Neuropharmacology 55 (2008) 994–1005

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Serotonin transporter transgenic (SERT^{cre}) mouse line reveals developmental targets of serotonin specific reuptake inhibitors (SSRIs)

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ARTICLE INFO

Article history:
Received 13 May 2008
Received in revised form 8 August 2008
Accepted 11 August 2008

Keywords:
5-HTT
Serotonin
Brain development
Recombinase
Mouse models
Thalamus
Cortex
Prefrontal
Hippocampus
Cingulate

ABSTRACT

The serotonin transporter gene (SLC6A4; synonyms, SERT, 5-HTT) is expressed much more broadly during development than in adulthood. To obtain a full picture of all sites of SERT expression during development we used a new mouse model where Cre recombinase was inserted into the gene encoding the serotonin transporter. Two reporter mouse lines, ROSA26R and the Tau^{mGFP}, allowed to map all the cells that express SERT at any point during development. Combined LacZ histochemistry and GFP immunolabelling showed neuronal cell bodies and axon fiber tracts. Earliest recombination in embryos was visible in the periphery in the heart and liver by E10.5 followed by recombination in the brain in raphe serotonergic neurons by E12.5. Further, recombination in non-serotonin neurons was visible in the choroid plexus, roof plate, and neural crest derivatives; by E15.5, recombination was found in the dorsal thalamus, cingulate cortex, CA3 field of the hippocampus, retinal ganglion cells, superior olivary nucleus and cochlear nucleus. Postnatally, SERT mediated recombination was visible in the medial prefrontal cortex and layer VI neurons in the isocortex. Recombined cells were co-labelled with Neu-N, but not with GAD67, and were characterized by long range projections (corpus callosum, fornix, thalamocortical). This fate map of serotonin transporter expressing cells emphasizes the broad expression of SERT in non-serotonin neurons during development and clarifies the localization of SERT expression in the hippocampus and limbic cortex. The identification of targets of SSRIs and serotonin releasers during embryonic and early postnatal life helps understanding the very diverse physiological consequences of administration of these drugs during development.

1. Introduction

The high affinity serotonin transporter (SERT, gene name: solute carrier SLC6A4) is an essential actor of serotonin clearance and is critical for serotonin (5-HT) homeostasis both at central 5-HT synapses and in the periphery, in blood, lung or gut (Blakely et al., 1991; Blakely, 2001). As such, SERT is a major target of different classes of psychotropic drugs. The 5-HT specific reuptake inhibitors (SSRIs) are the most widely prescribed drugs for depression, eating disorders, or obsessive-manic disorders (Vaswani et al., 2003; Lesch and Gutknecht, 2005; Murphy and Lesch, 2008). Several monoamine releasers, in particular fenfluramine, or the recreative drug MDMA, ecstasy, also target the 5-HT transporter (Bengel et al., 1998).

In the adult brain, SERT is expressed in serotonin producing neurons of the brain and gut, the raphe nuclei and the enterochromaffin cells, respectively. In addition, several serotonin accumulating cell types such as platelets, medullary chromaffin cells, or crypt cells in the gut express SERT (rev. in Blakely, 2001). During development SERT is expressed much more broadly than in adults in several non-serotonin neurons of the brain and in neural crest derivatives (rev. in Gaspar et al., 2003). This developmental expression of the SERT gene, together with the developmental expression of the vesicular monoamine transporter gene (Hansson et al., 1999) explains the early observations of serotonin accumulating cells in a number of tissues such as the heart, the cranial mesenchyme, the notochord, and neural crest derivatives (Wallace, 1982; Shuey et al., 1993) and the brain (Fujimiya et al., 1986; D'Amato et al., 1987). High affinity uptake of 5-HT during these periods could serve the purpose of controlling locally the level of 5-HT-allowing to regulate the state of activation of serotonin

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doi:10.1016/j.neuropharm.2008.08.020

receptors. Thus, developmental SERT expression could be an important regulator of the developmental effects of serotonin which have been observed in different systems and numerous organisms (for reviews see Lauder, 1993; Whitaker-Azmitia, 2001; Gaspar et al., 2003; Vitalis and Parnavelas, 2003). Administration of SSRIs during pregnancy or infancy could directly target SERT expressing neurons and modify their adult properties, either by altering specific wiring, or by changing gene expression.

Previous studies of SERT expression during development focused on sensory pathways. These studies showed the existence of transient SERT expression in the thalamocortical pathways with in situ hybridization (Lebrand et al., 1996, 1998; Hansson et al., 1998, 1999), autoradiographic binding (D'Amato et al., 1987; Bennett-Clarke et al., 1997) and immunocytochemistry (Lebrand et al., 1998; Zhou et al., 2000). SERT expression was noted in the cortex and in different limbic brain areas but was not analyzed in great detail despite the importance of these areas for emotional control.

Here, we used a SERT^{cre} knock-in mouse (Zhuang et al., 2005) that allows to visualize all the cells that have expressed SERT at any point during their lifetime. SERT^{cre} mice were mated with ROSA26R (R26R) mice that express beta-galactosidase in response to expression of Cre recombinase (Soriano, 1999) and with Tau^{mGFP} mice that express both beta-galactosidase and a membrane targeted GFP in response to Cre recombinase (Hippenmeyer et al., 2005), allowing to visualize the fiber tracts originating from SERT expressing neurons.

We show that the cumulated sites of transient SERT expression in non-5-HT neurons clearly outnumber the SERT expression in 5-HT neurons of the raphe during the late embryonic period and early postnatal life. Thus the cellular targets of SSRIs are multiple and vary over different developmental periods. The possible actions of SSRI on non-serotonergic neurons have to been taken into consideration in the therapeutic and side effects of these compounds.

2. Material and methods

2.1. Animals

The production of the SERT^{cre} mouse line has been reported previously. In brief, exon 2 of the SERT gene was replaced by the CRE recombinase (Zhuang et al., 2005). SERT genomic DNA fragments that contained the 5′-region and the first two exons were excised from phage DNA isolated from a mouse 129 Sv/J genomic library. The gene targeting vector was constructed by inserting into the 5′-UTR region of the SERT genes a cassette containing the Cre recombinase coding sequence with a nuclear localization signal and the neomycin-resistance gene, flanked by FRT sites. The inserted transgene disrupts one copy of the SERT gene. The mice were maintained on a mixed 129 Sv-C57-Black6 background. Germline recombination was found to occur sporadically, causing all the embryonic cells to express beta-galactosidase. This was observed in 25% of cases when crossings were performed with homozygote Cre mouse lines. However the frequency of such events was reduced to 1 out of 20 pups when male SERT$^{cre/+}$ were crossed to female reporter mice. Germline recombination is probably due to the expression of several serotonin-related genes, including SERT in murine ovarian cells (Amireault and Dubé, 2005).

Two different mouse reporter lines ROSA26R (R26R) (Soriano, 1999) and Tau^{mGFP} (Hippenmeyer et al., 2005) were used in the present study.

The R26R mouse line targeting of the ROSA26 locus and the derivation of a reporter line for monitoring Cre expression was obtained by the insertion of a neo expression cassette flanked by *loxP* sites, a LacZ gene and a polyadenylation (bpA) sequence at approximately 300-bp 5′ of the original gene-trap integration site. A triple polyadenylation sequence was added to the 3′ end of the neo expression cassette to prevent transcriptional read-through.

The Tau^{mGFP} line was obtained by inserting into exon 2 of the *Tau* (microtubule associated protein) genomic locus a *lox-STOP-lox-mGFP-IRES-NLS-LacZ-pA* targeting cassette: this is a sequence containing a transcriptional stop cassette surrounded by loxP sites, followed by the construct mGFP coding sequences consisting in a MARCKS protein (membranous) fused to the green fluorescent protein (GFP) and then an IRES-NLS-LacZ cassette. This insertion removes the Tau ATG start codon. The animals were of mixed genetic background 129/Ola and C57Bl6.

Animal care was conducted in compliance with the standard ethical guidelines (European Community guidelines on the care and use of Laboratory animals and French Agriculture and Forestry Ministry guidelines for handling animals-decree 87849).

2.2. Histology

X-gal staining in toto was done at embryonic ages, E8.5, E10.5, E12.5, E13.5, E14.5 and E16.5 ($n = 5$ per age from 2 different litters): the specimens were photographed before and after skin ablation, included in paraffin and sectioned to 10 μm thick sections. In addition, brains from E15.5, E18.5, P0, P5, P15 and frozen sectioned (60–90 μm-thick) before X-gal revelation. Briefly: SERT^{cre}; R26R mouse embryos were collected from stage E8.5–E16.5. X-gal staining for ß-gal activity was performed on whole mount preparations or brain sections using the procedure as previously described (Pavone et al., 2007). Briefly, brains were dissected in cold phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 15 min at 4 °C rinsed in PBS, and then immersed in X-gal buffer containing 5 mM EGTA, pH 8.0, 2 mM $MgCl_2$, 0.2% NP40, 0.1% deoxycholate, 2 mM $CaCl_2$. After washing, they were stained with X-gal staining solution (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS) at 30 °C for 2–3 h in the case of embryos that were to be photographed as whole mounts or overnight in the case of embryos and brains that were paraffin embedded and sectioned. Embryos and brains were washed in pre-warmed PBS for 10 min at room temperature, post-fixed in 4% paraformaldehyde in PBS for 10 min at 4 °C, rinsed in PBS, and stored in 70% ethanol.

ß-gal-stained embryos and brains were dehydrated through ethanol grades, cleared in xylene, paraffin embedded, sectioned at 10 μM, and counterstained with Nuclear Fast Red (Vector Laboratories). X-gal-stained sections from whole embryos and brains were observed under a light microscope, and photographed (Zeiss binocular stereomicroscope and microscope). Double staining with mouse Neu-N (Millipore; 1:500) or GAD67 (Millipore; 1:1000) antisera, and rabbit ß-gal antiserum (Rockland; 1/10000) was done at P7 and in adults.

SERT^{cre} × Tau$^{mGFP\text{-}IRES\text{-}LacZ}$ crossings: four to eight pups from 2 litters were analysed at P7, P12, P21 and as adults. Mice were perfused transcardially with paraformaldehyde, cryoprotected in 30% sucrose, sectioned to 52 μm thick frozen sections. Alternate serial sections were used for GFP-immunostaining (Molecular Probes; 1:2000). LacZ histochemical, or both. If needed, revelation was counterstained with a 0,01% safranine solution in water (Michrome, London), before dehydration.

3. Results

The fate map of the SERT expressing neurons was analyzed using two different reporter mouse lines: (1) the ROSA26R, which drives LacZ expression in the cell's cytoplasm indifferently of its lineage (Soriano, 1999) and (2) the Tau^{mGFP} mouse line which is specific to neurons (Hippenmeyer et al., 2005). In the latter, Cre-mediated recombination drives beta-galactosidase only in the nucleus of neurons, because the LacZ gene has a nuclear targeting sequence (NLS) which drives the beta-galactosidase protein to the nucleus. Furthermore, recombined neurons express a GFP fused MARKS protein, that is anchored to the cell's membrane and allows to visualize the whole extent of the cells which have undergone recombination, including axonal branches.

Comparison of the R26R and the Tau^{mGFP} reporter mouse lines showed a somewhat more abundant labelling of cells with the Tau^{mGFP} mouse line than with the ROSA26R reporter mouse line, this could be due to a different strength of the promoter, or to the fact that concentration of the X-gal signal in the nucleus can enhance its visibility.

The time line for the onset of LacZ histochemical staining in each structure is shown in Table 1.

3.1. SERT-induced recombination in raphe serotonin neurons

Serotonergic neurons start being generated by E10.5 in mice (Wallace and Lauder, 1983; Lidov and Molliver, 1982) and SERT gene expression has been noted by E11.5 (Schroeter and Blakely, 1996; Hansson et al., 1998). Labelled X-gal neurons were first found in the raphe, on either side of the midline by E12.5 (Fig. 1a,b), indicating that effective recombination occurs at least 24 h after gene expression. In embryos examined from E14.5 on, X-gal labelling in the raphe was clearly visible and extended to all raphe cell groups (Fig. 1c). In addition, GFP-labelled ascending raphe projections could be clearly visualized on tissue sections from Tau^{mGFP} mouse line (Fig. 1f).

Table 1
Time line for onset of SERT mediated Cre recombination

	E10.5	E12.5	E14.5	E15.5	P0	P5	Adult
CNS							
Raphe	0	+	+	+	+	+	+
Thalamus (VB)	0	0	0	+	+	+	+
Cingulate Cx	0	0	0	+	+	+	+
Hippocampus	0	0	0	+	+	+	+
Sup. olive	0	0	+	+	+	+	+
Retinal GC	0	0	0	+	+	+	+
Prefrontal Cx	0	0	0	0	+	+	+
Inf. colliculus	0	0	0	+	+	+	+
Neocortex (LVI)	0	0	0	0	+	+	+
DRG	0	0	+	+	+	+	+
Choroid plexus	0	0	+	+	+	+	+
Neural crest							
Roof plate	0	0	+	+	0	0	0
Merkel cells	0	+	+	+	+	+	+
Olf. epith.	0	0	+	+	+	+	+
Pharyng. epith.	0	+	+	+	+	+	+
Face cartilage	nd	nd	nd	nd	+	+	+
Non-neural							
Heart	+	+	+	+	+	+	+
Liver	+	+	+	+	+	+	+

Post-natally, X-gal labelling outlined clearly all the rostral and caudal serotonin cell groups of the raphe (Fig. 3g–i). However, GFP-labelled raphe efferents then constituted a minority of the labelled fiber tracts making them more difficult to identify than on embryonic material.

3.2. SERT-induced recombination in non-neural and neural crest derived structures

Intense X-gal staining was noted the heart, liver, lung and gut (Fig. 2g,g′,h), confirming previous reports of early SERT gene expression in these structures (Schroeter and Blakely, 1996; Sari and Zhou, 2003; Pavone et al., 2007). The SERT^{Cre}-mediated recombination was particularly precocious in heart and liver, beginning at E10.5 (Table 1).

The choroid plexus was another structure in which SERT mediated recombination was both precocious (E13.5) and intense, appearing along the lateral ventricles (shown at E14.5 in Fig. 1d,e), and at the mid-hindbrain boundary, corresponding to the roof of the fourth ventricle.

A large number of neural crest derivatives express the SERT gene during late embryonic development. This was initially observed by 5-HT uptake experiments (rev. in Lauder, 1993; Wallace, 1982) and later detailed by Hansson et al. (1999) using in situ hybridization in rats. SERT^{cre}-mediated expression of LacZ made these structures particularly prominent on in toto preparations. For instance labelled migrating neural crest cells were visible in the skin (Fig. 2b,c,f), in Merkel cells in the whisker follicles (Fig. 2c), in the cranial mesenchyme, the olfactory and pharyngeal epithelium, and the taste buds in the tongue (Fig. 2d,e). In addition, face cartilages seem to widely express beta-galactosidase. X-gal labelling was also seen in the dorsal root ganglia and in midline cells of the roof plate, extending from the mesencephalon to the spinal cord (Fig. 2f). The labelling of the roof plate, which may correspond to remnants of the neural crest, disappeared at late embryonic and postnatal (Fig. 3h) stages, possibly due to migration or death of these cells.

3.3. SERT-induced recombination in non-serotonin neurons of the CNS

In the brain, X-gal became visible in several non serotonergic neurons, and the projections of these cells became GFP positive in the Tau^{mGFP} reporter mice. Thus, by E15.5, the reporter genes were expressed in the dorsal thalamus, the hippocampal anlage (Fig. 1h), the cingulate cortex, a fraction of retinal ganglion cells (Fig. 1g), and in the superior olivary complex. At birth (P0) additional sites of recombination were observed in the medial prefrontal cortex and isocortex (Table 1).

3.3.1. Cerebral cortex

The areas containing the highest amount of recombined X-gal positive neurons were the medial prefrontal cortex (MFC), the infralimbic cortex (IL), the prelimbic cortex (PrL), the anterior cingulate (CgA), and the retrosplenial (Rspl) cortices (Fig. 3a–f). Labelling was found in the deep cortical layer VI, with scattered labelled cells in layer V. In addition, in the retrosplenial cortex, layer II neurons were intensely labelled. This clear transition in the labelling of layer II neurons is best seen in parasagittal sections (Fig. 4b). In the lateral isocortical areas, X-gal labelling was limited to interspersed neurons in layer VI (Fig. 3a–f). Interestingly, the lateral limbic areas (ventral to the rhinal sulcus) such as the piriform and orbital cortices, the claustrum and the amygdala showed no SERT-driven recombination at the different ages examined (Fig. 3a–e).

Co-localization analyses of beta-galactosidase with Neu-N showed that all the beta-galactosidase labelled cells in the medial prefrontal cortex and in layer VI of the isocortex were co-labelled with Neu-N (Fig. 5a) indicating their neuronal identity. Co-labelling with GAD67 showed a complete exclusion of GAD67-immunolabelled profiles (Fig. 5d,e), indicating that the SERT-recombined cortical neurons are not GABA interneurons.

GFP labelling showed the axon tracts and axon terminals derived from the SERT-recombined neurons. GFP could be directly visualized with fluorescence but was most clearly observed after immunolabelling and DAB revelation. This allowed simultaneous revelation with the X-gal staining (Fig. 4). The thalamocortical axon tracts were the most clearly labelled and formed dense fascicles (Fig. 1h, Fig. 4a,e). In the layer IV of the somatosensory cortex, the barrel pattern, formed by thalamocortical axons, was recognizable at P7 (Fig. 4d,e,e′). The pattern was still visible in more mature mice but tended to be blurred because of the presence of other GFP-labelled processes corresponding to apical dendrites of neurons in the deep layers, and to axon terminals of cortico-cortical, and raphe cortical origins. In the corpus callosum, GFP-labelled axons were visible in the caudal and dorsal-most sections (Fig. 4e,e″) probably corresponding to the commissural axons of the retrosplenial cortex (Vogt et al., 1981) that were recombined as assessed by X-gal staining (Fig. 4e,e″).

3.3.2. Hippocampus

X-gal nuclear labelling was essentially limited to the principal neurons of CA3 (extending from the hilus of the dentate gyrus to the border with CA2). The pre-subiculum also contained an abundant contingent of recombined neurons, whereas the other hippocampal fields such as the dentate gyrus, the CA1, the subiculum, contained no or rare recombined neurons (Fig. 3d,e).

Co-localization analyses with Neu-N confirmed the neuronal nature of the recombined hippocampal cells, showing that only a subpopulation of the CA3 neurons are recombined (Fig. 5c). As in the cerebral cortex, co-localization analyses with GAD67 showed no co-localization with GFP or beta-galactosidase immunostaining (not shown).

The major hippocampal fiber tracts, the fornix and hippocampal commissure, were heavily labelled with GFP (Fig. 4d,e). These likely correspond to the efferent fibers of CA3 and pre-subiculum recombined neurons (Amaral and Witter, 1989). Within the hippocampus itself (Fig. 4f,f′) the axon terminal fields of CA3 neurons were clearly labelled – CA3 axons have a broad distribution in the hippocampal formation, where they form Schaeffer collaterals to contact the apical dendrites of CA1 neurons, and

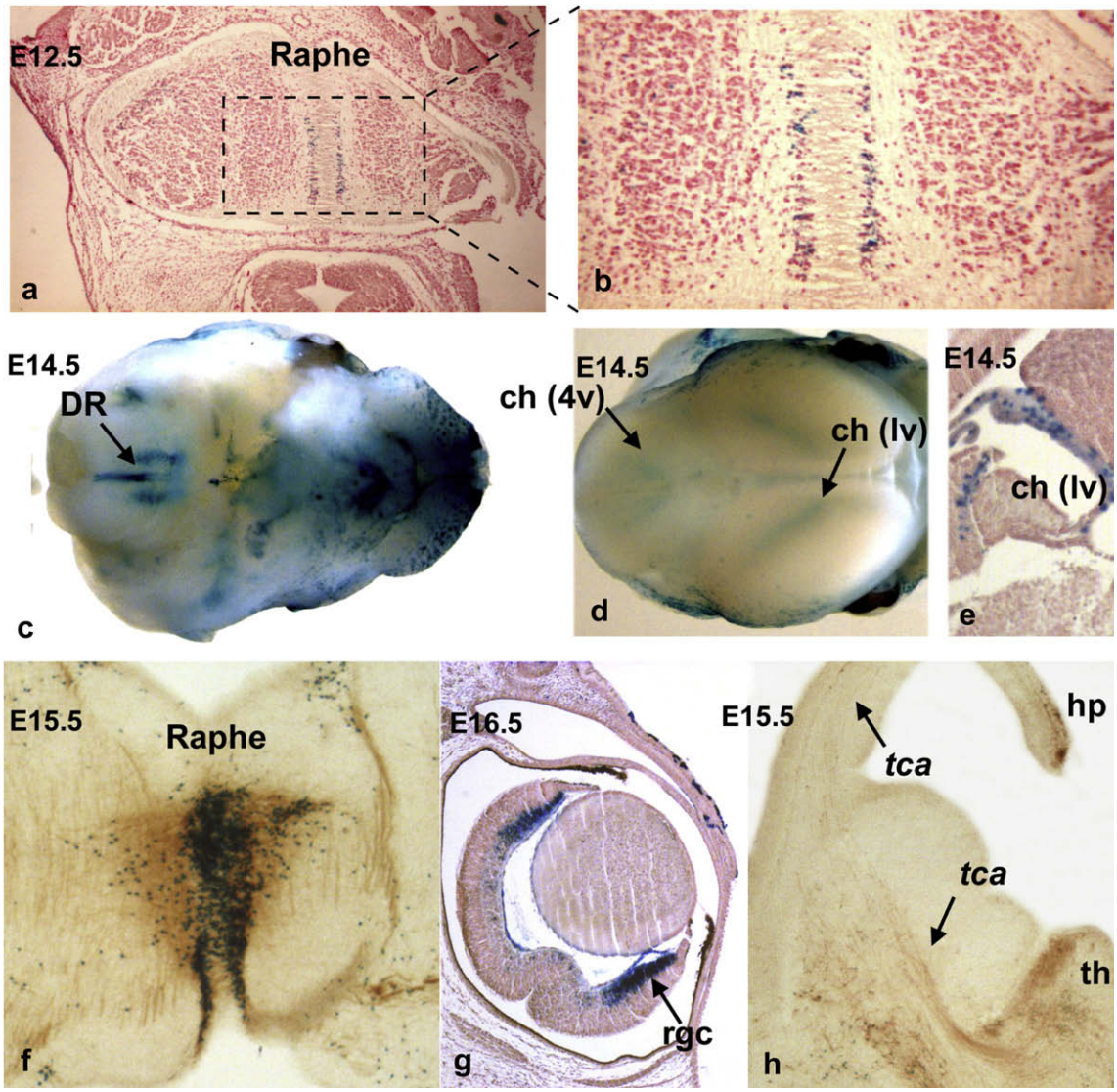

Fig. 1. Embryonic reporter expression driven by SERT^{cre} mice. (a–e) In toto revelation of beta-galactosidase; sections were obtained after paraffin inclusion and counterstained with neutral red. (a,b) At E12.5, LacZ is expressed only in the raphe in the brain. (c,d) At E14.5 expression is well visible in all the raphe cell groups, and becomes visible in the choroid plexus along the cerebral ventricles (arrows) (e) shows a section through the choroid plexus of the lateral ventricle (ch). (f–h) At E15.5, labelling of the raphe with X-gal and efferent raphe axons are labelled with gfp (f), LacZ expression is visible in the retina, ganglion cells are labelled in a peripheral crescent (g). Dorsal thalamus and hippocampal neurons are labelled with X-gal in Tau^{mGFP} mice, thalamic axons extending towards the cortex are labelled with gfp (h). cg: cingulate cortex, ch(4v): choroid plexus of the fourth ventricle, ch(lv): choroid plexus of lateral ventricles, hp: hippocampus, rgc: retinal ganglion cells, th: thalamus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

commissural connexions that reach the contra-lateral hippocampal fields (CA3-DG). Axon terminals in the dorsolateral septum could also be clearly visualized (Fig. 4c) that may correspond to the CA3 terminal projections.

3.3.3. Thalamus

The major site of LacZ expression was in the three main sensory relay structures of the dorsal thalamus, the ventrobasal thalamic nuclei (sensory), the lateral geniculate nucleus (visual) and the medial geniculate nucleus (auditory) (Fig. 3d–f). In these subnuclei, LacZ expression concerned almost every neuron as shown with Neu-N costaining (Fig. 5b). In the mediodorsal and the viscerosensory nuclei, a high density of neurons labelled with X-gal was also seen. More scattered X-gal positive cells were found in the anterior thalamic (limbic) and posterior thalamic (association) groups. Some thalamic nuclei showed no X-gal labelling, this is the case of the reticular, the ventral lateral geniculate nucleus and the midline thalamic nuclei. A less clear-cut labelling was visible in the medial habenula: punctate labelling was observed that did not correspond in an obvious manner to neurons.

The major thalamocortical efferent tracts arising from these recombined neurons were visualized with GFP labelling. Main axon tracts crossing through the striatum towards the cortex were noted (Fig. 4a,c–f). The thalamocortical axon terminal fields endings were noted in layers VIa and in layer IV of the cerebral cortex (Fig. 4d,e,e′).

3.3.4. Hypothalamus

Recombined cells were found in the preoptic areas. These were essentially scattered throughout the preoptic field and rostral hypothalamic groups (Fig. 3b,c), rather than being concentrated into one subnucleus. No recombined cells, other than background were visible in the suprachiasmatic (Fig. 3c) or in the infundibular and the caudal hypothalamic cell groups (Fig. 3d,e).

GFP immunolabelling in the hypothalamus revealed the presence of the fornix, which contains essentially hippocampal afferents to the hypothalamus (Fig. 4d).

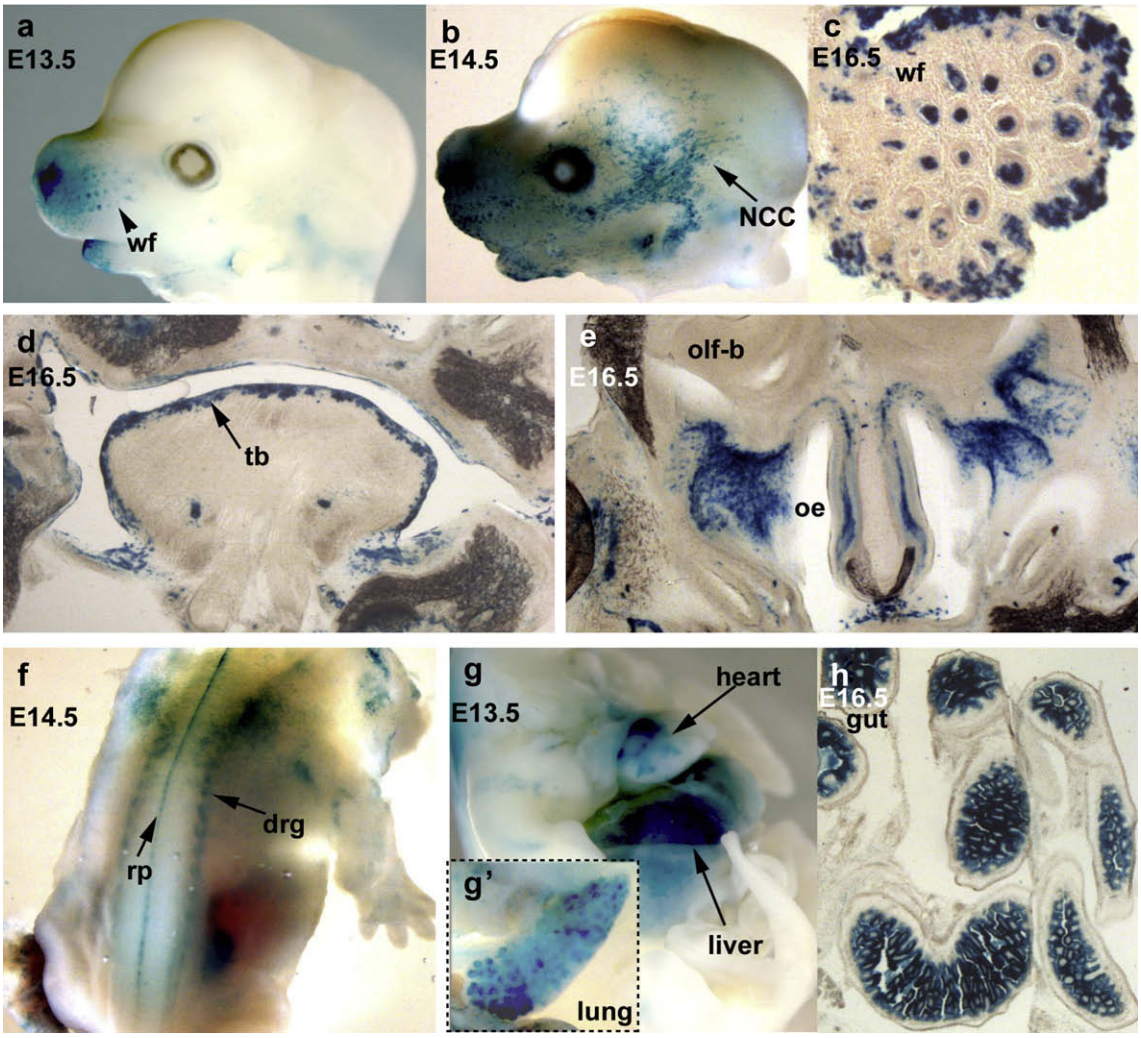

Fig. 2. Neural crest derivatives and peripheral structures showing LacZ expression. In toto revelation of beta-galactosidase using the R26R reporter mouse line. Embryos are shown at E13.5 (a,g,h); E14.5 (b,f) and E16.5 (c–e). (a–c) Numerous labelled cells are visible in the dermis, these correspond to Merkel cells in the mystacial area (a arrowhead); (b) a migrating stream of labelled cells is visible at E14.5 (b, arrow); Merkel cells are most concentrated at the basis of the hair follicles (c). (d,e) The oral and nasal cavities display a very intense beta-galactosidase expression that lines the endothelium of the tongue and palate; in the nasum, there is a clear labelling of cartilaginous structures, as well as labelling of the olfactory nerve. (f) Expression is visible in the all the dorsal root ganglia and in the roof plate; (g) among peripheral organs, expression appears earliest and most intensely in the liver, heart, lung (g′), and gut (h) (on a 10 μm thick section). *Abr.* drg: dorsal root ganglia, NCC: neural crest cells, oe: olfactory epithelium, olf-b: olfactory bulb, rp: roof plate, tb: taste bud, wf: whisker field.

3.3.5. Brainstem

In addition to raphe neurons, beta-galactosidase expression was particularly noteworthy in the auditory sensory relays: in the cochlear neurons (Fig. 3i) in the lateral part of the superior olivary nucleus (Fig. 3h), and in the inferior colliculus (Fig. 4a).

With GFP labelling, the axonal tracts that interconnect the two inferior colliculi at the midline, as well as axon tracts between the superior olivary complex and the inferior colliculus were clearly visible (not shown).

No recombination was visible in the other monoamine cell groups of the brainstem.

GFP labelling also revealed part of the optic tract (ot, Fig. 4d–f) and axon terminal fields in the superior colliculus (Fig. 4a), corresponding to recombined retinal ganglion cells in the retina (Fig. 1g).

4. Discussion

The present genetic tracing of the SERT expressing neurons provides a comprehensive map of the cells that express the serotonin transporter at any time point in development. We show that the cumulated sites of transient SERT expression in non-5-HT neurons outnumber SERT expression in 5-HT producing neurons of the raphe. Since SERT expression extinguishes in a number of brain areas over the first two postnatal weeks of life, the ratio of 5-HT to non-5-HT neurons that are targeted by SSRIs, similarly changes over time. This expression has to been taken into consideration in the therapeutic and side effects of these compounds, during development.

4.1. Specificity and reliability of the genetic labelling approach

Conditional recombination of reporter genes that are not normally expressed in the mammalian genome has proven to be a powerful method for labelling specific neuronal subtypes. In the present study the expression of bacterial beta-galactosidase and of the green fluorescent protein were both driven by the excision of a stop sequence, by the activity of a recombinase that cuts double stranded DNA at selected loxp sites. The selectivity of gene reporter expression is therefore dependent on the site of expression of the Cre recombinase. In the present case *Cre* was inserted in exon 2 of

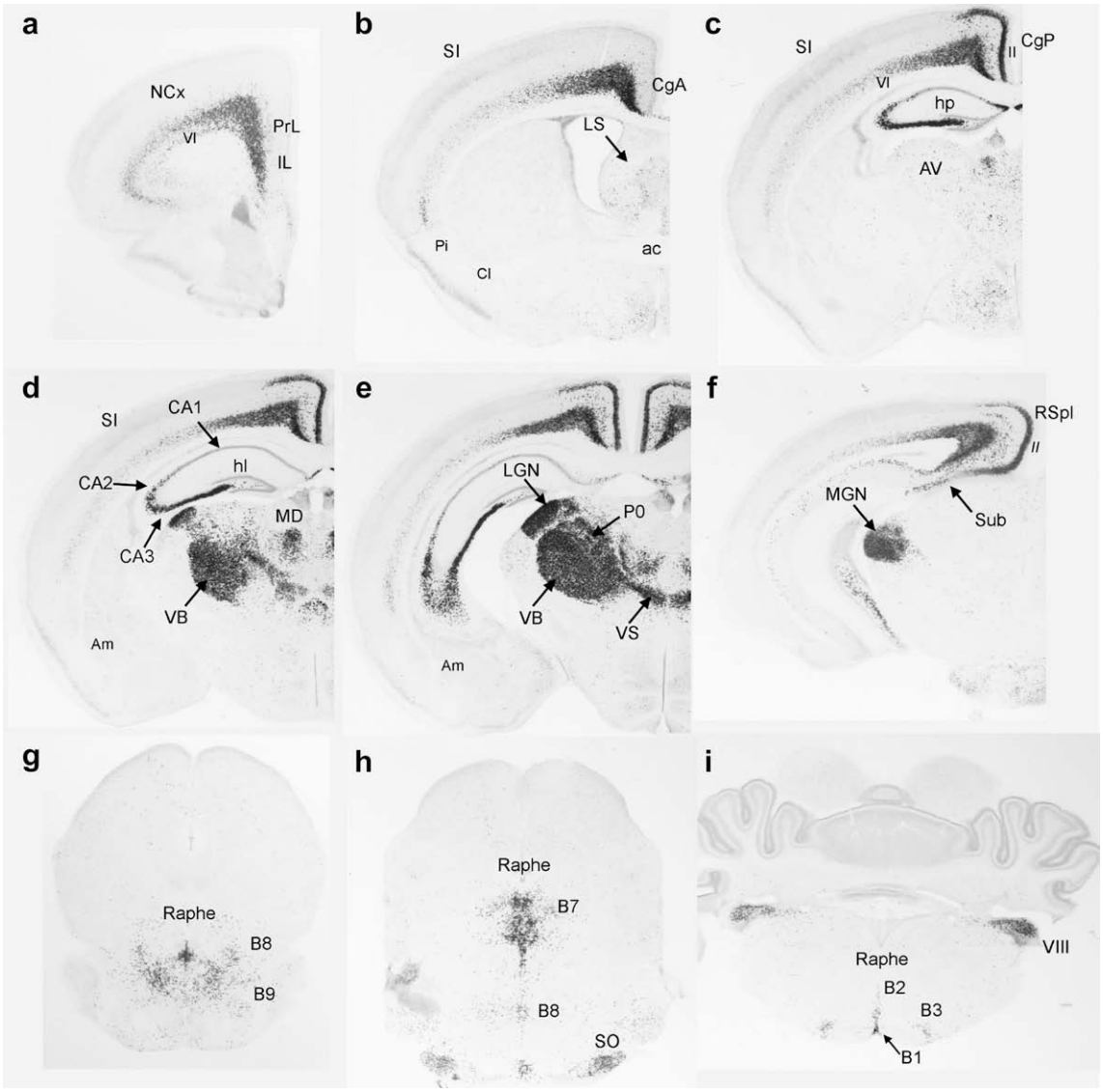

Fig. 3. Recombination pattern of SERT^{cre}-Tau^{mGFP} mice at P7 in the central nervous system. (a–i) Serial sections (52 μm thick) are arranged from rostral (a) to caudal (i), recombination was revealed by X-gal staining and sections were counterstained with safranine. In the cerebral cortex, recombination is restricted to deep layers of the cerebral cortices V and VI (V, VI) (a–f), except in the posterior cingulate cortex where it is also present in layer II (c–f). Specific thalamic nuclei, in particular visual, auditory and somatosensory contain recombined cells (d–f). Caudally, the raphe serotonin neurons are X-gal positive (g–i). *Abr.* ac: anterior commissure, Am: amygdala, av: anteroventral nucleus, B1–8: raphe nucleus 1–8, CA1–3: hippocampal CA1–3 field, CgA: anterior cingulate, CgP: posterior cinglulate, Cl: claustrum, CN: cochlear nucleus, Hl: hilus, Hp: hippocampus, II: cortical layer II, IL: infralimbic cortex, LGN, lateral geniculate nucleus, LS: lateral septum, MD: mediodorsal nucleus, MFC: medial prefrontal cortex, MGN: dorsal geniculate nucleus, MVB: medial ventrobasal nucleus, NCx Neocortex, Pi: piriform cortex, PO: posterior complex, PrL: prelimbic cortex, RSpl: retrosplenial cortex, SI: primary somatosensory area, SO: superior olive, Sub: subiculum, VB: lateral ventrobasal nucleus, VI: cortical layers VI, VIII: dorsal cochlear nucleus, VS: viscerosensory.

the SERT gene, thereby avoiding unwanted, aberrant expression patterns that are due to insertional effects of the transgene in classical transgenic lines. Moreover, the Cre construction comprises an "NLS" sequence which ensures nuclear targeting and thereby a more active recombination. Indeed, the pattern of LacZ expression, and therefore of active recombination, matched accurately, with a one day delay, the spatio-temporal pattern of SERT gene expression in both the serotonin neurons of the raphe (E12.5), and the major non-serotonergic sites of SERT expression, such as the thalamus (E15.5). This 24 h delay may be related to the level of Cre expression since SERT^{cre} heterozygous mice were used here. More likely 24 h could be the time necessary for sufficient beta-galactosidase accumulation within the cells after recombination. Moreover, as assessed by the expression of the reporter genes in these areas, recombination was highly reproducible and complete. This mouse model can therefore be used as an efficient tool for targeting the ablation of genes within specific neuronal circuits of the sensory thalamus, in limbic neurons of the cingulate cortex and in the CA3 region of the hippocampus. The spatio-temporal map of occurrence of recombination driven by the SERT promoter can thus be predicted accurately to target gene ablation in these neurons. The more obvious use and initial purpose of this Cre mouse line for targeting genes in the raphe neurons, will however be obscured, in the case of ubiquitous genes, by the abundance of non-raphe recombination. However, since SERT expression extinguishes in non-raphe areas by P14, specific targeting of the raphe using the SERT gene could be obtained by temporally restricting Cre expression. In the meantime, it is likely that other mouse lines with more restricted Cre expression to the raphe such as those obtained under the pet1 (Scott et al., 2005) and the TPH2 gene (Dusan Bartsch, personal communication) will be more useful for raphe specific invalidation.

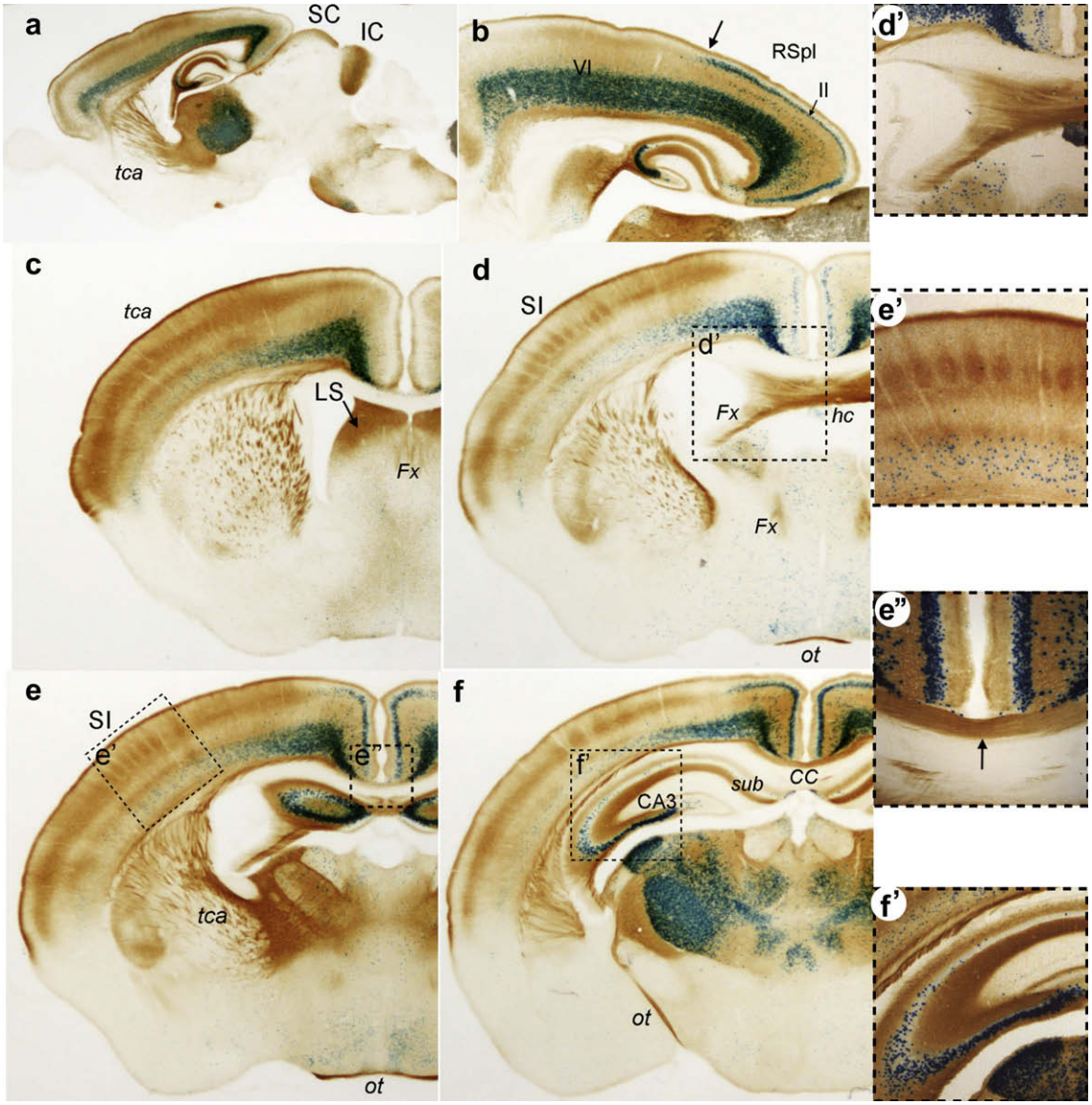

Fig. 4. Projection of SERT^{cre} recombined neurons visualized via membranous-anchored GFP. Sections were immunostained for GFP in addition to X-gal revelation to trace recombined neurons projections. (a,b) Sagittal sections of a P7 brain and (c–f′) coronal sections of a P7 brain. At P7 the major axonal projection linked to SERT expressing neurons are the thalamocortical axons (a–f), organized in columns in the barrel cortex (e′). Hippocampus CA3 neurons project broadly (f,f′), crossing the midline (d,d′). Smaller axon tracts are also notable. In the corpus callosum the caudal and dorsal-most parts contain gfp-labelled axons probably originating from the cingulate cortex (e,f,e″). Superior and inferior colliculus show dense axon labelling, corresponding to inputs from retinal and auditory fibers (a). *Abr.* CA3: hippocampal CA3 field, *CC*: corpus callosum, DR: dorsal raphe, *fx*: fornix, *hc*: hippocampal commissure, IC: inferior colliculus, II: cortical layer II, LS: lateral septal nucleus, RSpl: retrosplenial cortex, *ot*: optical tract, SC: superior colliculus, SI: primary somatosensory area, *sub*: subiculum, *tca*: thalamocortical axon, VI: cortical layers VI.

4.2. Fate map versus SERT expression studies

The present fate map provides a useful complement to previous reports that described the localization of the SERT gene and protein during development. It offers a more comprehensive view of all the regions expressing SERT during development, and a more accurate cell localization of SERT expression in limbic brain areas. Thus, although previous studies had noted the presence of SERT mRNA in the hippocampus (Lebrand et al., 1998; Hansson et al., 1998), it was unclear which hippocampal fields were labelled because of frequent background effects with in situ hybridization studies in this brain area. The present study clearly outlines the CA3 hippocampal field as the major site expressing SERT, with an onset of expression at E15. Moreover, all the efferent axons of the CA3 field were clearly labelled with GFP. In contrast, the CA1–CA2 hippocampal field, the dentate gyrus, the striatum, the piriform cortex and amygdala that were previously suspected to express transiently SERT, based on in situ hybridization or immunocytochemistry (Lebrand et al., 1998; Hansson et al., 1998; Cases et al., 1998; Zhou et al., 2000) do not in fact appear to express the SERT gene at any time during development.

Furthermore, the present study allowed to assess the existence of transient SERT expression in areas that had previously been partly overlooked such as the medial prefrontal cortex. This labelling was particularly marked and appeared to be part of a continuum covering the entire medial limbic cortex, extending to the anterior cingulate cortex and the retrosplenial cortex. Interestingly, the SERT expressing neurons participated to long corticocortical projections as indicated by the labelling of callosal axons, at least at the level of the retrosplenial cortex.

Finally, confirming and extending previous observations (Cases et al., 1998; Thompson and Lauder, 2005), the present description outlined a marked expression of SERT in the entire auditory pathway; in the cochlea, the medial cochlear nucleus, lateral

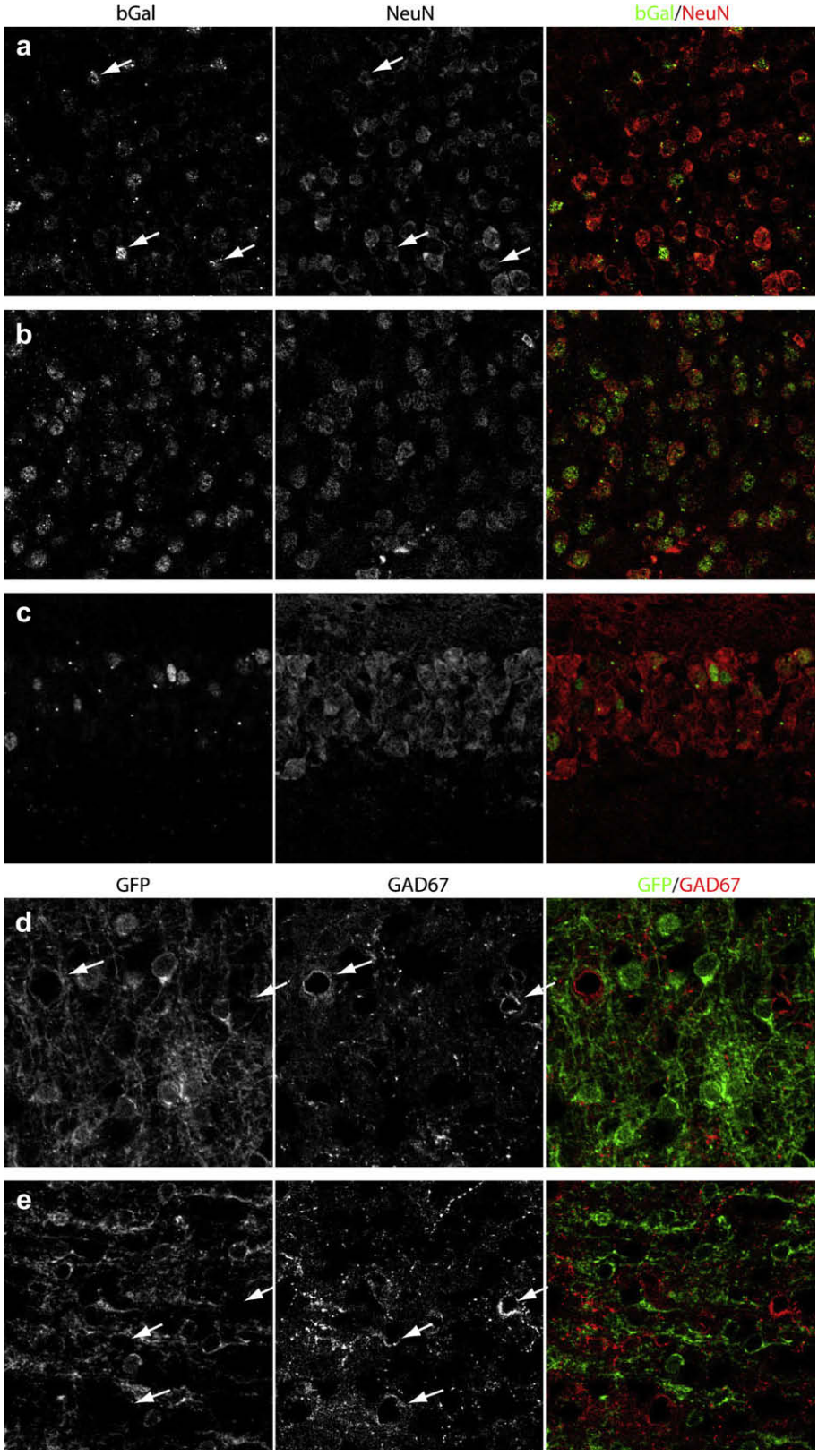

Fig. 5. SERT^{cre} recombined cells are principal neurons. Section of P7 SERT^{cre}-R26R brain was co-immunostained with beta-galactosidase and Neu-N (a–c). In layer VI isocortex (a), VB nucleus of the thalamus (b) and CA3 field of the hippocampus (c), all beta-galactosidase positive cells coexpressed Neu-N, though the proportion of beta-galactosidase positive neurons among the total Neu-N labelled population is very different depending on the region. Section of a P7 SERT^{cre}-Tau^{mGFP} brain were immunostained with GFP and GAD67 (d,e). In the layer VI of the isocortex (d) and in the prefrontal cortex (e), no GFP expressing neuron coexpressed GAD67.

portion of the superior olivary nucleus and the inferior colliculus. These observations underline potentially other important cellular targets of SSRIs during critical developmental periods that would require closer investigation.

The functional role of transient SERT expression in non-serotonergic neurons of the brain has been most extensively investigated in the sensory systems, in thalamocortical and the retino-geniculate projections (rev. in Gaspar et al., 2003). In both thalamic and retinal neurons, SERT expression appears to be needed for maintaining the homeostasis of 5-HT at developing axon terminals during the critical period. In particular SERT is required to set the level of activation of presynaptic 5-HT receptors. The presynaptic 5-HT1B receptors and SERT are expressed simultaneously and at similar locations in thalamic and retinal axons. 5-HT1B receptors have two main downstream effects, one is a powerful effect on glutamate release (Rhoades et al., 1994; Laurent et al., 2002), the other is a modulation of levels of cAMP, which in turn can modulate the response of axons to attractive guidance factors such as netrins

Table 2
Review of the effects of administration of antidepressants during development in rodents

	Period	Drug/dose/administration	Effect in adults	Reference
Rats	E14–P7	Fluoxetine 10 mg/kg minipumps	- Reduced SERT in raphe - Decrease activity (elevated maze) - Reinforcing effects of cocaine	Forcelli and Heinrichs (2008)
Mice	P4–P21	Fluoxetine 10 mg/kg Clomipramine 20 mg/kg Citalopram 10 mg/kg IP	- Increased anxiety behaviour	Ansorge et al. (2008)
Mice	P5–P19	Escitalopram 10 mg/kg Subcutaneous	- Increased REM sleep - Anhedonia - Increased 5-HT1A autoreceptor function	Popa et al. (2008)
Mice	Pregnancy and lactation	Fluoxetine 7.5 mg/kg Gavage	- Reduced impulsivity - Immobility in Forced swim test	Lisboa et al. (2007)
Rats	E6–E20	Fluoxetine 8–12 mg/kg orally	- Delay in motor development - Reduced birth weight - Reduced weight gain during preweaning	Bairy et al. (2007)
Rats	E11–E21	Fluoxetine 10 mg/kg Gastric gavage	- Fetal pulmonary hypertension - Increased pulmonary arterial smooth muscle proliferation rate - Increased postnatal mortality	Fornaro et al. (2007)
Rats	P8–P21	Citalopram 5 mg/kg/12 h Subcutaneous	- Reduced TPH2-IR in raphe - Reduced SERT axons in cortex - Increased locomotor activity - Reduced sexual behaviour	Maciag et al. (2006)
Guinea-pig	Pregnancy	Fluoxetine 3.5 or 7 mg/kg/D Osmotic pump	- Increased thermal pain threshold	Vartazarmian et al. (2005)
Mice	P4–P21	Fluoxetine 10 mg/kg IP	- Decreased exploratory behaviour - Increased stress response	Ansorge et al. (2004)
Rats	Pregnancy	Fluoxetine 8 mg/kg Subcutaneous	- Maternal aggressive behaviour - Increased ocytocin receptor number with lower affinity	Johns et al. (2004)
Rats	P1–P19	Citalopram 20 mg/kg/3D Subcutaneous	- Reduced aggressive behaviour	Manhães de Castro et al. (2001)
Hamsters	P8–P21	Clomipramine 15 mg/kg	- Increased Locomotor activity - Anxiety related behaviour-Decreased 5-HT hypothalamus, cortex	Yannielli et al. (1999)
Rats	E13–E20	Fluoxetine10 mg/kg Subcutaneous	- SERT density decreased in hypothalamus, hippocampus and amygdala in prepubescent offspring	Cabrera-Vera and Battaglia (1998)
Rats	E13–E20	Fluoxetine 10 mg/kg Subcutaneous	- 28% reduction of 5-HT in the frontal cortex of prepubescent but not adult rat	Cabrera-Vera et al. (1997)
Rats	P8–P21	Clomipramine 20 mg/kg/12 h Subcutaneous	- Lower firing rate in raphe neurons	Kinney et al. (1997)
Rats	E7–E20	Fluoxetine 1, 5, or 12 mg/kg Gavage	- Maternal weight loss - Increased neonatal mortality	Vorhees et al. (1994)
Rats	E13–E20	Fluoxetine 10 mg/kg Subcutaneous	- Reduced weight at birth and at P70 - Reduction of 5-HT2A/2C receptors - Attenuated adrenocorticotropin response to DOI	Cabrera and Battaglia (1994)
Rats	P7–P21	Clomipramine 25 mg/kg IP	- Neonatal REM suppression - Increased ethanol intake	Hilakivi et al. (1986)

(Bonnin et al., 2007) or to repellent molecules as ephrins (Nicol et al., 2006). When SERT function is disrupted, excessive 5-HT1B receptor activation could cause reduced glutamate release and altered response to guidance cues. Similar functions of SERT as gatekeeper for activation of different 5-HT receptors could exist in other cell types in which SERT is developmentally expressed. For instance in the heart, embryonic blockade of SERT function alters heart development (Yavarone et al., 1993; Sari and Zhou, 2003), possibly by modulating the activation of 5-HT2B receptors (Choi et al., 1997). Interestingly, in the limbic areas in which we find a developmental expression of SERT, such as the medial prefrontal, the anterior cingulate cortical areas and the CA3 field of the hippocampus, high levels of the 5-HT2A and 5-HT2C receptors are expressed, seemingly in the same neurons as those expressing the SERT (Cornea-Hebert et al., 1999; Stankovski et al., 2007). Activation of these receptors modulates prefrontal cortical network activity (Lambe and Aghajanian, 2006) by increasing neuronal excitability. Thus, the level of activation of 5-HT2 receptors could be finely modulated by SERT expression in the prefrontal cortical neurons thereby influencing the level of activity in these neuronal circuits during critical time points in development.

Interestingly, limbic neuronal circuits in the prefrontal and cingulate cortex are implicated in emotional control, and reinforcing behaviours, that have been found to be modified after early treatment of mouse pups with different SSRI compounds administered during late embryonic or early postnatal life (detailed in Table 2). Besides controlling neuronal excitability in the cortical neurons, 5-HT2 receptors could also have trophic effects (rev. in Vitalis and Parnavelas, 2003). Dendritic maturation (Bou-Flores et al., 2000) and developmental cell death in the upper cortical layers of the cingulate cortex (Stankovski et al., 2007) can be modulated by 5-HT2 receptor activation. Other maturation events, particularly in the hippocampus, could involve an interaction between SERT and the 5-HT1A receptors during critical developmental periods, since several developmental effects linked to SERT dysfunction could be corrected by 5-HT1A antagonists (Alexandre et al., 2006), that are also known to have trophic effects on neurite and dendrite outgrowth (Durig and Hornung, 2000).

Given the widespread expression of SERT in development, one may question the safety of administration of SSRIs during pregnancy or infancy in humans. SERT is also expressed more broadly in the human embryonic brain compared to adults (Verney et al., 2002) but a full description of these localizations could not be obtained. In non-human primates, transient SERT expression is found in major sensory afferents, such as retinal inputs and cochlear inputs and in sensory ganglia but not in thalamocortical neurons (Lebrand et al., 2006). Thus, the spatio-temporal characteristics of transient SERT expression differ between primates and rodents, although the general trend for more widespread expression of SERT during development is conserved. Potential hazards of administration of SSRIs during embryonic or postnatal periods have been repeatedly analysed in prospective or retrospective studies in humans. Most of these studies concluded to the lack of major fetal anomalies or any later effects on child development, including intellectual performance (Nulman et al., 2002), although several recent studies emphasized potential risk of serotonin withdrawal syndrome (Sanz et al., 2005) or of pulmonary hypertension (Chambers et al., 2006). In contrast, numerous developmental effects of SSRI administration during gestation or early postnatal life have been found in rodent models showed conspicuous effects (reviewed in Table 2). These effects range from global effects on adult behaviour, such as change in sleep patterns (Hilakivi et al., 1986; Popa et al., 2008), modified alcohol intake, anxiety responses (Yannielli et al., 1999; Ansorge et al., 2004, 2008; Forcelli and Heinrichs, 2008), reduced aggressivity (Manhães de Castro et al., 2001; Lisboa et al., 2007), increased helplessness (Popa et al., 2008). The neurochemical bases for these behavioural changes may be related to changes in the serotonin metabolism since reduced SERT expression (Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998; Forcelli and Heinrichs, 2008; Maciag et al., 2006), reduced firing rates of raphe neurons (Kinney et al., 1997), and reduced expression of 5-HT2A receptors (Cabrera and Battaglia, 1994) were reported. Minor changes in the development of the somatosensory cortex were also noted (Zhou et al., 2000) that partly mimicked the altered development of thalamocortical projection in SERT KO mice (Persico et al., 2001; Salichon et al., 2001). Interestingly, the behavioural phenotype caused by a functional blockade of SERT during development is different from the SERT blockade in adults (Ansorge et al., 2004) suggesting that transient SERT expression could contribute to the developmental effects of SSRIs. Interestingly, similar conditions could exist in human diseases, since the result of a genetic reduction in SERT function increase the risk for developing depression or anxiety traits, countering the known therapeutic effects of SSRIs (rev. in Lesch and Gutknecht, 2005; Murphy and Lesch, 2008). This suggests a major influence of the developmental effects of SERT function in disease pre-disposition.

5. Conclusion

The present re-assessment of developmental SERT expression with novel genetic tools allowed to characterize more accurately the sites of SERT expression in the limbic areas of the brain, such as the prefrontal cortex, the cingulate cortex and the hippocampus. The broad expression of SERT during development should arouse a greater awareness of the multiple cellular targets of SSRIs during development besides the serotonin neurons themselves.

Acknowledgements

This work was supported by the Inserm, and University Paris 6. Funding for this project was from Agence Nationale de la Recherche (ANR605-neur-046), NeRF and from the European Commission (FP7-health-2007-A-201714). We thank Luc Maroteaux for discussions and Gaelle Angenard for technical help.

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