Expression Analysis of Sulf1 in the Chick Forebrain at Early and Late Stages of Development

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Sulfatase 1 is a secreted enzyme that modulates the sulfation state of heparan sulfate proteoglycans (HSPGs), which are potential key regulators of diverse developmental signals during embryonic patterning. In the present work, we have analyzed the Sulf1 gene expression pattern during chicken forebrain development. Our results indicate that, at early developmental stages, chicken Sulf1 is expressed in the alar and basal plate of the secondary prosencephalon (telencephalon and hypothalamus, respectively) as well as in the diencephalic basal and floor plates. Later in development, Sulf1 is expressed by a subset of nuclei derived from these regions. Developmental Dynamics 238:2418 –2429, 2009. © 2009 Wiley-Liss, Inc.

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ABBREVIATIONS 3DM oculomotor nucleus, dorsomedial part 3v third ventricle AEP entopeduncular area AH anterior hypothalamic area APT anterior pretectal nucleus APTp anterior pretectal nucleus superficial cell plate AuL1 auditory area of nidopallium, shell L1 field Cb cerebellum ccs caudocentral septal area chp choroid plexus dCaPa caudal paraventricular area, dorsal part DIA dorsal intermediate anterior nucleus of the thalamus DIP dorsal intermediate posterior nucleus of the thalamus DLN dorsolateral anterior nucleus of the thalamus DMA dorsomedial anterior nucleus of the thalamus DMP dorsal medial posterior nucleus of the thalamus DTF1p dorsal tegmental area of prosomere 1 ERot epirotundic nucleus ExM external mammillary nucleus Hb habenular nucleus HGF hepatocyte growth factor HPO hypothalamic periventricular organ Hyp hypothalamic IGL intergeniculate leaft InC interstitial nucleus of Cajal IPT intermediate pretectal nucleus Is isthmus LA lateral anterior hypothalamic nucleus LHB lateral habenular nucleus LSHb lateral subhabenular nucleus LSt lateral striatum L1/TJ lateral terminal nucleus, juxtapostitial commissural part M1 mammillary nucleus MCPI magnocellular prethiestic nucleus Mes mesencephalon MG medial geniculate nucleus MJC medial juxtapostitial commissural nucleus of the pretectum MPG medial perigeniculate nucleus MPT medial pretectal nucleus MSHb medial subhabenular nucleus MV mesopallium, ventral part mVTA mesencephalic ventral tegmental area Nlcp nidopallium, intermediate part, corticoid plate NIF nidopallium, intermediate part, islet field OB olfactory bulb p1 prosomere 1 (pretectum) p1MT p1 medial terminal nucleus p1PAG p1 periaqueductal gray p1PEW p1 pre-Edinger-Westphal nucleus p1Rt p1 reticular formation p1SNC p1 substantia nigra, compact part p1Tg p1 tegmentum area p1VTA p1 ventral tegmental area p2 prosomere 2 (thalamus) p2PAG p2 periaqueductal gray p2Tg p2 tegmentum area p2VTA p2 ventral tegmental area p3 prosomere 3 (prethalamus) p3Tg p3 tegmentum area p3VTA p3 ventral tegmental area p4 posterior commissure Pe periventricular stratum PH posterior hypothalamic nucleus Pi pineal gland POA preoptic area PRot perirotundic area PrTPT principal pretectal nucleus of the commissural pretectum PThb basal prethlamic nucleus Rh rhombencephalon rh1 rhombomere 1 RM retromammillary nucleus RMC red nucleus, magnocellular part Rot rotundus nucleus RPC red nucleus, parvicellular part SAC stratum album centrale of the tectum ScH suprachiasmatic nucleus Se septum SGC stratum griseum centrale of the tectum SGFS stratum griseum et fibrosum superficiele of the tectum SHB subhabenular nucleus SLPG semilunar perigeniculate nucleus Smt stria medialis SMI superficial microcellular nucleus SP secondary prosencephalon SPha subparaventricular nucleus SPc superficial parvicellular nucleus of the thalamus Spl lateral spinothalamic nucleus SPT subpretectal nucleus SRT subrotundus nucleus of the thalamus STh subthalamic nucleus TC tuber cinereum area TEL telecephalon TGC tectal gray, central stratum vCaPa caudal paraventricular area, ventral part VisCo visual nidopallial nucleus, core region VisSh visual nidopallial nucleus, shell region VtgM ventral tegmental area of mammillary region VTgP1 ventral tegmental area of prosomere 1 VTgRM ventral tegmental area of retromammillary region xso supraspatic decussation ZLI intrathalamic limits zone

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INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are major components of the extracellular matrix that play a central role in controlling cell proliferation, differentiation, and morphological development through their interactions with signaling molecules and other extracellular matrix components (Perrinn and Bernfield, 2000; Selleck, 2000). HSPGs are composed of a protein core surrounded by covalently linked heparan sulfate (HS) chains composed of disaccharide repeats (Bernfield et al., 1999; Prydz and Dalen, 2000). Based on the nature of their core protein, they can be classified into three functionally distinct families: the transmembrane syndecans, the glycosylphosphatidylinositol (GPI) -anchored glypicans and the soluble perlecans. During synthesis of HS chains, a specific sulfation pattern of highly (S-domains), partially (transition zones), and nonsulfated regions is generated (Maccarana et al., 1996). This sulfation pattern is established in the Golgi apparatus by specific sulfotransferases at the 2-O position of uronic acid and 6-O, 3-O, and N positions of glucosamine (Ori et al., 2008). It has been proposed that this structural heterogeneity is specific of certain cell types or stages of development and plays an important role in regulating signaling pathways (Gallagher, 2006; Kreuger et al., 2006).

Recently, two additional HS-modifying enzymes that generate the sulfation HS pattern have been discovered. These enzymes, called Sulf1 and Sulf2, are extracellular endosulfatasases that have the unique ability to eliminate the sulfate group in position 6-O of glucosamine in highly sulfated regions of HS (Morimoto-Tomita et al., 2002; Ai et al., 2003, 2007). Genes encoding for Sulf enzymes have been identified in birds, mouse, rat, and human, and more recently in amphibian someres 1–3 (p1–p3; Puelles and J.R.L. Rubenstein, and coworkers (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles, 1995; Rubenstein et al., 1998; Puelles and Rubenstein, 2003). In this model, the anteroposterior (AP) regionalization first causes the forebrain to become subdivided into rostral secondary prosencephalon (hypothalamo-telecephalic complex) and caudal diencephalon (Puelles et al., 1987, 2004; Puelles and Rubenstein, 1993, 2003; Puelles, 1995). The diencephalic region next develops three prosomeric transverse units, known as prosomeres 1–3 (p1–p3; Puelles and Rubenstein, 2003). Our analysis is based on the prosomeric model developed by L. Puelles, J.R.L. Rubenstein, and coworkers (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles, 1995; Rubenstein et al., 1998; Puelles and Rubenstein, 2003). In the ventral spinal cord, Sulf1 has been detected in oligodendrocyte precursors, where it functions as a positive regulator of Shh signaling and contributes to trigger neural progenitors from a neuronal to glial fate (Danesin et al., 2006).

In this study, we have performed a detailed analysis of Sulf1 expression in the forebrain of chicken embryo. We have further compared Sulf1 expression with previously described genes such as Nkx2.2, pld/m20, and Pax6 to precisely position domains of Sulf1 expression within the longitudinal as well as transversal prosomeric limits. Our analysis is based on the prosomeric model developed by L. Puelles, J.R.L. Rubenstein, and coworkers (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles, 1995; Rubenstein et al., 1998; Puelles and Rubenstein, 2003). In the ventral spinal cord, Sulf1 has been detected in oligodendrocyte precursors, where it functions as a positive regulator of Shh signaling and contributes to trigger neural progenitors from a neuronal to glial fate (Danesin et al., 2006).

RESULTS

We first described the early gene expression pattern of Sulf1 at Hamburger and Hamilton (HH) stages HH10 (embryonic day [E] 10) to HH26 (E5) in whole-mounts. Next, we analyzed in consecutive series of coronal sections the molecular pattern at later stages (E8 [HH34], E14 [HH40], and E18 [HH44]). Representative results are displayed in Figure 1 for earlier stages and Figures 2–6 for later stages. We have used the anatomical nomenclature and abbreviations of the Chick Brain Atlas by Puelles et al., 2007 (see the list of abbreviations).

Sulf1 Gene Expression at Early Stages (HH10–HH26)

Sulf1 transcripts were first detected at stage HH10 (E2) in the mesoderm adjacent to the hindbrain (Fig. 1A). In the CNS, Sulf1-expressing cells were first observed at stage HH18 (E2.5; Fig. 1B).

At E2.5–E3 (HH18–HH20), Sulf1 expression was exclusively localized in the roof plate of the telencephalon and in the basal plate of the diencephalon, mesencephalon and rostral rhombencephalon (Fig. 1B–D). Even though it is expressed in the floor plate of the spinal cord, caudal rhombencephalon, (r2–r7) and diencephalon, Sulf1 transcripts were absent in the floor plates of the rostral rhombencephalon (r1) and mesencephalon (where the expression shifted from the floor to basal plate), as well as in the whole isthmic region, between r1 and mesencephalon (Fig. 1D).

At E4 and E5 (HH22–HH26), the expression pattern of Sulf1 changed significantly. Rostrally, new regions with Sulf1 + cells appeared inside the secondary prosencephalon: in the chorioid plexus at the roof plate (cph; Fig. 1E–G), as well as in the preoptic region (POA; Fig. 1F,G), subparaventricular area (SPa; Fig. 1E–H), and the caudal paraventricular areas (dCPA, vCPA; Fig. 1F,H), in the alar plate.

Ventrally, in the basal plate, Sulf1 was expressed in mammillary area (M; Fig. 1F–H), as well as in the mammillary and retromammillary regions of the ventral terminal area (VTgM, VTgRM; Fig. 1E–H). Note the weak Sulf1 signal in the dorsal retromammillary area (RM; Fig. 1E–H).

In the diencephalon, at E4.5 and E5 (HH24–HH26), Sulf1 signal was lo-
Fig. 1. A–J: Expression of Sulf1 at early stages of neural tube development (A,D: anterior is up; B,C,E–J: anterior is oriented to the left). Whole-mount chick embryos at embryonic day (E) 2 (HH10; A), E2.5 (HH18; B), E3 (HH20; C,D), E4.5 (HH24; E), and E5 (HH26; F–J). A: Dorsal view of the chick neural tube at HH10. B,C: Lateral view of the chick neural tube at HH18 and HH20. D: Dorsal view of the rombencephalon at HH20. E: In situ hybridization of Sulf1 at stage HH24. F: Lateral view of a chick neural tube at HH26. G,H: Double in situ hybridization with Sulf1 (labeled in blue) and Shh (G), Pax6 (H; labeled in red). I,J: Double in situ hybridization with Sulf1 (labeled in red) and plp/dm20 (I), Nkx2.2 (J; labeled in blue). The dashed lines identify the interprosomeric boundaries (transversal oriented lines) and the alar–basal limit (longitudinal oriented line). K: Schematic representation of expression patterns in a lateral view of neural tube at stage HH24, color codes represent the expression patterns of Nkx2.2, Pax6, Shh, and Sulf1. L: Diagram of a section transversal to p1. b, basal plate; dap, dorsal alar plate; fp, floor plate; pb, parabasal plate; pm; paramedian band; rf, roof plate; vap, ventral alar plate.
calized only in ventral domains. Strong expression appeared in the floor and basal plates of p1 and p2, whereas in p3 the signal was weaker (Fig. 1E–J). Double in situ hybridization with Sulf1 (labeled in blue) and Shh (labeled in red) showed that Sulf1 expression was absent in the zona limitans intrathalamica (ZLI), a Shh-expressing domain that separates the prethalamus and thalamus (p3/p2 boundary; Bulfone et al., 1993; Puelles and Rubenstein, 1993; Fig. 1G). A Sulf1 weak area was detected, corresponding to p3 tegmentum (Tgp3), which was limited caudally by a negative strip, separating Tgp3 from the p2-positive tegmentum (Tgp2). This strip continued the p3/p2 limit into the basal and roof plates of the diencephalon (Fig. 1F–H). Note that in prosomere 2, we observed a Sulf1 weakly expressing domain progressing from the basal plate into the alar plate, ending caudal to the ZLI in the anterovernal region of the thalamus (Fig. 1F). In prosomere 1 (p1), Sulf1 selectively labeled an additional band dorsal to the continuous tegmental expression in p1 (VTgp1) and p2 (Tgp2; Fig. 1E–J). At these early stages of development, the alar/basal boundary in p1 is underlined by the ventral edge of Pax6 expression in the alar plate; and the dorsal border of Nkx2.2 expression in the parabasal band of the basal plate (Puelles et al., 2000; Ferran et al., 2007). Thus, to adequately localize the DTgp1 band, we carried out double in situ hybridization for Sulf1 and Nkx2.2, plp/dm20, or Pax6. Precctal Pax6 transcripts appeared in the entire alar plate adjacent to the alar–basal boundary, while an additional positive strip appeared separated in the basal mantle layer (Fig. 1H; Ferran et al., 2007). Double in situ hybridization with Sulf1 (labeled in blue) and Pax6 (labeled in red) showed that the basal Pax6+ cell band appeared ventral to the DTgp1 Sulf1-positive band (between DTgp1 and VTgp1; arrow in Fig. 1H). In addition, it is interesting to note how the strong expression of Pax6 in the alar plate of p3 allows clear identification of this domain and recognition of its corresponding basal region, between two weak Sulf1-expressing stripes. While the anterior limit of basal p3 was localized between VTgRM and Tgp3, its caudal limit curved between Tgp3 and Tgp2 (Fig. 1H). When double in situ hybridization with plp/dm20 (labeled in blue) and Sulf1 (labeled in red) was performed, plp/dm20 signal appeared ventral to the DTgp1-positive band (Fig. 1I). Finally, comparison of Nkx2.2 (labeled in blue) and Sulf1 (labeled in red) expression patterns showed that DTgp1 overlapped with the dorsal part of the Nkx2.2-expressing band (Fig. 1J). We then developed a Sulf1 expression maps in relation to Shh, Pax6, plp/dm20, and Nkx2.2 expression patterns between stages HH24 and HH26 (Fig. 1K,L).

**Sulf1 Gene Expression at Later Stages in the Telencephalon**

While the early expression of Sulf1 in the telencephalon was restricted to the roof plate, presumptive region of the choroid plexus and the septum (Pombero and Martinez, 2009), new areas appeared in the subpallium at HH26 (E5): the POA and Pa. Then, between E8 (HH34) and E18 (HH44), we observed new pallial domains expressing Sulf1 in the telencephalon.

**Telencephalic Pallium**

*Regional specific expression in the ventral pallium*

The ventral pallium (VPall) corresponds to the old “neostriatum” and was recently renamed as “nidopallium” (Reiner et al., 2004). The ventral pallium includes, rostrally, the olfactory bulb and several associated areas. The olfactory bulb strongly expressed Sulf1 in the mitral cell layer (Fig. 2E,F). The VPall encloses several thalamorecipient neural formations, as well as associative areas, classified as nidopallial subregions (Puelles et al., 2000, 2007; Reiner et al., 2004), including the so-called island fields (Redies et al., 2001). High level of Sulf1 expression was found at the intermediate and caudal nidopallium whereas the frontal areas of VPall appeared negative for Sulf1. On the other hand, the intermediate nidopallium presents the visual core nuclei (VisCo), corresponding to the old “ectostriatum,” or “entopallium” of Reiner et al. (2004). The entire complex, consisting of a core portion (VisCo) plus an associative shell (VisSh), strongly expressed Sulf1 at E12 (HH38, data not shown) and at later stages (E14, HH40 and E18, HH44; Fig. 2E–G,I,J). The nidopallial island field (NIF) that surrounds VisCo also showed some positive Sulf1-expressing islands (Fig. 2F,I,J). In the caudomedial part of the VPall (caudal nidopallium), we detected the auditory core nuclei and its associative shell formation (AuL), a large ovoid region classically called “L field.” AuL1 domain strongly expressed Sulf1 at E18 (HH44) while AuL2 and AuL3 expression levels were less intense and irregular (AuL1; Fig. 2K,L).

**Regional specific expression in the lateral pallium**

The lateral pallium, adjacent to dorsal pallium, and previously known as “ventral hyperstriatum,” was recently renamed mesopallium (Reiner et al., 2004; Puelles et al., 2007). In the mesopallium, Sulf1 was transiently expressed in the anterior areas of its ventral and dorsal parts (MV; Fig. 2E–G). This signal was only observed at E14 (HH40) and disappeared at E18 (HH44, Fig. 2I–L).

**Expression in the Telencephalic Subpallium**

The subpallium derivatives develop into the striatal, pallidal-peduncular, and preoptic regions (Puelles et al., 2000, 2004, 2007). In the striatum, we observed scattered Sulf1+ cells in its lateral part (LSt; Fig. 2H,K), while, in the septum (Se), located in the medial telencephalic wall, ependymal expression of Sulf1 was detected at all stages analyzed (Fig. 2A–C,H–K).

**Sulf1 Gene Expression at Later Stages in the Hypothalamus**

The hypothalamus forms the non-telencephalic region of the secondary prosencephalon (Puelles et al., 1987, 2004, 2007; Puelles, 1995, 2001a; Puelles and Rubenstein, 2003). Most of the caudal hypothalamic ventricular epithelium strongly expressed Sulf1, with the exception of the floor plate that appeared negative except
Fig. 2. In situ hybridization of Sulf1 expression in the chick telencephalon. A–L: Coronal sections at embryonic day (E) 8 (HH34; A–D), E14 (HH40; E–H), and E18 (HH44; I–L) are shown in a rostral-to-caudal sequence and dorsal is to the top. Whereas in early stages of development (E8), Sulf1 is mainly detected in the subpallial and olfactory bulb neuroepithelium (A–D), later in development (E–L) Sulf1 becomes activated in the extended telencephalic pallial derivatives of ventral pallium (VisCo, VisSh, and AuL1) and lateral pallium (MV).

Fig. 3. A–G: In situ hybridization of Sulf1 in E10 (HH36) chick brains, showing coronal sections from rostral (A) to caudal levels (G). Basal plate ependymal cells strongly express Sulf1, together with abundant cells in the hypothalamus, diencephalic and mesencephalic tegmental areas. A–C: Dorsally, in the alar plate, strong expression of Sulf1 was detected in the habenular region, where ependymal cells of the subhabenular nucleus (SHb) were strongly positive. A–C: From this SHb region, a superficial stream of Sulf1-expressing cells forms the SMI and SPC nuclei. C–F: Some pretectal nuclei contain Sulf1-expressing cells. Dashed lines in E–G delimit the interprosomeric boundaries in diencephalic tegmental regions.
for the mammilar region (MM). In addition, several nuclei in the mantle layer expressed this gene.

**Regional specific expression in the alar hypothalamus**

The alar hypothalamus is divided longitudinally into an upper optopeduncular paraventricular area and a subparaventricular area underneath (Puelles et al., 2007). Maintaining the early expression domains, *Sulf1* transcripts were detected in subparaventricular ependymal cells, crossing the midline at the level of the posterior supraoptic decusation (x sod; Figs. 3B–D, 4E–I, 5B). In the mantle layer, we observed *Sulf1* expression in the caudal paraventricular nucleus (CaPa; Figs. 3A,B, 4C,D, 5A) and the suprachiasmatic nucleus (SCh; Puelles et al., 2007; Figs. 3B, 4E,F, 5B,C). Moreover, at the analyzed stages (E10, E14, and E18), we also observed *Sulf1*+ cells in the tuber cinereum area (TC; Figs. 3B, 4E, 5B–F), anterior hypothalamic area (AH; Figs. 3A–C, 4C–E, 5A,B), and lateral anterior hypothalamic nucleus (LA; Figs. 3A, 4D, 5A).

![Fig. 4. A–K: Coronal sections at embryonic day 14 (HH40), showing rostral (A) to caudal (K); (A–E,G–K) hybridized for *Sulf1* and (F) depicts double in situ hybridization with *Sulf1* (labeled in blue) and *Gbx2* (labeled in red). Both ependymal and nuclear cells maintain *Sulf1* expression in this more mature stage of development. F: *Gbx2* expression allows to better localize and identify *Sulf1* expression in the thalamus.](image)
Regional specific expression in the basal hypothalamus

The basal hypothalamus is divided into the rostral or prepeduncular hypothalamic domain and caudal or peduncular hypothalamic domain (Puelles et al., 2007). In the prepeduncular domain Sul1 expression was not detected, whereas some nuclei of the peduncular domain expressed Sul1 during these stages: the hypothalamic periventricular organ strongly expressed Sul1 (HPO; Figs. 3E–G, 4H–K, 5F,H). Finally, while the medial mammillary nuclei (MM) presented a weak signal, high level of Sul1 expression was detected within the external mammillary nuclei (ExM) at these stages (Figs. 3E, 4I–K, 5G). We have also observed Sul1 in cells in the subthalamic nucleus (STh; Figs. 3C, 4E–G, 5B–E).

Sul1 Gene Expression at Later Stages in the Diencephalon

According to the prosomeric model, the diencephalon proper represents the continuation of the forebrain caudal to the hypothalamus, down to the border with the midbrain (Puelles and Rubenstein, 2003; Puelles et al., 2004). The diencephalic region develops three segments or neuromeres, known as prosomeres 1–3 (p1 lying caudally, adjacent to the midbrain, and p3 rostrally, contacting the hypothalamus and the telencephalon; Puelles et al., 1987; Puelles and Rubenstein, 2003). Each prosomere displays characteristic dorsal and ventral domains, namely, the dorsalized roof and alar plates and the ventralized basal and floor plates. The alar territory produces three anatomical regions: alar p1 = pretectum; alar p2 = thalamus and habenula (the old dorsal thalamus and epithalamus); alar p3 = prethalamus (prior ventral thalamus; see fate map in García-López et al., 2004).

Regional specific expression in Prosomere 3

Prethalamus (alar plate). The prethalamus was negative for Sul1 in the analyzed stages (from E8, HH34 to E18, HH44; Figs. 4A–G, 5A–F).

Prethalamic tegmentum (basal and floor plates). In the reticular tegmentum (p3Tg; Puelles et al., 2007), weak expression of Sul1 was detected at E10 (HH36, Fig. 3D), where at later stages (E14, HH40 and E18, HH44) the signal disappeared (Figs. 4G, 5D) except from the most anterior ventral tegmental area (p3VTA; Fig. 4K). Otherwise, p3 ependymal tegmentum was strongly positive for Sul1 transcripts (Fig. 3E–G).

Regional specific expression in Prosomere 2

Thalamus (alar plate). This large nuclear complex can be subdivided into 5 main histogenetic areas (Redies et al., 2000; Puelles, 2001b; Martínez-de-la-Torre et al., 2002; García-López et al., 2004). These areas are the habenular/subhabenular complex or epithalamus, the dorsal tier group, the intermediate tier group, the ventral tier group, and the anteroventral group (Redies et al., 2000; Puelles, 2001b; Martínez-de-la-Torre et al., 2002; Puelles et al., 2007).

In the habenular/subhabenular complex, Sul1 was strongly expressed in the subhabenular ependymal region, the lateral and medial subhabenular nuclei (LSHb, MSHb), as well as in the periventricular stratum and lateral habenular nucleus (LHb; Figs. 3A–C, 4A–C, 5A,B).

Ventral to the subhabenular nuclei appears the dorsal tier domain, which produces a voluminous mass of derivatives in its mantle layer that represent the largest components of the avian thalamus (Redies et al., 2000; Puelles, 2001b; Puelles et al., 2007). High levels of Sul1 expression were detected in several of the nuclear components in the dorsal tier: dorsomedial anterior (DMA; Figs. 3A, 4A–C, 5A), dorsal intermediate anterior and posterior nuclei (DIA, DIP; Figs. 3A,B, 4A–C, 5A,B) and in the dorsolateral anterior nuclei (DLA; Figs. 3A, 4A,B, 5A).

Superficially, a continuous stream of Sul1-expressing cells was observed from the subhabenular nuclei to the superficial parvicellular nucleus (SPC; Figs. 3A–C, 4A–C, 5B) and in the superficial microcellular nucleus (SMi; Figs. 3A,B, 4A,B, 5A,B).

The intermediate tier is larger caudally, where it makes extensive contact with the pretectum (Redies et al., 2000; Puelles, 2001b; Martínez-de-la-Torre et al., 2002; Puelles et al., 2007). In this tier, Sul1 expression was restricted to the largest part of this complex, the rostrum nucleus (Rot), which is pushed outward ventrolaterally by the disproportionate growth of the dorsal tier group. Faint Sul1 expression in the scattered rotundal cell was detected at stage E12 (HH38, data not shown). At E14 (HH40), the low level of Sul1 expression was apparent (Fig. 4C–G) but at E18 (HH44), we observed high expression levels compared with previous stages (Fig. 5B–E).

Ventral to the intermediate tier is a smaller histogenetic domain called the ventral tier. This domain contains the avian medial geniculate nucleus (MG, Puelles et al., 2007), formerly called “ovoidal nucleus” (Papez, 1935, 1936). It is surrounded by inner and outer perigeniculate formations: medial perigeniculate nucleus (MPG) and semilunar perigeniculate nuclei (SLPG). To better locate Sul1 expression in the intermediate and ventral thalamic tiers, we carried out double in situ hybridization experiments for Sul1 (labeled in blue) and Gbx2 (labeled in red). Gbx2 was strongly expressed in the ventral tier derivatives (i.e., the MG) and in the subrotundus nucleus (SRot; Puelles et al., 2007), a derivative of the anteroventral area (Martínez-de-la-Torre et al., 2002). We observed that the MG and SRot, stained with the Gbx2 probe, were negative for Sul1 (positive for Gbx2, Fig. 4F) while MPG and SLPG were strongly positive (Figs. 3B–D, 4D–F, 5B,C). In addition, the diffuse perigenulate area also expressed Sul1 (DPG; Figs. 3D, 4G, 5D,E).

In the chicken, cells originating in the boomerang-shaped anteroventral histogenetic zone of the dorsal thalamus migrate to the surface, partially filling the area of origin in depth and partially dispersing tangential-caudally in the superficial strata of p2, to finally cover the most of the dorsal, intermediate, and ventral tiers (Rendahl, 1924; Puelles et al., 1991; Yoon et al., 2000; Puelles et al., 2007). The known derivatives of this region (subrotundus nucleus, perirotundic area and intergeniculate leaflet) differentiate in close spatial relation to the rotundus nucleus. The expression of Sul1 was restricted at the perirotundic area (PROT), pirotundic nucleus...
The complex formed by IGL plus PrOt corresponds to the “n.supernialis magnocellularis” of Rendahl (1924). This was then renamed “n.interstitialis tractus opticus” by Puelles et al. (1991) and Martínez et al. (1991). Also, we observed expression in the posteroverentral and posterocaudal thalamic nuclei (PVTh, PCTh; Figs. 4H, 5F).

Thalamic tegmentum (basal and floor plates). Sulf1 was expressed in the basal p2 epithelium as well as in disperse reticular cells of the thalamic tegmentum (p2Tg), including the anterior pole of the ventral tegmental area (p2VTA; Figs. 3E, 4H).

Regional specific expression in Prosomere 1

Prosomere 1 consists of the pretectal region in its alar plate, as well as a distinct underlying tegmental region.

Pretectum (alar plate). The pretectum is wedged between the thalamic portion of the diencephalon and the midbrain. The alar pretectum appears anteroposteriorly divided into three main radial histogenetic areas called commissural (PTc), juxtacommissural (PTj), and precommissural domains (PTp; Yoon et al., 2000; Redies et al., 2000; Puelles et al., 2007; Ferran et al., 2007). These terms refer to their positions relative to the posterior commissure. The sets of nuclei derived from the three subdivisions of the pretectum displayed differences regarding to Sulf1 expression.

Commissural pretectum. We mapped Sulf1-positive cells in migrated nuclei of the commissural domain, the principal pretectal nucleus (PrPT; Puelles et al., 2007), the intermediate pretectal nucleus (IPT; Puelles et al., 2007), and subpretectal nucleus (SPT; Puelles et al., 2007; Figs. 3C–F, 4E,F,H,I, 5D–H).

Juxtacommissural pretectum. Sulf1 expression was prominent in the medial juxtacommissural nucleus (MJc; Figs. 4F, 5E), and scattered cells of the lateral spiriform nucleus (SpL; Figs. 3E, 4G, 5F), as well as the juxtacommissural portion of the lateral terminal nuclei of the accessory optic tract.
varied in size, morphology, and distribution in ependymocytes, mantle layer cells later stages of development. In contrast, the ependymal expression at laterities in the ependymal expression at the developmental stages (Fig. 1E,F,H,K), we did observe weak Sul1 expression in two layers of the previously known-as “superficial synencephalic nucleus,” the superficial cell plate of the APT (APTp; Puelles et al., 2007; Figs. 3B, 4C,D) and superficial plexiform layer of the APT (APTs; Puelles et al., 2007; Figs. 4B,C, 5C). Sul1 was strongly expressed in the p1 portion of the medial terminal nucleus of the accessory optic tract (p1MT; Puelles et al., 2007; Figs. 3G, 4J, 5H,I). The MT in birds was named “n. ectomammillaris” or “n. of the basal optic root” (Puelles et al., 2007). We also detected high levels of Sul1 expression in the interstitial nucleus of Cajal (InC; Puelles et al., 2007; Figs. 3F, 4I, 5F).

Pretectal tegmentum (basal and floor plates). Sul1 was strongly expressed throughout all p1 tegmentum (p1Tg; Puelles et al., 2007): the parvocellular red nucleus (RPC), the p1 reticular formation (p1Rt), the p1 Edinger-Westphal nucleus (p1PEW), the p1 substantia nigra, pars compacta (p1SNC), and the p1 ventral tegmental area (p1VTA; Figs. 3E–G, 4H–K, 5F–I).

Phenotype of Sul1 Expressing Cells
While early expression was localized in the neural tube epithelium, Sul1-expressing cells migrated and differentiated in various regions of the neural parenchyma. We then studied the mature morphological phenotype of Sul1-expressing cells. Epithelial expression was retained in the majority of the basal plate ependymal areas: caudal hypothalamus, diencephalon, and mesencephalon. Even though interprosomeric limits were evident at early stages of development (Fig. 1E,F,H,K), we did not detect clear differences or heterogeneities in the ependymal expression at later stages of development. In contrast to the strong homogeneous expression in ependymocytes, mantle layer cells varied in size, morphology, and distribution. Small Sul1-positive cells that invaded the axonal tracts from positive ependymal regions were strongly reminiscent of oligodendrocyte progenitors (Fig. 6A,C), while the second population of cells, much larger in size and with polygonal cell bodies, was distributed in nuclear areas, suggesting that they could be neurons (Fig. 6B).

DISCUSSION
Sul1 Expression at Early Stages
In the present study, we have addressed the precise distribution of Sul1 transcripts during forebrain development in chick embryos. At early stages, our in situ hybridization experiments revealed a restricted pattern of Sul1 expression in the neural tube. Rosstrally, in the secondary prosencephalon, the expression pattern was distributed in alar and basal neuroepithelial domains, while in the diencephalon Sul1 expression was restricted to the basal plate epithelium. Although Sul1 expression in the basal plate, telencephalic POA and SPr could be directly related to the regulation of Shh signaling due to the expression of both genes in nearby domains (Puelles et al., 2007; Bardet, 2007; Garcia-Lopez et al., 2008), Sul1 expression throughout the choroids plexus (chp) seems to be unrelated to Shh signaling.

In the diencephalic prosomeres, the Sul1 signal was restricted to basal plate domains and was weakly expressed at the level of interprosomeric boundaries. This expression pattern is evidence that there is a continuity of the interprosomicerous boundaries, which were easily detectable in the alar plate, into the basal plate. Thus, Sul1 expression in the basal plate brings additional evidence supporting the segmental character of diencephalic prosomeres and its metameric structure, and clearly argues against alternative interpretation in favor of a columnar distribution of the basal plate diencephalic neuroepithelium (Larsen et al., 2001).

In this work, we compared the expression of Sul1 with that of Nkx2.2, plp/dm20, and Pax6, by double in situ hybridization to precisely locate Sul1-positive bands in prosomere 1 according to recent studies on pretectal regionalization (Ferran et al., 2007). The issue of molecularly defining the alar–basal boundary at diencephalic levels is still open for discussion. Shimamura and collaborators (1995), Puelles and Rubenstein (1993, 2003), and Puelles and collaborators (2004) tentatively postulated that the longitudinal band of progenitor cells expressing Nkx2.2 “approximates” the alar–basal boundary. Moreover, Ferran and collaborators (2007) recently observed that the molecular alar–basal diencephalic boundary corresponds to the limit between the Pax6 expression domain in the alar plate neuroepithelium and the Nkx2.2 expression domain in the basal plate. In this study, we observed that the dorsal Sul1+ band only present in p1 appeared ventrally compared with the dorsal Pax6-expressing domain, and partially overlapped with the Nkx2.2 domain. Accordingly, we have assumed in the present report that this Sul1+ band in prosomere 1 is localized in the basal plate.

Whether Sul1 is involved in oligodendroglial specification in chick brain remains an open question. In support of this, we observed that Sul1 expression in the Nkx2.2 domain of the ventral brain neuroepithelium, starting from E4, is earlier than the specification of oligodendrocyte progenitors in this territory and co-expression of both genes in migrated oligodendrocyte precursors. However, we do not observe any overlap between the plp/dm20 domain of expression and Sul1-positive bands in prosomere 1. In the chick developing brain, the ventricular domains of plp/dm20 expression correspond to restricted foci that give rise to oligodendrocytes, closely associated to the domain of Shh expression (Ponti et al., 1996; Pringle et al., 1996; Perez-Villagrasa et al., 1999). In mouse, PDGFRA and plp/dm20 have been shown to be expressed in distinct oligodendrogial precursors (Spaskey et al., 1988); therefore, we cannot exclude that plp/dm20 and Sul1 identify distinct neural precursors with oligodendroglial potential. Thus, in the rostral part of the CNS, such Sul1+/plp-dm20+ precursors may coexist with Sul1−plp-dm20− precursors. In chick, the positional information that controls the pattern of specification of neural stem cells has to account for mosaic-like patterns inside the ventral segments of the CNS. An
Explanatory model for such a mosaic pattern of specification has already been proposed for different subtypes of motor- and interneurons, which segregate within contiguous columnar domains along the ventrodorsal axis of the neuroepithelium (Pfaff et al., 1996; Ericson et al., 1997), as well as of neurons and oligodendrocytes in the diencephalon from plp/dm20-positive progenitors (Spassky et al., 2008).

**Sulf1 Expression at Late Stages of Development**

At late stages of development, Sulf1 was highly expressed in ependymal regions of the corresponding Sulf1-expressing neuroepithelial domains. In the alar plate, in addition to choroidal and septal expression, Sulf1 appeared in the ependymal regions of ventral habenular nuclei. This epithelial expression, together with the choroidalplexus, represents exceptions to the topological relation between Shh and Sulf1-expressing domains (Ericson et al., 1995; Vieira et al., 2005).

From these epithelial domains, Sulf1-positive cells further invaded different levels of the mantle layer, following migratory routes and entering into the axonal tracts (mainly small cells that suggest to be oligodendroglia progenitors) or nuclear neuroepiles (with heterogeneous sizes and shapes, possibly neuronal and glial fates).

In the telencephalon, the in situ hybridization study revealed a restricted pattern of Sulf1 expression into a subset of gray matter structures:

- In the ventral pallium, intense gene expressions was detected in the intermediate and caudal nidopallium (Vis-Co, VisSh, NIF, AuL; Reiner et al., 2004). The nidopallium is a large hypothalamic area with heterogeneous sizes and shapes, possibly neuronal and glial fates.

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- In the basal hypothalamus, we detected Sulf1-expressing cells in the subthalamus (STh). The avian STh is clearly an homolog of the mammalian nucleus of the same name (Jiao et al., 2000). STh originates from the retromammillary pouch, and its cells migrate dorsally briefly (Puelles et al., 2007).

- The diencephalic domain is subdivided into three segments or neuro- meres, known as prosomeres 1, 2, and 3 (Puelles et al., 1987, 2007; Puelles and Rubenstein, 2003). In prosomere 1 and 2 regions, we observed intense Sulf1 expression in alar and basal territories. In prosomere 3, we observed a Sulf1-negative alar domain as well as a weak signal at stage E10 (HH36). While an abundant amount of Sulf1-expressing cells, which would correspond to locally produced oligodendroglial progenitors and reticular neurons, were detected in the basal plates, the heterogeneous alar expression could represent both migrated Sulf1-positive cells from the basal plate or local Sulf1 activation. A recent clonal study of plp/dm20 cell distribution in developing mouse thalamus showed a generation of both alar and basal diencephalic neuronal and glial cells from the basal domain expressing this gene (Delaunay et al., 2009). Functional studies are now required to define the function of Sulf1 in the differentiation and functional maturation of thalamic cells.

The expression of Sulf1 in the neural tube and spinal cord has been previously reported both in quail and rat (Dhoot et al., 2001; Ohto et al., 2002). In chick, Sulf1 expression was detected in ventral neuroepithelial cells of the spinal cord (Braquart-Varnier et al., 2004). This study suggests that Sulf1 could represent a gene expressed by cells of the oligodendroglial lineage: both in oligodendrocyte neuroepithelial progenitors and in more mature oligodendrocytes. In the embryonic chick spinal cord, the specification of oligodendroglial precursors in the basal plate requires the morphogenetic signal of Sonic hedgehog (Shh) produced by the notochord and floor plate cells (Trousse et al., 1995; Orentas and Miller, 1996; Poncet et al., 1996; Braquart-Varnier et al., 2004; Danesin et al., 2006). Moreover, the creation of oligodendrocyte precursors from ventral Nkx2.2-expressing neural progenitors occur precisely when these progenitors stop generating neurons, indicating that the mechanism of the neuronal/oligodendroglial switch is a time related feature that is most likely dependent of ventral specification. Chick-Sulf1 is expressed in the ventral neuroepithelium of the spinal cord just before oligodendrocyte specification and seems to be involved in the regulation of Shh signaling (Danesin et al., 2006).

**EXPERIMENTAL PROCEDURES**

**Chick Embryos**

Fertilized chicken eggs (*Gallus gallus domesticus*) were obtained from commercial sources and incubated at 37°C in a forced air incubator until the desired embryonic stage. The embryos were staged according to Hamburger and Hamilton (1951).
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REFERENCES


In Situ Hybridization to Whole-Mount Embryos or to Tissue Sections

The head of the chick embryos was fixed overnight by immersion in 4% paraformaldehyde in phosphate-buffered saline solution (PBS; 0.1 M, pH 7.4). In addition, E18 embryos were transcardially perfused with the same fixative solution. All brains were dissected out and post-fixed for 48 hr at 4°C. Early embryos (HH10–HH26) were processed as whole-mounts, whereas the brains of older embryos (HH33–HH44) were dissected, embedded in 4% agarose in PBS, and transversally sectioned in 100-μm-thick sections with a Vibratome (Leica). Several embryos were embedded in paraffin and 8 μm were obtained.

In Situ Hybridization

Antisense digoxigenin-labeled riboprobes for Sulf1, Gbx2, Pax6, plp-dm20, Nkx2.2, and Shh were synthesized and subsequently processed for in situ hybridization as previously described by Henrique and collaborators (1995). RNA labeled probes were detected by an alkaline-phosphatase-coupled antibody (Roche Diagnostics, Mannheim, Germany), and NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) were used as a chromogenic substrate for BCIP solution, whereas the fluorescein-labeled probes were revealed in dark blue using an NBT/BCIP solution, whereas the fluorescein-labeled probes were revealed in red, using an INT/BCIP solution, following a standard procedure. After hybridization, embryos were washed in PBT, photographed under a dissecting microscope (Leica) and stored at 4°C in PBT/0.1% sodium azide.


Developmental Dynamics.