ORIGINAL ARTICLE

Dynamic expression of tyrosine hydroxylase mRNA and protein in neurons of the striatum and amygdala of mice, and experimental evidence of their multiple embryonic origin

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Abstract Emotional and motivational dysfunctions observed in Parkinson's disease, schizophrenia, and drug addiction are associated to an alteration of the mesocortical and mesolimbic dopaminergic pathways, which include axons projecting to the prefrontal cortex, the ventral striatum, and the amygdala. Subpopulations of catecholaminergic neurons have been described in the cortex and striatum of several mammals, but the presence of such cells in the adult amygdala is unclear in murine rodents, and in other rodents appears to show variations depending on the species. Moreover, the embryonic origin of telencephalic tyrosine hydroxylase (TH) cells is unknown, which is essential for trying to understand aspects of their evolution, distribution and function. Herein we investigated the expression of TH mRNA and protein in cells of the striatum and amygdala of developing and adult mice, and analyzed the embryonic origin of such cells using in vitro migration assays. Our results showed the presence of TH mRNA and protein expressing cells in the striatum (including nucleus accumbens), central and medial extended amygdala during development, which are persistent in adulthood although they are less numerous, generally show weak mRNA expression, and some appear to lack the protein. Fate mapping analysis showed that these cells include at least two subpopulations with different embryonic origin in either the commissural preoptic area of the subpallium or the supraopto-paraventricular domain of the alar hypothalamus. These data are important for future studies trying to understand the role of catecholamines in modulation of emotion, motivation, and reward.

Abbreviations

A	Amygdala
aac	Anterior limb of the anterior commissure
AAd	Anterior amygdala, dorsal subdivision
AAv	Anterior amygdala, ventral subdivision
ac	Anterior commissure
Ac	Nucleus accumbens
AcC	Nucleus accumbens, core
ACo	Anterior cortical amygdalar area
AcSh	Nucleus accumbens, shell
Bas	Nucleus basalis (Meynert)
BC	Basal amygdalar complex
BL	Basolateral amygdalar nucleus
BSTL	Lateral bed nucleus of the stria terminalis
BSTLp	Posterior part of BSTL
BSTM	Medial bed nucleus of the stria terminalis
BSTMpl	Posterolateral part of BSTM
Ce	Central amygdala
CeC	Central amygdala, capsular subdivision
CeCvm	Central amygdala, capsular subdivision, ventromedial part
CeL	Central amygdala, lateral subdivision
CeM	Central amygdala, medial subdivision
CPu	Caudate-putamen
DP	Dorsal pallium

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EAce	Central extended amygdala
ec	External capsule
EMT	Thalamic (or prethalamic) eminence
f	Fornix
gl	Glomerular cell layer of the olfactory bulb
GP	Globus pallidus
GrO	Granular cell layer of the olfactory bulb
HDB	Horizontal limb of the diagonal band nucleus
ic	Internal capsule
IPAC	Interstitial nucleus of the posterior limb of the anterior commissure
L	Lateral amygdalar nucleus
LGE	Lateral ganglionic eminence
LGEc	Lateral ganglionic eminence, caudal part
LGEd	Lateral ganglionic eminence, dorsal subdivision
LGEd + v	Lateral ganglionic eminence, dorsal and ventral subdivisions
lot	Lateral olfactory tract
LOT	Nucleus of the lateral olfactory tract
LPO	Lateral preoptic area
Me	Medial amygdala
MeA	Medial amygdala, anterior subdivision
MCPO	Magnocellular preoptic complex (it includes MPO and Bas)
MePD	Medial amygdala, posterodorsal subdivision
MePV	Medial amygdala, posteroventral subdivision
MGE	Medial ganglionic eminence
MPO	Magnocellular preoptic area
nsp	Nigrostriatal projection
OB	Olfactory bulb
pac	Posterior limb of the anterior commissure
pG	Periglomerular cells of the olfactory bulb
Pir	Piriform cortex
PO	Preoptic area
POB	Basal/ventral preoptic area
POC	Commissural preoptic area
PVN	Paraventricular hypothalamic nucleus
Se	Septum
SNc	Substantia nigra, pars compacta
SPV	Supraopto-paraventricular domain of the alar hypothalamus
TH	Tyrosine hydroxylase
Tu	Olfactory tubercle
VP	Ventral pallium
VTA	Ventral tegmental area

Introduction

Dopamine and other catecholamines play a key role as neuromodulators in the central nervous system, and their depletion or dysregulation in the cerebral hemispheres is behind several neurological or psychiatric pathologies such as Parkinson's disease and schizophrenia (Greengard 2001; Carlsson 2006; Iversen and Iversen 2007). For example, in Parkinson's disease there is a progressive depletion of the dopaminergic innervation of the somatomotor part of the striatum (caudate nucleus and putamen), due to neurodegeneration of tegmental dopaminergic neurons that project to it (in particular those of the substantia nigra pars compacta or A9 group) (Alexander 2004). In addition to the motor symptoms, idiopathic Parkinson's disease is characterized by emotional dysfunction (depression, anxiety, apathy), which appears associated to abnormalities involving mesolimbic and mesocortical dopaminergic pathways, such as those reaching the ventral striatum (nucleus accumbens), the amygdala and the prefrontal cortex (Blonder and Slevin 2011). The mesocorticolimbic dopaminergic system is also one of the systems altered in schizophrenia (Howes and Kapur 2009; Heinz and Schlagenhauf 2010). This disorder involves dopamine hypofunction in the prefrontal cortex that is associated to deficient stimulation of dopamine D1 receptors (Howes and Kapur 2009), and dopamine hyperfunction at both amygdalar (Reynolds 1983) and striatal levels (Davis et al. 1991; Howes and Kapur 2009). Dopamine hyperfunction in the striatum of schizophrenics appears associated to excessive stimulation of dopamine D2 receptors and increased levels of D2 receptor binding in the dorsal striatum (Laruelle et al. 2003; Howes and Kapur 2009), and to increased dopamine D3 receptor binding in the ventral striatum (Joyce and Gurevich 1999). Catecholamines, including dopamine released from incoming axons that originate in neurons of the ventral tegmental area (VTA or A10 group), are known to modulate reward, motivation and emotions at the level of the amygdala and the nucleus accumbens (Haber and Fudge 1997; Hasue and Shammah-Lagnado 2002; Rosenfeld et al. 2011; Lintas et al. 2011). Similar to the striatum (including caudate-putamen and accumbens), the major catecholaminergic innervation in the amygdala is observed in its "striatal-like" part, including the central amygdala, although other amygdalar parts are also lightly innervated (Alheid et al. 1995). As with the striatum (reviews by Reiner et al. 1998; Smeets and González 2000), the origin of catecholaminergic innervation of the central amygdala is thought to be extratelencephalic (Haber and Fudge 1997), and includes: dopaminergic axon terminals that primarily originate in neurons of A10 (VTA), with lesser contribution of A9 (nigral) and A8 (retrorubral) neurons; noradrenalinergic terminals that originate in cells of the A6 (locus coeruleus) and A2 groups; and adrenergic terminals that originate in the cells of the C2 group (Fallon et al. 1978; Riche et al. 1990; Zardetto-Smith and Gray 1995; Hasue and Shammah-Lagnado 2002; Delaney et al. 2007). However, the

precise role of catecholamines in modulation of non-motor functions at the level of the striatum and amygdala is still unclear.

Interestingly, recent data have shown the presence of catecholaminergic neurons inside the striatum and some parts of the amygdala (Dubach et al. 1987; Tashiro et al. 1989; Prensa et al. 2000; Baker et al. 2003; Cossette et al. 2005a, b; Marín et al. 2005; Huot and Parent 2007; Northcutt et al. 2007; Ugrumov 2009). The presence of these cells is extremely important and relevant for attempts trying to understand the role of catecholamines in modulation of motor and non-motor functions (including emotions, emotion-related learning, motivation and reward) in normal and pathological conditions. In the striatum, these cells are a small subpopulation of dopaminergic interneurons, expressing tyrosine hydroxylase (TH, rate-limiting enzyme for the synthesis of catecholamines), aromatic L-amino acid decarboxylase (AADC, involved in the synthesis of dopamine), dopamine membrane transporter (DAT), and the transcription factor Nurr1 (which is essential for the differentiation of tegmental dopaminergic neurons) (Huot and Parent 2007; Cossette et al. 2005b; Ugrumov 2009). Notably, the number of these cells is smaller in rodents than in primates, but in both mammalian groups the cell number increases upon lesion of the dopaminergic nigrostriatal pathway (rodents: Tashiro et al. 1989; Meredith et al. 1999; primates: Betarbet et al. 1997; Palfi et al. 2002). This has also been observed in human patients affected by Parkinson's disease (Porritt et al. 2000), although this finding is controversial (Huot et al. 2007). Regarding the amygdala, cells immunoreactive for TH have been described in the medial extended amygdala in some rodents, but their presence and abundance show variations depending on the species and the sex (Northcutt et al. 2007). TH-expressing cells are abundant in the medial extended amygdala of prairie voles, specially in males (Northcutt et al. 2007), and are thought to play a role in monogamous behavior, which is typical in this species (Northcutt and Lonstein 2011). Such cells are absent in the rat medial extended amygdala (Northcutt and Lonstein 2011), and no published data on them exist in the mouse. On the other hand, only transient TH immunoreactive cells have been described in the central amygdala of rats (Verney et al. 1988), while there is no published data in other mammals. Moreover, apart from those of the olfactory bulb, no published data exist on the embryonic origin of catecholaminergic neurons of the telencephalon, although this information may be relevant in order to understand some aspects of their evolution, phenotype and function (Yamamoto and Vernier 2011). In this study, we have analyzed the distribution of TH-expressing neurons (containing mRNA and/or protein) in the striatum and extended amygdala of developing and adult mice, and investigated their embryonic origin using in vitro fate mapping assays combined with immunofluorescence. Our data show novel evidence for the existence of catecholaminergic cells in the extended amygdala of adult mice, and provide experimental demonstration for multiple embryonic origins of the TH-expressing telencephalic cells. Preliminary reports of this study have been presented at the annual meetings of the Spanish Society for Neuroscience (2011) and the American Society for Neuroscience (2012).

Materials and methods

Mouse (Swiss; CD1) embryos from embryonic day 12.5 (E12.5) until day 18.5 (E18.5), neonates (P0), and adult mice (8 females and 4 males; 1.5–5 months old) were used in the present study. All animals were treated according to the regulations and laws of the European Union (86/609/EEC) and the Spanish Government (Royal Decree 1021/2005) for care and handling of animals in research. The protocols used were approved by the Committee for handling and care of research animals of the University of Lleida. Adult mice were maintained in standard conditions in the rodent Facility of our University, at 22–23 °C, 45–55 % humidity, in a 12:12 light–dark cycle, with water and food ad libitum, living in groups of 3–5 animals per cage. The mouse embryos were obtained from pregnant females, and their brains were processed either for in situ hybridization, immunohistochemistry or for preparing organotypic cultures for in vitro migration assays. Neonates and adult mice were deeply anesthetized (with 0.1 ml/100 g weight of 0.02 % sodium pentobarbital) and perfused transcardially with cold saline solution (0.9 % NaCl), followed by phosphate-buffered 4 % paraformaldehyde. Embryonic brains (until E15.5) to be processed for in situ hybridization or immunohistochemistry were rapidly decapitated and fixed by immersion as previously described (García-López et al. 2008). Older embryos (E16.5–E18.5) were first anesthetized by cold, and then perfused transcardially with phosphate-buffered 4 % paraformaldehyde. After fixation, brains were embedded in 4 % low-melt agarose and sectioned (70- to 110-μm thick) using a vibratome (Leica VT 1000S).

In situ hybridization

Frontal or horizontal brain sections were processed for in situ hybridization using digoxigenin-labeled riboprobes, following a procedure previously described (Medina et al. 2004; García-López et al. 2008; Abellán and Medina 2008, 2009). The riboprobes were synthesized from cDNA of rat tyrosine hydroxylase (TH; bp 1–1770; Genbank accession no: NM_012740; the cDNA was kindly provided by

Dr. Faustino Marín, who obtained it from Blanchard et al. 1994; Marín et al. 2005). This fragment showed 98 % identity in their sequence between rat and mouse. Recent findings show that two copies of the *TH* gene (*TH1* and *TH2*) are present in most jawed vertebrates, but only the *TH1* copy is present in placental mammals (Yamamoto and Vernier 2011). The general mRNA expression pattern observed in the mouse brain during early embryonic stages using this probe is identical to that published previously in the mouse (Marín et al. 2005), although we analyzed in more detail the extended amygdala and found novel sub-populations of TH-expressing cells.

We used PCR to obtain the DNA template employed for synthesizing the riboprobe, either the antisense for studying the mRNA expression or the sense for controls. We synthesized the digoxigenin-labeled riboprobes using Roche Diagnostics's (Mannheim, Germany) protocols for the genes mentioned above. Before hybridization, the sections were washed in PBS containing 0.1 % Tween-20 (PBT 1×), prehybridized in hybridization buffer (HB) for 2 h at 58 °C, and then hybridized in HB containing the riboprobe overnight at 58 °C (0.5–1 μg/ml, depending on the probe and brain size). The hybridization buffer contained 50 % of deionized formamide, 1.3× standard saline citrate (SSC; pH 5), 5 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0; Sigma-Aldrich, Steinheim, Germany), 1 mg/ml of yeast tRNA (Sigma-Aldrich), 0.2 % Tween-20, 100 μg/ml of heparin (Sigma-Aldrich), completed with water (free of RNAase and DNAase; Sigma-Aldrich). Following hybridization, the sections were washed with a mix 1:1 of MABT 1× (1.2 % maleic acid, 0.8 % NaOH, 0.84 % NaCl and 0.1 % Tween-20) and HB at 58 °C during 20 min and washed abundantly at room temperature with MABT 1× (about 2 h). Following this, the sections were blocked with a solution containing blocking reagent (Roche), MABT 1× and sheep serum (Sigma) for 4 h at room temperature, then incubated in an antibody against digoxigenin (alkaline phosphatase coupled anti-digoxigenin; diluted 1:3,500; Roche Diagnostics) overnight at 4 °C, later washed with MABT 1× and finally revealed with BM purple (Roche Diagnostics). Sections were then mounted on glycerol gelatine (Sigma).

As a control, parallel series of sections from some adult animals, from rostral to caudal levels, were hybridized using either the sense or the antisense, following otherwise identical conditions of incubation, washing and final staining to reveal the signal. The sections processed using the antisense showed TH-expressing neurons in the brain following the typical pattern described in previous studies in the mouse and/or other mammals, including subpopulations of TH-expressing cells in the cortex, striatum, and amygdala (Hökfelt et al. 1984; Smeets and Reiner 1994; Marín et al. 2005; Björklund and Dunnett 2007; Huot and Parent 2007; Northcutt et al. 2007; Ugrumov 2009). In contrast, the sections processed with the sense showed no signal in the brain at any single section level (Fig. 1).

Organotypic cultures

For the migration assays, we prepared organotypic cultures of forebrain slices from E13.5 to E16.5 mice following a previously described procedure (Soria and Valdeolmillos 2002; slightly modified according to Legaz 2006; Bupesh et al. 2011a, b). Upon extraction, embryos were placed in a standard ice cold, oxygenated culture medium artificially resembling cerebrospinal fluid (containing 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4 \cdot 7H_2O$, 26 mM $CHNaO_3$, 2.4 mM $CaCl_2 \cdot 2H_2O$, and 10 mM D(+)-glucose; Soria and Valdeolmillos 2002), where brains were dissected out. The brains were sectioned at 300 μm in the frontal or horizontal plane using a vibratome (Leica VT 1000S), and the slices were mounted onto porous culture plate inserts (Millicell-CM, 0.4 μm pore diameter; 30 mm insert diameter; Millipore, Molsheim, France; Soria and Valdeolmillos 2002) and placed in culture medium DMEM F-12 (Gibco; supplemented with 5 % fetal bovine serum, 0.1 mM glutamine, 6.5 mg/ml D-glucose, 1 % supplement N2, and 1 % penicillin) (Soria and Valdeolmillos 2002; Legaz 2006). Slices were allowed to recover in a CO_2 incubator (5 % CO_2; 37 °C) for 1 h before application of the tracer dye. After that, tungsten particles coated with the fluorescent dye CMFDA (Cell Tracker Green 5-chloromethylfluorescein diacetate; InVitrogen-Molecular Probes, Paisley, UK; excitation peak: 490 nm; emission peak: 514 nm; Alifragis et al. 2002) were applied to the ventricular/subventricular zone of either the lateral ganglionic eminence (at intermediate or caudal levels), the commissural preoptic area (POC), or the supraopto-paraventricular hypothalamic domain. The slices were then transferred to culture medium Neurobasal (Gibco, Grand Island, NY, USA; supplemented with 5 % fetal bovine serum, 0.1 mM, glutamine, 6.5 mg/ml D-glucose, 1 % supplement B27 (Gibco), and 1 % penicillin; Soria and Valdeolmillos 2002; Legaz 2006) and incubated in a CO_2 incubator (5 %; 37 °C) for 24–48 h. Following incubation, the slices were fixed with phosphate-buffered 4 % paraformaldehyde (pH 7.4) for 8 min, and then rinsed and stored in phosphate buffer (0.1 M, pH 7.4) containing 0.1 % sodium azide until microscopic observation. The labeling was analyzed and images were captured using a confocal scanner microscope (Olympus FV500).

Fig. 1 Images of parallel frontal sections of the brain of an adult female mouse (case MA17), at the level of the olfactory bulb (**a, b**), striatum (**c, d**) and substantia nigra (**e, f**), processed for in situ hybridization using either the antisense or sense probe for TH. Note the absence of signal in the sections processed with the sense. For abbreviations see list. *Scale*: 200 μm (*bar* in **a**, applies to all)
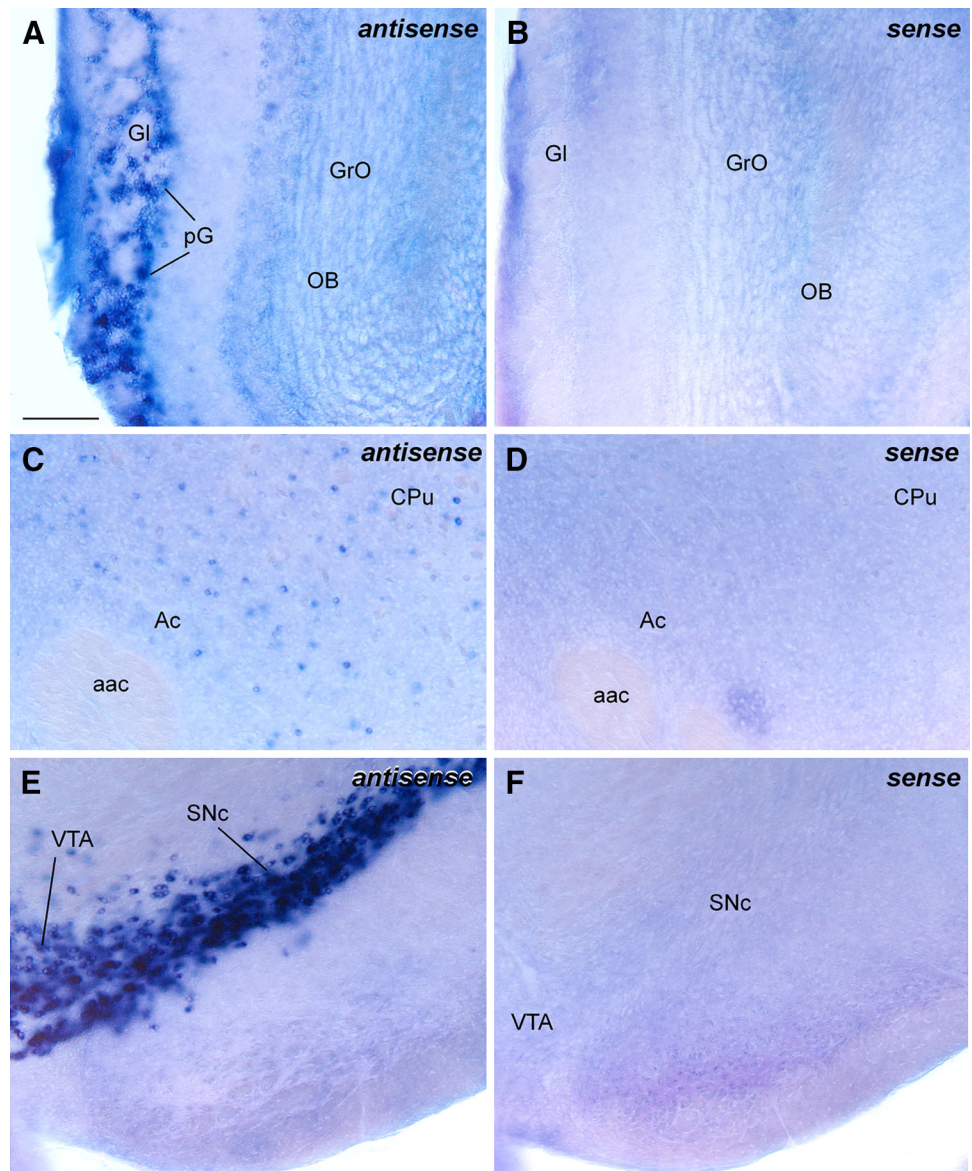
Immunohistochemistry and immunofluorescence

Some embryonic brains were sectioned in frontal or horizontal planes and processed for immunohistochemistry to detect tyrosine hydroxylase (rabbit anti-tyrosine hydroxylase; Millipore, Temecula, CA, USA). Moreover, selected slices from the migration experiments were processed (directly or, more often, after re-sectioned at 40–50 μm-thick) for immunofluorescence to detect TH. See section below for details on the primary antibody. The primary antibody was diluted at 1:1,000 in PBS containing 0.3 % Triton X-100, and the tissue was incubated for 2–3 days at 4 °C, under constant and gentle agitation. To block unspecific binding of the secondary antisera, 10 % normal goat serum (Sigma) and/or 10 % normal horse serum

(Sigma) was added to the solution containing the primary antibody.

Following this incubation and standard washes in PBS-Triton, the sections were incubated in a secondary antiserum for either 1 h (for immunohistochemistry) or 2 h (for immunofluorescence) at room temperature. For immunohistochemistry, the secondary antisera used was biotinylated goat anti-rabbit (diluted 1:200), purchased from Vector (Burlingame, CA, USA). After washing, the sections were incubated in the avidin–biotin complex (ABC kit; Vector; 0.003 % dilution) for 1 h at room temperature. The immunolabeling was revealed by 0.05 % diaminobenzidine (DAB; Sigma-Aldrich, Steinheim, Germany) in 0.05 M Tris (pH 7.6), containing 0.03 % H_2O_2. For immunofluorescence, we used a donkey anti-rabbit

conjugated to Alexa 568 (diluted 1:500) from Molecular Probes. Following incubation, the sections were rinsed and stored at 4 °C, in the darkness, until analysis using a confocal microscope. To check the specificity of our secondary antisera, some sections were processed omitting the primary antibody. Following this, no labeling was observed.

Antibody characterization

The rabbit anti-tyrosine hydroxylase antibody (Millipore, Temecula, CA, USA) was raised against denatured tyrosine hydroxylase from rat pheochromocytoma, and by Western blotting it recognizes a band of approximately 62 kDa on PC12 lysates, which corresponds to the enzyme tyrosine hydroxylase (manufacturer's datasheet). In the developing mouse brain, it produces a staining pattern identical to that observed in previous reports in mouse, rat, and other mammals (Smeets and Reiner 1994; Jacobowitz and Abbott 1997), and the distribution of immunoreactive perikarya seen with this antibody is generally identical to that observed by in situ hybridization in the mouse brain (Marín et al. 2005; present results). However, in nuclei densely innervated by TH axon terminals, such as the striatum and the central extended amygdala (specially in adult animals), it is difficult to discern the presence of TH-expressing perikarya, and for that the in situ hybridization technique is more useful.

Digital photographs and figures

Digital photographs from hybridized and immunostained sections were taken on a Leica microscope (DMR HC) equipped with a Zeiss Axiovision digital camera, while serial images from fluorescent material were taken using a confocal microscope (Olympus FV500). Selected digital images were adjusted for brightness/contrast using Adobe PhotoShop and figures were mounted and labeled using FreeHand.

Identification of cell masses and nomenclature

For identification of forebrain cell masses during development, we used well-known atlas of developing mouse (Jacobowitz and Abbott 1997) and rat (Paxinos et al. 1994; Foster 1998) brain, as well as our own publications on the subject (especially Legaz et al. 2005; García-López et al. 2008; Abellán et al. 2010; Bupesh et al. 2011a, b). For BST and amygdalar subdivisions, we followed the brain atlas by Paxinos and cols. (Paxinos et al. 1999; Paxinos and Franklin 2004; which followed the scheme of Alheid et al. 1995).

Results

Distribution of cells expressing tyrosine hydroxylase (TH) in the striatum and extended amygdala of mice during embryonic development and in neonates

Early and intermediate stages

At E12.5 and later, the preoptic area of mouse contained abundant perikarya showing strong mRNA signal for TH and moderate TH immunoreactivity (Figs. 2a, 3a, e), corroborating previous findings in mouse, rat, and other mammals (Specht et al. 1981a; Foster 1994; Tillet 1994; Ugrumov 1994; Marín et al. 2005). At E12.5, the TH expression of the preoptic area was clearly continuous with a stream of TH-expressing cells that appeared to extend into the ganglionic eminences (thick arrow in Fig. 2a; also seen by Marín et al. 2005). At E12.5, two other TH expression domains were observed in the mouse telencephalon: (1) one located at the surface of the lateral ganglionic eminence (LGE), in the developing olfactory tubercle (Fig. 2a, b), resembling the superficial expression of Pax6 at this same place (Puelles et al. 2000; Bupesh et al. 2011b); (2) another one located at caudal telencephalic levels, in the primordium of the amygdala, that appear to be continuous with TH-expressing cells located in the supraopto-paraventricular hypothalamic domain (SPV; Fig. 2b, c; thin arrow in b points to the stream of cells apparently spreading from SPV into the caudal telencephalon).

At E15.5–E16.5, the TH expression in the preoptic area and other parts of the telencephalon was better defined (Fig. 3). In the preoptic area, expression was stronger in the commissural preoptic subdivision (POC), and from here appeared to spread into three directions: (1) radially toward the surface of this domain to populate the lateral preoptic area (LPO) and adjacent primordium of the corticopetal cell groups (including the magnocellular preoptic area and horizontal diagonal band nucleus); (2) ventrally into the periventricular zone of the basal/ventral preoptic subdivision (POB); (3) dorsally into the periventricular zone and mantle of the ganglionic eminences (Fig. 3a, e). At intermediate embryonic stages, the latter stream was weaker than previously. In the mantle of the ganglionic eminences, TH-expressing cells were observed in the developing olfactory tubercle, striatum, and extended amygdala (including the bed nucleus of the stria terminalis, the central amygdala and the medial amygdala) (Fig. 3). The TH-expressing perikarya of the olfactory tubercule were continuous with those of the piriform cortex, and were clearly detected with both immunohistochemistry and in situ hybridization (Fig. 3a, b, d). In the developing striatum and extended amygdala, TH-expressing perikarya
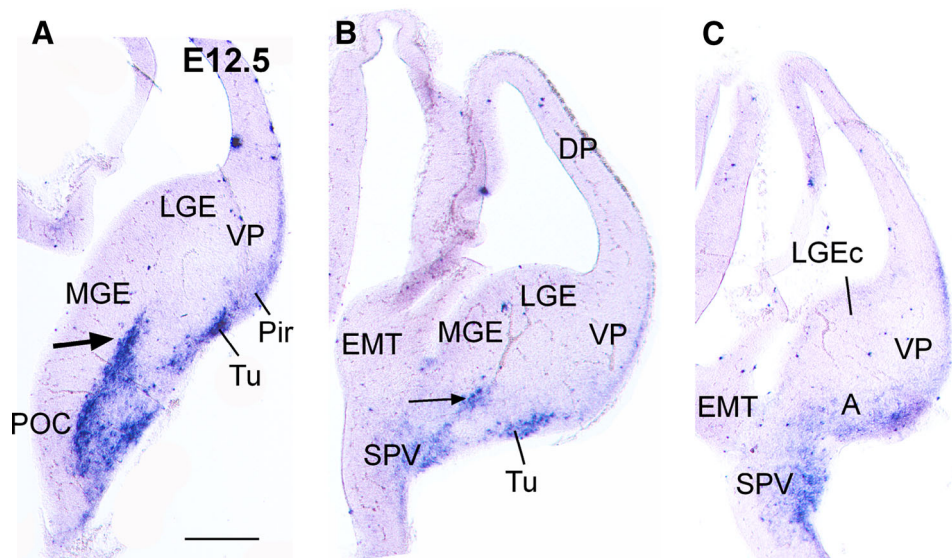
Fig. 2 Images of frontal sections through the telencephalon of an E12.5 mouse embryo, from intermediate (**a**) to caudal levels (**c**), hybridized for TH. Note the strong expression in the commissural preoptic area (POC), from where a stream of TH-expressing cells extend into the ganglionic eminences (*thick arrow* in **a**). Additional expression is observed in the developing olfactory tubercle (Tu, at the surface of the lateral ganglionic eminence) and the primordium of the amygdala (**a**). Expression in the latter is continuous (*thin arrow* in **b**) with that in the supraopto-paraventricular domain of the alar hypothalamus (SPV, **b**, **c**). For abbreviations see list. *Scale*: 300 μm (*bar* in **a**, applies to all)

generally showed a scattered distribution (Fig. 3b–f). These cells were clearly detected with in situ hybridization (Fig. 3b, b', c, c', c''), but they were hardly visible with immunohistochemistry due to their low number, faint immunoreactivity and/or their overlap with strongly TH immunoreactive (TH-ir) fibers of the nigrostriatal dopaminergic projection system (including incoming axons from cells of the substantia nigra, but also from ventral tegmental area and other tegmental catecholaminergic cell groups) (Fig. 3d, d', f, f'). In the striatal primordium, particularly in the developing caudate-putamen, the TH-ir perikarya show a trend to be intermingled between the TH-ir incoming axons of the nigro- or tegmento-striatal projection (Fig. 3d, d'). In the extended amygdala, TH-expressing cells (with both mRNA and protein) were mainly located in the primordia of the bed nucleus of the stria terminalis and the medial amygdala (Fig. 3c, c'', e, f, f''), but a few cells were also observed in the medial aspect of the central amygdala, accompanying the TH-ir fibers of the tegmento-amygdalar projection (Fig. 3c, c', f, f'). In addition, scattered TH-expressing cells were also observed in the pallial, cortical part of the amygdala (Fig. 3c, f).

Late embryonic (prenatal) stages and neonates

At prenatal stages (E17.5–E18.5), the telencephalon already showed a dense innervation by TH-ir fibers and varicosities (Fig. 4), partially resembling the pattern observed in adult mice. Numerous TH-ir fibers were observed in the nigrostriatal projection tract (nsp, Fig. 4a–c) and in the tegmento-amygdalar projection tract (arrow in Fig. 4f), both of which include ascending axons of TH-r cells located in the tegmentum. Moreover, abundant TH-ir fibers and varicosities were present in the major telencephalic targets of these axons, specially the striatum (caudate-putamen, nucleus accumbens), olfactory tubercle (Fig. 4a, b) and central extended amygdala (central amygdala and lateral bed nucleus of the stria terminalis; Fig. 4c–f). Nevertheless, the innervation pattern by TH-ir fibers was not fully mature, since this was strong only at rostrointermediate striatal levels, but at caudal levels of the caudate-putamen it was still patchy with areas of poor innervation (Fig. 4c', e').

Importantly, the number of TH-expressing perikarya in the mouse telencephalon at prenatal stages was higher compared to that in previous stages (Figs. 4, 5). Numerous TH-expressing perikarya were observed in the preoptic region (Figs. 4a, a', 5b), the striatum (Figs. 4b, 5a–c), the amygdala (Figs. 4d–f, 5e–f'), the bed nuclei of the stria terminalis (Figs. 4a, c, 5b–d), and the olfactory bulb (not shown). In most areas/nuclei, TH-expressing cells were clearly observed with both immunohistochemistry and in situ hybridization. However, with immunohistochemistry clear or unequivocal identification of TH-ir cells was complicated in nuclei with dense TH-ir innervation, such as
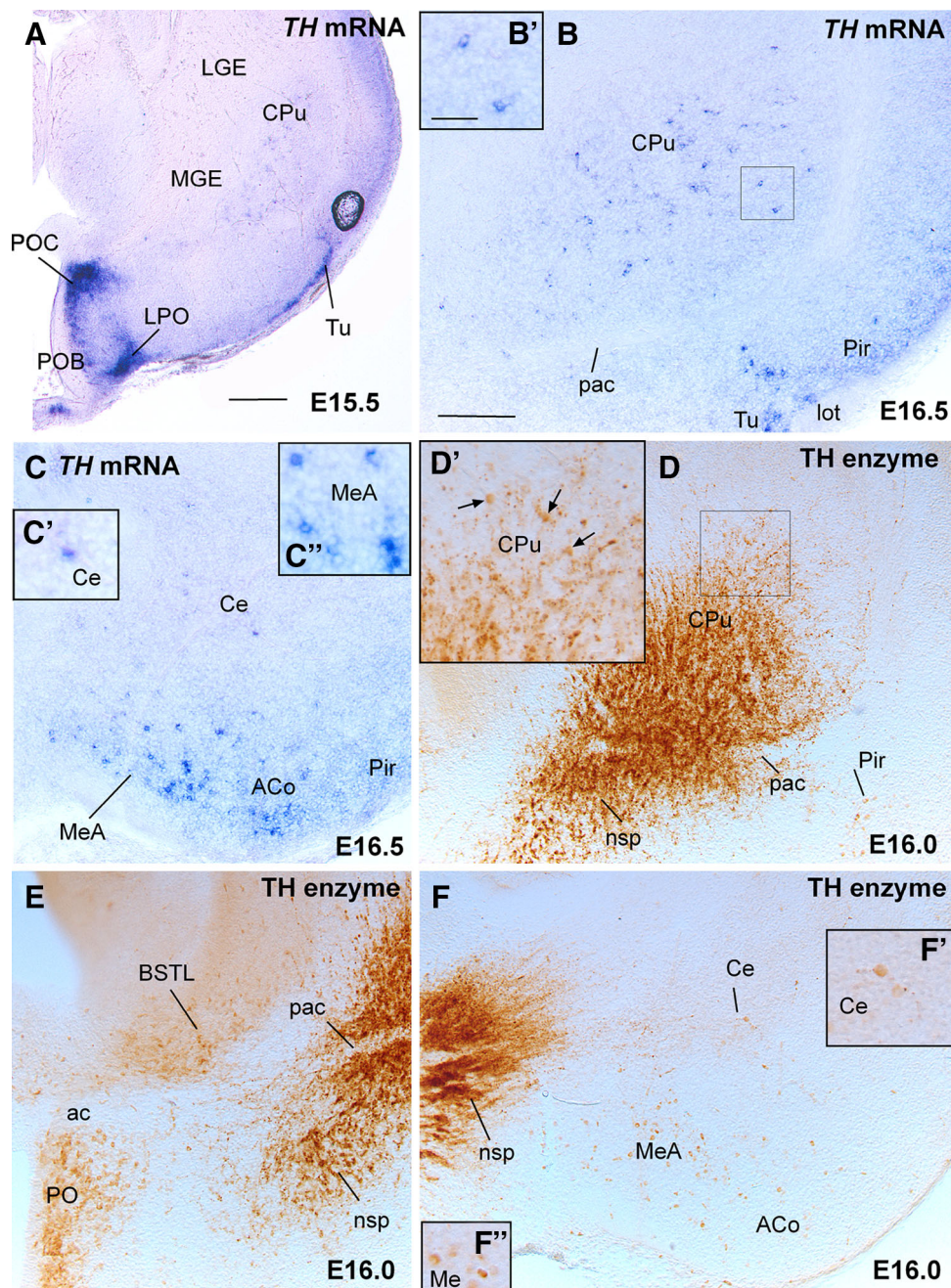
Fig. 3 Images of frontal sections through the telencephalon of E15.5–E16.5 mice, hybridized (**a–c"**) or immunostained (**d–f"**) for TH. Details of the square areas in each image are shown in **b', c', c"**, **d', f'** and **f"**. Note the strong expression of TH mRNA (**a**) in the commissural preoptic area (POC), from where it appears to extend into the lateral preoptic area (LPO) and the periventricular part of the basal/ventral preoptic area (POB). The preoptic area also contains numerous cells showing moderate protein expression, as seen in **e**. A few cells, expressing both mRNA and protein, also appear to extend into the mantle of the ganglionic eminences, where subpopulations of cells are observed in the caudate-putamen (CPu; **b, d**, and details in **b', d'**), the lateral BST (BSTL; **e**) and the central amygdala (Ce; **c, f**, and details in **c', f'**). In the developing striatum, note the relationship between TH-ir cells and the TH-ir incoming axons of the nigrostriatal (or tegmentostriatal) projection (**d'**). The medial amygdala (Me) also contains a subpopulation of cells expressing TH mRNA and protein (**c, f**, and details in **c", f"**). In addition, other subpopulations of TH-expressing cells (both mRNA and protein) are observed in the piriform cortex (Pir), the olfactory tubercle (Tu), and the cortical amygdalar area (ACo). For other abbreviations, see list. *Scales*: *bar* in **a** 300 μm; *bar* in **b** 200 μm (it applies to **b, c, d, e, f**); *bar* in **b'** 50 μm (it applies to **b', c', c", d', f', f"**)

the caudate-putamen. In this situation, in situ hybridization was essential to assure the presence of TH-expressing cells.

Using the latter technique, we observed TH-expressing cells in several parts of the striatum, including subpopulations in the caudate-putamen (specially abundant at its
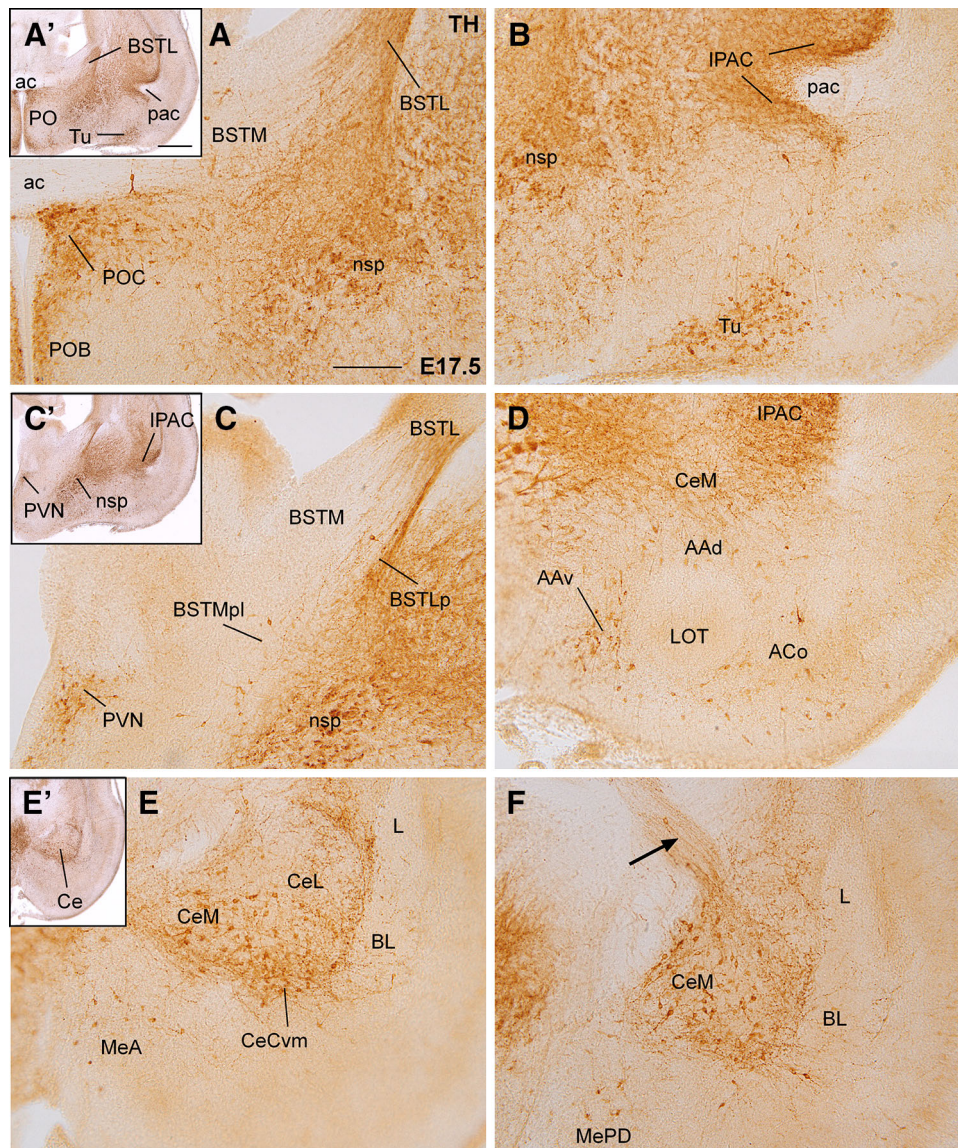
Fig. 4 Images of frontal sections through the telencephalon of an E17.5 mouse, from intermediate (a, b) to caudal levels (f), immunostained for TH (as indicative for protein expression). **a** and **b** are details of **a'**, **c** is a detail of **c'**, and **e** is a detail of **e'**. Note the generally strong TH-ir innervation in the striatum and the central extended amygdala (BSTL, the interstitial nucleus of the posterior limb of the anterior commissure or IPAC, and the central amygdala). Subpopulations of TH-ir perikarya are clearly visible in the preoptic region, olfactory tubercle, part of the central extended amygdala (in particular, the medial and ventromedial capsular part of the central amygdala; a few cells are also seen in the BSTL and anterodorsal amygdala or AAd) and part of the medial extended amygdala (including a few cells in the BSTM, the anteroventral amygdala or AAv, and the anterior and posterodorsal medial amygdala). However, in the areas with the strongest TH-ir innervation, it is difficult to discern the presence of TH-ir perikarya. The *arrow* in **f** points to the tegmento-amygdalar TH-ir projection (note the TH-ir cells intermingled with the incoming axons). *Scales*: *bar* in **a** = 200 μm (it applies to **a–f**); *bar* in **a'** 500 μm (it applies to **a'**, **c'**, **e'**)

rostrointermediate and ventral parts, but scarce to very scarce dorsally and caudally; Fig. 5a–d), nucleus accumbens (mainly in its ventrolateral part; Fig. 5A) and olfactory tubercle (abundant in its lateral part, adjacent to the boundary with the piriform cortex; Fig. 5a–c). TH-ir cells were also clearly observed in the olfactory tubercle using immunohistochemistry (Fig. 4b).

In the extended amygdala, TH-expressing cells were observed in the medial and lateral subdivisions of the bed nucleus of the stria terminalis (BSTM, BSTL; Figs. 4a, c, 5b, d), in the sublenticular extended amygdala (mainly its central part or EAce), as well as in the anterior, medial and central amygdala (Figs. 4d–f, 5e–f). In the BSTM, a group of scattered TH-expressing cells was located in its anterior

Fig. 5 Images of frontal sections through the telencephalon of E17.5–E18.5 mice, from rostral (**a**) to caudal levels (**f**), hybridized for TH. **c** is a detail of **b**, and **f'** is a detail of **f**. Note the presence of TH mRNA-expressing cells in the accumbens, caudate-putamen, and different parts of the central and medial extended amygdala. See text for more details. *Scales*: *bar* in **a** 300 μm (it applies to **a**, **b**, **d**–**f**); *bar* in **c** 200 μm (it applies to **c**, **f'**)
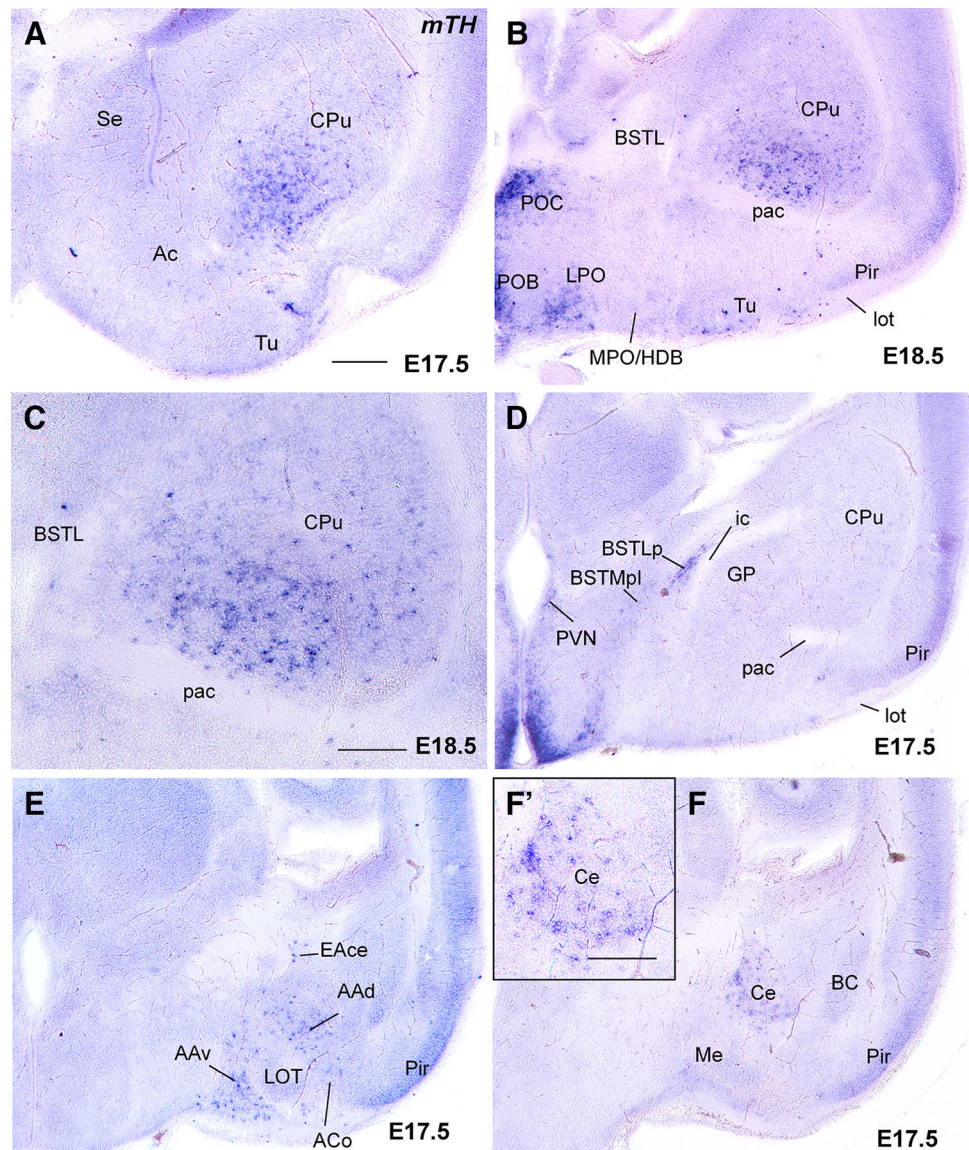
subnucleus (Figs. 4a, 5b) and appeared to correspond to those described above the anterior commissure in different mammals (Specht et al. 1981b; Foster 1994; Ugrumov 1994). These cells were clearly visible with both immunohistochemistry and in situ hybridization. Both techniques also revealed another group of scattered TH-expressing cells in the posterolateral subnucleus of the BSTM (Figs. 4c, 5d). In the BSTL, TH-expressing cells were difficult to see with immunohistochemistry because they were partially hidden by the abundant TH-ir fibers and varicosities that innervate this nucleus (Fig. 4c). With in situ hybridization these cells were clearly distinguished, being located in the posterior subnucleus of BSTL (Fig. 5d). In the anterior amygdala numerous TH-expressing cells were observed in both ventral and dorsal subdivisions with both immunohistochemistry and

in situ hybridization (Figs. 4d, 5e). A number of TH-expressing cells were observed in the anterior and posterodorsal subdivisions of the medial amygdala with both immunohistochemistry (Fig. 4e, f) and in situ hybridization (not shown). Moreover, an important subpopulation of TH-expressing cells was observed in the central amygdala with both immunohistochemistry and in situ hybridization (Figs. 4e–f', 5f, f'). These cells appeared to be specifically located in the medial or ventromedial aspect of the central amygdala. In addition to these cells in the extended amygdala, TH-expressing cells were also observed in the pallial amygdala, which were mostly located in the anterior cortical amygdalar area (Figs. 4d, 5e). Overall, the distribution of TH-expressing cells observed in the mouse striatum and amygdala with in situ hybridization at prenatal stages was identical to that in neonates.

Distribution of cells expressing TH in the striatum and extended amygdala of adult mice

To determine whether the TH-expressing cells observed in the striatum and extended amygdala at prenatal stages and neonates were still present in the adult brain, we carried out in situ hybridization and immunohistochemistry in adult mice. In both males and females, TH mRNA-expressing cells were observed in all the previously recognized catecholaminergic cell groups of the forebrain and midbrain, such as the A8–A10 groups of the diencephalic-midbrain tegmentum (retrorubral area, substantia nigra, and ventral tegmental area), the A11 group in the periaqueductal gray of the diencephalon and midbrain, the A12 hypothalamic group (arcuate nucleus), the A13 group (zona incerta, in the prethalamus), the A14 hypothalamic groups (in periventricular hypothalamus, from alar to basal regions), the A15 preoptic groups, and the A16 group of the olfactory bulb (Hökfelt et al. 1984; Björklund and Dunnett 2007). For consistency with the embryonic subdivisions of the telencephalon and hypothalamus defined by distinct combinatorial patterns of developmental regulatory genes and fate mapping (Puelles and Rubenstein 2003; García-López et al. 2008; Medina and Abellán 2012; Puelles et al. 2012), we restricted the use of A15 group to the cells of the preoptic region (which is now considered a part of the telencephalic subpallium), thus excluding the cells of the supraoptic nucleus and other lateral hypothalamic nuclei/areas (Marín et al. 2005; Björklund and Dunnett 2007). In addition to the classical groups, we observed subpopulations of TH mRNA-expressing cells in the cortex (not shown), claustrum (not shown), striatum (Fig. 6), extended amygdala (Figs. 7, 8) and the diagonal band/basal magnocellular corticopetal nuclei of the basal telencephalon (Fig. 8).

In the striatum, we observed a group of scattered, small cells showing weak or moderate TH mRNA expression distributed throughout the caudate-putamen and in the accumbens (Fig. 6). Notably, these cells were visibly more abundant and densely grouped in the nucleus accumbens, particularly the core, than in the accumbens shell or the adjacent and dorsal parts of the caudate-putamen (Fig. 6b, c). Cells were also more abundant in the caudoventral aspects of the caudoputamen and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) compared to surrounding areas (Fig. 6c). All of these striatal nuclei showed a very dense innervation by TH immunoreactive fibers (Fig. 6a), which impeded clear visualization of possible TH immunoreactive perikarya.

The extended amygdala also contained minor subpopulations of cells showing weak TH mRNA expression (Figs. 7, 8). A moderate number of TH mRNA-expressing cells was observed in the posterolateral subdivisions of the BSTM (Fig. 7b) and other parts of the medial extended amygdala, including the medial sublenticular extended amygdala, the anterior amygdala (ventral part; not shown), and the medial amygdala (anterior and posterodorsal subdivisions) (Fig. 8e, e'). None of these nuclei appeared to contain TH immunoreactive perikarya (Fig. 7a), suggesting that in adult mice catecholaminergic cells in these locations contained only the mRNA but not the protein. On the other hand, few cells expressing TH mRNA were also observed in the BSTL (mostly located in its posterior part or BSTLp), the central sublenticular extended amygdala and the central amygdala (its medial aspect or CeM) (Figs. 7b, 8d). The moderate to dense innervation of the central extended amygdala by TH immunoreactive fibers make it difficult to clearly identify the presence of TH immunoreactive perikarya in the BSTL or CeM (Figs. 7a, 8a–c), although observation at high magnification suggested the presence of such cells (Fig. 8c'). Nevertheless, at least in the forebrain the number of TH immunoreactive perikarya was generally (if not always) lower than that of cells expressing the TH mRNA (for example, compare Fig. 7a, b for the BST complex and the paraventricular hypothalamic nucleus).

Embryonic origin of TH-expressing cells of the basal telencephalon

To know the embryonic origin of the TH-expressing cells of the basal telencephalon, we carried out in vitro cell migration assays in telencephalic slices from E13.5 to E16.5 mice, using the fluorescent cell tracker CMFDA combined with immunofluorescence for TH. For our cell migration experiments, we placed the cell tracker in one of the following three progenitor domains: (a) The dorsal lateral ganglionic eminence (LGEd), because this is a known source of dopaminergic neurons for the olfactory bulb and olfactory tubercle (Yun et al. 2003), and has been suggested to produce dopaminergic cells for the striatum (reviewed by Medina 2008; Abellán and Medina 2009). (b) The POC, because this area is known to contain catecholaminergic neurons since early development (Specht et al. 1981a; Foster 1994), and produces other types of neurons that tangentially migrate to the basal ganglia and amygdala (García-López et al. 2008; Nóbrega-Pereira et al. 2010; Carney et al. 2010; Bupesh et al. 2011a). (c) The supraopto-paraventricular domain of the alar hypothalamus (SPV), because this domain contains catecholaminergic neurons since early development (Specht et al. 1981a; Foster 1994), and is known to produce other cell types for the medial extended amygdala (García-Moreno et al. 2010; Bupesh et al. 2011a). In addition, the distribution of the TH-expressing cells in the mouse forebrain during early and intermediate embryonic stages (present results explained in previous sections) also suggests that LGEd,
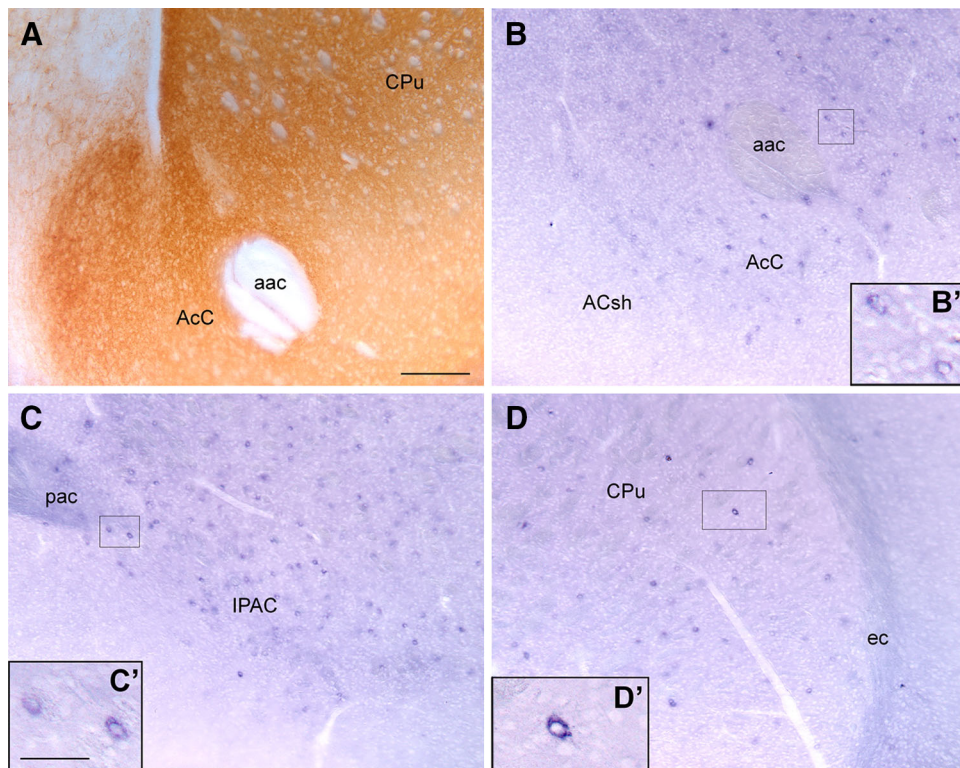
Fig. 6 Images of frontal sections through the telencephalon of two adult female mice immunostained (**a**, case MA18) or hybridized (**b–d**'; case MA09) for TH. TH mRNA-expressing cells are observed in the nucleus accumbens (Ac; **b**, and detail in **b**'), caudate-putamen (CPu; **d**, and detail in **d**'), and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; **c**, and detail in **c**'). Note that the density of TH-expressing cells appears to be higher in the accumbens core (AcC) and the IPAC than in the accumbens shell (AcSh) and the dorsal parts of CPu. Note also the high density of innervation by TH fibers in the Ac (**a**; a similar situation occurs in IPAC and CPu), which impedes the identification of TH immunoreactive perikarya. *Scales*: *bar* in **a** 200 μm (it applies to **a**, **b**, **c**, **d**); *bar* in **b**' 50 μm (it applies to **b**', **c**', **d**')

POC and SPV are likely sources of TH-expressing cells of the telencephalon.

When the cell tracker was placed in the LGE, involving its dorsal subdomain (LGEd), numerous CMFDA-labeled cells were seen in the caudate-putamen and reached the olfactory tubercle (Fig. 9), as previously described (Bupesh et al. 2011b). When the cell tracker involved caudal levels of dLGE, numerous labeled cells were observed in the central amygdala (as reported by Bupesh et al. 2011b). In all cases, a number of cells were also observed to reach the pallium, presumably by tangential migration. Some of these cells were seen in the piriform cortex, as described by De Carlos et al. (1996). Following immunolabeling for TH, however, we could not see any double-labeled cell inside the caudate-putamen nor the central amygdala (Fig. 9). At the ages examined (E14.5 until E16.0, plus 24 h in culture), the central amygdala only contained few TH-immunolabeled cells, which clearly constituted a handicap for searching for double-labeling cases.

When the cell tracker was placed in the POC, in addition to cells in the preoptic area, we observed a fair amount of cells invading the striatum and parts of the extended amygdala, including the lateral BST and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) (Figs. 10, 11), as previously described (Bupesh et al. 2011a). Following immunolabeling for TH, many of the POC-derived cells were double-labeled, including some of those invading the ventral aspect of the developing caudate-putamen and the IPAC (Figs. 10e–f", 11c–f").

When the cell tracker was placed in the SPV, we observed some cells invading by tangentially migration parts of the medial extended amygdala, including the medial BST and the medial amygdala (Fig. 12), as previously reported (García-Moreno et al. 2010; Bupesh et al. 2011a). Following immunolabeling for TH, we could detect only extremely few cases of double-labeled cells (Fig. 12c, c').

Discussion

The major findings of this study are: (1) the observation of subpopulations of catecholaminergic cells (expressing TH mRNA and protein) in the striatum, diagonal
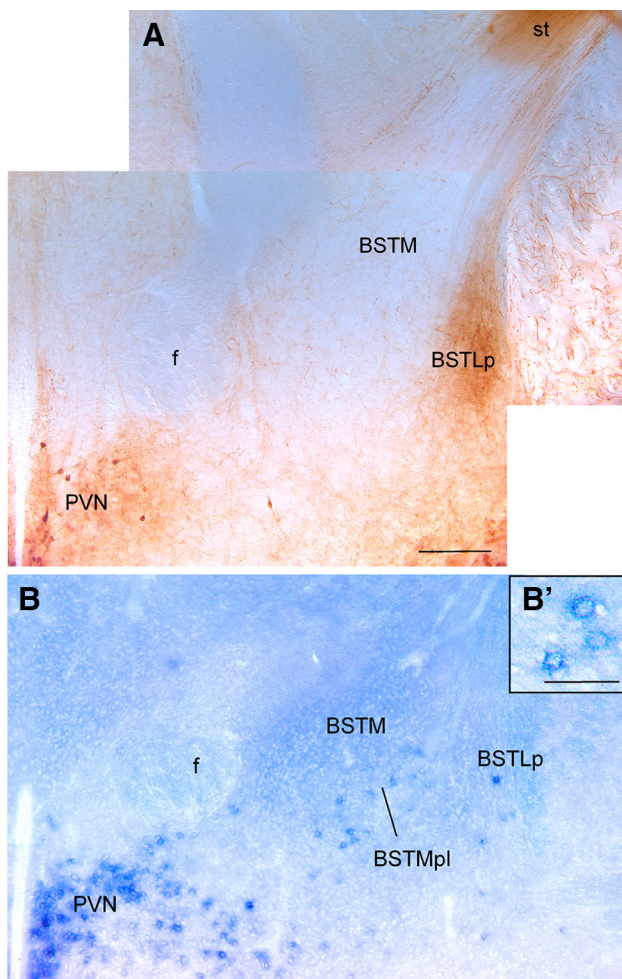
Fig. 7 Photomontages of images of frontal sections through the telencephalon of two adult mice, at the level of the lateral and medial nuclei of the stria terminalis (BSTL, BSTM), immunostained (**a**; case MA18, female) or hybridized (**b**; case MA10, male) for TH. Subpopulations of cells expressing TH mRNA are observed in the posterior BSTL (BSTLp) and the posterolateral BSTM (BSTMpl) (**b**, and detail in **b'**). No TH immunoreactive perikarya are visible in the BSTM, but a few may be present in BSTL (**a**). However, the BSTLp, as other parts of the central extended amygdala, shows moderate to dense innervation by TH fibers, which makes it difficult the clear visualization of TH immunoreactive perikarya. Note that in the paraventricular hypothalamic nucleus (PVN), while many cells express TH mRNA, only few show strong TH protein expression, and most show either weak or no immunoreactivity. For other abbreviations see list. See text for more details. *Scales: bar in* **a** 200 μm (it applies to **a**, **b**); *bar in* **b'** 50 μm

band/corticopetal basal telencephalic nuclei, central extended amygdala and medial extended amygdala of mouse during development, which are persistent in the adult as scattered cells generally showing weak to moderate mRNA expression (except those in the corticopetal groups, which show moderate to strong mRNA expression), but with variations regarding the protein expression (see summary in Table 1); and (2) the experimental demonstration that these cells include at least two distinct subpopulations that originate either in the commissural preoptic subdivision of the subpallium or the supraopto-paraventricular domain of the alar hypothalamus (summary in Table 2). The TH-expressing cells in the striatum (caudate-putamen and nucleus accumbens) and diagonal band nuclei were previously observed in developing and adult rodents and primates (Dubach et al. 1987; Dubach 1994; Prensa et al. 2000; Marín et al. 2005; Cossette et al. 2005a, b; Björklund and Dunnett 2007; Ugrumov 2009). However, data on catecholaminergic cells in the amygdala were quite scarce. In the central extended amygdala, there was only a report on transient cells in developing rats (Verney et al. 1988). In the medial extended amygdala, there is a recent description of numerous cells in this part of the amygdala of prairie voles, but the number of cells appears to be sex- and species-dependent, and they are not observed in rats (Northcutt et al. 2007). Therefore, our study provides the first description of catecholaminergic cells in the adult central extended amygdala in mammals, and the first description of such cells in the medial extended amygdala of developing and adult mice. The discrepancy in the finding of such cells in the amygdala between mouse and rat may be due to species differences, to the different technique employed (in situ hybridization for mRNA detection versus immunohistochemistry for protein detection; we used both techniques, but previous studies in the amygdala of rats only used immunohistochemistry), or to other problems related to differences in the age, sex, social interactions, stress, or diet of the animals, all of which have been proposed to influence the expression level of catecholamines in several brain centers in different vertebrates (Sabban and Kvetnanský 2001; Northcutt et al. 2007; Braun et al. 2012; Lynch et al. 2012; Vucetic et al. 2012). In fact, there is increasing evidence for a complex regulation of the TH expression at all levels, transcriptional, post-transcriptional, translational and post-translational, which may account for differences in expression of mRNA versus protein, or to other expression differences related to sex, stress, etc. (Kumer and Vrana 1996; Xu et al. 2007).

Regarding the differences based on the technique, it is important to mention that in the medial extended amygdala of adult mice, we detected cells expressing TH mRNA (we did controls using the sense to check the specificity of the signal), but not TH protein. Our data on TH mRNA in the amygdala of adult mice agrees with that shown at the Allen Brain Atlas web site. This may be one of the reasons why such cells of the mouse medial extended amygdala expressing only TH mRNA but not the protein were not observed in previous studies in rats using immunolabeling (Northcutt et al. 2007). On the other hand, in brain nuclei densely innervated by TH fibers, such as the striatum and the central extended amygdala, in situ hybridization is
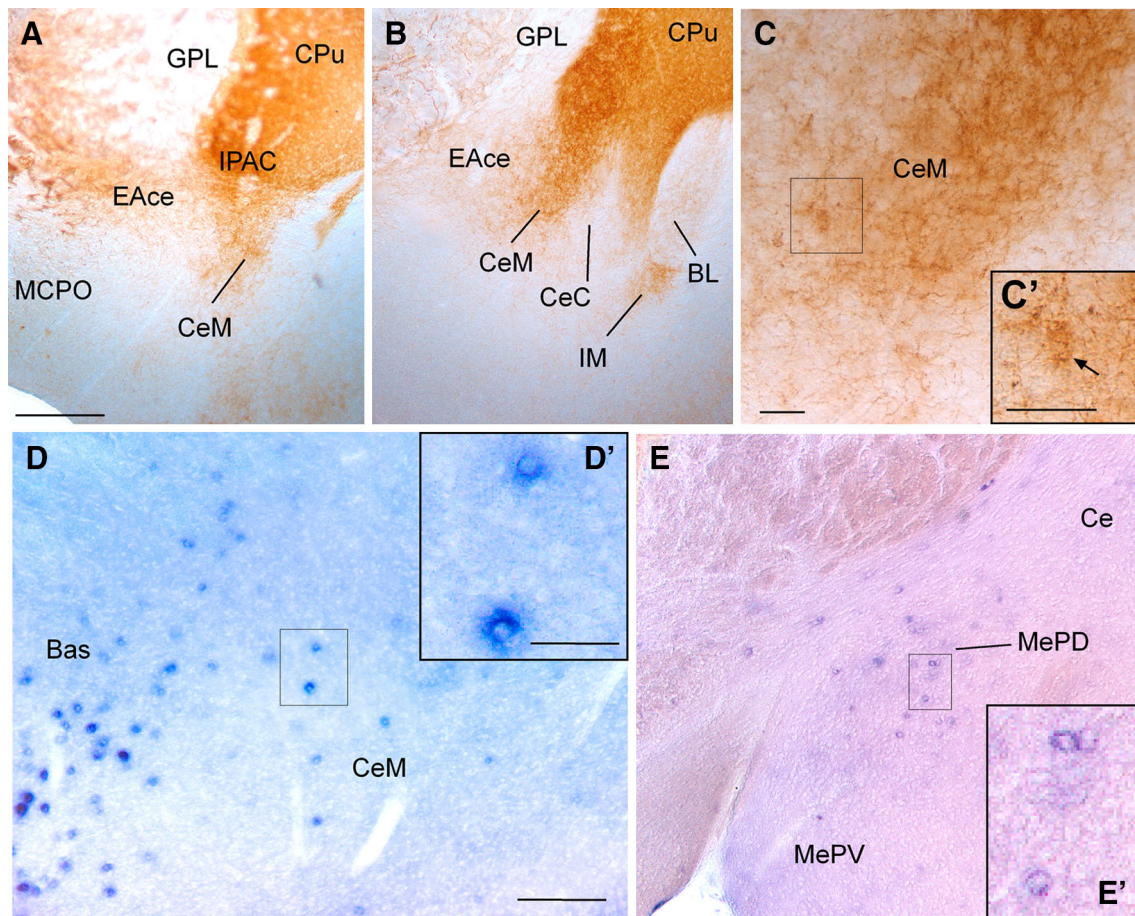
Fig. 8 Images of frontal sections through the telencephalon of three adult mice immunostained (**a–c**'; case MA10, female) or hybridized (**d, d**': case MA10, male; **e, e**': case MA06, female) for TH. In addition to the TH-expressing cells in the corticopetal groups of the basal telencephalon (such as the magnocellular preoptic complex or MCPO, which includes the nucleus basalis or Bas), a number of cells showing weak TH mRNA expression are observed in medial part of the central amygdala (CeM) and anterior and posterodorsal parts of the medial amygdala (MePD) (**d, e**, and details in **d**', **e**'). No TH immunoreactive perikarya are observed in the medial amygdala (not shown), but a few may be present in the central amygdala, intermingled with the moderate to dense TH immunoreactive innervation (**c**, and detail in **c**'). *Scales: bar* in **a** 400 µm (it applies to **a, b**); *bar* in **c** 50 µm; *bar* in **c**' 50 µm; *bar* in **d** 200 µm (it applies to **d, e**); *bar* in **d**' 50 µm (it applies to **d**', **e**')

usually better than immunodetection for determining the presence of positive perikarya.

In addition, as noted above the expression level of catecholamines is known to vary depending on several factors (Sabban and Kvetnanský 2001; Northcutt et al. 2007; Braun et al. 2012; Lynch et al. 2012; Vucetic et al. 2012), which in the case of TH may affect differently mRNA versus protein expression (Kumer and Vrana 1996; Xu et al. 2007). This may be the reason (or one of the reasons) for the presence of cells in the medial extended amygdala having the mRNA but not the protein. At basal physiological conditions in extended amygdalar cells, the protein may not be produced or may be present at very low, undetectable levels, but at certain conditions (different depending on the type of function in which the cell is involved) the protein may be produced (or may be produced at a higher rate) turning the cell detectable with standard immunochemical methods.

On the other hand, the expression of many genes, including the TH gene and other genes related to monoamine neurotransmission and drug responses, shows important interstrain variations in mice, affecting particularly their expression in cells of higher order centers of the forebrain, such as the striatum (Morris et al. 2010). These gene expression variations may contribute significantly to differences in behavior and responses to neuroactive drugs in different laboratory mouse strains (Morris et al. 2010). If this is so within one single species, it is not surprising that such variations may also be present between different species, even between close-related species such as mouse and rat.

Fig. 9 Digital images from two representative organotypic cultures of telencephalic frontal slices of mouse embryo (MB133B and MB138A), in which the fluorescent cell tracker CMFDA (*green*) was involving the dorsal lateral ganglionic eminence (LGEd). The slices were resectioned and immunolabeled to detect TH (shown in *red*). Note the LGE-derived cells (*green*) and the TH-ir cells (*red*) in the caudate-putamen (CPu). In our experiments, we did not see any double-labeled cell. *Scales*: bar in **a** 500 μm (it applies to **a**, **d**); bar in **b** 200 μm (it applies to **b**, **e**); bar in **c** 100 μm (it applies to **c**, **c'**)
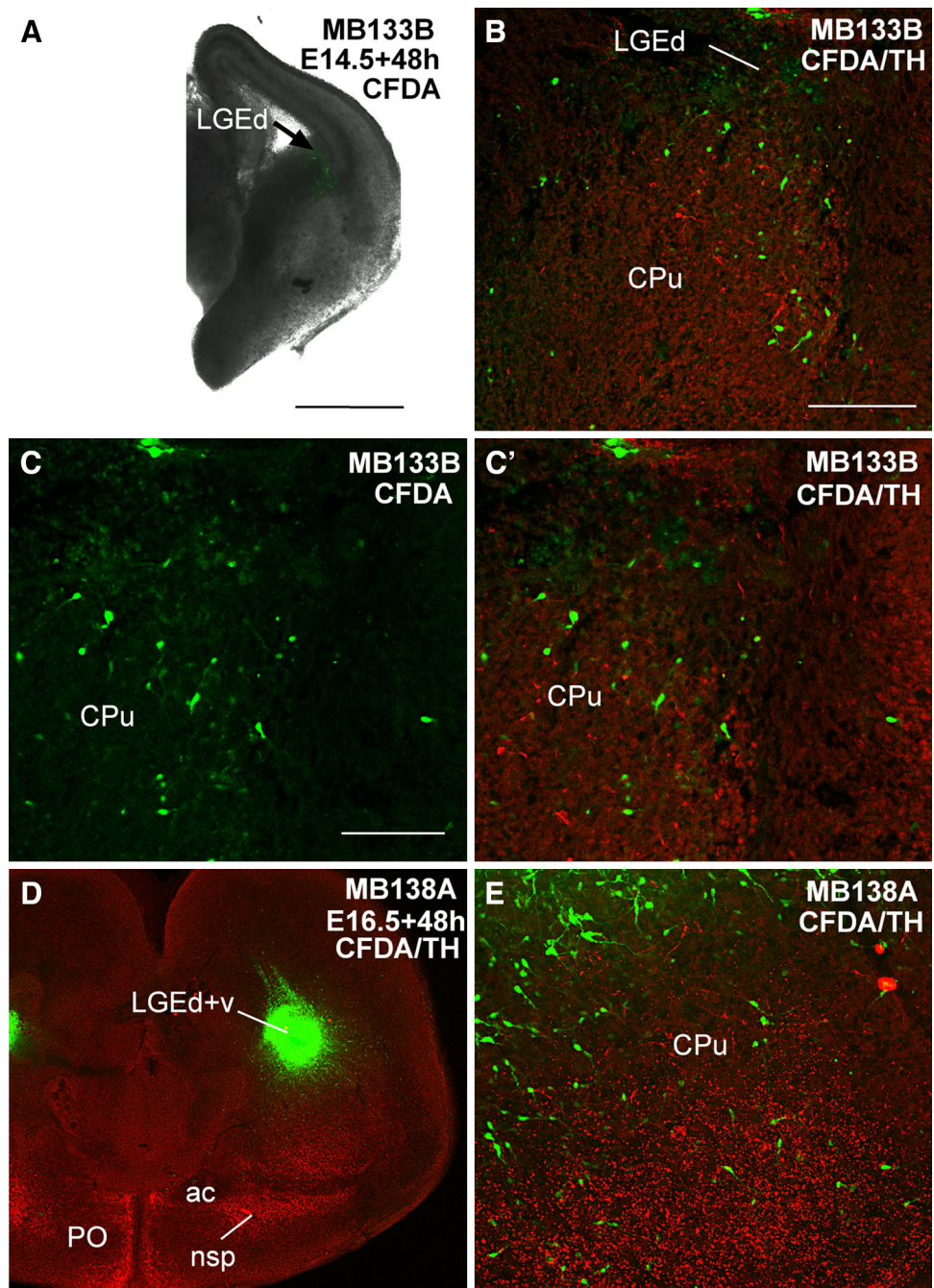
Two or more embryonic origins for the TH-expressing cells of the striatum and amygdala?

Our fate mapping study shows that part of the catecholaminergic cells of the basal telencephalon originate in the commissural preoptic subdivision (POC) of the subpallium, the same subdivision that appears to produce the A15 preoptic cell group (summary in Table 2). The observation of a continuum of TH-expressing cells extending from the POC into the ganglionic eminences also agrees with this (Marín et al. 2005; present results). Our fate mapping data showed that many TH-expressing cells derived from POC invade the striatum (most of which are located ventrally in the striatum at this early age) and at least the medial part of the central extended amygdala (such as the IPAC). The POC is also known to produce nitrergic and other cell types for the medial amygdala (Carney et al. 2010; Bupesh et al. 2011a), and it is possible that some of the POC-derived cells of the medial amygdala may be TH-ir (although we did not obtain any evidence for that). During development,
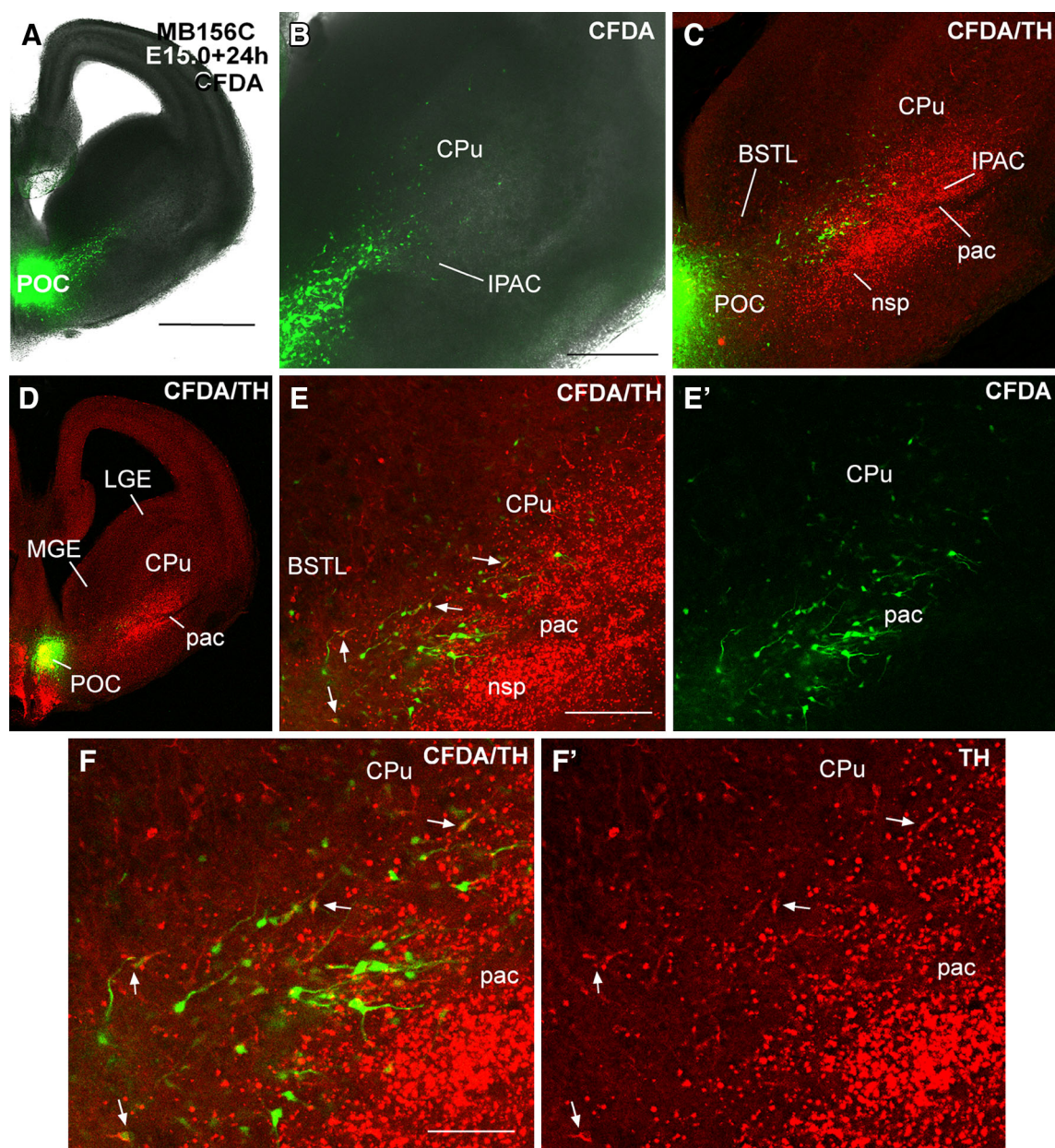
Fig. 10 Digital images from one representative organotypic culture of a telencephalic frontal slice of mouse embryo (MB156C), in which the fluorescent cell tracker CMFDA (*green*) was involving the commissural preoptic area (POC). The slice was resectioned and immunolabeled to detect TH (shown in *red*). Note the POC-derived cells (*green*) and the TH-ir cells (*red*) in the caudate-putamen (CPu), the IPAC, and BSTL. Many of the cells in the CPu and medial IPAC region were double-labeled (*arrows* in **e**, **f**, **f'**; **f**, **f'** are details of **e**). *Scales*: *bar* in **a** 500 μm (it applies to **a**, **d**); *bar* in **b** 200 μm (it applies to **b**, **c**); *bar* in **e** 100 μm (it applies to **e**, **e'**); *bar* in **f** 50 μm (it applies to **f**, **f'**)

the preoptic ventricular zone (partially included as part of AEP in some previous studies) expresses the signaling protein Shh and the transcription factors Nkx2.1, Dbx1 and Lhx7/8, while the subventricular zone and mantle express Shh, Lhx7/8 and Islet1 (Flames et al. 2007; García-López et al. 2008; Elshatory and Gan 2008; Abellán and Medina 2009). Lhx7/8 and Islet1 have been involved in the differentiation of cholinergic neurons produced in POC,

which become corticopetal neurons of the preoptic magnocellular complex/basal nucleus (Meynert) or cholinergic interneurons of the striatum (Zhao et al. 2003; Manabe et al. 2007; Elshatory and Gan, 2008). On the other hand, a previous study has shown that Shh is necessary and sufficient to induce dopaminergic neurons in the anterior neural plate, and cooperates with Fgf8 for the production of such cells at specific locations of the anterior neural tube (Ye
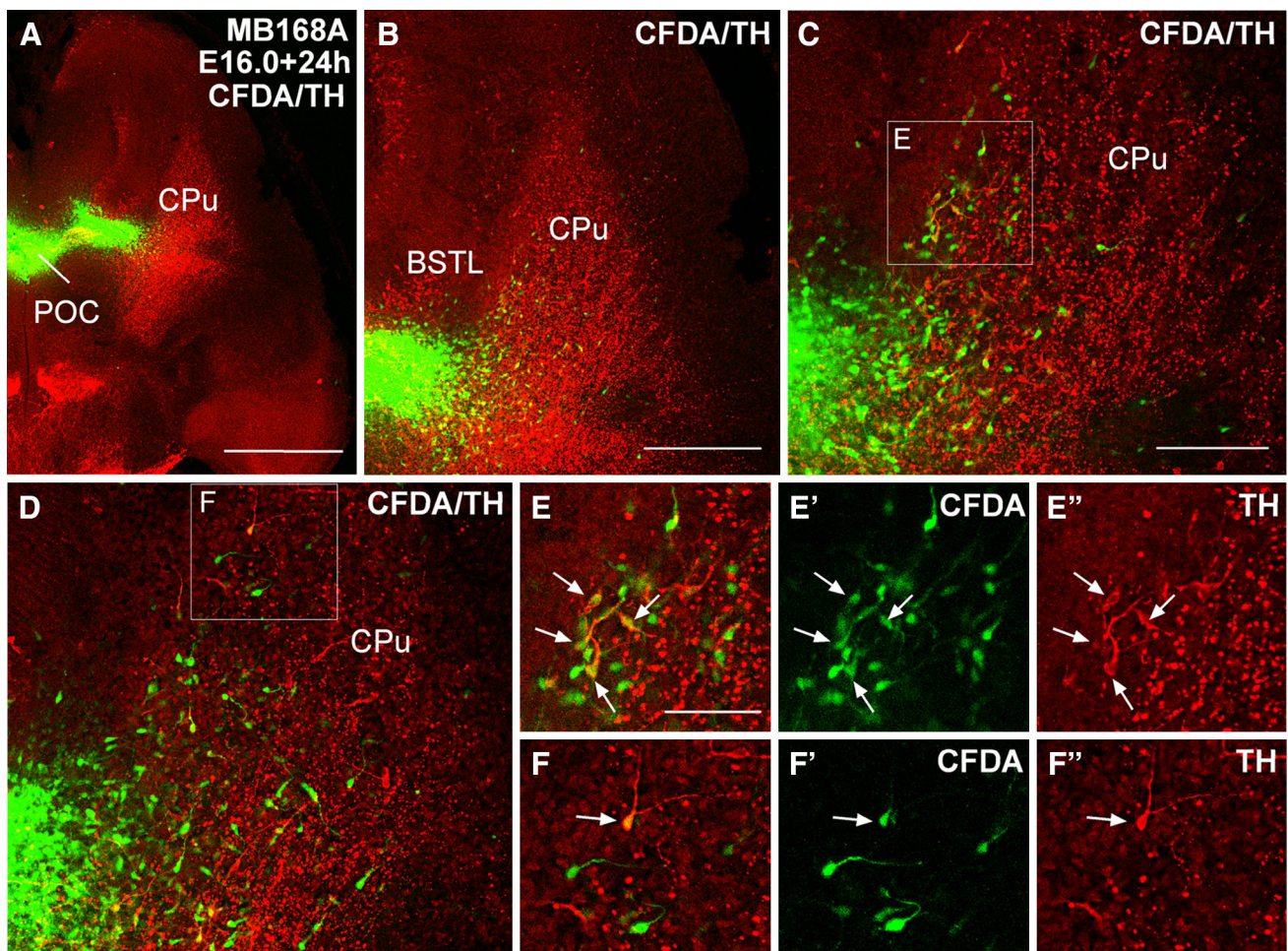
Fig. 11 Digital images from one representative organotypic culture of a telencephalic horizontal slice of mouse embryo (MB168A), in which the fluorescent cell tracker CMFDA (*green*) was involving the commissural preoptic area (POC). The slice was resectioned and immunolabeled to detect TH (shown in *red*). Note the POC-derived cells (*green*) and the TH-ir cells (*red*) in the caudate-putamen (CPu) and BSTL. Many of the cells in the CPu were double-labeled (*arrows*; see details in **e–e"** and **f–f"**, from the areas squared in **c** or **d**, respectively). *Scales*: *bar* in **a** 500 μm; *bar* in **b** 200 μm; *bar* in **c** 100 μm (it applies to **c**, **d**); *bar* in **e** 50 μm (it applies to **e–e"** and **f–f"**)

et al. 1998). During early development, the preoptic area is under the influence of both types of signals, Fgf8 (produced at the anterior neural ridge; Ye et al. 1998) and Shh, and may cooperate for the production of dopaminergic neurons from this progenitor domain.

On the other hand, our fate mapping data also indicate at least part of the TH cells of the medial extended amygdala originate in the supraopto-paraventricular domain (SPV) of the alar hypothalamus. This agrees with our observation at early stages of a continuum of TH-expressing cells apparently extending from SPV into the medial extended amygdala region. During development, the SPV of different vertebrates also appears to produce TH cells for the supraoptic and paraventricular hypothalamic nuclei (Foster 1994; Smeets and Reiner 1994; Marín et al. 2005; present results). In addition, it appears that SPV produces TH cells (present results) as well as other cell subpopulations,

including calbindin neurons, for the medial extended amygdala (Soma et al. 2009; García-Moreno et al. 2010; Bupesh et al. 2011a). While calbindin neurons invade both BSTM and medial amygdala (Bupesh et al. 2011a), herein we present evidence of a few TH cells invading the medial amygdala. The development of these hypothalamic-derived TH and calbindin neurons appears to depend on the transcription factor Orthopedia (Otp) (Acampora et al. 1999; Ryu et al. 2007), which is expressed in the SPV domain and in SPV-derived amygdalar neurons in different vertebrates (Bardet et al. 2008; García-Moreno et al. 2010).

In addition to the two sources mentioned above, previous studies showed that the dorsal subdivision of the lateral ganglionic eminence (LGEd, characterized by giving rise to cells that keep postmitotic expression of Pax6) produces the catecholaminergic (dopaminergic) interneurons of the olfactory bulb, as well as those at the surface of the LGEd

Fig. 12 Digital images from one representative organotypic culture of a telencephalic frontal slice of mouse embryo (MB126B), in which the fluorescent cell tracker CMFDA (*green*) was involving the supraopto-paraventricular domain of the alar hypothalamus (SPV). The slice was resectioned and immunolabeled to detect TH (shown in *red*). Note the SPV-derived cells (*green*) and the TH-ir cells (*red*) in the BSTM and medial amygdala (MeA). The *arrow* in **c**, **c'** points to a double-labeled cell in the MeA. *Scales*: bar in **a** 500 µm; bar in **b** 100 µm; bar in **c** 50 µm (it applies to **c**, **c'**)
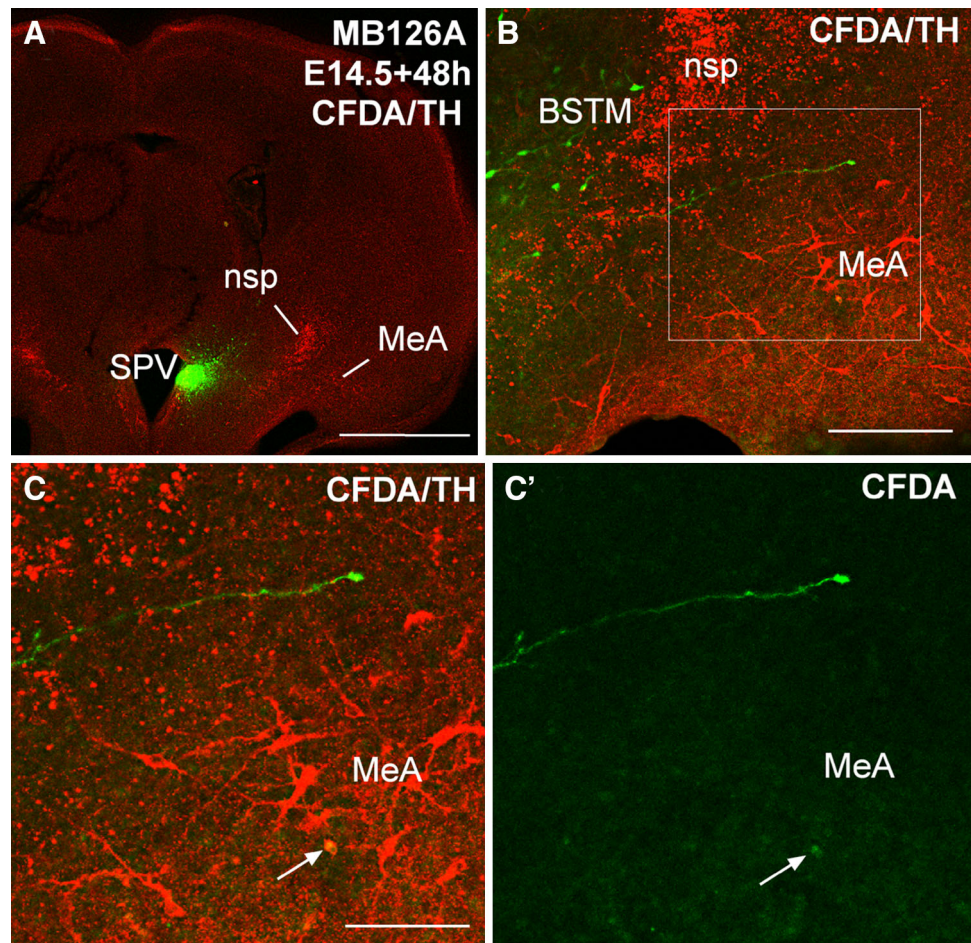
Table 1 Expression of TH mRNA or protein in cells of selected subpallial centers of the telencephalon during development and in adult mice

	E16.5 TH mRNA	E16.5 TH protein	E18.5 TH mRNA	E18.5 TH protein	Adult TH mRNA	Adult TH protein
Ac	–	–	+	+?	+	?
CPu	+	+	++	+?	+	?
IPAC	+	+	+	+?	+	?
Ce	+	+	++	++	+	+?
Me	+/++	+/++	++	+/++	+	–
AA	+/++	+/++	++	+/++	+	–
BSTL	+	+	+	+	+	+?
BSTM	+	+	+	+	+	–
PO	+++	++	+++	++	++	+
Tu	++	+	++	++	+	+
MCPO	+	+	++	+	++/+++	+

–, no expression; +, weak expression; ++, moderate expression; +++, strong expression; +?, possible immunoreactive cells, but unclear due to the presence of immunoreactive fibers; ?, unable to determine due to the dense and strongly immunoreactive innervation

radial domain, apparently including the olfactory tubercle (Yun et al. 2003). Based on this evidence, we proposed that LGEd was likely a source for other catecholaminergic cells of the telencephalon, such as those of the caudate-putamen (reviewed in Medina 2008; Abellán and Medina 2009). In our study, the distribution of TH-expressing cells in the olfactory tubercle highly resembles that of the transcription factor Pax6 (Bupesh et al. 2011b), which agrees with the origin of these cells in LGEd. In fact, Pax6 has been involved in the prenatal development of TH/dopaminergic

Table 2 Summary of fate mapping results following cell tracker (CMFDA) application in mouse LGEd, POC or SPV at early or intermediate embryonic ages (E13.5–E16.5)

	CMFDA in LGEd		CMFDA in POC		CMFDA in SPV	
	Migrated CMFDA cells	Doubled labeled CMFDA/TH	Migrated CMFDA cells	Doubled labeled CMFDA/TH	Migrated CMFDA cells	Doubled labeled CMFDA/TH
CPu	Yes	No	Yes	Yes	No	No
Tu	Yes	No	No	No	No	No
IPAC	Yes	No	Yes	Yes	No	No
Ce	Yes	No	No*	No*	No	No
Me	No	No	Yes	No	Yes	Yes
BSTL	No	No	Yes	Yes	No	No
BSTM	No	No	Yes	No	Yes	No

* Ce not visible in the experimental slices for POC of present study

neurons, which are produced in LGEd and migrate radially to the olfactory tubercle, and tangentially to the olfactory bulb (Yun et al. 2003). Pax6 is also involved in the post-natal production of TH/dopaminergic neurons of the olfactory bulb, which originate the striatal subventricular zone (Kohwi et al. 2005). However, in our migration experiments, we have been unable to observe TH-ir cells in the striatum or central amygdala derived from LGEd. This may be due to a technical problem since at the embryonic ages when we prepared the organotypic cultures, TH-ir cells are still scarce in the dorsal parts of the striatum, and in the central amygdala. Therefore, although our results show that the POC produces many TH immunoreactive cells for the striatum (but note that, at this age, these cells are mostly located ventrally in the striatum) and medial parts of the central extended amygdala (such as the IPAC), we cannot discard another contribution from LGEd.

In any case, our results together with previous data (Yun et al. 2003) indicate that the TH-expressing cells of the telencephalon originate from at least three distinct embryonic domains: LGEd, which produces TH cells for at least the olfactory bulb and olfactory tubercle (Yun et al. 2003); the POC, which produces at least part of the TH cells of the striatum and central extended amygdala (present results); the hypothalamic SPV, which appears to produce at least part of the TH cells of the medial extended amygdala (present results). Future studies will need to analyze whether TH cells from each distinct origin coexist in single nuclei of the striatum or the amygdala, and the specific connections and function of each cell subpopulation.

The data on the embryonic origin of different TH cells are surely useful for trying to understand the evolution and some features of the phenotype and function of the cate-cholaminergic cells of the telencephalon (Yamamoto and Vernier 2011). The telencephalon of fishes is known to include subpopulations of catecholaminergic cells (Smeets

and Reiner 1994). It was only recently shown that these cells are also found in mammals (see references above), and it is now mandatory to re-study their presence in the striatum, amygdala and other parts of the telencephalon in amphibians, reptiles and birds using in situ hybridization. Only when this picture is clear, we can start to analyze the correspondence between different subpopulations across species, their specific phenotype and function, and the level of evolutionary conservation and/or variation in the different progenitor domains that produce these cells.

TH-expressing cells in centers of the reward-related mesocorticolimbic circuitry

One striking finding of our study is that the core of the nucleus accumbens contains a higher density of cathol-aminergic neurons than the accumbens shell or the caudate-putamen (except its most ventral part), and this was so in both females and males. This appears to be positively correlated with the relatively high density of TH-expressing cells in other centers related to reward, such as the prefrontal cortex and the ventral tegmental area (A10 group or VTA) (Dubach 1994; Ugrumov 2009). The VTA contains densely packed cells that strongly express TH and dopamine and project to both the nucleus accumbens and prefrontal cortex (Björklund and Dunnett 2007), being this dopaminergic projection and the interaction between the three centers critical for reward processing (Schultz 1998; Schott et al. 2008).

The TH-expressing neurons of the striatum and neo-cortex have been shown to be interneurons (Cossette et al. 2005b; Huot and Parent 2007; Ugrumov 2009; Asmus et al. 2008, 2011). Cortical neurons expressing TH are reported to belong to the calretinin-containing subpopulation of interneurons (Asmus et al. 2008), shown elsewhere to originate in the caudal ganglionic eminence, in the part corresponding to the caudal LGE (Nery et al. 2002; Xu

et al. 2004). At least postnatally, some TH cortical neurons also appear to express the enzyme choline acetyltransferase (CHAT) or the vasoactive intestinal peptide (VIP) (Asmus et al. 2011). Calretinin and VIP cells may represent a single subset of cortical TH-expressing neurons (Asmus et al. 2011) since both calretinin- and VIP-expressing cells also originate in the caudal LGE (Xu et al. 2004; Miyoshi et al. 2010) and many of the VIP cells in the rat cortex co-contain calretinin (Cauli et al. 1997; Porter et al. 1998). On the other hand, cholinergic neurons of the telencephalon have been shown to originate in the Shh-expressing progenitor zone of the preoptic area (this domain also produces Islet1-expressing cells, and was previously included as part of anterior peduncular area) and in part of the medial ganglionic eminence (Zhao et al. 2003; García-López et al. 2008; Elshatory and Gan 2008; Abellán and Medina 2009; Medina and Abellán 2012). Here we have shown that the preoptic progenitor area is an embryonic source of TH cells for the striatum and parts of the extended amygdala, but perhaps it may produce TH cells for the cortex as well (if so, these may be the TH/CHAT cells reported in the cortex).

On the other hand, striatal neurons expressing TH are considered to represent a small subpopulation of dopaminergic interneurons (Huot and Parent 2007). In addition to TH, these cells express aromatic L-amino acid decarboxylase (AADC, the enzyme that converts L-DOPA to dopamine), dopamine membrane transporter (DAT) and the transcription factor Nurr1 (which is essential for the differentiation of tegmental dopaminergic neurons) (Mura et al. 1995; Betarbet et al. 1997; Cossette et al. 2005b; Huot and Parent 2007). These cells lack the enzyme dopamine beta-hydroxylase, which discards a noradrenergic or adrenergic phenotype (Cossette et al. 2005b). In primates, TH-expressing cells of the striatum are more abundant than in other mammals, and they fall into several morphologically distinct classes, including both aspiny as well as spiny neurons (variable depending on the species; Tashiro et al. 1989; Betarbet et al. 1997; Cossette et al. 2005b). As noted above, these cells appear to have at least two different embryonic origins, the POC (present results) and possibly the dorsal LGE (Yun et al. 2003). If confirmed, future studies will need to analyze more details of the phenotype and function of each subtype of TH striatal cells. In addition, studies of TH cells focusing in the ventral, viscerolimbic striatum (including the nucleus accumbens) are needed. As noted above, in the mouse we found a higher density of TH-expressing cells in the core of nucleus accumbens than in most of the caudate-putamen. We also observed a relatively higher density of TH-expressing cells in the ventral aspects of the striatum surrounding the posterior limb of the anterior commissure, that include the IPAC and other areas belonging to the extended amygdala

(see next section). In humans, the ventral aspect of the human striatum, including the region that surrounds the anterior commissure, also contains a higher density of TH-expressing cells as compared to the dorsal striatum (Cossette et al. 2005b). However, according to the same study, the nucleus accumbens of humans does not appear to contain many cells. Perhaps this is due to a species difference, but additional studies are needed in order to discard (or not) other possible causes, such as aging, diet, stress, or other factors that are known to have an influence on the expression level of catecholamines in the brain (Asmus and Newman 1993b; Sabban and Kvetnanský 2001; Northcutt et al. 2007; Braun et al. 2012; Lynch et al. 2012; Vucetic et al. 2012). In any case, our results show that in the adult mouse (1.5–5 months), the core of the nucleus accumbens, which is a key center involved in motivation and reward, appears to contain a relatively higher number of TH-expressing cells than other striatal areas. The TH cells in this center may be interneurons (based on what is known in other striatal parts; see above). However, their exact role in modulation of principal neurons in the accumbens, whether these TH cells receive direct dopaminergic input from the VTA, and if so the type of dopamine receptors they express remain unknown. Such information is essential in order to fully understand the regulation of motivation and reward under normal conditions, and the dysregulations that occur in this system in addiction or other disorders.

TH-expressing cells in the extended amygdala: relation to stress, diet and social behavior

In the medial extended amygdala of mouse, we observed TH-expressing cells in the BSTM (mainly the posterolateral part), the anterior ventral amygdala and the medial amygdala (anterior and posterodorsal parts). This is similar to the findings in prairie voles, meadow voles and hamsters (Asmus et al. 1992; Asmus and Newman 1993a, b; Northcutt et al. 2007). However, TH-expressing cells are more abundant in the BSTM and posterodorsal amygdala of prairie voles (rodents that show monogamous behavior and both sexes rear their offspring together), than in meadow voles and hamsters, both with promiscuous behavior and in which only females take care of the offspring (Northcutt et al. 2007). Moreover, such cells (at least based on protein expression) are absent in these nuclei of rat, but this should be reanalyzed using in situ hybridization to detect the mRNA. In the mouse, such TH cells of the medial extended amygdala showed mRNA expression but not protein expression (present results; Table 1). In the prairie voles, TH cells are more abundant in virgin males than in virgin females or in gonadectomized males (in the latter, the effect is reversed by testosterone) (Northcutt

et al. 2007). Since such a difference between males and females is not observed in hamsters, it has been suggested that there is a species-specific sex difference in the presence of TH-expressing cells in the medial extended amygdala (Northcutt et al. 2007). Moreover, these TH cells express androgen receptors, are influenced by gonadal hormones, and project to the medial preoptic area, and it has been suggested that they play a role in reproduction and other aspects of social behavior related to pair-bonding and paternal care (Asmus et al. 1992; Asmus and Newman 1993b; Northcutt et al. 2007; Northcutt and Lonstein 2011). In the mouse, we did not observe differences in the expression of TH between males and females, and the TH cells—although moderately abundant in the posterolateral BSTM—show generally weak mRNA signal, and no protein expression. In terms of distribution and abundance of TH-expressing cells, it appears that the pattern in the mouse is similar to that found in prairie females or in castrated prairie males. However, the absence of detectable protein in these cells turns the mouse pattern more similar to that in the rat.

Our data show that in the mouse TH cells of the BSTM primarily locate in the posterolateral subdivision (but not in the posteromedial or principal BSTM subdivision, in contrast to that described in other rodents by Northcutt et al. 2007). The location is important, because our previous studies showed that the posteromedial BSTM is rich in cells expressing Lhx6, which originate in the caudoventral part of the pallidal embryonic domain (caudoventral part of the medial ganglionic eminence or MGEcv), while the posterolateral BSTM appears to include many cells of preoptic and hypothalamic (SPV) origins (García-López et al. 2008). Since we did not find evidence for a preoptic contribution of TH cells to the medial extended amygdala, perhaps most of the TH cells in this medial extended amygdalar corridor originate in the SPV. However, although in our fate mapping experiments we observed numerous SPV-derived cells in the BSTM and a few in the medial amygdala (as in previous reports, Bupesh et al. 2011a), only very few of these cells were double-labeled for TH, and none was found in the BSTM. Perhaps this was due to a technique problem related to the early embryonic stage when we prepared the cultures, or to the late differentiation of TH neurons (in particular, timing for producing the TH enzyme after leaving the cell cycle). As noted above, during development the SPV produces TH cells (Marín et al. 2005; present results) as well as oxytocin and vasopressin cells of the paraventricular and supraoptic hypothalamic nuclei (Wang and Lufkin 2000). A few SPV-derived vasopressin cells also appear to migrate to the medial extended amygdala (see "Discussion" in Bupesh et al. 2011a). It appears that a similar situation occurs with the TH cells produced in the SPV, so that some of them

invade the medial extended amygdala (present results). The TH cells and the vasopressin cells of the medial extended amygdala may represent the same neuron subtype, since TH and vasopressin are coexpressed in the hypothalamic nuclei derived from SPV (Ugrumov 2009). Vasopressin and oxytocin cells play a key role in several aspects of social behavior including social recognition, maternal care and pair-bonding (Hammock and Young 2006). As the vasopressin cells, the TH cells of the medial extended amygdala are also postulated to play a role in sociosexual behavior (Northcutt and Lonstein 2011).

On the other hand, TH cells were only observed transiently during late embryonic and postnatal development in the central amygdala of the rat (Verney et al. 1988). Such cells have never been observed or described in adult rodents or other mammals, at least using immunohistochemistry for protein detection. Therefore, our results provide the first description of TH cells (expressing the mRNA and perhaps the protein) in this location in adult rodents. As noted above, our data on TH mRNA in the amygdala agree with that shown in the Allen Brain Atlas. The expression of TH mRNA in these cells is weak, and this may have been the reason for being undetected previously using immunohistochemical techniques. In adult mice, a small to moderate number of TH-expressing cells are observed in the medial part of the central amygdala, in the sublenticular part of the central extended amygdala, and in the medial IPAC, and very few cells were also present in the BSTL. These cells originate, at least partially, from the preoptic progenitor zone, and may invade the central amygdala following the incoming tegmental TH-expressing axons. Whether these cells are projection neurons or interneurons is unknown. The central extended amygdala is involved in control of fear responses, stress/anxiety, and ingestion (LeDoux 2000; Swanson 2000; Paré et al. 2004; Petrovich and Gallagher 2007; Martínez-García et al. 2012). TH cells may play a role in these behaviors and their effect may be complementary to that exerted by the mesolimbic or other incoming catecholaminergic axons. Considering that in several brain centers, including the centromedial amygdala, the level of catecholamine expression is affected by stress and diet (Savard et al. 1983; Sabban and Kvetnanský 2001; Vucetic et al. 2012), it would be important to investigate whether the TH perikaryal expression is altered in the central extended amygdala in these situations.

Notably, emotional and motivational dysfunctions have been reported in Parkinson's disease and schizophrenia (Blonder and Slevin 2011; Rosenfeld et al. 2011; Lo Bianco et al. 2012). The bases for these dysfunctions are still unclear although they appear related to an alteration of the mesolimbic/mesocortical pathways (Blonder and Slevin 2011; Howes and Kapur 2009; Heinz and Schlagenhauf

2010). In particular, in schizophrenia these dysfunctions are related to alterations in dopamine receptors in the targets of the mesolimbic/mesocortical pathways in the telencephalon (Buchsbaum et al. 2006; Joyce and Gurevich 1999; Kessler et al. 2009). For example, D3 dopamine receptors are generally enriched in the viscerolimbic striatum and extended amygdala in normal conditions, and their number is increased in schizophrenics (Joyce and Gurevich 1999). In general, these studies have not considered the presence of TH cells inside the viscerolimbic striatum and extended amygdala, which is now critical in order to fully understand the role of catecholamines in the emotional/motivational dysfunctions observed in these human diseases or disorders.

Phenotype of TH-expressing telencephalic neurons: dopaminergic versus monoenzymatic or bienzymatic neurons

As previously mentioned, in rodents and primates the TH-expressing cells of the striatum and the olfactory bulb are considered to be dopaminergic (Cossette et al. 2005b; Björklund and Dunnett 2007). However, in the striatum, olfactory bulb and other parts of the telencephalon, many cells appear to be monoenzymatic or bienzymatic, only partially expressing the dopaminergic phenotype (Ugrumov 2009). The whole enzymatic machinery of a dopaminergic cell includes TH (the rate-limiting enzyme of the catecholamine synthesis, that converts L-tyrosine into L-3,4-dihydroxyphenylalanine [L-DOPA]), AADC (that converts L-DOPA into dopamine; this enzyme is also abbreviated DDC), vesicular membrane transporter, type 2 (VMAT2, needed for cytosol capture of dopamine into the secretory vesicles), and DAT (for recapturing dopamine from the synaptic cleft for reutilization) (Ugrumov 2009; see Yamamoto and Vernier 2011, for other functions of VMAT). Monoenzymatic cells only express either TH (these cells do not produce dopamine but only L-DOPA, as a final synthetic product) or AADC (these cells may be able to produce dopamine from L-DOPA taken from the extracellular medium) (Ugrumov 2009). Bienzymatic neurons express TH and AADC, but lack VMAT2. Based on the data published in the Allen Brain Atlas web site, the telencephalic areas or nuclei of adult mouse that contain TH mRNA-expressing neurons, such as the striatum and extended amygdala, also contain cells expressing AADC (also called DDC), VMAT2 or DAT, suggesting a dopaminergic phenotype for at least some of them. Nevertheless, in both rodents and primates, monoenzymatic and (less frequently) bienzymatic cells have also been shown or suggested to be present in the cortex, striatum, olfactory bulb, nucleus of the horizontal diagonal band, preoptic area, hypothalamus, and many other catecholaminergic cell groups of the brain, including the substantia nigra, pars compacta (A9) and the VTA (A10) (Ugrumov 2009). Considering this, it is possible that some TH cells of the extended amygdala are monoenzymatic or bienzymatic. This is suggested to be the case for TH-ir cells of the vole medial amygdala (Ahmed et al. 2012). Moreover, in the medial extended amygdala of mouse there are pools of cells expressing TH mRNA that appear to lack the protein (present results). It has been suggested that monoenzymatic and bienzymatic TH cells may represent a pool of immature dopaminergic neurons, present not only during development but also in adulthood (Weihe et al. 2006; Ugrumov 2009). As for dopamine, it is possible that the L-DOPA plays a different role during development (if present, being involved in neuron differentiation) than in the adult (Ugrumov 2009). In adulthood, the role of L-DOPA is controversial, and while some authors suggest that it plays a role in intracellular signaling, other authors have provided convincing data for a role in intercellular communication (reviewed by Ugrumov 2009). According to the latter, L-DOPA produced in monoenzymatic TH neurons is secreted and plays a role as neurotransmitter or neuromodulator acting on target neurons via catecholamine receptors, such as dopamine D2 (reviewed by Ugrumov 2009). Further investigation focused in the TH cells of the viscerolimbic striatum and the extended amygdala is surely needed in order to fully understand the secondary effects of L-DOPA treatments in Parkinson's disease, but also the emotional and motivational dysfunctions observed in this disease, in schizophrenia, or in drug addiction.

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