organización de los sistemas aferentes a la médula espinal (Capítulos 2 y 3), en el **Capítulo 4** describimos la secuencia temporal de aparición de las vías supraespinales descendentes así como el desarrollo de las aferencias CA espinales. Para llevar a cabo estos estudios, utilizamos *Xeno-*

pus laevis como modelo debido a la existencia de una tabla detallada de su desarrollo (Nieuwkoop y Faber, 1967) y la disponibilidad de larvas y embriones en nuestro laboratorio. En primer lugar, mediante técnicas de trazado retrógrado *in vitro*, describimos la ontogenia de las conexiones descendentes supraespinales durante el desarrollo de *Xenopus*. Nuestros resultados demuestran que las proyecciones a la médula espinal desde diversos grupos celulares están bien desarrolladas desde *estadios embrionarios* tardíos. Así, a partir del estadio 40 están presentes las conexiones desde la formación reticular, los núcleos del rafe, las neuronas de Mauthner, los núcleos vestibulares, el locus coeruleus, el núcleo intersticial del flm, el tubérculo posterior y el núcleo periventricular de la zona incerta. Al inicio del *período premetamórfico* (estadio 46), aparecen las proyecciones supraespinales desde el núcleo supraquiasmático, el torus semicircularis, la región pretectal y el telencéfalo ventral. Durante la premetamorfosis, se desarrollan también las aferencias tectoespinales y cerebeloespinales (estadio 48), la vía rubroespinal (estadio 50) y las conexiones espinales desde el área preóptica (estadio 51). Finalmente, durante el *período prometamórfico*, aparecen las proyecciones desde el núcleo del tracto solitario, el área de la línea lateral y el núcleo mesencefálico del nervio trigémino.

Como segundo objetivo en esta parte del estudio, analizamos el desarrollo de la inervación CA de la médula espinal mediante el empleo de métodos de doble marcaje, basados en el trazado retrógrado *in vitro* con dextranaminas en combinación con inmunohistoquímica para la enzima TH. Nuestros resultados muestran que a partir del estadio 40/41, las neuronas CA del tubérculo posterior son las primeras en proyectar a la médula espinal. Posteriormente, en el estadio 43, aparecen nuevas conexiones desde el núcleo periventricular de la zona incerta y el locus coeruleus. La última proyección CA a la médula espinal se origina en las neuronas del núcleo del tracto solitario al inicio de la prometamorfosis (estadio 53). Estos resultados demuestran la existencia de una secuencia temporal rostrocaudal en la aparición de los grupos celulares CA que proyectan a la médula espinal. En cuanto al desarrollo de la inervación CA en la médula espinal, se caracteriza por la presencia de fibras THi desde estadios embrionarios que se distribuyen inicialmente en las zonas marginales, y que a medida que avanza el desarrollo van invadiendo la sustancia gris espinal.

En el **Capítulo 5**, hemos llevado a cabo un estudio detallado y comparado de la distribución de fibras y terminales CA mediante el empleo de métodos inmunohistoquímicos para detección de la DA y las enzimas TH y dopamina -hidroxilasa (DBH), que revela la existencia de un patrón complejo en la inervación CA del techo mesencefálico de anuros (*Rana perezi*) y urodelos (*Pleurodeles waltl*). En general, la distribución de fibras y terminales inmunoreactivos en el techo óptico presenta una organización selectiva de acuerdo con un patrón laminar. En *Rana*, las fibras THi/DAi se distribuyen principalmente en las capas tectales profundas, en particular en las capas 3, 5 y 7, mientras que en *Pleurodeles* las capas 4, 5 y 7 son las que presentan una inervación más densa. Por el contrario, las fibras DBHi se localizan de forma mayoritaria en las capas tectales superficiales, tanto en anuros como en urodelos.

La aplicación de trazadores retrógrados en el techo óptico de *Rana* y *Pleurodeles* reveló las conexiones aferentes a esta estructura mesencefálica. Aunque nuestros resultados corroboran en gran medida datos previos sobre la conectividad del techo en anfibios (Wilczynski y Northcutt, 1977; Finkenstädt y cols., 1983; Rettig, 1988; Hofmann y cols., 1990), demostramos la existencia de nuevas proyecciones desde centros que no se habían descrito con anterioridad. Así, en la rana encontramos células de proyección al techo óptico en el estriado, la amígdala central, el pálido dorsal, el área preóptica anterior y el núcleo supraquiasmático. Además, los experimentos de trazado revelan la presencia de células dispersas dentro del hipotálamo dorsal y el tubérculo posterior ventrolateral. Sin embargo, los grupos más numerosos se localizan dentro del tálamo dorsal y el tálamo ventral, y en la región pretectal. En particular, existe una importante proyección ipsilateral al techo desde los núcleos talámicos anterior, central y lateral anterior. Dentro del tálamo ventral, se encontraron células marcadas retrógradamente en los núcleos ventromedial y ventrolateral, y el núcleo periventricular de la zona incerta. La proyección pretecto-tectal se origina fundamentalmente desde los núcleos yuxtacomisural y posterodorsal lateral, y en menor medida desde los núcleos precomisural y lentiforme. Hay que destacar también la presencia de una conexión intratectal desde las capas 6 y 7 del techo contralateral, junto con una importante conexión istmo-tectal, tegmento-tectal y toro-tectal. Finalmente, se demostró la presencia de células en la formación reticular y el núcleo del tracto solitario. En el urodelo, las proyecciones aferentes al techo óptico son similares a las demostradas en la rana, aunque no encontramos una conexión desde el estriado o la amígdala en esta especie.

Con objeto de determinar el origen de las aferencias CA en el techo mesencefálico, utilizamos la aplicación *in vivo* de dextranaminas en forma de cristales en combinación con la inmunodetección de la TH. Los resultados de estos experimentos demostraron que las neuronas DA de los núcleos supraquiasmático y yuxtacomisural (en *Rana*) o el núcleo pretectal (en *Pleurodeles*), junto con las células NA del locus coeruleus

Abreviaturas

Amyg	amígdala
DCN	núcleo de la columna dorsal
Jc	núcleo yuxtacomisural
Lc	locus coeruleus
LDT	núcleo tegmental laterodorsal
Lpd	núcleo posterodorsal lateral
LS	septo lateral
MesV	núcleo mesencefálico del nervio trigémino
MS/DB	complejo del septo medial/banda diagonal
nCb	núcleo cerebeloso
Nflm	núcleo intersticial del flm
Nsol	núcleo del tracto solitario
PC	núcleo precomisural
POa	área preóptica anterior
Ra	núcleo del rafe
Ri	núcleo reticular inferior
Rm	núcleo reticular medio
Rs	núcleo reticular superior
Rub	núcleo rojo
SC	núcleo supraquiasmático
tegm	tegmento mesencefálico
tm	techo mesencefálico
Tor	torus semicircularis
TPvl	tubérculo posterior ventrolateral
TPdm	tubérculo posterior dorsomedial
VIIIa	núcleo vestibular anterior
VIIIc	núcleo vestibular caudal
VIIIvl	división lateral del núcleo vestibular ventral
VIIIvm	división medial del núcleo vestibular ventral
VL	núcleo talámico ventrolateral
VM	núcleo talámico ventromedial
Zip	núcleo periventricular de la zona incerta

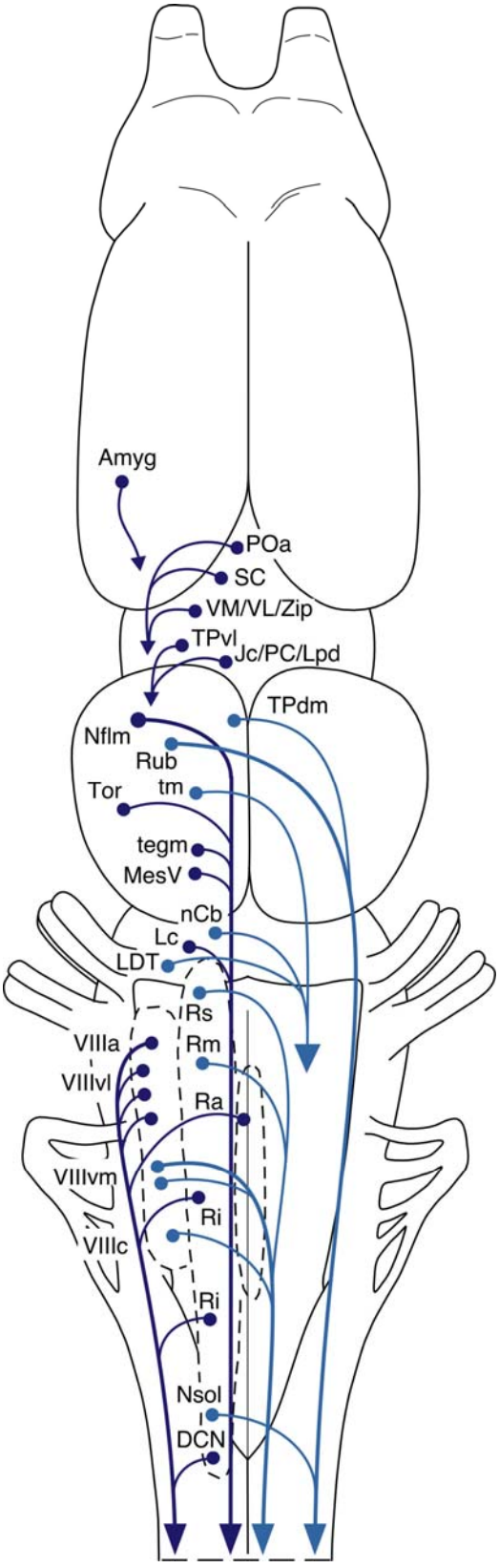

Fig. 1. Resumen de las principales vías aferentes a la médula espinal en el anuro *Rana perezi*.

constituyen la principal fuente de fibras y terminales CA en el techo óptico de anfibios (Fig. 2). Sólo ocasionalmente se encontraron células doblemente marcadas en la porción ventrolateral del tubérculo posterior de *Rana perezi*.

Finalmente, en el **Capítulo 6** estudiamos la organización de la inervación catecolaminérgica en la región septal del anuro *Rana perezi* mediante el empleo de inmunohistoquímica para la DA y las enzimas TH y DBH. Nuestros resultados demuestran que existe un patrón selectivo y específico en la distribución de las fibras y terminales CA dentro de la región del septo. Así, encontramos que las fibras DA se distribuyen principalmente en la porción dorsal del septo lateral, mientras que las fibras NA (DBHi) están predominantemente localizadas en el complejo del septo medial/banda diagonal.

Para determinar los centros de origen de esta inervación CA, en este trabajo utilizamos diferentes técnicas de trazado neuronal. En unos casos se emplearon métodos de trazado retrógrado con dextranaminas siguiendo una aproximación *in vivo* idéntica a la utilizada en trabajos previos para individuos adultos (Capítulos 2, 3 y 5), o una aproximación *in vitro* similar a la empleada en los estudios de desarrollo (Capítulo 4) pero adaptada para individuos adultos (ver Consideraciones Metodológicas). Tanto las aplicaciones *in vivo* como las realizadas en condiciones *in vitro*, se combinaron con la inmunodetección de la enzima TH. Los resultados de estos experimentos demuestran que cuatro grupos celulares CA proyectan a la región del septo en la rana: 1) un grupo DA localizado dorsal al núcleo periventricular de la zona incerta, dentro del tálamo ventral; 2) el tubérculo posterior dorsomedial y su continuación caudal en el tegmento mesencefálico; 3) el locus coeruleus; y 4) el núcleo del tracto solitario (Fig. 2). Mientras que los dos primeros grupos son responsables de la inervación DA del septo, el locus coeruleus y probablemente también el núcleo del tracto solitario, proporcionan las aferencias NA a la región septal.

Discusión General

Consideraciones Metodológicas

Técnicas Inmunohistoquímicas para la Detección de las Catecolaminas

El empleo de anticuerpos frente a las enzimas de síntesis de las catecolaminas supuso un gran avance en el conocimiento de la distribución de los sistemas catecolaminérgicos (CA) en el SNC de los vertebrados. La especificidad en el marcaje con estos anticuerpos obtenidos en mamíferos, resultó ser muy alta cuando se aplicaron en el encéfalo de otras especies de vertebrados no-mamíferos. En particular, los anticuerpos contra la enzima TH se han empleado de forma generalizada en numerosas especies representativas de las distintas clases de vertebrados, demostrando su especificidad en el marcaje de las estructuras CA (Smeets y Steinbusch, 1990; Smeets y González, 2000). Por el contrario, la reactividad cruzada interespecífica de los anticuerpos frente a las enzimas DBH y PNMT, implicadas en la conversión de la DA en NA y de ésta en adrenalina respectivamente, ha resultado ser menos efectiva. Con el desarrollo de anticuerpos frente a la dopamina y la noradrenalina fue posible la demostración directa de estos neurotransmisores en el encéfalo de vertebrados. Sin embargo, los anticuerpos frente a la adrenalina resultaron menos efectivos en el marcaje de las estructuras adrenérgicas, de manera que los datos existentes sobre la distribución de este neurotransmisor se deben fundamentalmente a los estudios realizados con inmunohistoquímica para la enzima PNMT.

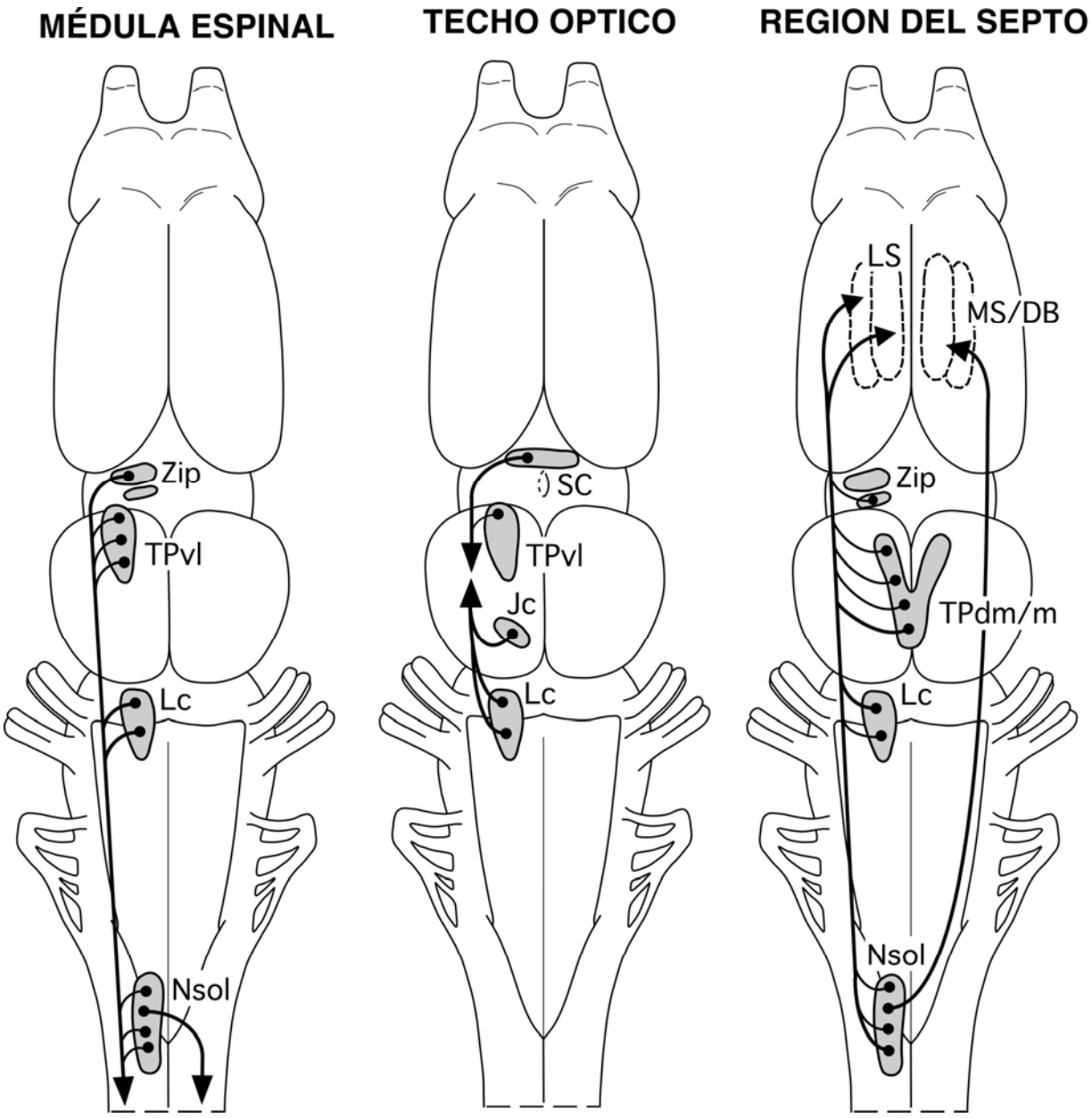

Fig. 2. Esquemas representativos de las principales aferencias catecolaminérgicas a la médula espinal, el techo mesencefálico y la región septal en el anuro *Rana perezi*.

En los anfibios, los anticuerpos frente a la DA y las enzimas TH y DBH dan lugar a un patrón específico y constante de inmunomarcaje de los sistemas CA en todas las especies analizadas hasta el momento, lo que demuestra la utilidad de estos anticuerpos (González y cols., 1993, 1994, 1995; González y Smeets 1991, 1993, 1994a,b 1995). En nuestro estudio, la inmunohistoquímica para la enzima TH se ha utilizado para demostrar la distribución de las fibras y terminales CA en la médula espinal, el techo mesencefálico y el septo de varias especies de anfibios (*Rana perezi*, *Xenopus laevis*, *Pleurodeles waltl* y *Dermophis mexicanus*). Mediante la inmunodetección de esta enzima no se puede distinguir entre las distintas estructuras que contienen DA, NA o adrenalina en el SNC. Sin embargo, ya que las células dopaminérgicas (DA) y noradrenérgicas (NA) constituyen poblaciones celulares separadas en el encéfalo, su auténtica naturaleza se puede inferir en base a su localización topográfica, excepto en el núcleo del tracto solitario donde se han encontrado células DA, NA y adrenérgicas. El uso de anticuerpos frente a DA revela la presencia de una subpoblación de células TH positivas responsables de la inervación dopaminérgica. Del mismo modo, el empleo de inmunodetección de la enzima DBH revela la presencia de estructuras NA y adrenérgicas en el SNC (Smeets y Steinbush, 1990).

Es importante destacar que hemos encontrado diferencias en la morfología de las fibras y terminales marcados mediante el empleo de inmunohistoquímica para la TH o la DBH. En general, la inmunodetección de la enzima TH revela fibras largas y varicosas, con segmentos intervaricosos claramente

visibles. Por el contrario, las fibras inmunopositivas para DBH presentan una morfología típica que se caracteriza porque estos segmentos son difícilmente visibles, marcando solamente las varicosidades. Una posible explicación para estas diferencias morfológicas es la distribución axonal de estas enzimas. Así, la TH se localiza en el citosol, mientras que la DBH está presente en las vesículas sinápticas que se pueden acumular específicamente en las varicosidades (Venter y cols., 1988; Pickel y cols., 1996). Este hecho podría explicar la presencia de inmunorreactividad para la TH en toda la fibra, mientras que el marcaje para la DBH aparece sólo en las varicosidades.

Técnicas de Trazado Axonal con Dextranaminas

En el presente trabajo se han utilizado diferentes aproximaciones en el empleo de las técnicas de trazado axonal, variando tanto el tipo de trazador utilizado como el modo de aplicación del mismo. Hemos empleado una aproximación *in vivo* para la aplicación del trazador en regiones de fácil acceso mediante microcirugía, como es el caso de la médula espinal o el techo mesencefálico (Capítulos 2, 3 y 5). La aplicación de las dextranaminas *in vivo* se ha utilizado con gran éxito en estudios previos, y ha demostrado ser una herramienta muy útil y eficaz para el trazado axonal, comparado con otras macromoléculas como la peroxidasa de rábano o el complejo cobalto-lisina (Marín y cols., 1997a). En el análisis del desarrollo ontogenético de las conexiones descendentes y las aferencias CA espinales, empleamos técnicas de trazado *in vitro* con embriones y larvas de *Xenopus laevis* (Capítulo 4). Finalmente, en el estudio de las aferencias a la región septal (Capítulo 6), debido a la dificultad de acceder al sitio de interés así como de realizar aplicaciones restringidas sin contaminar zonas cercanas o de paso de fibras, combinamos aproximaciones *in vivo* e *in vitro* en la aplicación de las dextranaminas, tanto en forma de cristales como en solución inyectada iontoforéticamente. El uso de preparaciones del SNC *in vitro* en anfibios se ha utilizado en trabajos previos para experimentos de trazado no sólo en el SNC adulto (Luksch y cols., 1996; Roth y Westhoff, 1999) sino también en estudios de desarrollo (A. Muñoz y cols., 1996; Marín y cols., 1997c). En nuestro trabajo, esta nueva aproximación en la aplicación de las dextranaminas se ha probado mediante inyecciones iontoforéticas en el cerebro adulto de *Rana*. El uso de trazado axonal en aplicaciones *in vitro* presenta una serie de ventajas que hacen que sea un método de trazado muy atractivo. En primer lugar, no existen problemas de superviencia y prácticamente todas las partes del cerebro son accesibles al mismo tiempo. Asimismo, ofrece la ventaja de conseguir aplicaciones restringidas y más precisas del trazador que siguiendo una aproximación *in vivo*. Además, la preparación aislada del SNC completo en anfibios se puede mantener "viva" durante varios días permitiendo estudios inmunohistoquímicos, electrofisiológicos y de trazado sin signos de degeneración en el tejido (Luksch y cols., 1996). Nuestro trabajo también demuestra que el patrón de marcaje en los experimentos *in vitro* es comparable con los resultados obtenidos siguiendo una aproximación *in vivo*. Sin embargo, hay que destacar que en los experimentos en condiciones *in vitro*, el marcaje retrógrado de proyecciones largas es menos numeroso que en la aproximación *in vivo*. Una posible explicación es que, a pesar de que el trazador utilizado en estos experimentos es de menor peso molecular, el tiempo de transporte está también limitado. Además, las aplicaciones iontoforéticas utilizadas en los experimentos *in vitro* siempre son más restringidas que los cristales, y por tanto, se libera menos cantidad de trazador en el lugar de inyección.

El modo de aplicación de las dextranaminas es también una variable experimental importante (Marín y cols., 1997a). Así, la aplicación de estos trazadores en forma de cristales resulta más eficaz en un transporte retrógrado, mientras que las inyecciones iontoforéticas dan lugar a un transporte anterógrado más efectivo. Como hemos mencionado anteriormente, las aplicaciones iontoforéticas resultan más apropiadas para un marcaje más restringido, particularmente cuando se quiere obtener información detallada sobre conexiones dentro de una estructura determinada. Por el contrario, la aplicación de cristales da lugar a un mayor número de neuronas marcadas retrógradamente, aunque tiene la desventaja de que los sitios de aplicación son mayores.

Por último, la eficacia de diferentes trazadores también varía en función de su peso molecular y de las moléculas con las que están conjugadas para su posterior visualización. En el presente trabajo hemos empleado como trazadores dextranaminas conjugadas con biotina (BDA) o asociadas a moléculas fluorescentes como Texas Red™ (TRDA). Las dextranaminas biotinadas presentan la ventaja de que dan lugar a un marcaje de las neuronas que recuerda a las tinciones de Golgi, mostrando claramente la morfología del soma y sus dendritas. Por el contrario, las dextranaminas fluorescentes dan mejor resultado cuando se aplican como cristales, aunque la morfología de las células marcadas es menos clara. Por otro lado, la utilización de dextranaminas de bajo peso molecular ha resultado más apropiada para estudios de desarrollo o en individuos adultos siguiendo una aproximación *in vitro*, debido a que se transportan más rápidamente que las de mayor peso molecular empleadas para aplicaciones *in vivo* (Fritzsch, 1993).

Métodos de Doble Marcaje

Mediante el empleo de técnicas de trazado retrógrado en combinación con inmunohistoquímica para la enzima TH, en el presente trabajo hemos determinado los centros de origen de la inervación catecolaminérgica en la médula espinal, el techo mesencefálico y la región septal. En estos experimentos de doble marcaje, el trazador TRDA presenta la ventaja de que es directamente fluorescente y por tanto, el tejido se puede procesar inmediatamente después de ser cortado para la inmunofluorescencia de la TH. Sin embargo, como hemos mencionado anteriormente, el uso de BDA como trazador resulta también muy apropiado, aunque su visualización requiere la incubación del tejido con una estreptavidina fluorescente que nos permita revelar la presencia de las células doblemente marcadas.

Vías Descendentes a la Médula Espinal

El análisis de la organización de las vías descendentes supraespinales ha sido objeto durante décadas de numerosos estudios neuroanatómicos en representantes de prácticamente todos los grupos de vertebrados (ver revisiones: Kuypers y Martin, 1982; ten Donkelaar, 2000, 2001; Cruce y Newman, 1984; Nudo y Masterton, 1988). El principal objetivo de estos trabajos ha sido determinar los grupos celulares localizados en el tronco encefálico y el prosencéfalo que proyectan a la médula espinal, analizando su papel en el control supraespinal de la actividad motora y sus efectos moduladores sobre el procesamiento de la información sensorial. De manera adicional, el análisis comparativo en los distintos grupos de vertebrados de estas vías descendentes espinales ha proporcionado información sobre determinados aspectos en la evolución del SNC (ten Donkelaar, 2000, 2001).

En los anfibios, los datos disponibles hasta el momento han puesto de manifiesto que este grupo comparte un patrón común en la organización de las proyecciones descendentes a la médula espinal que está presente en todos los vertebrados tetrápodos (ten Donkelaar, 1982, 2000, 2001), incluyendo una subdivision general en sistemas descendentes lateral y medial. Así, las vías intersticioespinal, reticuloespinal y vestibuloespinal descienden en el funículo ventral y la parte ventral del funículo lateral, y terminan en la porción mediodorsal del asta ventral y partes adyacentes de la zona intermedia. Este sistema medial está funcionalmente relacionado con las actividades posturales, y constituye un sistema básico a través del cual el cerebro ejerce control sobre los movimientos. El sistema lateral está constituido por fibras que descienden a través del funículo lateral hasta la médula espinal, y está formado principalmente por las fibras rubroespinales. El tracto rubroespinal termina en las regiones lateral y dorsal de la zona intermedia, y está implicado en los movimientos de las extremidades. Además, existen evidencias que apuntan a la existencia de un tercer sistema, un componente emocional del sistema motor, que incluiría las proyecciones coeruleo-espinal y rafe-espinal en anuros que, al igual que en amniotas, estaría bajo el control del sistema límbico (Kuypers, 1981; Holstege y Kuypers, 1987; ten Donkelaar, 1990; Holstege, 1991). Aunque nuestros resultados en anuros (*Rana perezi* y *Xenopus laevis*) han confirmado en gran medida los datos previos en estas especies (ten Donkelaar y cols., 1981; Tóth y cols., 1985), demuestran además la existencia de una mayor distribución de células de proyección espinales, aclarando las controversias existentes hasta el momento acerca de la presencia de determinadas conexiones descendentes como, por ejemplo, las vías telencéfalo-espinal, tecto-espinal, rubro-espinal o cerebelo-espinal. Por el contrario, en urodelos (*Pleurodeles waltl*) y ápodos (*Dermophis mexicanus*), nuestros resultados proporcionan por primera vez, información detallada sobre la organización de estas vías, demostrando que, en estos anfibios las conexiones descendentes son tan elaboradas como en anuros, y comparables a las de otros vertebrados, a pesar de que poseen un encéfalo aparentemente poco diferenciado debido a un proceso de "paedomorfosis" o "simplificación secundaria" (Roth y cols., 1993).

Las proyecciones descendentes más prominentes en anfibios se originan en el rombencéfalo, en particular en la porción vestibular del área octavolateral y la formación reticular. En las cuatro especies estudiadas se demostró la existencia de dos *vías vestibuloespinales* distintas, una ipsilateral desde el núcleo vestibular lateral, y una proyección contralateral que se origina en el núcleo vestibular medial y, cuando está presente, desde el núcleo vestibular caudal. En anuros además, observamos una pequeña conexión ipsilateral desde el núcleo vestibular anterior. En general, las proyecciones vestibuloespinales están altamente conservadas en todos los vertebrados, distinguiéndose también un componente ipsilateral y contralateral en agnatos (Ronan, 1989), peces cartilaginosos (Smeets y Timerick, 1981; Cruce y cols., 1999), teleósteos (Oka y cols., 1986; Prasada Rao y cols., 1987), reptiles (ten Donkelaar y cols., 1980; Woodson y Künzle, 1982), aves (Cabot y cols., 1982) y mamíferos (Nudo y Masterton, 1988). En *Xenopus*, que retiene el sistema de la línea lateral en el adulto, se observó además una proyección espinal desde los núcleos de la línea lateral. En cuanto a las conexiones desde la formación reticular, existen dos *vías reticuloespinales* principales, una que se origina en el núcleo reticular inferior y desciende en el funículo lateral, y otra que parte desde niveles reticulares más rostrales que incluyen el núcleo intersticial del flm, y que cursa a través del funículo ventral. En *Pleurodeles*, las células de Mauthner inervan la médula espinal contralateralmente. Aunque se ha descrito que estas neuronas mantienen su conexión espinal en las ranas adultas (Will, 1986, 1991; Davis y Farel, 1990), nosotros no encontramos dicha proyección ni en *Rana* ni en *Xenopus*, de acuerdo con otros estudios previos en anuros (ten Donkelaar y cols., 1981; Tóth y cols., 1985), ni tampoco en el ápodo *Dermophis mexicanus*. En cuanto a la vía rafe-espinal serotoninérgica, se ha demostrado que la porción rostral del núcleo del rafe inerva el asta dorsal, la zona intermedia y el asta ventral de la médula espinal, mientras que la porción caudal de este núcleo sólamente inerva la zona intermedia y el asta ventral (Tan y Miletic, 1990). De acuerdo con los datos previos existentes en anfibios, demostramos también la existencia de proyecciones espinales desde los núcleos sensitivos principal y descendente del nervio trigémino, el núcleo del tracto solitario y el núcleo de la columna dorsal (Muñoz y cols., 1995, 1998).

En todas las especies estudiadas, se ha demostrado la existencia de una *proyección cerebeloespinal* principalmente contralateral, que se origina en el núcleo cerebeloso, localizado lateralmente en el pedúnculo del cerebelo (ten Donkelaar y cols., 1981; Tóth y cols., 1985; Naujoks-Manteuffel y Manteuffel, 1988). Sin embargo, nuestros resultados demuestran además que tanto en anuros como en urodelos, se puede distinguir una población lateral de células de proyección espinal situadas dentro de la capa granular del cuerpo cerebeloso. Esta proyección desde el cerebelo a la médula espinal está ausente en agnatos, peces cartilaginosos y teleósteos, y por tanto, podría considerarse como una característica exclusiva de tetrápodos (Nudo y Masterton, 1988).

En niveles ístmicos, demostramos la existencia de proyecciones desde el locus coeruleus y el núcleo laterodorsal tegmental. Este último núcleo se ha caracterizado en anfibios mediante histoquímica para la NADPH-diaforasa e inmunohistoquímica para la óxido nítrico sintasa (NOS) y la colina acetil-transferesa (ChAT) (González y cols., 1996; M. Muñoz y cols., 1996; Marín y cols., 1997d). En base a sus características neuroquímicas y a su proyección espinal se ha comparado con el núcleo laterodorsal tegmental colinérgico de amniotas (Vincent y Kimura, 1992). Por otro lado, se ha propuesto la existencia de una proyección coeruleospinal como una característica común en todos los vertebrados (lampreas: Pierre y cols., 1994; peces cartilaginosos: Stuesse y Cruce, 1992; Cruce y cols., 1999; teleósteos: Meek, 1994; reptiles: Smeets, 1994; aves: Reiner y cols., 1994; Puelles y Medina, 1994; mamíferos: Kitahama y cols., 1994).

Las proyecciones espinales desde el mesencéfalo incluyen una *proyección tectoespinal*, principalmente contralateral a la médula cervical. La conexión con el techo óptico es una característica general de anfibios, que está implicada en el control de los músculos del cuello y de la dirección y amplitud del movimiento sacádico de los ojos (Naujoks-Manteuffel y Manteuffel, 1990). La existencia de una pequeña conexión tectoespinal contralateral a la médula espinal cervical parece ser una caraterística constante en los vertebrados (elasmobranquios: Smeets y Timerick, 1981; peces pulmonados: Ronan y Northcutt, 1985; reptiles: Woodson y Künzle, 1982; mamíferos: Nudo y Masterton, 1988). Sin embargo, no se han identificado neuronas tectoespinales en algunos elasmobranquios (Cruce y cols., 1999), teleósteos (Oka y cols., 1986; Prasada Rao y cols., 1987), algunos reptiles (ten Donkelaar y cols., 1980), y aves (Cabot y cols., 1982; Gross y Oppenheim, 1985; Webster y cols., 1990), probablemente debido a que en estas especies la vía tectoespinal no se extiende más allá de niveles rombence-

fálicos caudales. Nuestro estudio ha demostrado también la existencia de una proyección espinal desde el *núcleo mesencefálico del nervio trigémino* en los tres órdenes de anfibios. Anteriormente, se habían publicado datos contradictorios en cuanto a la existencia de esta proyección. Así, aunque en urodelos esta vía supraespinal se había descrito con anterioridad (Naujoks-Manteuffel y cols., 1988; Roth y cols., 1990), en anuros no se pudo demostrar su existencia (ten Donkelaar y cols., 1981; Muñoz y cols., 1993). Las proyecciones espinales desde el núcleo mesencefálico del nervio trigémino parecen ser una característica común en los vertebrados anamniotas (Smeets y Timerick, 1981; Ronan y Northcutt, 1985; Pombal y cols., 1997), y están presentes también en algunos amniotas (ten Donkelaar y cols., 1980; Ebbesson, 1981; Woodson y Künzle, 1982).

En el presente trabajo hemos demostrado la existencia de aferencias desde el *torus semicircularis* a la médula espinal, que no se habían puesto de manifiesto en trabajos previos (Naujoks-Manteuffel y cols., 1988; Feng y Lin, 1991; Matesz y Kulik, 1996). La proyección desde esta estructura mesencefálica se origina principalmente en el núcleo laminar del torus, al igual que sucede en reptiles (ten Donkelaar y cols., 1980; Butler y Bruce, 1981; Woodson y Künzle, 1982). Al menos parte de esta proyección correspondería con las conexiones desde la sustancia gris periacueductal de mamíferos, que inerva la médula espinal cervical y que está implicada en los movimientos de giro de la cabeza, vocalización, locomoción y modulación del dolor (Holstege, 1991). Dentro de las conexiones mesencefálicas, encontramos también una importante *proyección tegmento-espinal* que se origina desde los núcleos tegmentales anterodorsal, anteroventral y posteroventral que forman parte de la formación reticular mesencefálica en anfibios. La formación reticular mesencefálica también inerva la médula espinal en todos los vertebrados (agnatos: Ronan, 1989; peces cartilaginosos: Smeets y Timerick, 1981; Cruce y cols., 1999; teleósteos: Behrend y Donicht, 1990; Ronan y Northcutt, 1985; reptiles: ten Donkelaar y cols., 1980; Woodson y Künzle, 1982; Newman y cols., 1983; aves: Cabot y cols., 1982; Gross y Oppenheim, 1985; mamíferos: Nudo y Masterton, 1988). En anuros además, se ha identificado el homólogo del núcleo de Edinger-Westphal, situado dorsolateral al núcleo oculomotor (Matesz y Székely, 1977; Marín y cols., 1997d). Nuestro trabajo demuestra la existencia de proyecciones a la médula espinal desde este grupo en *Rana* y *Xenopus*, mientras que en *Pleurodeles* y *Dermophis* no se ha podido identificar este núcleo, posiblemente porque se encuentra incluído dentro del núcleo del flm. Proyecciones espinales desde este núcleo se han descrito también en reptiles (ten Donkelaar y cols., 1980; Woodson y Künzle, 1982), aves (Cabot y cols., 1982; Gross y Oppenheim, 1985) y mamíferos (Nudo y Masterton, 1988).

En las cuatro especies de anfibios estudiadas pudimos constatar la existencia de una importante *proyección rubroespinal*. Estudios previos habían demostrado la existencia de aferencias espinales desde el núcleo rojo en *Xenopus* y en *Salamandra*, pero no en el ápodo *Ichthyophis* (ten Donkelaar y cols., 1981; Naujoks-Manteuffel y cols., 1988). En cuanto a la presencia de esta vía descendente en otras especies de vertebrados, existen datos contradictorios tanto en peces cartilaginosos (Smeets y Timerick, 1981; Cruce y cols., 1999) como en teleósteos (Oka y cols., 1986; Prasada Rao y cols., 1987; Behrend y Donicht, 1990; Becker y cols., 1997). Del mismo modo, aunque en la mayoría de reptiles se ha identificado un tracto rubroespinal (ten Donkelaar y cols., 1980; ten Donkelaar, 1982; Woodson y Künzle, 1982; Cruce y cols., 1983), no se ha demostrado su presencia en determinadas especies de serpientes (ten Donkelaar, 1982; ten Donkelaar y Bangma, 1983). La vía rubroespinal está presente también en aves (Wild y cols., 1979; Cabot y cols., 1982; Gross y Oppenheim, 1985; Webster y cols., 1990) y mamíferos (Nudo y Masterton, 1988), pero aparentemente ausente en el hombre (Nathan y Smith, 1982). Hay que destacar que, aunque en un principio se postuló que la existencia de esta vía estaba directamente relacionada con la presencia de extremidades (ten Donkelaar, 1988), los datos existentes hasta el momento no apoyan dicha idea.

Las *proyecciones diencéfalo-espinales* en anfibios se originan en el tálamo ventral, la región pretectal, el tubérculo posterior y el núcleo intersticial del flm. Además, encontramos proyecciones espinales desde el área preóptica anterior, el núcleo preóptico magnocelular y el núcleo supraquiasmático (incluídos dentro del diencéfalo según la definición clásica de Neary y Northcutt, 1983). En general, todas estas conexiones espinales son principalmente ipsilaterales, y se extienden caudalmente hasta la médula espinal lumbar. En cuanto a las proyecciones pretectoespinales, en anuros se originan fundamentalmente desde el núcleo yuxtacomisural y la división posterolateral del núcleo lateral. En urodelos, las neuronas de proyección del pretecho se sitúan en el núcleo pretectal y un grupo denominadao núcleo de Darkschewitsch (Naujoks-Manteuffel y Manteuffel, 1988). En amniotas, esta proyección pretectoespinal está ausente (reptiles: ten Donkelaar y cols., 1980; Cruce y Newman, 1981; aves: Webster y cols., 1990; mamíferos: Nudo y Masterton, 1988). Por último, las proyecciones intersticioespinales se originan en el núcleo intersticial del flm, y están presentes en todos los vertebrados.

Finalmente, hemos demostrado la existencia de *proyecciones telencéfalo-espinales* en *X. laevis*, *R. perezi*, *P. waltl*, y *D. mexicanus*. En todas estas especies, observamos una proyección ipsilateral a la médula espinal cervical desde células localizadas en la porción ventrocaudal del subpalio, que muy probablemente pertenecen a la amígdala central. Proyecciones amigdaloespinales se han demostrado en reptiles (Follett, 1989; Siemen y Künzle, 1994), y mamíferos (Hopkins and Holstege, 1978; Nudo and Masterton, 1988). En aves, el denominado tracto occipitomesencefálico se extiende desde la porción sensorimotora del arquiestriado hasta la médula espinal rostral (Dubbeldam y cols., 1997). Este circuito recuerda al tracto corticobulbar de mamíferos y el componente caudal, "amigdalar", del arquiestriado podría formar parte de este sistema descendente. Recientemente, se ha descrito en aves paseriformes la existencia de un "tracto piramidal" que se originaría desde el Wulst rostral y tendría importantes proyecciones al tronco encefálico y a la médula espinal cervical (Wild y Williams, 2000).

En conjunto, nuestros resultados han demostrado la existencia de proyecciones descendentes a la médula espinal desde las principales regiones encefálicas. Así, la presencia de las vías telencéfalo-espinal y diencéfalo-espinal, y junto con una importante proyección troncoencefálico-espinal, constituyen una característica común en todas las especies de anfibios analizadas en el presente trabajo. Este sistema de conexiones descendentes espinales está presente en especies de amniotas y anamniotas, demostrando la existencia de un patrón básico en la organización de las vías descendentes supraespinales que se ha conservado evolutivamente en los vertebrados.

Desarrollo de las vías descendentes a la médula espinal

Un aspecto muy importante en la organización de las vías descendentes a la médula espinal en los vertebrados corresponde al desarrollo y la secuencia temporal de aparición de dichas proyecciones. Nuestros resultados en *Xenopus laevis* han corroborado en gran medida los datos existentes en estudios previos (ten Donkelaar y de Boer-van Huizen, 1982; van Mier y ten Donkelaar, 1984; Nordlander y cols., 1985; Hartenstein, 1993). Sin embargo, dada la sensibilidad de las dextranaminas como trazadores retrógrados, pudimos demostrar la existencia y desarrollo de aferencias espinales desde regiones más rostrales como el hipotálamo, el área preóptica o el hemisferio telencefálico caudal, que no se habían descrito con anterioridad. Más aún, nuestros datos demuestran que más que una secuencia temporal caudorostral en el desarrollo de estas conexiones como se había aceptado de forma general hasta el momento (van Mier y ten Donkelaar, 1984), existe un patrón ventrodorsal (o de la placa basal a la placa alar) en la aparición de las células de proyección a la médula espinal. Así por ejemplo, en el rombencéfalo, las proyecciones espinales desde grupos basales como la formación reticular o los núcleos del rafe se desarrollan antes que las procedentes de grupos situados en la placa alar como los núcleos vestibulares o el núcleo del tracto solitario. Esta secuencia ventrodorsal también se ha observado en otras especies de vertebrados, en particular en el desarrollo de las proyecciones reticuloespinal y vestibuloespinal (peces: Mendelson, 1986; Sharma and Berthoud, 1992; aves: Okado and Oppenheim, 1985; mamíferos: Cabana y Martin, 1984; Wang y cols., 1992; Auclair y cols., 1993; Kudo y cols., 1993; Martin y cols., 1993). Por otro lado, debemos destacar que la proyección telencéfalo-espinal es más numerosa durante el desarrollo larvario, y que aparentemente se reduce durante la metamorfosis, ya que en el individuo adulto solamente observamos algunas células marcadas retrógradamente en la porción ventrolateral del hemisferio telencefálico caudal (Sánchez-Camacho y cols., 2001a). Este hecho sugiere la existencia de proyecciones transitorias desde el telencéfalo basal a la médula espinal durante el desarrollo de *X. laevis.*

En general, las proyecciones descendentes supraespinales en *Xenopus* se desarrollan siguiendo un patrón que es común en una amplia variedad de vertebrados, desde peces a mamíferos (ten Donkelaar, 2000). En todas las especies estudiadas, estas conexiones espinales están presentes desde estadios tempranos del desarrollo. Así, los estudios de trazado axonal han demostrado que, al igual que sucede en *Xenopus*, las fibras reticuloespinales e interticioespinales son las primeras en alcanzar la médula espinal, seguidas por las proyecciones vestibuloespinales, y las fibras rubroespinales y, cuando están presentes, las proyecciones corticoespinales. Todos estos datos sugieren una constancia filogenética en el desarrollo y maduración de las vías descendentes supraespinales de los vertebrados (ten Donkelaar, 2000). Más aún, el hecho de que estas proyecciones descendentes se desarrollen en estadios en los que las células diana en la médula espinal son todavía relativamente inmaduras, sugiere la posibilidad de que estas conexiones puedan mediar interacciones celulares importantes implicadas en la neurogénesis de la médula espinal (Okado y Oppenheim, 1985).

Inervación y Aferencias Catecolaminérgicas a la Médula Espinal

La presencia de una abundante inervación CA en la médula espinal es una característica común en todos los vertebrados (Smeets y Reiner, 1994; Smeets y González, 2000), sin embargo, sólo existen datos dispersos sobre la naturaleza de esta inervación en la médula espinal de especies de no-mamíferos. En la lamprea, las fibras DA se distribuyen en la mitad dorsal de la médula espinal rostral, y en la columna ventromedial a lo largo de toda la extensión rostrocaudal espinal (Schotland y cols., 1996). En los peces cartilaginosos, la inmunohistoquímica para DA ha revelado la presencia de fibras inmunorreactivas localizadas principalmente alrededor del canal central, con un menor número de fibras dentro del asta dorsal y ventral (Roberts y Meredith, 1987). Resultados similares se han obtenido en peces teleósteos (Roberts y cols., 1989). Los trabajos realizados en reptiles, revelan la presencia de fibras DAi y NAi principalmente localizadas en las láminas I y II del asta dorsal, y la porción dorsal de la capa X, así como fibras dispersas, mayoritariamente NAi, dentro del asta ventral (Smeets, 1994). En aves, se ha demostrado la presencia de plexos de fibras THi elaborados tanto en el asta dorsal como en el asta ventral (Okado y cols., 1991; Reiner y cols., 1994). El plexo más abundante se localiza en la columna de Terni (neuronas simpáticas preganglionicas), en la lámina X y la parte medial de las láminas V-VII de la médula espinal cervical y torácica.

Por el contrario, existen numerosos trabajos en mamíferos que han analizado en detalle la distribución de la inervación CA en la médula espinal, demostrando la abundante presencia de fibras DA y NA, y en menor medida terminales adrenérgicos, todos ellos implicados en el control motor, nocicepción y funciones autónomas. Las fibras DA se distribuyen fundamentalmente en las capas profundas del asta dorsal (láminas III-V) y en la lámina X de Rexed. De manera adicional, existe un denso plexo de fibras DAi que inerva la columna celular intermediolateral, además de una moderada inervación en el asta ventral (Shirouzu y cols., 1990; Mouchet y cols., 1992; Ridet y cols., 1992; Weil-Fugazza y Godefroy, 1993; Holstege y cols., 1996). Las fibras NA se distribuyen de manera profusa en la lámina I y la porción más externa de la lámina II del asta dorsal (Mouchet y cols., 1992). Además, existe un fuerte plexo de fibras NAi presente en la lámina X, y en la columna celular intermediolateral en niveles torácicos. En el asta ventral, las fibras y terminales NA están principalmente localizados en la lámina IX. En comparación con la distribución de fibras DA y NA, la inervación adrenérgica de la médula espinal es mucho más restringida, y el plexo más denso se localiza en niveles torácicos, en la columna intermediolateral del sistema autónomo (Hökfelt y cols., 1984; Carlton y cols., 1991). Un plexo adenérgico menos denso se distribuye en la región que rodea al canal central, en la parte superficial del asta dorsal (lámina I) y la sustancia gelatinosa (Carlton y cols., 1991).

Nuestros resultados en especies representativas de los tres órdenes de anfibios han demostrado la presencia de una inervación CA muy rica en todos los niveles de la médula espinal. Además, la distribución de fibras y terminales dentro del campo intermediolateral espinal, donde recientemente se ha descrito la presencia de grupos colinérgicos en niveles torácicos (Muñoz y cols., 2000), sugiere la implicación de las CA sobre esta columna de neuronas simpáticas preganglionares. Por otro lado, la fuerte inervación CA demostrada en urodelos y ápodos dentro de la sustancia gris ventral, formando terminales perisomáticos sobre grandes neuronas del asta ventral, recuerda a los datos publicados en mamíferos que demuestran la presencia de fibras CA que se concentran alrededor de motoneuronas en la lámina IX, principalmente en niveles torácicos y lumbares (Pindzola y cols., 1988; Yoshida y Tanaka, 1988). Sin embargo, datos previos de nuestro laboratorio demuestran que esta inervación no se produce sobre motoneuronas como

sucede en mamíferos, ya que no se trata de neuronas espinales colinérgicas. En general, comparando los resultados obtenidos en anfibios en el presente trabajo con los datos existentes en mamíferos podemos concluir que ambos grupos comparten una fuerte inervación CA en la sustancia gris dorsal profunda y el área dorsal al canal central, mientras que la inervación del asta ventral es sólo débil o moderada.

Aunque una pequeña proporción de las fibras CA presentes en la médula espinal de anfibios es de origen intraespinal, nuestros datos corroboran la existencia de proyecciones CA supraespinales. Estas conexiones constituyen la mayor parte de la inervación CA presente en la médula espinal, y se originan desde cuatro centros celulares distintos: el tubérculo posterior ventrolateral, el núcleo periventricular de la zona incerta, el locus coeruleus y el núcleo del tracto solitario. Estos resultados demuestran que la organización de las aferencias CA a la médula espinal es similar a la descrita en mamíferos, aunque aplicando una aproximación segmental a la interpretación de nuestros datos encontramos algunas diferencias que discutimos más adelante. Los estudios de doble marcaje en mamíferos han puesto de manifiesto que las fibras DA espinales tienen su origen en el grupo celular A11 (Björklund y Lindvall, 1984; Skagerberg y Lindvall, 1985; Takada y cols., 1988; Shirouzu y cols., 1990), mientras que las proyecciones NA a la médula espinal se originan en el locus coeruleus (A6) y los grupos A5 y A7 (Westlund y cols., 1983, 1984; Lyons y cols., 1989; Clark y cols., 1991). El origen supraespinal de las fibras adrenérgicas se localiza en los grupos C1 y C3 (Ross y cols., 1984; Carlton y cols., 1991; Guyenet y cols., 1994). Con la excepción de un par de trabajos, no existen experimentos de doble marcaje llevados a cabo en especies de no-mamíferos. Sin embargo, en base a estudios de trazado axonal y datos inmunohistoquímicos es posible que las aferencias CA supraespinales en reptiles y aves sean idénticas a las que se han demostrado en mamíferos (Chikasawa y cols., 1983; Coote, 1985).

El análisis segmental del encéfalo, ha puesto de manifiesto que la mayor parte de las regiones clásicamente denominadas "hipotalámicas", no pertencen al diencéfalo, sino que están incluidas dentro del prosencéfalo secundario (Puelles y Rubenstein, 1993). De esta manera, de acuerdo con el modelo neuromérico, el grupo A11 inicialmente considerado como un núcleo hipotalámico, se localizaría dentro de la placa alar diencefálica de los prosómeros 1 y 2 y se continuaría caudalmente en el mesencéfalo (Puelles y Verney, 1998). Del mismo modo, el grupo A13 (zona incerta) se desarrolla en la placa alar de p3. En conjunto, la columna formada por los grupos A13-A11 sería el origen de las proyecciones diencéfalo-espinales en mamíferos, y se situaría dentro de la placa alar de los prosómeros p1, p2 y p3. Los homológos de estos grupos DA se han identificado también en aves y reptiles (Medina y cols., 1994; Puelles y Medina, 1994). En anfibios, existen dos poblaciones celulares DA que se localizan dentro de la placa alar de p3: el núcleo periventricular de la zona incerta y una población de células más pequeñas situada dorsalmente, dentro del tálamo ventral. Nuestros resultados demuestran que la proyección desde el núcleo periventricular de la zona incerta constituye la única fuente diencefálica de fibras DA en la médula espinal de anfibios. Según estos datos, se podría comparar la proyección que encontramos desde este núcleo con la demostrada desde el grupo A11 en mamíferos, aunque existiría una diferencia en la localización prosomérica de esta proyección espinal. Así, en mamíferos se desarrollaría desde p1-p2, mientras que en anfibios tendría un origen más rostral situado en el prosómero 3. Hay que destacar que, aunque inicialmente se describió la existencia de fibras DA espinales desde el grupo A13 de mamíferos (Blessing y Chalmers, 1979), estudios posteriores demostraron que las proyecciones eferentes desde la región de la zona incerta medial que contine las células DA del grupo A13, no alcanzan la médula espinal (Wagner y cols., 1995), y por tanto, no existe una proyección DA espinal desde p3. Por otro lado, los datos inmunohistoquímicos disponibles en mamíferos han demostrado que las células DA del grupo A11 que proyectan a la médula espinal son inmunorreactivas para el péptido CGRP (Orazzo y cols., 1993), mientras que las células del grupo A13 coexpresan DA y somatostatina (Meister y cols., 1987). En anfibios, el núcleo periventricular de la zona incerta, también presenta células que continen CGRP (Petkó y Sánta, 1992), mientras que el grupo dorsal de células DA coexpresan TH y somatostina (Inagaki y cols., 1981).

La proyección DA encontrada desde la porción ventrolateral del tubérculo posterior se sitúa en el prosencéfalo secundario. La topografía segmental de este grupo correspondería con los núcleos supramamilar y mamilar de la placa basal del prosómero 4. Aunque su localización parece diferir de los datos existentes en mamíferos, debemos destacar que aplicando un análisis segmental de las aferencias CA a la médula espinal de mamíferos, parte del sistema que se ha descrito clásicamente como "hipotálamo-espinal" podría ser comparable con nuestros resultados en anfibios.

El locus coeruleus (grupo A6) en mamíferos se desarrolla dentro de la porción caudal del segmento ístmico y se encuentra claramente en esta misma localización en todos los vertebrados. Algunas células de mayor tamaño se continúan caudalmente con este grupo, y se extienden dentro de los rombómeros 2-3, en el llamado locus subcoeruleus. En los anfibios, las células CA localizadas dentro de la región ístmica ocupan porciones de r1 y r2, y podrían considerarse en base a su contenido en NA, su localización topográfica y sus conexiones con la médula espinal y el telencéfalo basal el homólogo del locus coeruleus/subcoeruleus de mamíferos (González y Smeets, 1993, 1995; Marín y cols., 1996, 1997b).

Finalmente, el núcleo del tracto solitario en anfibios se podría considerar el equivalente de los grupos C1/A1-C3/A3 de amniotas. Sin embargo, atendiendo a una aproximación segmental (Smeets y González, 2000), podemos concluir que las proyecciones espinales demostradas desde este núcleo en anfibios, serían comparables sólamente a las descritas desde el grupo A1 en mamíferos.

Desarrollo de las aferencias CA a la médula espinal

La existencia de fibras y terminales CA de origen supraespinal que inervan la médula espinal desde estadios tempranos del desarrollo parece ser una característica constante en todos los vertebrados (Smeets y González, 2000; ten Donkelaar, 2000). En particular, en los anfibios se ha demostrado la existencia de fibras CA en la médula espinal desde estadios embrionarios tardíos (González y cols., 1994, 1995). La aparición temprana de esta inervación CA podría jugar un papel importante en la organización del desarrollo de la médula espinal, influyendo de manera directa sobre la maduración de las neuronas espinales (Specht y cols., 1981; Voorn y cols., 1988).

Durante el desarrollo de *Xenopus*, el patrón de inervación CA de la médula espinal se caracteriza por la distribución gradual de las fibras THi en la zona marginal, dentro de la sustancia blanca, seguida por la invasión progresiva de la sustancia gris espinal. En mamíferos, se ha demostrado la existencia de similitudes con anfibios en el desarrollo de la inerva-

ción CA espinal (Pindzola y cols., 1990; Rajaofetra y cols., 1992). Estos resultados sugieren que el crecimiento de axones THi y NAi en la sustancia gris espinal sigue una secuencia temporal de rostral a caudal durante el desarrollo. Al igual que sucede en anfibios, existe un retraso entre la llegada de las fibras supraespinales a la sustancia blanca y la invasión de la sustancia gris espinal.

En cuanto al desarrollo de las proyecciones CA descendentes a la médula espinal en embriones y larvas de *Xenopus*, nuestros resultados demuestran que existe una secuencia rostrocaudal. De esta manera, las proyecciones desde el tubérculo posterior, el núcleo periventricular de la zona incerta y el locus coeruleus alcanzan la médula espinal al final del período embrionario, mientras que la conexión espinal desde el núcleo del tracto solitario no se desarrolla hasta el comienzo de la prometamorfosis. En *Xenopus* se ha descrito también una secuencia rostrocaudal similar en el desarrollo de las aferencias CA a los ganglios basales (Marín y cols., 1997c). Más aún, comparando la secuencia de aparición de los grupos CA con el momento en que se detectan las primeras fibras CA espinales, parece posible que la inmunorreactividad para TH se desarrolle primero en las neuronas CA que inervan la médula espinal, e inmediatamente después se identifiquen sus proyecciones descendentes espinales.

En amniotas, solamente el estudio realizado por Pindzola y cols. (1990) en *Didelphis virginiana*, ha tratado el desarrollo de estas conexiones mediante métodos de doble marcaje. Comparando los datos obtenidos en este estudio con nuestros resultados en anfibios, podemos llegar a la conclusión de que las proyecciones CA que se han demostrado en *Didelphis* son comparables a las que encontramos desde el tubérculo posterior, el locus coeruleus y el núcleo del tracto solitario en *Xenopus*. Sin embargo, no se ha demostrado en mamíferos una proyección comparable a la que observamos desde el núcleo periventricular de la zona incerta.

Inervación y Aferencias Catecolaminérgicas al Techo Mesencefálico

El techo mesencefálico tiene un papel muy importante en la integración de la información visual y multisensorial que resulta esencial en el comportamiento normal de los anfibios. Aunque existen importantes diferencias en la estructura laminar del techo óptico entre anuros y urodelos, se han identificado tipos funcionales y morfológicos de neuronas tectales muy similares en ambos grupos en base a su arborización dendrítica y al patrón de proyecciones ascendentes y descendentes (Lázár y cols., 1983; Roth y cols., 1990, 1999; Dicke y Roth, 1996; Dicke, 1999; Sánchez-Camacho y cols., 2001a). Así, las capas periventriculares 6-9 en urodelos son homólogas a las capas 1-6 en la rana. El conjunto de las fibras tectofugales cursa en las capas 4 y 5 en urodelos y en la capa 7 en anuros. Por otro lado, las aferencias desde la retina terminan en las capas 1-3 en anuros y en las capas 8 y 9 del techo de urodelos. Nuestros resultados apoyan esta homología, demostrando un patrón comparable en la inervación CA del techo óptico en las dos especies estudiadas. Así, la distribución de fibras THi localizadas en las capas tectales 3 y 5 en *Rana* sería equiparable a las localizadas dentro de la capa 7 en *Pleurodeles*, mientras que la inervación de la capa 7 en la rana correspondería con la presente en las capas 4 y 5 del urodelo.

La actividad sináptica en el techo mesencefálico está modulada por la influencia de aferencias de diversa naturaleza neuroquímica, entre las que se incluyen los sistemas catecolaminérgicos. Los resultados de este trabajo demuestran que el patrón de inervación CA en el techo de anfibios presenta un alto grado de organización, y sugiere que las proyecciones CA tendrían un papel modulador similar en el techo tanto de anuros como de urodelos. Así, la amplia distribución de fibras y terminales DA y NA presente en casi todas las capas del techo óptico, indica que las catecolaminas podrían estar implicadas en todos los niveles tectales, modulando así las proyecciones tectofugales y las aferencias sensoriales desde otros centros encefálicos, así como circuitos locales intratectales implicados en el procesamiento de la información visual y no visual. En particular, la presencia de una gran inervación en las capas de las principales vías eferentes del techo de *Rana* (capa 7) y *Pleurodeles* (capas 4 y 5), sugiere que las catecolaminas podrían intervenir en la modulación de las vías descendentes tectobulbar y tectoespinal, que median distintos aspectos del procesamiento visual y visuomotor.

En general, la presencia de una abundante distribución de fibras y terminales CA en el techo mesencefálico (colículo superior en mamíferos) parece ser una característica común en todos los vertebrados, aunque existen diferencias en el patrón laminar de esta inervación. Así, en los peces teleósteos existe una escasa inmunorreactividad que está presente sólo en las capas profundas e intermedias, pero no en las capas más superficiales que reciben la información visual (Meek y cols., 1989; Roberts y cols., 1989). De acuerdo con los resultados de nuestro trabajo, las fibras DA se distribuyen principalmente en las capas tectales profundas, mientras que las fibras NA predominan en las capas superficiales, tanto en anuros como en urodelos. Por el contrario, en aves la inervación CA más densa se distribuye en las capas superficiales del techo (Rodman y Karten, 1995), al igual que sucede en reptiles (Smeets y cols., 1986; Medina y Smeets, 1992). Finalmente, en contraste con el techo de los vertebrados no-mamíferos, el colículo superior en mamíferos presenta una escasa laminación, y la distribución laminar en la inervación CA no es tan pronunciada. Así, las fibras NA predominan en las capas superficiales más que en las láminas profundas del colículo (Morrison y Foote, 1986; Mooney y cols., 1990).

En cuanto a los centros de origen de dicha inervación DA en el techo óptico de anfibios, nuestro trabajo ha demostrado que se localizan en la región pretectal y el núcleo supraquiasmático, y ocasionalmente también en el tubérculo posterior en la rana. La proyección DA al techo más importante se origina en la región pretectal, en particular desde el núcleo yuxtacomisural en anuros, y el núcleo pretectal en urodelos. La proyección tectal DA desde el núcleo supraquiasmático es mucho menor, tanto en anuros como en urodelos. Por otro lado, el locus coeruleus es el único centro que proporciona inervación NA al techo mesencefálico de anfibios, y proyecta principalmente a las capas tectales superficiales que reciben la información de la retina.

En anfibios, los ganglios basales actúan sobre el techo mesencefálico a través de una vía directa que proporciona un efecto modulador sobre las respuestas del techo a estímulos visuales, dando lugar en último término a comportamientos de orientación o de escape (Marín y cols., 1997e, 1998). Además, la información estriatal influye en la función del techo mediante rutas indirectas a través del pretecho y el tegmento mesencefálico (Marín y cols., 1997e, 1998, 1999). Así, a través de la vía estriado-pálido-pretecto-tectal, la estimulación estriatal da lugar a una inhibición de las neuronas tectofugales. Por el contrario, a través de la vía estriado-pretecto-tectal el resultado final es la desinhibición de las neuronas tectales. De esta forma, el núcleo yuxtacomisural con su proyección DA al

techo de anuros, funcionaría como un centro de relevo en estas vías indirectas, y posiblemente también el núcleo pretectal en urodelos. Asimismo, se ha sugerido la implicación del locus coeruleus en la vía indirecta estriadopalidal-tegmento-tectal (Marín y cols., 1999). Con todos estos datos, proponemos que las proyecciones CA desde el núcleo DA yuxtacomisural (en *Rana*) o el núcleo pretectal (en *Pleurodeles*), y el locus coeruleus mediarían parte de las aferencias estriatales al techo, proporcionando un efecto inhibitorio sobre el procesamiento sensorial en el techo óptico de anuros y urodelos. Además, nuestro trabajo sugiere la existencia de otra posible ruta indirecta de la información estriatal al techo mediada por DA, que se localizaría en el núcleo supraquiasmático. Así, se ha demostrado que una región de este núcleo recibe fibras aferentes estriatales (Marín y cols., 1999), que a su vez proyectan al estriado (Allison y Wilczynski, 1994), mientras que otra porción distinta recibe las fibras procedentes de la retina (Tuinhof y cols., 1994). Nuestros resultados indican que la proyección DA al techo tiene su origen en un grupo de células dentro del núcleo supraquiasmático que contactan con las fibras estriatales y no con las aferencias retinofugales.

El análisis comparado de nuestros resultados con los datos existentes en amniotas, demuestran que existen diferencias en el origen de la inervación DA del techo en los vertebrados. Así, las aferencias DA al colículo superior en mamíferos se originan en grupos celulares DA mesencefálicos, en particular desde la sustancia negra pars reticulata (Campbell y Takada, 1989; Mooney y cols., 1990), mientras que en aves y anfibios se localizan en la región pretectal (Rodman y Karten, 1995) y, de manera adicional en anfibios, en el núcleo supraquiasmático. Aunque no existen estudios de doble marcaje en reptiles, la comparación de los datos hodológicos e inmunohistoquímicos, sugiere que tanto la región pretectal como el tegmento mesencefálico podrían ser los centros de origen de las aferencias CA al techo (Medina y Smeets, 1992). Sin embargo, son necesarios estudios de doble marcaje en este grupo y otras especies de anamniotas para poder encontrar características comunes en el origen de las proyecciones CA al techo en las distintas clases de vertebrados. Por el contrario, la proyección NA al techo mesencefálico estaría muy conservada ya que tiene su origen exclusivamente en el locus coeruleus en todos los vertebrados.

Inervación y Aferencias Catecolaminérgicas a la Región Septal

La región del septo es una estructura telencefálica primitiva que ocupa una posición estratégica en el sistema límbico de todos los vertebrados terrestres. En anfibios, la organización de la inervación CA en la región septal presenta algunas características comunes con la de amniotas. Así, en *Rana perezi*, las fibras y terminales DA se localizan principalmente en la porción dorsal del septo lateral, mientras que las fibras NA se distribuyen en niveles caudales del complejo del septo medial/banda diagonal. En reptiles (Smeets y cols., 1986, 1987), aves (Reiner y cols., 1994; Wynne y Güntürkün, 1995) y mamíferos (Moore, 1978; Gall y Moore, 1984; Gaspar y cols., 1985) también se ha descrito la existencia de un gran plexo de fibras DA en la región septal lateral, caracterizado por la presencia de cestas pericelulares de terminales DA que rodean a los somas neuronales. Además, se ha descrito la presencia de fibras DA que no forman estas cestas, y que inervan de manera más difusa el septo lateral. En el septo de anuros y urodelos no se ha demostrado la presencia de dichas cestas perisomáticas, que si se han descrito sin embargo, en el septo lateral de los ápodos (González y Smeets, 1991, 1994b; González y cols., 1993). Por otro lado, la inervación DA del septo medial es mucho más escasa que en el septo lateral en todos los amniotas, al igual que sucede en anuros. Finalmente, las fibras NA en el septo de amniotas se distribuyen principalmente en el complejo del septo medial/banda diagonal, y de forma menos conspícua en el septo lateral caudoventral (Smeets y Steinbusch, 1989; Risold y Swanson, 1997). Debemos destacar que, a pesar de la abundante presencia de fibras y terminales CA en el septo, en todas la especies estudiadas hasta ahora no se ha demostrado la presencia de somas neuronales CA en esta región, y por tanto, podemos asumir que el origen de las fibras CA es extraseptal. La única excepción hasta el momento, se ha encontrado en el telencéfalo basal de primates, donde se ha descrito una subpoblación de neuronas THi en el núcleo septal medial y la banda diagonal (Gouras y cols., 1992).

Estudios sobre el origen de la inervación CA en el septo mediante métodos de doble marcaje se han realizado solamente en mamíferos, y por tanto, no disponemos datos similares en otras clases de vertebrados. Sin embargo, existen diversos trabajos sobre la conectividad y las características neuroquímicas de la región del septo en aves y reptiles, que demuestran la existencia de características comunes en la organización de esta estructura telencefálica en todos los vertebrados amniotas (Reiner y cols., 1994; Font y cols., 1995, 1997, 1998; Wynne y Güntürkün, 1995). Más aún, recientemente se han realizado trabajos de doble marcaje en peces teleósteos que demuestran la presencia una proyección desde el núcleo tuberal posterior en el diencéfalo al telencéfalo ventral, similar a las proyecciones CA mesoestriatal y mesolímbica de tetrápodos (Rink y Wullimann, 2001, 2002; Wullimann y Rink, 2002). Sin embargo, en este trabajo solamente se demuestra una proyección al área ventral telencefálica, sin poder identificar claramente si esta conexión representa parte de las aferencias al septo de teleósteos.

En mamíferos, se ha demostrado que el *grupo incerto-hipotalámico* (que incluye los grupos celulares A11, A12 y A13) proporciona inervación DA al septo (Lindvall y Stenevi, 1978). Nuestros resultados demuestran la existencia de células doblemente marcadas en un grupo CA situado dorsal al núcleo periventricular de la zona incerta, dentro del tálamo ventral. Siguiendo una aproximación segmental en el análisis topográfico de este grupo diencefálico, junto con los datos existentes sobre sus conexiones y los del presente trabajo, este núcleo CA podría compararse con la zona incerta de mamíferos, localizada dentro de la placa alar del prosómero 3 (Puelles y Rubenstein, 1993; Puelles y cols., 1996; Milán y Puelles, 2000; Smeets y González, 2000; Sánchez-Camacho y cols., 2001b).

La mayor fuente de inervación DA en el septo de mamíferos tiene su origen en el *área tegmental ventral* mesencefálica (grupo A10), que forma parte del sistema mesolímbico (Lindvall, 1975; Lindvall y Stenevi, 1978, Swanson, 1982). En la rata, las neuronas de este grupo celular presentan una disposición topográfica en las proyecciones a diferentes regiones del telencéfalo basal (Fallon y Moore, 1978). Aunque en la rana no existe una distinción clara entre la sustancia negra y el área tegmental ventral (los grupos A9 y A10, respectivamente), en base a sus conexiones con los ganglios basales y a su localización neuromérica, se ha propuesto que las células DA de la porción dorsomedial del tubérculo posterior y su continuación caudal en el tegmento mesencefálico son homólogas de los grupos A9-A10 de amniotas (Marín y cols., 1998). Los resultados del presente trabajo, que demuestran su conexión con la región septal, como un componente importante de la ruta mesolímbica también presente en anuros, apoyan dicha homolo-

gía. Más aún, se ha demostrado que existe un cierto grado de somatotopía en este grupo en la proyección de sus neuronas DA a distintas áreas de los ganglios basales (Marín y cols., 1997b). Por tanto, dentro de la extensión del tubérculo posterior-grupo mesencefálico, la porción caudal estaría principalmente relacionada con las proyecciones mesolímbicas, al núcleo accumbens y al septo, y sería comparable al grupo A10 de mamíferos.

En cuanto a las proyecciones NA al septo en mamíferos, la mayor parte se originan en el complejo del *locus coeruleus* (Lindvall y Stenevi, 1978; Moore, 1978; Risold y Swanson, 1997; Senatorov y Renoud, 1999). En nuestro trabajo, encontramos células doblemente marcadas en el locus coeruleus, dentro de la región del istmo, que proyectan al septo de *Rana*. Como ocurre con las conexiones demostradas desde este grupo a los ganglios basales, la médula espinal y el techo mesencefálico, solamente un escaso número de neuronas proporciona la inervación NA de la región septal en anfibios (Marín y cols., 1997b; Sánchez-Camacho y cols., 2001b, 2002a-c). Este hecho apunta a que los axones de las células del locus presentan una amplia colateralización que permite que única neurona proyecte a varias regiones muy distantes en el encéfalo.

Por último, se ha demostrado en la rata que una considerable proyección NA a la banda diagonal de Broca se origina en el *núcleo del tracto solitario* (grupo A2; Senatorov y Renoud, 1999). De acuerdo con estos resultados, en experimentos con aplicaciones restringidas al núcleo de la banda diagonal, encontramos células marcadas retrógradamante en el núcleo del tracto solitario. En anfibios, este grupo CA posee neuronas inmunoreactivas no sólo para NA, sino también células DA y adrenérgicas. Sin embargo, en base a la topografía y la morfología de las células marcadas, es muy probable que la proyección septal desde este núcleo sea también de naturaleza NA al igual que sucede en mamíferos.

Hodología de los Grupos CA en el SNC de Anfibios

El presente trabajo proporciona los primeros datos sobre la conectividad y ontogenia de varios grupos CA en el SNC de diversas especies de anfibios. Así, el empleo de técnicas de doble marcaje nos ha permitido determinar la existencia de una proyección desde un núcleo concreto y la naturaleza neuroquímica de dicha conexión. Datos previos de nuestro laboratorio han demostrado con idéntica metodología las conexiones de algunos grupos CA, aunque se han limitado al análisis de la inervación CA de los ganglios basales en anfibios anuros y urodelos (Marín y cols., 1997b,c, 1998). De los distintos grupos CA descritos en el SNC de anfibios, nuestro estudio ha demostrado una variedad de conexiones desde siete de estas poblaciones celulares:

- el núcleo supraquiasmático.
- la porción ventrolateral del tubérculo posterior.
- el núcleo periventricular de la zona incerta y un grupo DA dorsal, ambos localizados en el tálamo ventral.
- el tubérculo posterior dorsomedial y su continuación caudal en el tegmento mesencefálico.
- el núcleo yuxtacomisural.
- el locus coeruleus.
- y el núcleo del tracto solitario.

El *núcleo supraquiasmático* recibe información desde la retina y proyecta a diferentes regiones encefálicas entre las que se incluyen el estriado, el área preóptica o el hipotálamo ventral (Allison y Wilczynski, 1994). A través de estas conexiones, este núcleo proporcionaría información sobre el ambiente, en particular acerca del fotoperíodo, para coordinar y regular la actividad sexual y reproductora en los anfibios. Por otro lado, a través de su conexión con la hipófisis, el núcleo supraquiasmático controla la liberación de la hormona estimulante de melanocitos (MHS), implicada en el mecanismo denominado “de adaptación al fondo”, por el cual el animal cambia el color de su piel para adaptarlo a las condiciones de luz del ambiente (Tuinhof y cols., 1994; Kramer y cols., 2001a,b). Se ha demostrado que la DA procedente de este núcleo tiene un importante papel en este proceso, ejerciendo un control inhibitorio en la secreción de la MHS desde las células melanotropas de la hipófisis (Tuinhof y cols., 1994). Pero además, los datos de nuestro trabajo han demostrado la existencia de una conexión adicional desde el núcleo supraquiasmático con el techo mesencefálico. En base a estos resultados, proponemos que la población DA que forma parte de este grupo podría también actuar como centro de relevo de la información estriatal al techo óptico, junto con el núcleo yuxtacomisural y el locus coeruleus (Sánchez-Camacho y cols., 2002c).

La información disponible acerca de la hodología de la *porción ventrolateral del tubérculo posterior* es bastante escasa. Los resultados de nuestro estudio demuestran la existencia de diversas proyecciones desde este núcleo DA localizado en la región mamilar y supramamilar del prosencéfalo secundario. En particular, encontramos una importante conexión descendente con la médula espinal, y sólo de manera ocasional con el techo mesencefálico de anfibios (Sánchez-Camacho y cols., 2001b, 2002a-c).

En el presente trabajo demostramos también la conexión del *núcleo periventricular de la zona incerta* con la médula espinal (Sánchez-Camacho y cols., 2001b, 2002a,b). Además, encontramos que la población DA situada más dorsalmente dentro del tálamo ventral proyecta hacia la región septal (Sánchez-Camacho y cols., 2002d). A pesar de que ambos grupos DA se localizan dentro de la placa alar del prosómero 3, en base a sus conexiones podrían constituir dos poblaciones celulares diferentes dentro del tálamo ventral. En este caso resulta difícil establecer homologías con otros grupos CA de mamíferos. Sin embargo, es posible que el núcleo periventricular de la zona incerta, con su conexión descendente a la médula espinal, represente parte de las proyecciones diencéfalo-espinales de mamíferos que se originan desde el grupo A11, aunque con un origen segmental más rostral en el caso de anfibios. Por otro lado, la conexión ascendente desde la población DA dorsal con la región septal, sería comparable con la demostrada desde el grupo incerto-hipotalámico de mamíferos, en particular desde el grupo celular A13.

En cuanto a las proyecciones desde el *tubérculo posterior dorsomedial* y su extensión caudal en el tegmento mesencefálico, nuestro estudio demuestra su conexión con la región del septo (Sánchez-Camacho y cols., 2002d). Datos previos revelan además la existencia de aferencias CA desde este núcleo a los ganglios basales (Marín y cols., 1997b,c, 1998). En conjunto, estas proyecciones al telencefálo basal constituirían parte del sistema DA ascendente mesoestriatal y mesolímbico, que estaría incluído dentro del circuito de integración de la información motora. En base a su localización topográfica y al patrón de eferencias, este núcleo DA se ha homologado a los grupos A9-A10 de amniotas.

Nuestro estudio revela la existencia de una proyección desde el *núcleo yuxtacomisural* al techo mesencefálico (Sánchez-Camacho y cols., 2002c). Se ha demostrado que las células DA de este núcleo, implicadas en la vía pretecto-tectal,

ejercen un efecto inhibitorio sobre las neuronas del techo óptico. Esta modulación DA de la actividad tectal, influye en último término en diferentes patrones visuomotores, como las estrategias de captura de presas, en las que se requiere información relacionada con la presa y con su localización en el campo visual. Además, trabajos previos revelan la importancia de este grupo DA como centro de relevo de la información estriatal al techo óptico de anfibios, y su implicación en el circuito motor que integra la información visual (Marín y cols., 1997e).

El *locus coeruleus* es probablemente uno de los grupos CA más conservados a lo largo de la evolución de los vertebrados. Mediante métodos de doble marcaje, se han demostrado diversas proyecciones NA desde este grupo ístmico, rostralmente hacia regiones telencefálicas, en particular el septo y los ganglios basales**,** junto con una conexión con el techo mesencefálico y la proyección descendente coeruleoespinal (Marín y cols., 1996, 1997b,c, 1998; Sánchez-Camacho, 2001b, 2002a-d). Así, en base a su patrón de eferencias, su localización dentro del segmento ístmico y su naturaleza noradrenérgica, permiten considerarlo como el homólogo del grupo A6 de amniotas. A pesar de que este grupo está constituido únicamente por un escaso número de neuronas, sus axones presentan una amplia arborización y colateralización que alcanza prácticamente todas las regiones del encéfalo. Este elevado número de conexiones implica que el locus coeruleus estaría relacionado con funciones muy diversas. Así por ejemplo, nuestros datos revelan que este núcleo NA ejercería un efecto modulador en el procesamiento sensorial de la información visual a nivel del techo óptico. Por otro lado, mediante sus conexiones con la médula espinal, los ganglios basales o el septo, participaría de manera directa sobre el comportamiento motor.

Por último, el *núcleo del tracto solitario* está implicado en el procesamiento de la información visceral ascendente y descendente. Nuestro trabajo demuestra la existencia de una conexión hacia la región del septo, y una proyección descendente con la médula espinal desde este grupo celular (Sánchez-Camacho y cols, 2001b, 2002a,b,d). Asimismo, se han demostrado aferencias desde el núcleo del tracto solitario al estriado y el núcleo accumbens (Marín y cols., 1997b,c). Finalmente, hay que recordar que este grupo CA está formado por una población mixta de células DA, NA y adrenérgicas, por lo que no es posible determinar la naturaleza de sus proyecciones en base solamente a la inmunodetección de la enzima TH.

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Conclusiones

La organización de las proyecciones descendentes a la médula espinal presenta un patrón común en los tres órdenes de anfibios. Nuestros resultados revelan la presencia de numerosas conexiones desde distintas regiones encefálicas, destacando la existencia de una proyección telencéfalo-espinal, posiblemente desde la amígdala central, característica de amniotas. Asimismo, el estudio de la ontogenia de las vías descendentes supraespinales, demuestra que estas conexiones se desarrollan desde estadios muy tempranos de acuerdo con una secuencia temporal de aparición, y siguiendo un patrón básico que sería comparable entre los vertebrados. En conjunto, este sistema de conexiones espinales parece ser una constante filogenética durante la evolución de los vertebrados, que ha permitido el desarrollo de diversos patrones de locomoción, controlados a nivel supraespinal por las vías descendentes desde centros del tronco encefálico y el prosencéfalo.

El origen y la distribución de la inervación catecolaminérgica en la médula espinal se organiza de una manera similar en todos los grupos anfibios. Su desarrollo, según nuestros resultados en *Xenopus laevis,* se establece siguiendo una secuencia temporal rostrocaudal. De esta manera, la presencia de las catecolaminas en la médula espinal desde estadios embrionarios podría ser fundamental en el control temprano de la locomoción. Aunque sólo disponemos de datos previos en mamíferos, es posible que la organización de estas aferencias catecolaminérgicas espinales presente características comunes en todos los tetrápodos.

La inervación catecolaminérgica del techo mesencefálico en anfibios presenta un patrón laminar en la distribución de las fibras y terminales dopaminérgicos y noradrenérgicos. El origen de dichas aferencias tectales se localiza fundamentalmente en la región pretectal y el locus coeruleus, tanto en anuros como en urodelos. Este patrón de conexiones dopaminérgicas al techo óptico es comparable al existente en aves, mientras que en mamíferos se localiza en la sustancia negra. Por el contrario, las proyecciones noradrenérgicas desde el locus coeruleus estarían muy conservadas entre los tetrápodos.

Se ha demostrado que la organización de la inervación catecolaminérgica en la región septal de anuros comparte muchas características en común con la de vertebrados amniotas, en cuanto a su organización topográfica y neuroquímica. Asimismo, el origen de dichas aferencias catecolaminérgicas es muy similar al presente en mamíferos. En particular, la proyección desde el tubérculo posterior/tegmento mesencefálico que forma parte del circuito dopaminérgico ascendente mesolímbico, representaría una característica ancestral en el SNC de los vertebrados.

El presente trabajo ha demostrado la existencia de una amplia variedad de conexiones ascendentes y descendentes desde distintos grupos catecolaminérgicos que alcanzan numerosas regiones encefálicas, formando un patrón amplio y específico de inervación en el SNC. De esta forma, las catecolaminas tendrían una gran importancia funcional, y estarían implicadas en el procesamiento de la infomación sensorial y el control motor, en el comportamiento visual y visuomotor, y posiblemente durante el desarrollo, en la neurogénesis y maduración de las neuronas espinales. Aunque existen pocos datos acerca de la conectividad de sus grupos en otros vertebrados, y la mayor parte se han centrado en mamíferos, nuestros datos apuntan a que las catecolaminas constituyen un grupo de neurotransmisores altamente conservado a lo largo de la evolución, siendo posiblemente uno de los sistemas neuroquímicos más antiguos filogenéticamente del encéfalo de vertebrados, no sólo en base a la distribución de sus grupos, sino también en base a su hodología y ontogenia.

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