Comparative Aspects of Subplate Zone Studied with Gene Expression in Sauropsids and Mammals

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There is currently a debate about the evolutionary origin of the earliest generated cortical preplate neurons and their derivatives (subplate and marginal zone). We examined the subplate with murine markers including nuclear receptor related 1 (Nurr1), monooxygenase Dbh-like 1 (Moxd1), transmembrane protein 163 (Tmem163), and connective tissue growth factor (Ctgf) in developing and adult turtle, chick, opossum, mouse, and rat. Whereas some of these are expressed in dorsal pallium in all species studied (Nurr1, Ctgf, and Tmem163), we observed that the closely related mouse and rat differed in the expression patterns of several others (Dopa decarboxylase, Moxd1, and thyrotropin-releasing hormone). The expression of Ctgf, Moxd1, and Nurr1 in the oppossum suggests a more dispersed subplate population in this marsupial compared with mice and rats. In embryonic and adult chick brains, our selected subplate markers are primarily expressed in the hyperpallium and in the turtle in the main cell dense layer of the dorsal cortex. These observations suggest that some neurons that express these selected markers were present in the common ancestor of sauropsids and mammals.

Keywords: amygdala, cerebral cortex, chick, claustrum, Cplx3, Ctgf, Monodelphis domestica, Moxd1, Nurr1, progesterone receptor, subplate, Tmem163, Trh, turtle

Introduction

The development of the mammalian cerebral cortex starts with the generation of the preplate zone. The preplate is subsequently split into a superficial marginal zone (future layer 1) and a deeper subplate due to the arrival of later generated cortical plate neurons (Marín-Padilla 1971; Luskin and Shatz 1985; Pearlman and Sheppard 1996; Bystron et al. 2008). The cells of the preplate and its derivatives (marginal zone and subplate) arrive in the dorsal cortex through 2 migratory pathways: radial migration from the cortical ventricular zone and tangential migration from the ventral pallium, specifically from the border of the pallium and the subpallium (Parnavelas 2000; Marín and Rubenstein 2003; Bielle et al. 2005; García-Moreno et al. 2007). The earliest preplate neurons arrive through tangential migration (Bystron et al. 2006; Espinosa et al. 2009). The subplate layer contains a variety of cell types with different developmental origins, survival, connectivities, and structural and functional characteristics (Antonini and Shatz 1990; Kostovic and Rakic 1990; Allendoerfer and Shatz 1994; Hanganu et al. 2001; Hevner and Zecevic 2006; Hoerder-

Suabedissen et al. 2009; Luhman et al. 2009; Kanold and Luhmann 2010). Arguably, the 3 most distinct features of the subplate are its deep anatomical location below the cortical layer, the presence of some of the earliest neurons in the cortex and the transient targeting of thalamocortical projections. Subplate cells have been shown to pioneer the corticofugal pathway and to be involved in the guidance of thalamocortical afferents (TCAs) to the cortex (McConnell et al. 1989; De Carlos and O'Leary 1992; Molnár and Blakemore 1995). Neurons in the subplate establish functional synapses with the TCAs during the "waiting period" (Higashi et al. 2002; Molnár et al. 2003). They play a role in the establishment of functional modules such as monocular dominance columns in the cat and the barrel formation in the mouse (Lund and Mustari 1977; Shatz and Luskin 1986; Kostovic and Rakic 1990; Herrmann et al. 1991; Ghosh and Shatz 1992; Piñon et al. 2009).

There are marked differences in the thickness of the subplate zone in rodents, carnivores, humans, and nonhuman primates. A thicker subplate is observed in relation to the cortical plate in the larger brained mammals (Kostovic and Rakic 1990). This suggests that the evolutionary expansion of the neocortex and the expansion of the subplate zone are linked. In addition, Aboitiz (1999) and Supèr and Uylings (2001) hypothesized that the evolutionary appearance of the subplate zone led to a change in the axonal ingrowth pattern from above to below the cortical plate. They suggested that this change was crucial for the radial expansion of the dorsal cortex and the development of the 6-layered mammalian neocortex.

The questions we want to address in this study are: What are the evolutionary origins of the subplate neuronal populations and how are they associated with the evolution of a larger and more complex dorsal cortex?

Phylogenies based on morphological, fossil, and sequence data are all in agreement that the sauropsid and therapsid lineages diverged from a common amniote ancestor approximately 310 million years ago (MYA). The sauropsids evolved into the extant reptile and bird lineages and the therapsids into the mammals (Hedges 2002; Xia et al. 2003; Butler and Hodos 2005; Zhang et al. 2005; see Fig. 1 for phylogenetic relationships).

There are currently 3 distinct hypotheses about the phylogenetic origin of subplate neurons. The first hypothesis is that these neurons were all already present in the common
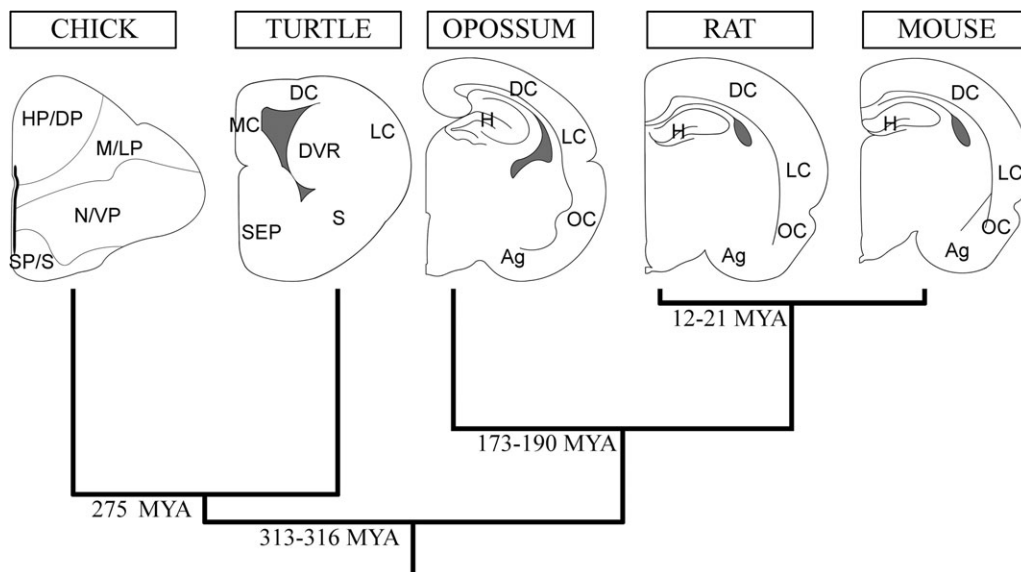
Figure 1. Cladogram showing the phylogenetic relationship of the species examined (turtle, chick, mouse, rat, and opossum) in our study (based on Gibbs et al. 2004; Murphy et al. 2004).

ancestor of mammals and sauropsids. Previous studies, mainly based on morphological observations, have proposed that cells comparable with the mammalian preplate and subplate cells are present in the reptile dorsal pallium (Marín-Padilla 1971, 1998; Goffinet 1983; Nacher et al. 1996; Cordery and Molnár 1999; Aboitiz et al. 2005). Marín-Padilla (1978) proposed that the dorsal cortex in reptiles is equivalent to the preplate in mammals, while the mammalian cortical plate has no equivalent structure in reptiles. On the other hand, it has been suggested that the external plexiform layer corresponds to the marginal zone, the inner plexiform layer to the subplate and the cell dense layer (CDL) to the infragranular layers (Reiner 1991; Karten 1997; Supèr et al. 1998; Aboitiz et al. 2005). In either case, these observations support the idea that the subplate evolved early in vertebrate evolution, before the divergence of mammals and sauropsids some 300 MYA.

The second alternative hypothesis is that the subplate is unique to mammals and increases in anatomical complexity as the neocortex increases in size in different lineages. According to this hypothesis, the subplate evolved to support the development of cortical connectivity in mammals and does not have a homologue in the reptilian cortex. Thus, the subplate zone is considered an embryonic adaptation that appeared in mammals, after the divergence of mammals and sauropsids (Kostovic and Rakic 1990; Supèr and Uylings 2001; Molnár et al. 2006) and possibly only after the divergence of marsupials and eutherian mammals, as it has been suggested that there is no subplate layer in marsupials, either. In marsupials, the earliest generated cells are instead incorporated into the lower cortical plate. Consistently, the TCAs appear to pass directly into layer 4, without a waiting period in marsupials (Harman et al. 1995; Reep 2000; Pearce and Marotte 2003). Eutherian mammals, on the other hand, have a distinct subplate layer located between the cortical plate and the white matter suggesting that specialized subplate cells only evolved in this line of mammals (Reep 2000).

Primates have the largest and most complex subplate that is generated over a longer period of cortical neurogenesis (Smart et al. 2002; Lukaszewicz et al. 2005) and is largely transient in nature (Kostovic and Rakic 1990; Dehay and Kennedy 2007; Bayatti et al. 2008; Wang et al. 2010). It is not known whether the increase in subplate size relative to cortex in primates is due to the expansion of a specific subplate cell population or due to the increase in diversity and complexity of several subpopulations.

A third hypothesis is a combination of the 2 hypotheses above and states that the subplate in mammals is a combination of new and ancestral cell populations (Aboitiz 1999; Aboitiz et al. 2005). This would imply that embryonic subplate cells were present in the common ancestor of both mammals and sauropsids. Following the divergence of these 2 lineages, additional populations of subplate cells evolved in mammals as the dorsal cortex (neocortex) became progressively larger and more complex.

Recent advances in cell sorting and separation combined with microarray- or sequencing-based approaches have provided excellent opportunities for establishing the molecular taxonomy of cortical neuronal subtypes (Nelson et al. 2006; Sugino et al. 2006; Molyneaux et al. 2007). Lately, several laboratories have developed valuable markers to reveal subplate neurons (Heuer et al. 2003; Hoerder-Suabedissen et al. 2009; McKellar and Shatz 2009; Osheroff and Hatten 2009; Wang et al. 2009). These new markers are selectively present in murine subplate cells at different developmental stages, and at least some of them change their position in reeler mutant where the cortical organization is altered (Hoerder-Suabedissen et al. 2009) and colocalise with bromodeoxyuridine label in early born neurons (Heuer et al. 2003).

In this study, we utilize a combination of these new molecular markers (Cplx3, *Ctgf*, *Moxd1*, Nurr1, and *Tmem163*) in a comparative analysis of developing and adult sauropsids (chick and turtle), marsupials (opossum), and eutherians (rat and mouse). An overview of the predicted functions of these genes is summarized in Table 1. We aim to determine if there are common features of the subplate organization that all of these species share due to common ancestry.

Materials and Methods

Animals

All animal experiments performed in the United Kingdom were approved by a local ethical review committee and conducted in accordance with personal and project licenses under the UK Animals (Scientific Procedures) Act (1986). All protocols from University of California Davis (UCD), USA were approved by IACUC and conformed to NIH guidelines. Protocols from University of Melbourne, Australia were approved by the Animal Experimentation Ethics Committee and conformed to NHMRC guidelines. Protocols in Chile have been authorized by the Ethics Committee of the Center for Medical Research, Ponticia Universidad Católica de Chile, the Center for Biomedical Research, Faculty of Medicine, Diego Portales University, Santiago, Chile and under authorization from Chilean Servicio Agricola y Ganadero. Table 2 gives numbers and developmental stages of all animals used in this study.

Mice (Mus musculus) and Rats (Rattus norvegicus)

C57/BL6 mice were obtained from the Oxford University Animal Facility, Wistar rats from Harlan Laboratories, UK and maintained in the Oxford University Animal Facility. At least 3 brains were collected for each age (postnatal day (P) 8 and adult) and species. For in situ hybridization, animals were killed by cervical dislocation, brains were dissected in ice-cold phosphate-buffered saline (PBS) and fresh-frozen in TissueTek OCT compound (Sakura Finetek). For immunohistochemistry, animals were deeply anesthetized with pentobarbitone (Euthatal 150 mg/kg intraperitoneally; Merial Animal Health Ltd) and perfused through the heart with 4% paraformaldehyde (PFA). Brains were dissected and postfixed in 4% PFA for 24–48 h.

South American Gray Short-Tailed Opossum (Monodelphis domestica)

Brains from P20, P44, and adult animals were obtained from N. Saunders (University of Melbourne) and L. Krubitzer (UCD). Mothers were

Table 1

Overview of the subplate markers studied

Gene symbol	Gene name	Function	Expression in murine subplate	References
Cplx3	Complexin 3	Regulation of Ca^{2+}-triggered vesicular release at retinal ribbon synapses	Only postnatal expression; in non-GABAergic neurons	Reim et al. (2005) and Hoerder-Suabedissen et al. (2009)
Ctgf	Connective tissue growth factor	Secreted matricellular protein; regulation of extracellular matrix production; angiogenesis	From late embryonic (E17) into adulthood	Frazier et al. (1996), Babic et al. (199), Heuer et al. (2003), Watakabe et al. (2007), and Hoerder-Suabedissen et al. (2009)
Ddc	Dopa decarboxylase	Production of dopamine and serotonine	Only postnatal expression; in non-GABAergic and nondopaminergic cells	Hoerder-Suabedissen et al. (2009)
Moxd1	Monooxygenase D1	Copper type II ascorbate-dependent monooxygenase; homologue to dopamine beta-hydroxylase; substrate unknown	Only postnatal expression	Chambers et al. (1998) and Hoerder-Suabedissen et al. (2009)
Nurr1/Nr4a2	Nuclear receptor related 1	Orphan nuclear receptor; primarily investigated for its role in the differentiation and survival of dopaminergic neurons	From early embryonic (E15) into adulthood in glutamatergic neurons.	Zetterström et al. (1997), Saucedo-Cardenas et al. (1998), Arimatsu et al. (2003), Hoerder-Suabedissen et al. (2009), and Wang et al. (2009)
Tmem163	Transmembrane protein 163	Unknown	Peak of expression in early postnatal stages	Hoerder-Suabedissen et al. (2009)
Trh	Thyrotropin-releasing hormone	Hormone; excitatory neurotransmitter and/or neuromodulator	Not previously described	Nillni and Sevarino (1999), Broberger and McCormick (2005), and Hara et al. (2009)

Table 2

Overview of the sample analysis

Species	Stage	Number of specimens	Fixation procedure	Selecting procedure	Probes and antibodies	Figure related
Mouse (Mus musculus)	P8	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf, Moxd1, Tmem163, Trh	Figures 2–4 and Supplementary Figure 1
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Cplx3, Ddc, Nurr1	
	Adult	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf, Moxd1, Tmem163, Trh	
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Cplx3, Ddc, Nurr1	
Rat (Rattus rattus)	P8	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf, Tmem163 Rat probes for Moxd1, Trh	Figures 2 and 3 and Supplementary Figure 1
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Cplx3, Ddc, Nurr1	
	Adult	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf, Tmem163 Rat probes for Moxd1, Trh	
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Cplx3, Ddc, Nurr1	
Opossum (Monodelphis domestica)	P20	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf Opossum probe for Moxd1	Figure 6
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibody against: Nurr1	
	P44	n = 2 (IHC)	4% PFA	Paraffin section 10–14 μm	Antibody against: Nurr1	
	Adult	n = 2 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Mouse probes for Ctgf Opossum probe for Moxd1	
		n = 2 (IHC)	4% PFA	Vibratome 40 μm	Antibody against: Nurr1	
Chick (Gallus gallus)	E19	n = 4 (ISH)	Fresh frozen	Cryosections (14–16 μm)	Chick probes for Ctgf, Moxd1, Tmem163 Nurr1	Figure 8
		n = 2 (IHC)	Fresh frozen	Cryosections (14–16 μm)		
	Adult	n = 2 (ISH)	Fresh frozen	Cryosections (20 μm)	Chick probes for Ctgf, Moxd1, Tmem163	Supplementary Figure 3
		n = 2 (IHC)	4% PFA	Vibratome 40 μm	Antibody against: Nurr1	
Turtle (Pseudemys scripta elegans)	S25	n = 2 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Nurr1, Ctgf	Figure 7
	Adult	n = 2 (ISH)	Fresh frozen	Cryosections (16 μm)	Chick probes for Ctgf, Moxd1	Figure 7 and Supplementary Figure 2
		n = 4 (IHC)	4% PFA	Vibratome 40 μm	Antibodies against: Nurr1, Ctgf, Cplx3, Foxp2, Calretinin, GAD65/67	

Note: Antibodies supplies: Cplx3 (gift from Dr Kerstin Reim), Nurr1 (R&D Systems, AF2156), Ddc (Abcam, ab 3905), Ctgf (Abcam, ab6992), Foxp2 (Abcam, ab16046), Calretinin (Chemicon, AB149), and GAD65/67 (Millipore, AB1511). IHC, Immunohistochemistry; ISH, In situ hybridization.

anesthetized with isoflurane, while the pups were collected from their teats. For immunohistochemistry on paraffin sections, pups were terminally anesthetized with isoflurane, brains fixed in 4% PFA for 24 h, immersed in Bouin's fixative for 24 h, dehydrated in ethanol, and embedded in paraffin (Saunders et al. 1989). Adult opossums were terminally anesthetized with inhaled isofluorane, perfused through the aorta with 4% PFA and brains processed for paraffin embedding. In situ hybridization on opossum brains was performed using fresh-frozen tissues collected the same way as outlined above.

Chicken (Gallus gallus)
Fertile hen's eggs were incubated at 37 °C in a humidified chamber to E19 stage. The embryos were staged according to Hamburger and Hamilton (1992). The eggs were placed on ice to anesthetize the embryos, and brains were collected by decapitation. For immunohistochemistry and in situ hybridization, brains were immersion fixed in 4% PFA for 24 h and fresh-frozen, respectively. The adult chicken brains were obtained from a commercial abattoir. The brains were postfixed in 4% PFA for 2–3 days for immunohistochemistry and fresh-frozen for in situ hybridization. At least 2–4 brains from each stage were used in each experiment. We followed Puelles et al. (2007) for the nomenclature.

Turtle (Pseudemys scripta elegans, Freshwater Red-Eared Slider)
For the developmental stages, the turtle eggs were obtained from Harvey Kliebert's Reptile Farm in Louisiana with permission from the Louisiana Department of Agriculture and Forestry. The embryos were staged according to Yntema (1968) and Cordery and Molnár (1999). The adult turtle brains were obtained from the Center for Biomedical Research, Faculty of Medicine, Diego Portales University, Santiago, Chile. Adults and embryos were anaesthetized by chilling in ice water for 30 min and then decapitated. The brains were fixed in 4% PFA for 2–3 days for immunohistochemistry and fresh-frozen for in situ hybridization.

Analysis of Sequence Homologies
A brief overview of the selected markers for this study is summarized in Supplementary Table 1. Full-length cDNA and protein sequences of Cplx3, Ctgf, Moxd1, Nurr1, and Tmem163 were obtained from the NCBI database for the mouse, rat, human, chick, and opossum or from the UCSC genome browser (opossum) using the mouse sequence for the search. Adjacent genomic regions were compared for the presence of flanking genes to ensure that the correct gene had been identified in opossum. Pairwise comparison against the mouse protein sequences was performed using ClustalX, and the number of amino acid substitutions (or length of gaps) counted. Turtle sequences were not available for analysis.

Immunobistochemistry
Immunohistochemistry was performed against Nurr1 for mouse, rat, chick, turtle, and opossum, against Ddc for mouse and rat, and against Cplx3 for mouse, rat, and turtle. Turtle sections were additionally stained for Foxp2, Ctgf, calretinin, and Gad65/67. Table 2 shows details of numbers of animals used for various conditions. Brains fixed in 4% PFA were cut coronally at 40–50 μm thickness on a vibrating microtome (VT1000S; Leica Microsystems). Opossum brains embedded in paraffin wax were cut at 10–14 μm. For these sections, we used standard antigen retrieval protocols before immunostaining (Bayatti et al. 2008). For nonfluorescent permanent immunohistochemistry sections were quenched in 1.5% hydrogen peroxide and then blocked with 5% donkey or goat serum (Sigma) in Tris-buffered saline with (for Nurr1 and Ddc) or without (for Cplx3) 0.1% Triton-X100 (BDH) for 2 h at room temperature (RT). Sections were incubated with primary antibody (goat anti-Nurr1, 1:200, R&D Systems, AF2156; rabbit anti-Cplx3, 1:3000, gift K. Reim; rabbit anti-Ddc, 1:2000 Abcam, ab3905; Foxp2 1:3000, Abcam, ab16046; rabbit anti-calretinin 1:500, Chemicon, Ab1550; rabbit anti-GAD65/67 1:1000, Chemicon, Ab1511; and rabbit anti-Ctgf 1:1000, Abcam, ab6992) in 1% serum with or without 0.1% Triton-X100 overnight at 4 °C. Secondary antibody (biotinylated donkey antigoat IgG, 1:200, Ab6884 and biotinylated donkey antirabbit IgG, 1:200, Ab6720, both Abcam) in 1% serum was applied for 2 h at RT.

Sections were reacted with avidin–biotinylated enzyme complex using the Vectastain Elite kit (Vector) and diaminobenzidene (Vector) according to the manufacturer's instructions. For fluorescent immuno-histochemistry secondary antibodies labeled with Alexa Fluor 488 or 568 (Invitrogen, A21206, A10042) in 1% serum were applied for 2 h at RT and sections were counterstained with 4′,6-diamidino-2-phenyl-indole (DAPI).

In Situ Hybridization
In situ hybridization was performed against Ctgf and Moxd1 for mouse, rat, chick, turtle, and opossum, Tmem163 for mouse, rat, and chick, and Trh for mouse and rat. Fresh-frozen brains were sectioned to 14–16 μm coronally on a cryostat (Jung CM3000; Leica). Species-specific ribop-robes were synthesized from respective cDNAs. Total RNA was extracted from brains of individual species and the first strand cDNA was synthesized using Superscript III reverse transcriptase together with random hexamers (Invitrogen) following the manufacturer's instructions. Supplementary Table 2 lists the forward and reverse primers used to generate gene-specific cDNA fragments using poly-merase chain reaction (PCR). The resulting PCR products were individually ligated into the pST-Blue 1 plasmid (Novagen) and confirmed by sequencing. The antisense and sense (a negative control) cRNA probes were transcribed using T7 and SP6 RNA polymerase with digoxigenin (DIG)-labeled RNA mixture, respectively (Roche). The in situ hybridizations were performed as previously described (Hoerder-Suabedissen et al. 2009). Briefly, frozen sections were fixed with 4% PFA in PBS for 30 min, deproteinized with 0.1 N HCl for 5 min, acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride), and prehybridized at RT for at least 1 h in a solution containing 50% formamide, 10 mM Tris, pH 7.6, 200 μg/mL Escherichia coli transfer RNA, 1× Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% sodium dodecyl sulfate, and 1 mM ethylenediaminetetra-acetic acid. The sections were hybridized in the same buffer with the DIG-labeled probes overnight at 66–68 °C. After hybridization, sections were washed to a final stringency of 30 mM NaCl/3 mM sodium citrate at 66–68 °C and detected by anti-DIG-alkaline phosphatase antibody in conjunction with a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Roche). Apart from using species-specific probes, mouse Ctgf probe was also used on rat and opossum, mouse Tmem163 probe on rat. Chicken Ctgf and MoxD1 probes were used on turtle as these genes are likely to share certain degree of sequence homology (Supplementary Table 1).

Microscopy and Image Analysis
Sections were imaged on a DMR transmission light microscope (Leica) or confocal (Zeiss LSM710). Images for publication were contrast adjusted and compiled using Adobe Photoshop CS3 and Adobe Illustrator CS3. The schematic summary illustrations were constructed after detailed microscopic analysis of several serial sections from at least 3 individuals, and similarities and differences were color coded on schematic coronal sections using Illustrator.

Results

Sequence Homologies of Subplate Markers across Species
The predicted protein sequences for Cplx3, Ctgf, Moxd1, Nurr1, and Tmem163 were compared between mouse, rat, opossum, chicken, and human (Supplementary Table 1). Homologous protein sequences were found in all 5 species and the degree of conservation compared with mouse was high overall (above 70%). Nurr1 shows the greatest conservation; it is nearly identical between mouse, rat, and human and nearly 95% conserved between mouse and chick. The Ctgf protein is also very well conserved between mouse and rat and only diverges by around 10% for human, opossum, and chick. Cplx3 is identical between mouse and rat but diverges by over 20% between mouse and chick. MoxD1 and Tmem163 proteins are

predicted to be present in all species, with varying degrees (73–95% and 82–97%, respectively) of conservation. Of all proteins compared, *Moxd1* shows a slightly greater divergence between mouse and rat (see Supplementary Table 1). Functional domains, however, are well conserved, suggesting a similar function in both species. Figure 1 demonstrates a cladistic perspective of the species selected for examination in our current work based on Gibbs et al. (2004).

### Comparison of Selected Subplate Gene Expression Patterns in Mouse and Rat

We started our analysis by comparing gene expression patterns in the subplate of early postnatal and adult mice and rats. We investigated 7 murine subplate markers either using immuno-histochemistry (Cplx3, Ddc, and Nurr1) or in situ hybridization (*Ctgf*, *Moxd1*, *Tmem163*, and *Trh*). As reported previously (Hoerder-Suabedissen et al. 2009), in the P8 mouse neocortex

Cplx3 (Fig. 2*A,C*), *Ctgf* (Fig. 2*E,G*), Nurr1 (Fig. 2*I,K*), *Tmem163* (Fig. 2*M,O*), Ddc (Fig. 3*A,C*), and *Moxd1* (Fig. 3*E,G*) have a distinctive, narrow laminar distribution within the subplate. Nurr1 shows this restricted expression only in the dorsal cortex, additional layers are labeled in the lateral cortex (Fig. 2*I*). The expression pattern of *Tmem163* includes additional labeling in layer 5 in the dorsal neocortex and in all infragranular layers in the lateral neocortex (Fig. 2*M*). Ddc+ cells are also dispersed throughout the white matter (Fig 3*C*). In this study, we found that an additional gene, *thyrotropin-releasing hormone* (*Trh*), is also specifically and strongly expressed in the subplate of mice (Fig. 3*I,K*). All of these genes are also expressed in the subplate of adult mice with the exception of Ddc, which is drastically reduced in the adult (see Hoerder-Suabedissen et al. 2009).

In the P8 and adult rat cortex, the expression pattern of Cplx3 (Fig. 2*B,D*), *Ctgf* (Fig. 2*F,H*), Nurr1 (Fig. 2*J,L*), and
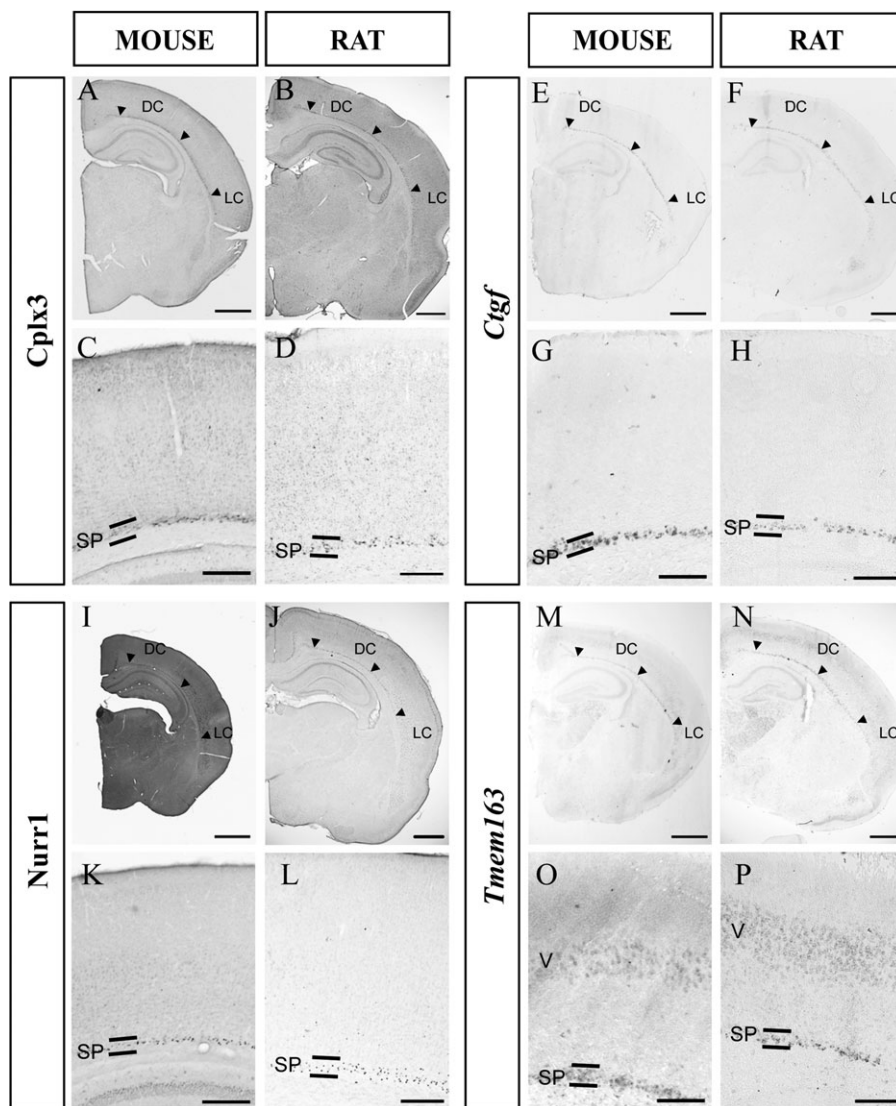
Figure 2. Genes that show similar expression patterns in mouse and rat at P8. The expression patterns of *Ctgf* and *Tmem163* mRNA and Cplx3 and Nurr1 protein in mouse (*A*, *E*, *I*, and *M*) are identical in rat (*B*, *F*, *J*, and *N*). In the dorsal cortex, Cplx3, *Ctgf*, and Nurr1 are all expressed in a distinctive narrow band within subplate both in mouse (*C*, *G*, and *K*) and in rat (*D*, *H*, and *L*). *Tmem163* is expressed in subplate as well as in layer 5 in both species (mouse: *M* and *O*; rat: *N* and *P*). Scale bars: 1 mm for *A*, *B*, *E*, *F*, *I*, *J*, *M*, and *N* and 250 μm for *C*, *D*, *G*, *H*, *K*, *L*, *O*, and *P*. DC: dorsal cortex; LC: lateral cortex; SP: subplate. In all figures, dorsal is at the top, and medial is to the left.
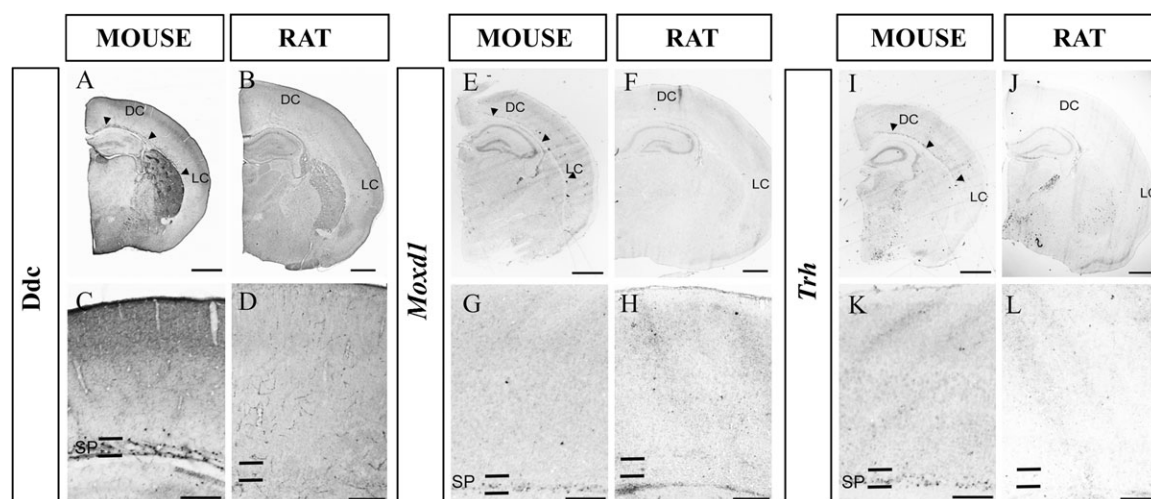
Figure 3. Genes that show differential gene expression patterns in subplate in mouse and rat at P8. Immunohistochemistry shows that Ddc+ cells are dispersed in the subplate and the white matter, forming a discernible band in mouse (*A* and *C*), while in rat, Ddc+ cells are rarely found in the subplate (*B* and *D*). Species-specific riboprobes for *Moxd1* and *Trh* are used to detect their expression. Although the extracortical expression patterns of *Moxd1* and *Trh* are the same in mouse (*E* and *I*) and rat (*F* and *J*), both genes are only detected in mouse subplate (*G* and *K*) and are absent in rat subplate (*H* and *L*). Scale bars: 1 mm for *A*, *B*, *E*, *F*, *I*, and *J* and 250 μm for *C*, *D*, *G*, *H*, *K*, and *L*. Orientation conventions and abbreviations are as in previous figure.

Tmem163 (Fig. 2*N,P*) are the same as in the mouse. Surprisingly, however, Ddc (Fig. 3*B,D*), *Moxd1* (Fig. 3*F,H*), and *Trh* (Fig. 3*J,L*) do not label any subplate cells in the rat even though the extracortical expression patterns are the same in both species (Fig 5; Supplementary Fig. 1). In order to confirm these differences in expression, we generated species-specific probes for *Moxd1* and *Trh*. We used mouse probes on rat brains and vice versa (probe homology of over 90%). Both mouse and rat probes label subplate in mouse but not in rat, which indicates that the observed difference in subplate expression is not due to different isoforms or probe specificity (data not shown).

In contrast to the observations in the mouse (Hoerder-Suabedissen et al. 2009), there are only very few dispersed Ddc+ cells in the subplate of rat, but there are some in the white matter (Fig 3*B,D*). There is no distinct Ddc+ band in the subplate in any of the examined rat brains (*n* = 5).

Extracortical Gene Expression in Mouse and Rat

Of all the selected genes, only Cplx3 is expressed exclusively in the subplate. For all other genes, additional expression in extracortical areas was observed. Overall, Ddc, *Moxd1*, *Tmem163*, and *Trh* show a wider extracortical expression than *Ctgf* and Nurr1 (Fig. 4). We focus here on their expression in the following structures: claustrum and endopiriform nuclei, piriform cortex, amygdala, basal ganglia, thalamus, and hypothalamus (these results are also summarized in Table 3; also see Supplementary Fig. 1).

In the P8 mouse, most of the genes are expressed in the lateral–ventral pallial extension of the subplate—the claustrum and the endopiriform nuclei (Fig. 4). There are a few exceptions: *Ctgf* is only expressed in the endopiriform nuclei, and *Moxd1* and *Trh* only in the claustrum. In the postnatal rat, most genes show very similar expression patterns in these structures. However, *Moxd1* is expressed in the rat endopiriform nuclei and *Trh* is not expressed in the rat claustrum. *Moxd1* and *Tmem163* are also expressed in the deep layers of

the piriform cortex, which is the main olfactory area, in both mouse and rat.

Expression of *Moxd1* and *Trh* is found in different sets of nuclei of the amygdaloid complex, both in the pallial and in the subpallial derivatives, with small differences between mouse and rat (for details, see Table 3). *Moxd1*-positive cells are scattered throughout the whole amygdaloid complex, while *Trh* expression is mostly restricted to the posteromedial cortical amygdaloid nucleus and the medial amygdaloid nucleus (MeA). In mice, but not in rats, *Tmem163* is expressed in the MeA.

Moxd1, *Tmem163*, and *Trh* genes show additional expression in the basal forebrain, in particular in the expansions of the amygdala—the bed nuclei of the stria terminalis (BST) and the substantia innominata (SI). Again, differential expression patterns are present in mouse and rat; in both species, *Moxd1* is expressed in the BST, but there is an additional expression in the striatum of the rat only. In the mouse, but not in the rat, *Tmem163*-positive cells are found in the SI and BST. *Trh* labeling is observed in the BST as well as in the striatum in rat only (Fig. 5).

Tmem163 and *Trh* show expression in the diencephalon in both species. *Trh* is expressed in the hypothalamus, the reticular thalamic nucleus, and the zona incerta, while *Tmem163* is expressed in several thalamic nuclei (Fig. 4*G,I*).

Most of these expression patterns are conserved in the adult rodents with a few differences that are summarized in Table 3. A schematic summary of these comparative gene expression patterns between rat and mouse is illustrated in Figure 5.

Expression of Ctgf, Moxd1 and Nurr1 in the Developing and Adult Cortex of *M. domestica*

In order to investigate, whether the presence of subplate is a general feature in mammals, we analyzed the expression of several subplate markers in postnatal and adult opossum brains (*M. domestica*). In this species, the existence of a distinct subplate zone has been questioned (Harman et al. 1995;
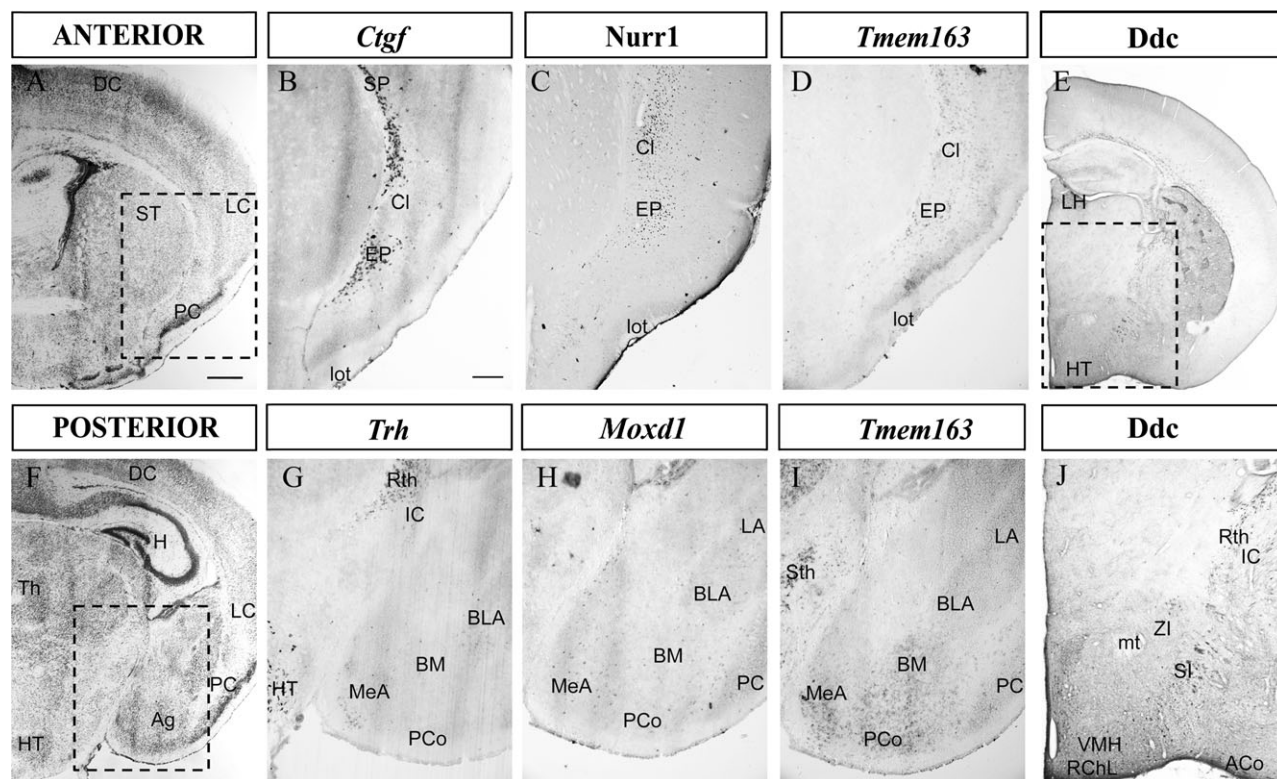
Figure 4. Extracortical expression of *Ctgf*, Nurr1, *Tmem163*, Ddc, *Trh*, and *Moxd1* in the P8 mouse in anterior (upper row) and posterior (lower row) sections. The expression of these genes extends beyond the cortical subplate toward the lateral–ventral pallial derivatives corresponding to divisions of the claustroamygdalar complex and for some, into the diencephalon. (*A*) Cresyl violet stained section through the anterior part of the hemisphere, the boxed area indicates the lateral–ventral pallial region enlarged in *B–D*. (*B*) *Ctgf* mRNA expression is only present in the endopiriform nucleus (EP). (*C*) Nurr1 immunoreactive cells are situated in the claustrum (Cl) and EP. (*D*) *Tmem163* mRNA expression is similarly in the Cl and EP. (*E* and *J*) Ddc immunoreactive cells are scattered in many areas including reticular nucleus (Rth), zona incerta (ZI), ventralmedial hypothalamus (VMH), retrochiasmatic area, lateral part (RChL), mammillothalamic tract (mt), anterior cortical amygdaloid nuclei (ACo), and substantia innominata (SI). (*F*) Cresyl violet stained section from the posterior brain. The gene expression shown in the right hand panels (*G–I*) was imaged in the boxed area of (*F*). (*G*) *Trh* is predominantly expressed in the medial amygdala (MeA) and additionally in the basolateral amygdala (BLA) but not in the basomedial amygdala (BM). *Trh* is also expressed in the Rth and the hypothalamus (HT). (*H*) *Moxd1*+ cells are scattered more evenly in the whole amygdaloid complex. (*I*) *Tmem163* expressing cells are found in the MeA, basomedial nucleus of the amygdala (BM), posterior cortical amygdaloid nuclei (PCo), and in subthalamus (Sth). Scale bars: 600 μm in *A* and applies to *E*. 250 μm in *B* and applies to *C*, *D*, and *F–H*. Ag: amygdalar; Cl: claustrum; DC: dorsal cortex; EP: endopiriform nucleus; IC: internal capsule; LA: lateral amygdaloid nuclei; LC: lateral cortex; LH: lateral habenular nuclei; lot: lateral olfactory tract; PC: piriform cortex; SP: subplate; ST: striatum; and Th: thalamus. Orientation conventions as in previous figures.

Marotte and Sheng 2000; Reep 2000). The expression patterns of *Ctgf*, *Moxd1*, and Nurr1 have been examined at developmental stages P20 and P44. P20 in opossum is estimated to be equivalent to early postnatal ages (P3–4) in rats and mice as far as cortical development is concerned (Molnár et al. 1998).

We found that all 3 genes are expressed at postnatal and adult stages in the deep compartment of the cortical plate, also called the loosely packed cortical zone (LCZ) (Fig. 6*J*-*O*). The different genes show distinctive patterns within this zone. At P20, *Ctgf*-positive cells form a dense band localized in a restricted dorsolateral area (Fig. 6*K,N*). In contrast, *Moxd1* labeled cells are scattered throughout the whole dorsolateral extension of the LCZ (Fig. 6*L,O*). In adult, both *Ctgf* and *Moxd1* labeled cells are dispersed in the deep part of the cortical plate (Fig. 6*E,F,H*, and *I*). Cortical Nurr1 expression is minimal at P20, but moderate Nurr1 staining is apparent in some cells in the deep layers of the cortex at P44 and in the adult (Fig. 6*J,D*). In both P44 and adult opossum cortex, Nurr1 labeling is mainly located in the cytoplasm around the nucleus (Fig. 6*G,M*) instead of inside the nucleus as found in extracortical regions in opossum and in all other species examined.

As in the rat and mouse, additional extracortical expression is found for all 3 genes, and again *Moxd1*-positive cells show

the widest distribution in opossum. *Moxd1* labeled cells are present in the claustrum, deep layers of the piriform cortex, basomedial nucleus of the amygdala, MeA, and BST. Nurr1 expression is found both in the claustrum and in the endopiriform nuclei, while *Ctgf* is restricted to the endopiriform nuclei (Table 4).

Ctgf, *Moxd1*, *Tmem163*, and Nurr1 Expression Patterns in Embryonic and Adult Turtle and Chick

We extended our analysis of subplate markers to nonmammalian vertebrates and looked at the expression patterns of *Ctgf*, *Moxd1*, *Tmem163*, and Nurr1 in embryonic (Stage 25) and adult turtle and embryonic (E19) and adult chick brains.

At Stage 25 in turtle, some cells within the CDL of the dorsal and medial cortex express *Ctgf* and Nurr1 (Fig. 7*Q–T*). In adult turtle, Nurr1, Ctgf/*Ctgf*, *Moxd1*, and Cplx3 are all expressed in a very similar pattern within the CDL of the dorsal and medial cortex. *Ctgf* and *Moxd1* are additionally expressed in some cells of the dorsal ventricular ridge (DVR) (Fig. 7*A–P*). Some cells in the CDL also express Foxp2 (Supplementary Fig. 2*A–D*), a transcription factor usually associated with layer 6 in mice (Ferland et al. 2003).

Table 3

Extracortical expression of the subplate murine expressed genes in rodents at P8 and adult stages

Gene	Specie	Age	Telencephalon						Diencephalon	
			Pallium				Subpallium		Hypothalamus	Thalamus
			Claustrum	Endopiriform nuclei	Piriform cortex	Pallial amygdala	Subpallial amygdala	Basal ganglia		
Ctgf	Mouse	Postnatal	−	+	−	−	−	−	−	−
		Adult	+ (caudal)	+	−	−	−	−	−	−
	Rat	Postnatal	−	+	−	−	−	−	−	−
		Adult	−	+	−	−	−	−	−	−
Ddc	Mouse	Postnatal	−	−	−	ACo	−	+	+	−
		Adult	−	−	−	−	−	−	+	−
	Rat	Postnatal	+	−	−	−	−	−	+	−
		Adult	−	−	−	−	−	−	+	−
Nurr1	Mouse	Postnatal	+	+	−	−	−	−	+	−
		Adult	+	+	−	−	−	−	+	−
	Rat	Postnatal	+	+	−	−	−	−	−	−
		Adult	+	+	−	−	−	−	−	−
Moxd1	Mouse	Postnatal	+	−	+ (deep layers)	+ (BM)	+ (MeA)	+ (BST)	−	−
		Adult	+	−	+ (deep layers)	+	+ (MeA)	+ (BST)	−	−
	Rat	Postnatal	+	+	+ (deep layers)	+ (BL; LA)	+ (MeA; CeA)	+ (BST; ST)	−	−
		Adult	+	+	+ (deep layers)	+ (BLP; BM)	+	−	−	−
Tmem163	Mouse	Postnatal	+	+	+ (deep layers)	+ (BM; Cortical)	+ (MeAV)	+ (SI; BST)	−	+ (RTh)
		Adult	+	+	+ (deep layers)	+	+ (MeA)	+ (SI; BST)	+	+
	Rat	Postnatal	+	+	+ (deep layers)	−	−	−	−	−
		Adult	+	+	+ (deep layers)	+ (BLP)	−	−	+	+
Trh	Mouse	Postnatal	+	−	−	+ (PMc)	+ (MeAR)	−	+ (PVH; LHA)	+ (RTh)
		Adult	+	−	−	+ (PMc)	+ (MeAR)	−	+ (PVH; LHA)	+ (RTh)
	Rat	Postnatal	−	−	−	+ (PMc)	+ (MeA)	+ (BST; ST)	+ (PVH; LHA)	+ (RTh)
		Adult	−	−	−	+ (PMc)	+ (MeA)	−	+ (PVH; LHA)	+ (RTh)

Note: ACo, anterior cortical amygdaloid area; BM, basomedial nucleus of the amygdala; BLP, basolateral nucleus of the amygdala, posterior part; BST, bed nucleus of the stria terminalis; CeA, central amygdaloid nucleus; LA, lateral amygdaloid nucleus; LHA, lateral hypothalamic area; MeAR, medial amygdaloid nucleus, rostral portion; MeAV, medial amygdaloid nucleus, ventral portion; nLOT, nucleus of the lateral olfactory tract; PMc, posteromedial cortical amygdaloid nucleus; PVH, paraventricular hypothalamic nucleus; RTh, reticular thalamic nucleus; SI, substantia innominata; and ST, striatum.
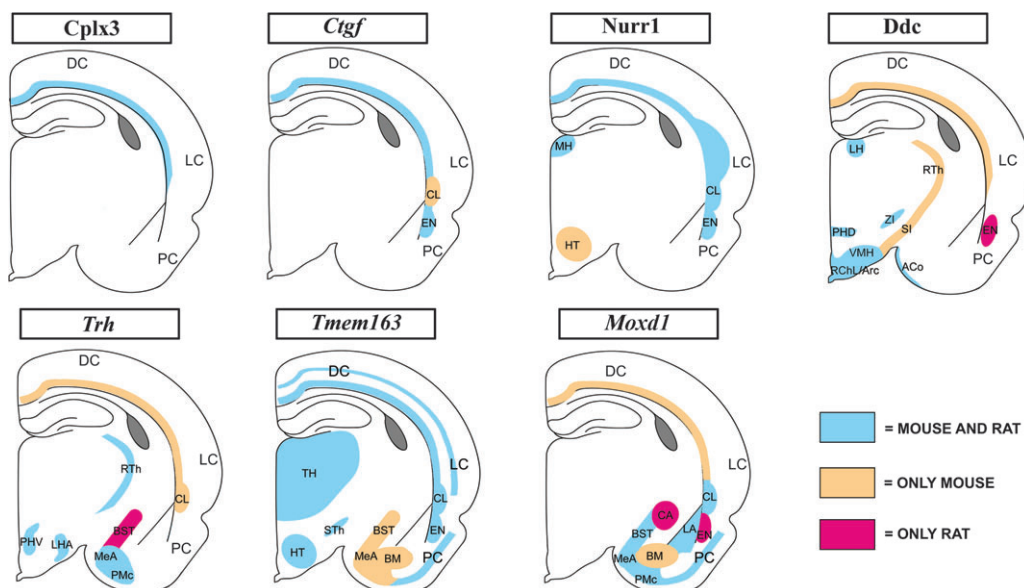

Figure 5. Schematic summaries of similarities and differences of gene expression patterns in cortical and extracortical areas in the mouse and rat. The areas where the gene expression patterns are conserved in both species are shown in pale blue. Regions colored in magenta or tan indicate expressions only found in rat or mouse, respectively. Cplx3 is exclusively expressed in the subplate of both mouse and rat. Ctgf and Nurr1 have similar dorsal cortical expression with slight differences in the extracortical patterns. Nurr1 is also expressed in layers 5 and 6 in lateral cortex in both mouse and rat. Ddc is expressed in mouse subplate and thalamic reticular nucleus (RTh), while in rat, it is found in endopyriform nucleus (EN). However, its expression in ventral hypothalamus is more conserved in both species. The expressions of Tmem163 in basomedial amygdala (BM), bed nucleus of the stria terminalis (BST), and medial amygdala (MeA) are only observed in mouse. The expression patterns of Moxd1 in EN, central amygdala (CA), and Trh, BST are only found in rat. Moxd1 and Trh are only expressed in the subplate of mouse. ACo: anterior cortical amygdaloid nuclei; Cl: claustrum; DC: dorsal cortex; HT: hypothalamus; LC: lateral cortex; LH: lateral habenular nuclei; LHA: lateral hypothalamic area; MH: medial habenular nuclei; PC: pyriform cortex; PHD: posterior hypothalamic area, dorsal; PHV: posterior hypothalamic area, ventral; PMc: posterior medial cortical amygdala; RChL: retrochiasmatic area, lateral part; STh: subthalamus; VMH: ventralmedial hypothalamus; and ZI: zona incerta.

It is interesting to note that calretinin and GAD65/67 antibodies, markers of inhibitory populations, label a different set of cells distributed in the external and internal plexiform layers. Calretinin is mostly expressed in the superficial external plexiform layer, but there are some immunoreactive fibers in the internal plexiform layer as well (Supplementary Fig. 2F–H). Gad65/67 expression is present in both the external and the internal plexiform layers, but it is largely
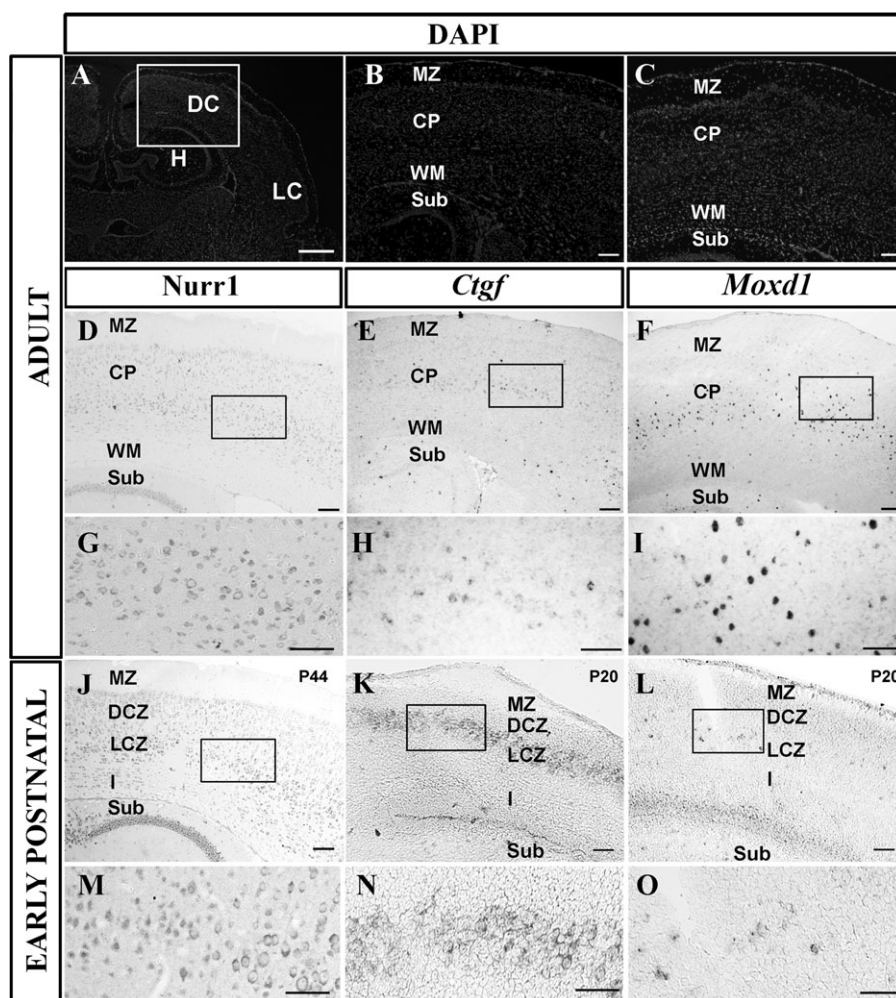
Figure 6. Nurr1, *Ctgf*, and *Moxd1* expression in adult (*A–I*) and developing (*K, L, N,* and *O* at postnatal age 20 and *J* and *M* at P44) *Monodelphis domestica* dorsal cortex. (*A*) Coronal section through the adult opossum brain stained with DAPI. *B* and *C* are higher power images from the region of the parietal cortex with the marginal zone, cortical plate, white matter and hippocampal subiculum indicated. (*D*) In adult opossum, the Nurr1 immunoreactive cells are widespread and extend throughout the cortex. The boxed area in *D* is shown in high power in G. The cortical expression of Nurr1 is observed in both the dorsal and the lateral cortex and claustrum and endopiriform nucleus. (*J* and *M*) At P44, Nurr1+ cells appear mostly in the lower part of the cortex. Boxed area in *J* is shown at higher magnification in *M*. Ctgf and Moxd1 probes label cells in a broadband within the adult opossum cortex (*E* and *F*, high power in *H* and *I*). Moxd1 is also expressed in the hippocampus and in the deeper layers of the piriform cortex in addition to dorsal and lateral cortex. The claustroamygdalar expression of Moxd1 is comparable with that observed in mouse and rat brains. At P20, *Ctgf*+ and *Moxd1*+ cells are seen in a broadband of cells in the loosely packed cortical zone (LCZ), below the dense cortical zone (DCZ) and above the intermediate zone. *Ctgf* labels more cells than *Moxd1*. Scale bars: (*A*) 600 μm; (*B*) 200 μm and applies to *C–F* and *J–L*; and (*G*) 100 μm and applies to *H, I,* and *M–O*. CP: cortical plate; DC: dorsal cortex; H: hippocampus; I: intermediate zone; LC: lateral cortex; MZ: marginal zone; Sub: hippocampal subiculum; and WM: white matter.

Table 4

Extracortical expression of the subplate murine expressed genes in opossum at P20 (*Ctgf* and *Moxd1*) and P44 (Nurr1)

Gene	Age	Telencephalon						Diencephalon	
		Pallium				Subpallium		Hypothalamus	Thalamus
		Claustrum	Endopiriform nuclei	Piriform cortex	Pallial amygdala	Subpallial amygdala	Basal ganglia		
Ctgf	Postnatal	−	+	−	−	−	−	−	−
	Adult	+ (caudal)	+	−	−	−	−	−	−
Nurr1	Postnatal	+	+	−	−	−	−	−	−
	Adult	+	+	−	−	−	−	−	−
Moxd1	Postnatal	+	−	+ (deep layers)	+ (BM)	+ (MeA)	+ (BST)	−	−
	Adult	+	−	+ (deep layers)	+	+ (MeA)	+ (BST)	−	−

absent from the CDL (Supplementary Fig. 2*J–L*). Some calretinin and Gad65/67 immunoreactive cells are scattered in the lateral and medial septal nuclei, the DVR, and the striatum.

In the embryonic chicken, the 4 genes, *Ctgf, Moxd1*, Nurr1, and *Tmem163*, show rather different expression patterns. However, they are all expressed in the pallium and overlap partially in the hyperpallium (Fig. 8). In the hyperpallium, *Ctgf*
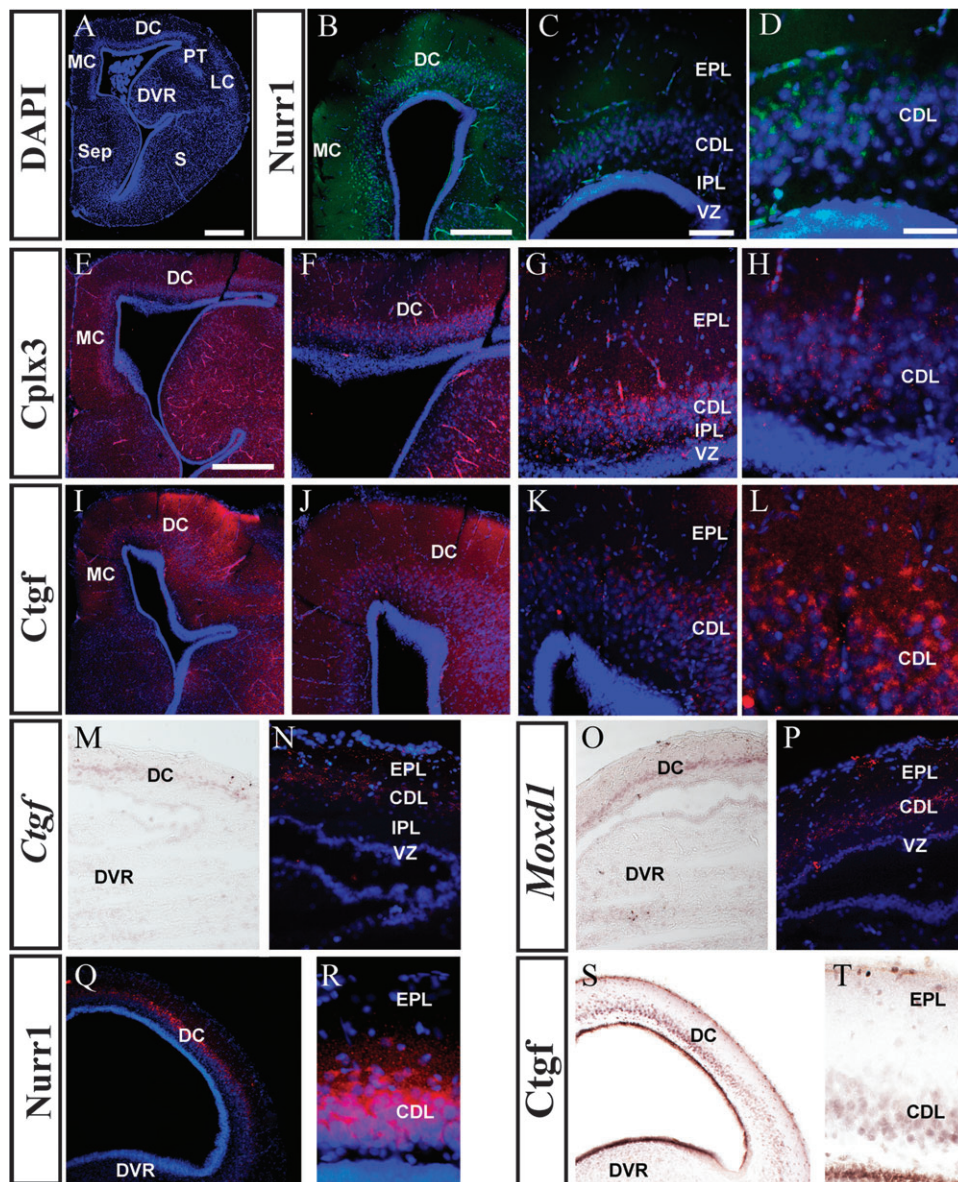
Figure 7. Expression of Nurr1, Cplx3, Ctgf (protein), *Ctgf* (mRNA), and *Moxd1* in the adult (*A–P*) and developing (Stage 25, *Q–T*) turtle dorsal cortex. (*A*) A DAPI stained coronal section of an adult turtle brain. The turtle dorsal cortex contains a single cell dense layer (CDL) flanked by 2 relatively cell sparse regions, the external and internal plexiform layers (EPL, IPL, see *C* and *G*). Nurr1 (*B–D*), Cplx3 (*E–H*), Ctgf (*I–L*), *Ctgf* (*M* and *N*), and Moxd1 (*O* and *P*) are all expressed in the CDL of the adult turtle dorsal cortex. *Ctgf* and *Moxd1* are additionally expressed in the dorsal ventricular ridge (DVR). Nurr1 and *Ctgf* (mRNA) expression are observed at embryonic Stage 25 (*Q–T*). Scale bars in *A* = 500 μm; *B* = 200 μm and also applies to *F*, *J*, *M*, and *O*; *C* = 100 μm also applies to *G*, *K*, *N*, *P*; *D* = 50 μm and also applies to *H*, *L*, *R*, and *T*; *E* = 500 μm also applies to *I*, *Q*, and *S*. DC: dorsal cortex; LC: lateral cortex; MC: medial cortex; PT: pallial thickening; S: striatum; Sep: septum; and VZ: ventricular zone.

expression is exclusively found in the central columns, that is, in the hyperpallium apical lateral (HAL) and in the interstitial part of the hyperpallium apicale (IHA) (Fig. 8*a*,*b*). In addition, there are strongly labeled cells in the dorsal and the ventral part of the mesopallium (dorsolateral pallium), specifically in the corticoid plate and core nucleus (Fig. 8*a*,*c*, and *d*). Very few *Moxd1*+ cells are found in the hyperpallium, in particular in the IHA (Fig. 8*e*,*f*, and *i*). Many more labeled cells are present in the intermediate part of the nidopallium (Fig. 8*g*) and in the basal somatosensory nucleus of the nidopallium (Fig. 8*h*). Nurr1-positive cells are more broadly distributed in the hyperpallium than the *Ctgf*-positive cells and are distributed throughout the entire apical part of the hyperpallium (hyperpallium apical medial, HAL, and IHA). No Nurr1 staining is present outside the

hyperpallium (Fig. 8*j*,*k*, and *l*). *Tmem163* shows the most widespread expression in chick and is present in all pallial regions, including the entire hyperpallium, mesopallium, nidopallium, and medial pallium, with a sharp boundary toward subpallial regions (Fig. 8*m–p*). These expression patterns remain largely conserved in the adult chicken (Supplementary Fig. 3).

Discussion

The subplate is a highly prominent laminar zone present during fetal stages of cerebral cortical development (Kostovic and Molliver 1974; Rakic 1977; Luskin and Shatz 1985). Our previous work has shown that subplate cells have selective
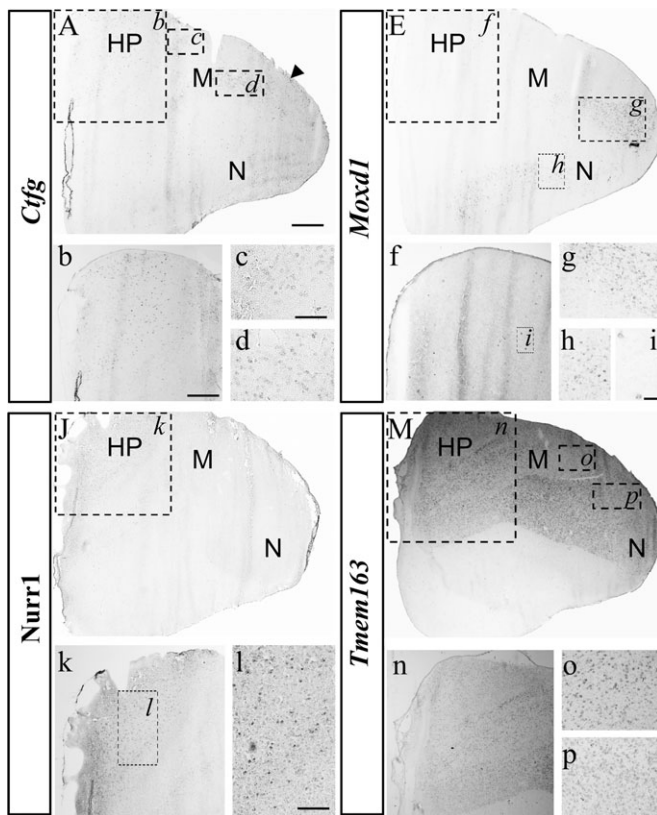
Figure 8. Expression of rodent subplate markers in the developing chick telencephalon at E19. *Ctgf*, *Moxd1*, Nurr1, and *Tmem163* are all expressed in the pallium of chick with distinct patterns. *A*, *E*, *J*, and *M* provide low-power view of coronal section through the chick brain. The boxed regions are magnified in corresponding panels for each gene. (*A*) *Ctgf* expression is present in the central columns of the hyperpallium as well as in the cortical plate and the core nucleus of the dorsolateral pallium (see box labeled *b*, *c*, and *d* in *A*, correspondingly). Respective high-power panels are shown in *b*, *c*, and *d*. (*E*) Only a small group of cells in the interstitial part of the hyperpallium apicale (IHA) express *Moxd1* (see *f*), while many Moxd1+ cells are found in the nidopallium, especially in the intermediate part (see *g*) and in the basal somatosensory nucleus (see *h*). High-power images are in panels *g*, *h*, and *i*. (*J*) Nurr1+ cells are found exclusively in the hyperpallium (see *k*), where they are evenly distributed throughout the entire region (see *k* and *l*). (*M*) *Tmem163* shows broad expression throughout the whole pallial region with a clear boundary between the pallial and the striatal domains (*n*–*p*). Scale bar in *A* = 500 μm, also applies to *E*, *J*, and *M*; *b* = 400 μm, also applies to *f*, *k*, and *n*; scale bar in *c* = 100 μm, also applies to *d*, *g*, *h*, *l*, *o*, and *p*; scale bar in *i* = 40 μm. HP: hyperpallium; M: mesopallium; and N: nidopallium.

gene expression patterns. In reeler and p35 knockout mice, 2 models of cortical laminar reorganization, the subplate markers (*Cplx3*, *Ctgf*, *Moxd1*, Nurr1, and *Tmem163*) change their location to the corresponding compartment as predicted by the altered migration (Hoerder-Suabedissen et al. 2009). This suggests that these genes are expressed by the preplate derived subplate cells and could serve as useful markers to compare cells between species with radically different cortical organization, such as mammals and sauropsids. In order to explore the evolutionary significance of subplate populations, we analyzed the telencephalic expression pattern of murine subplate markers in developing and adult turtle, chick, gray short-tailed opossum, mouse, and rat. Despite our efforts to use species-specific probes, some markers failed to give satisfactory and conclusive results in certain species. We are fully aware that the analysis of a marker alone cannot solve the absolute identity of a cell population. This is further underlined by our

finding that not all murine subplate markers are shared by rat. However, we hope that our approach of using several markers (*Ctgf*, *Moxd1*, Nurr1, and *Tmem163*) consistently in all species combined with topographical and functional information will provide a better understanding of the evolution of the subplate cells. A schematic summary of these comparative gene expression patterns is illustrated in Figure 9; Supplementary Figure 4 illustrates the expression of *Ctgf*, *Moxd1*, and Nurr1 in different species to facilitate direct comparisons.

Our study revealed 1) species-specific differences between rat and mouse in cortical and extracortical distribution of markers originally identified for the mouse subplate, 2) a much broader laminar position of subplate marker expression in marsupial cerebral cortex, and 3) a pallial expression of murine subplate markers in turtle and chick brains.

Conserved and Altered Patterns of Cortical Expression of Mouse Subplate Markers in Rat

Recent reports highlight the importance of the subplate in critical developmental processes of the cerebral cortex with emphasis on the differences for mammals that show a large variation in the size and complexity of the cortical sheet, such as human and rodents (Meyer et al. 2000; Meyer 2001; Ayoub and Kostovic 2009; Friedlander and Torres-Reveron 2009; Wang, Hoerder-Suabedissen, et al. 2010). In these studies, "rodents" generally refers to one species of mice (*M. musculus*) and one species of rats (*R. norvegicus*), and the 2 are often considered as very similar and nearly interchangeable. Comparing subplate gene expression in mouse and rat, however, we found species-specific differences for 3 of 7 genes examined. In rat, *Moxd1* and *Trh* expression were completely absent in the subplate and Ddc labeling was much reduced to only a few cells compared with mouse. The differences in the subplate gene expression patterns in mouse and rat are not that surprising if we consider that the mouse and rat lineages split between 12–24 MYA which is roughly at the same time as the split between human and orangutan lineages (Glazko and Nei 2003; Gibbs et al. 2004). Other species-specific differences in rodents, even within different strains of mice, have been described such as the different distributions of acetylcholinesterase in the barrels of primary somatosensory cortex present in mouse compared with rat or hamster (Sendemir et al. 1996; Morris et al. 2010) or the heterotopia formation in rat but not mouse neocortex after RNA interference knockdown of doublecortin (Ramos et al. 2006). Similarly, birthdating studies in mouse and rat suggest differences in the survival of subplate cells in these 2 species: in mice, all early born (labeled at E11) cells had disappeared from the postnatal subplate at P8 (Price et al. 1997; Del Río et al. 2000), whereas in rats many of these cells (labeled at E12 or E14) persisted into adulthood (Al-Ghoul and Miller 1989; Valverde et al. 1995; Robertson et al. 2000; Arias et al. 2002). However, it is noteworthy that all our markers, except for Ddc, are expressed in adult mouse subplate cells. Consistently, as mentioned earlier, rat adult subplate cells also express Cplx3, *Ctgf*, Nurr1, and *Tmem163*. The differences observed in the expression of Ddc, *Moxd1*, and *Trh* in rat and in mouse are therefore unlikely due to different survival of subplate cells in these 2 species.

At a larger level of organization, the size and laminar distinctions of cortical fields, the number of cortical fields,
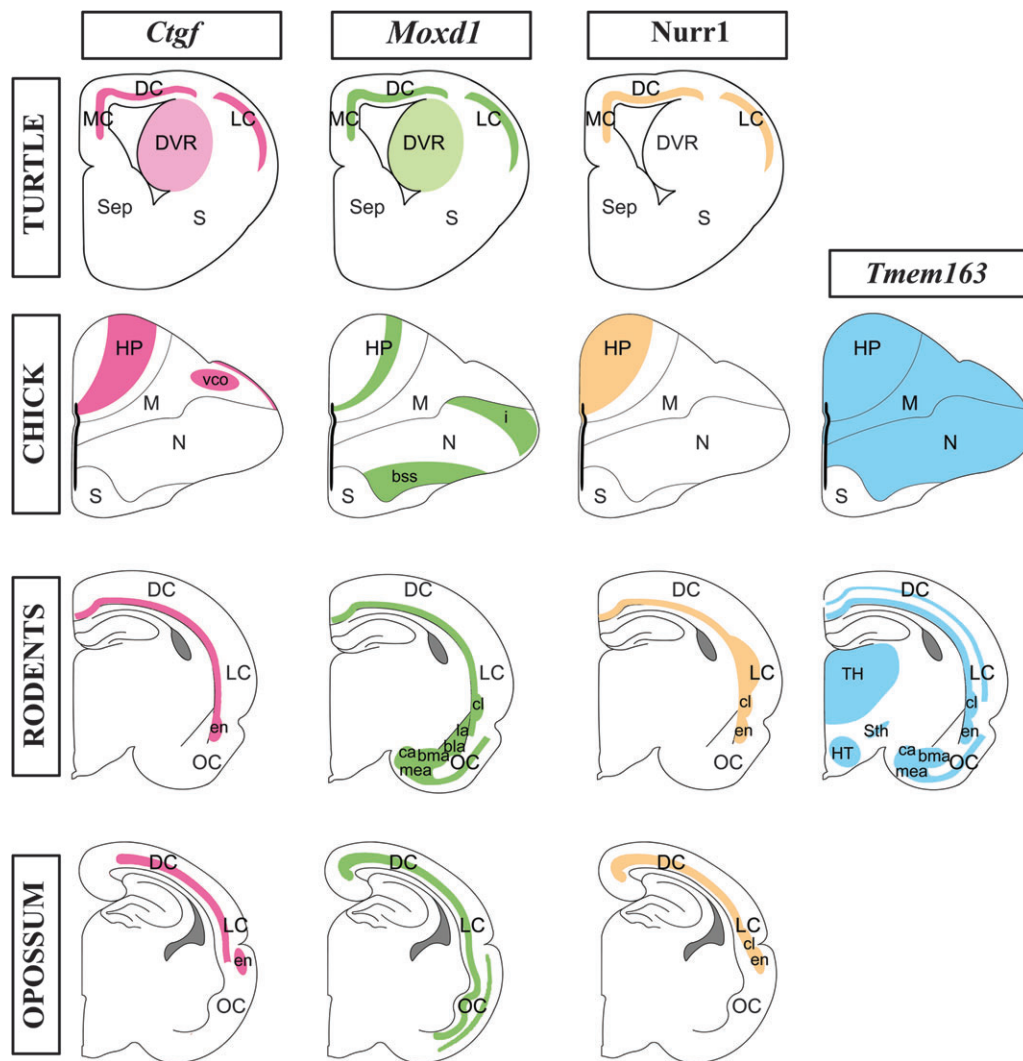
Figure 9. Schematic summary comparing the expression patterns of 4 selected murine subplate markers (*Ctgf*, *Moxd1*, Nurr1, and *Tmem163*) in turtle, chick, mouse/rat, and opossum. Comparing subplate gene expression in mouse and rat, we found some general overall patterns but also some noticeable species-specific differences for several genes. In *Monodelphis domestica*, the gene expression patterns suggest a more widespread subplate population scattered within the developing cortical plate. In chick brains, selected subplate markers are all expressed in pallial regions, mainly in the hyperpallium. The same genes are all expressed in the cell dense layer of the turtle dorsal cortex and parts of the DVR. bla: basolateral amygdala; bma: basomedial amygdala; bss: basal somatosensory nucleus; ca: central amygdala; Cl: claustrum; DC: dorsal cortex; DVR, dorsal ventricular ridge; en: endopyriform nucleus; HP: hyperpallium; HT: hypothalamus; i: intermediate part of nidopallium; la: lateral amygdala; LC: lateral cortex; M: mesopallium; MC: medial cortex; mea: medial amygdala; N: nidopallium; OC: olfactory cortex; S: striatum; Sep: septum; Sth: subthalamus; TH: thalamus; and VCO: visual core nucleus.

and the connections of cortical fields can be radically different in different rodents, such as squirrels and mice or rats (Krubitzer and Kaas 2005). Furthermore, both the sensory mediated behavior and the cognitive abilities of these species are quite different. Thus, our observations underscore that rat models and mouse models are not interchangeable, and that each species has evolved many unique characteristics in the neocortex.

Ctgf, *Moxd1*, and Nurr1 Reveal a Broad and Less Well-Defined Subplate Zone in *M. domestica*

Due to the lack of clear cytoarchitectonic distinctions and the absence of an obvious waiting period for TCAs below the cortical plate, the existence of the marsupial subplate has been questioned by some (Harman et al. 1995; Marotte and Sheng 2000; Reep 2000) but identified by others (Reynolds et al. 1985; Puzzolo and Mallamaci 2010). Whereas Harman et al. (1995)

proposed that there is no subplate in the quokka wallaby (*Setonix brachyurus*), others have described a subplate with distinctive morphological characteristics in a compartment deep to the cortical plate in the gray short-tailed opossum (*M. domestica*) and tammar wallaby (*Macropus eugenii*; Reynolds et al. 1985; Saunders et al. 1989).

By examining the patterns of the murine subplate markers *Ctgf*, Nurr1, and *Moxd1* in the developing short-tailed opossum brain, we found that these genes are all expressed in the LCZ, which later forms the deep layers of the cortex. We propose that these cells are the marsupial homologues of the eutherian subplate, although they are arranged differently than in postnatal mice where they form a distinct thin band below the cortical plate (Price et al. 1997; Hoerder-Suabedissen et al. 2009).

Recently, Puzzolo and Mallamaci (2010) described the distribution of calretinin and Foxp2 immunoreactivity and other layer-specific markers (Tle4, Cux1, and Tbr1) in the

developing opossum cerebral cortex. A broadband of calretinin-positive neurons are transiently apparent below the Foxp2 immunoreactive layer of cortical plate cells. However, we believe that the calretinin immunoreactive cell population and the populations identified by our markers are different. We hypothesize that the stream of calretinin immunoreactive cells are migrating interneurons that will eventually reside elsewhere. In contrast, the cells expressing *Ctgf*, Nurr1, and *Moxd1* constitute a postmigratory population of the developing marsupial cortex.

Interestingly, in the marsupial wallaby (*M. eugenii*), the LCZ has been described as the initial recipient compartment for TCAs similar to the subplate in other mammals. In the wallaby, the TCAs form the first synapses in the LCZ (at P30) and subsequently (at P65) the connections with layer 4 cells (Pearce and Marotte 2003).

Taken together, the results from the present and previous studies suggest that cells in the LCZ population in marsupials are homologous to the rodent subplate population. This proposition could help reconcile the controversy about the absence of a waiting period for TCAs in marsupials. TCAs might not "wait" or accumulate below the cortical plate as in other mammals but in the lower compartment within the cortical plate that also contains some of the neurons expressing murine subplate markers. Combined recording and gene expression studies in marsupial thalamocortical slices could confirm this experimentally.

Murine Subplate Markers are Expressed in Turtle and Chick Dorsal Pallium

We studied the expression of the murine subplate markers in developing and adult turtle and chick brains. In turtle and chick, the expression patterns of the subplate markers—*Ctgf*, *Moxd1*, Nurr1, and *Tmem163*—were most apparent in the dorsal cortex and the hyperpallium, respectively. In spite of regional differences in expression, all 4 markers were restricted to the pallium. In chick, despite marked differences in density and distribution of labeled cells, the expression of all genes overlapped in the hyperpallium (Fig. 8). These observations suggest that the pallium, and in particular the hyperpallium, contain cell populations homologous to cortical subplate subpopulations in mammals. We detected these cells mainly in the central columns of chick hyperpallium in particular in the IHA, which is the main recipient of thalamic afferents to the hyperpallium.

The columnar distribution of the subplate markers in chicken (Figs. 8 and 9) can be explained by the characteristic avian development of the hyperpallium. It has been shown that newly born neurons migrate from the ventricular zone guided by the radial glia in an outside-in neurogenetic gradient (Källén 1953; Tsai et al. 1981). In birds, the observed arrangement of radial glia implies that neurons of each radial column of the hyperpallium must be born in separate, adjacent regions of neuroepithelium, from where they migrate toward the pial surface. Distinguishable radial columns are formed parallel to the orientation of the radial glia and not perpendicular to them like the laminar organization in mammals and reptiles. The hyperpallium is therefore considered a nonlayered structure even though the radial columns are interconnected (Medina and Reiner 2000; Medina and Abellán 2009). The hyperpallial columnar subdivisions can be differentiated by distinct

expression patterns for calbindin and neural nitric oxide synthase (Suárez et al. 2006). Similarly, our results show that *Ctgf*, *Moxd1*, and Nurr1 only label certain columns in the hyperpallium. Recent studies by Karten and colleagues (Wang, Brzozowska-Prechtl, et al. 2010), however, identified a laminar structure in chick auditory cortex (Field L/CM complex) that is remarkably similar to the primary auditory cortex in mammals. Their findings suggest that both nucleus and laminar architectures coexist in avian telencephalon.

In turtles, the existence of a separate subplate layer is not observed. Cells labeled with subplate markers appear within the CDL across the entire length of the dorsal cortex. It has been reported that Cplx3+, *Ctgf*+, and Nurr1+ subplate cells are likely glutamatergic neurons in rodents and macaque (Arimatsu et al. 2003; Watakabe et al. 2007; Hoerder-Suabedissen et al. 2009). Consistently, in the turtle, we observed a clear neuronal subtype separation in a complementary distribution manner. The cells identified with subplate markers are localized within the central CDL of the turtle dorsal cortex, while calretinin and GAD65/67 immunoreactive interneurons are restricted to the external and internal plexiform layers.

Extracortical Expression of Murine Subplate Markers

In all species studied, the expression of the murine subplate markers *Ctgf*, *Moxd1*, *Tmem163*, and Nurr1 is not restricted to the dorsal pallium but also involves other telencephalic regions (Tables 3 and 4 and Figs. 4, 5 and 9). The extracortical expression of these markers makes it difficult to pin down possible homologous populations within ventral and dorsal pallium, since both telencephalic regions contain cells with similar neurochemical properties. Based on the expression profiles of several early patterning factors, Fernández, Puelles and their colleagues proposed that the telencephalic proliferative zone can be divided into 3 main domains: 1) A dorsal component (Emx1+, Pax6+, Tbr1+, and Dlx2−) from which the hyperpallium, the hippocampus, and the mesopallium of sauropsids will be derived. In mammals, this dorsal component gives rise to the dorsal neocortex (primary motor, somatic, and visual areas), part of the lateral neocortex (auditory area), and the hippocampus. 2) A ventral division (Emx1−, Pax6+, Tbr1+, and Dlx2−) forms the nidopallium and the arcopallium of sauropsids and in mammals, the pallial amygdala and parts of the claustral complex. Its contribution to parts of the lateral cortex is subject of current debate (Molnár and Butler 2002). 3) A subpallial territory (Emx1−, Pax6−, Tbr1−, and Dlx2+) will generate the subpallium (striatum) in sauropsids and the striatum, globus pallidus, and the subpallial amygdala in mammals (Fernandez et al. 1998; Puelles et al. 1999, 2000). The selective expression patterns of *Ctgf*, *Moxd1*, *Tmem163*, and Nurr1 in the present work are largely consistent with this model.

Ctgf and Nurr1 are exclusively expressed in dorsal derivates of the pallium in chicken, and we consistently find that both genes are mainly expressed in the neocortex in mammals (mouse, rat, and opossum). Interestingly, however, we also observed some expression of these genes in the claustrum and endopiriform nucleus, suggesting that at least some cell populations in these structures could have a dorsal origin (Figs. 2, 4, 8 and 9). Similarly, Puelles et al. (2000) have described a population of Emx1+ cells of probably dorsal origin in the dorsal claustrum.

Moxd1 and *Tmem163* show a broader expression pattern than *Ctgf* and Nurr1 and are expressed in both dorsal and ventral derivates of the pallium in chicken (Fig. 8). Consistently, in mammals, they show a widespread expression in the claustroamygdalar complex, in addition to their neocortical expression. However, we also find expression of these 2 genes in the subpallial amygdala (mostly in the MeA), whereas no expression is found in the subpallium of chicken (Figs. 2, 4, 8 and 9). This observation supports the idea that in mammals, the pallial–subpallial boundary is not as restrictive as it is in sauropsids (Striedter 1998).

The Ancestral Subplate Cells in a New Evolutionary and Developmental Scenario

In the developing mammalian cerebral cortex subplate neurons integrate into the intracortical and extracortical circuitry; they project locally to the overlying cortical plate, to the thalamus, and to the contralateral cortex through the corpus callosum (Friauf et al. 1990; Piñon et al. 2009). The presence of these neurons is necessary for the maturation of inhibition in cortical layer 4 in areas innervated by the thalamus (Kanold and Shatz 2006). In addition, they appear to drive oscillations in the gap junction-coupled early cortical syncytium (Dupont et al. 2006). It has been proposed that the excitatory component of the subplate surviving to postnatal stages provides glutamatergic drive to the overlying cortex, perhaps by innervating layer 4 neurons directly or via GABAergic neurons (Suárez-Solá et al. 2009; Kanold and Luhmann 2010). In carnivores, subplate cells are required for the establishment of ocular dominance columns in the primary visual cortex (Ghosh and Shatz 1992). These complex developmental steps are associated with enlargement of the subplate zone, but the addition of extra subplate populations is not yet supported by experimental evidence.

The preplate, which is the developmental precursor of the subplate and marginal zone, has been considered as the "reptilian framework of the mammalian brain" containing some of the most ancestral cell populations in mammals (Marín-Padilla 1971). Based on gene expression, we find that subplate-homologous cells are present in both sauropsids (turtle and chick) and therapsids (opossum and rodents). In turtles, we find these cells within the CDL of the cortical plate, in chicken in the hyperpallium, and in oppossum within the deep layers of the neocortex. Cells that reside in these locations have been suggested to be involved in the attraction of thalamic afferents to the dorsal pallium (Hall et al. 1977; Reiner and Karten 1983; Guirado and Dávila 2002; Pearce and Marotte 2003) suggesting a similar role as for eutherian mammalian subplate cells.

In eutherian mammals, subplate cells are amongst the earliest born cells in the telencephalon (Bayer and Altman 1990; Price et al. 1997). Furthermore, birthdating together with marker expressions in turtle, chick, and opossum could elucidate whether the populations we propose as subplate equivalent are also among the first generated cells.

Evolution of the mammalian cortex required the modification of the developmental programs that include a greater reliance on lager populations of subplate neurons (Supèr and Uylings 2001). We propose that the subplate is a phylogenetically ancient structure that takes a different form in sauropsids and mammals, particularly in human (Kostovic and Rakic 1990; Wang, Hoerder-Suabedissen, et al. 2010). It is tempting to believe that the mammalian subplate contains both ancestral and derived elements, which have been modified in the course of mammalian evolution to support the development of an increasingly large and complex cortical plate (Aboitiz 1999; Aboitiz et al. 2005).

The next challenge will be to distinguish between these ancestral populations that our study demonstrate in chicks, turtles, opossums, and rodents and the presumed newly evolved subplate cells in eutherian mammals. For this, we shall have to establish the subplate cell types and relate gene expression, connectivity, and functional properties in different species. These future experiments will help us to determine if and how the subplate has been altered in distinct mammalian lineages, and if such alterations are related to and possibly drive changes in the size and complexity of the mammalian neocortex.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

Notes

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