Development 128, 1983-1993 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2625

Cortical upper layer neurons derive from the subventricular zone as indicated by *Svet1* gene expression

Victor Tarabykin, Anastassia Stoykova, Natalia Usman and Peter Gruss*

Department of Molecular Cell Biology, Max-Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany
*Author for correspondence (e-mail: pgruss@gwdg.de)

Accepted 8 March 2001

SUMMARY

The cerebral cortex is composed of a large variety of different neuron types. All cortical neurons, except some interneurons, are born in two proliferative zones, the cortical ventricular (VZ) and subventricular (SVZ) zones. The relative contribution of both proliferative zones to the generation of the diversity of the cortical neurons is not well understood. To further dissect the underlying mechanism, molecular markers specific for the SVZ are required. Towards this end we performed a subtraction of cDNA libraries, generated from E15.5 and E18.5 mouse cerebral cortex. A novel cDNA, *Svet1*, was cloned which was specifically expressed in the proliferating cells of the SVZ but not the VZ. The VZ is marked by the expression of the *Otx1* gene. Later in development, *Svet1* and *Otx1* were expressed in subsets of cells of upper (II-IV) and deep (V-VI) layers, respectively. In the *reeler* cortex, where the layers are inverted, *Svet1* and *Otx1* label precursors of the upper and deeper layers, respectively, in their new location. Interestingly, in the *Pax6*/*small eye* mutant, *Svet1* activity was abolished in the SVZ and in the upper part of the cortical plate while the *Otx1* expression domain remained unchanged. Therefore, using *Svet1* and *Otx1* as cell-type-specific molecular markers for the upper and deep cortical layers we conclude that the *Sey* mutation affects predominantly the differentiation of the SVZ cells that fail to migrate into the cortical plate. The abnormality of the SVZ coincides with the absence of upper layer cells in the cortex. Taken together our data suggest that while the specification of deep cortical layers occurs in the ventricular zone, the SVZ is important for the proper specification of upper layers.

Key words: Cell migration, Cortical plate, Corticogenesis, Differentiation, Neocortex, *Otx1*, *Pax6*, Small eye mutant, Subventricular zone

INTRODUCTION

The cerebral cortex of the mammalian brain consists of six morphologically distinguishable cell layers. The majority of the cortical neurons are born in the neuroepithelium of the dorsal telencephalon lining the surface of the lateral ventricles of the brain. After the last mitosis, young neurons start to migrate along the radial glial cells towards the cortical plate to occupy their final position (Rakic, 1988). They are born in a deep layers-first/upper layers last gradient. In the neocortex the proliferative zone contains two mitotically active areas (Angevine and Sidman, 1961). Cells that are oriented perpendicular to the ventricular surface make-up the ventricular zone (VZ). These cells undergo a series of interkinetic nuclear migrations that correlate with their cell cycle progression, so that the progenitors' nuclei move to the ventricular surface to complete mitosis (Sauer and Walker, 1959). A secondary proliferative population (SPP) or subventricular zone (SVZ) is easily distinguished from VZ by the variable orientation of its cells and because it is not in contact with the ventricular lumen. The SVZ is very prominent in the telencephalon, but also present in some other compartments of CNS (The Boulder Committee, 1970). It is made up of at least two cellular populations: (1) proliferating cells that do not migrate to the ventricular lumen to undergo mitosis, and (2) young neurons migrating from the VZ towards the cortical plate. Interestingly, proliferative cells in the VZ and SVZ show distinct mitosis kinetics in response to GABA and glutamate (Haydar et al., 2000).

It has been shown that normal development of the striatal SVZ is necessary for proper differentiation of the late born striatal neurons (Anderson et al., 1997). The most anterior part of the telencephalic SVZ gives rise postnatally to olfactory bulb neurons (Zigova et al., 1996; Betarbet et al., 1996; Lois et al., 1993). The great majority of glial cells of the telencephalon are generated perinatally in the SVZ (Privat, 1975). As far as the neocortex is concerned, it is not clear which fate will be adopted by the cells generated within the SVZ. The predominant view is that the mitotic cells of the neocortical SVZ are the source of glial cells only, while the VZ is the sole source of all cortical neurons (Bayer and Altman, 1991). The alternative view states that the neurons in the upper layer (layers II-IV) are produced in the SVZ (Smart and McSherry, 1982). This conclusion is based on the fact that the peak neurogenesis of the upper layers is partially overlapping with the expansion of the size of the SVZ. As shown for the rat telencephalon, the size of the SVZ exceeds that of the VZ between embryonic day (E) 18 and E21

concomitantly with the peak of the layer II-IV neurogenesis (Bayer and Altman, 1991). However, such a correlation does not exclude the Bayer and Altman hypothesis, because the peaks of upper layer neurogenesis and gliogenesis overlap.

Only one marker, *Otx1*, has been reported to specifically label the deep layer cells and their progenitors (Frantz et al., 1994). To date no molecular markers of the SVZ have been reported. In addition none of the existing mouse/human mutants have been shown to disrupt the normal development of the cortical SVZ without severe disruption of the VZ. Another problem of tracing the destination of cells originating in the SVZ is that with existing methods, cells that are produced by the SVZ cannot be distinguished from cells derived from the VZ but migrating to the cortical plate through the SVZ.

To find SVZ-specific molecular markers, we performed cDNA subtraction. We isolated a novel cDNA, *Svet1*, labeling cells of the subventricular but not the ventricular zone. *Svet1* specifically marks a subpopulation of future cortical upper layer cells. Analyzing their expression profile in the developing cortex, we found that *Otx1* and *Svet1* expression domains do not overlap. Our data suggest that they label two precursor cell subpopulations – the deep layer and upper layer precursors, respectively, and we have therefore used them as lineage markers. We subsequently examined some mutants known to affect normal cortical development in order to find a mutation affecting the development of subventricular cells, but not the ventricular cells. In the *Pax6*/*small eye* mutant *Svet1* activity was abolished in the SVZ and in the upper part of the cortical plate while the *Otx1* expression domain remained unchanged. This suggests that the normal development of the cortical SVZ in the *Sey* mutant is disturbed. The abnormality of the SVZ coincides with the absence of upper layer cells in the cortex. Taken together our data suggest that while the specification of deep cortical layers occurs in the ventricular zone, the SVZ is important for the proper specification of upper layers.

MATERIALS AND METHODS

Animals

The allele of the *Small eye* used here is *Sey* (Hogan et al., 1988) on a C57BL/6J × DBA/2J background. Heterozygous *Sey* were crossed to obtain homozygous, heterozygous and wild-type embryos in the same litter. The day of appearence of the vaginal plug was considered embryonic day (E) 0.5. The *Emx2*$^{-/-}$ and *Otx1*$^{-/-}$ mutants have been described previously (Pellegrini et al., 1996; Acampora et al., 1996). *Reeler* mice were obtained from The Jackson Laboratory. Genotyping was performed as described previously (D'Arcangelo et al., 1996; Pellegrini et al., 1996).

cDNA subtraction

The neocortex tissue of E15.5 and E18.5 embryos was isolated in Dulbecco's modified medium (DMEM) containing 10 mM Hepes and collected in separate vials on ice. Tissue samples were then processed for RNA isolation and cDNA amplification. Amplified cDNA for cDNA subtraction was prepared as described previously (Tarabykin et al., 1995) or by Smart cDNA amplification kit (Clontech) according to the manufacturer's instructions. Subtractive hybridization of cDNA was performed as described previously (Lukianov et al., 1994; Gurskaya et al., 1996).

An E15.5 telencephalon-specific unidirectional cDNA library was constructed using cDNA library construction kit (Gibco-BRL). 10^6 independent clones were plated and screened with the *Svet1* probe.

In situ hybridization and BrdU immunohistochemistry

Sectioning, in situ hybridization, washing and emulsion autoradiography were performed as described (Stoykova and Gruss, 1994). Two independent in situ analyses were performed for each stage on serial sections from wild-type and mutant littermates. Signals were compared at corresponding levels in wild-type and mutant brains processed in the same in situ hybridization experiment.

Non-radioactive in situ hybridization on frozen sections was performed essentially as described by Schaeren-Wiemers et al. (Schaeren-Wiemers et al., 1993). Detection of digoxigenin-labeled RNA was performed using either the NBT-BCIP (Roche) or TSA-direct cyanine-5 kit for fluorescence detection (NEN Life Science Products) according to manufacturers' protocols.

For the BrdU plus in situ labeling, pregnant mice were injected with 100 μg/g bodyweight bromodeoxyuridine (BrdU). One (in the case of E13.5) or 2 hours later the animals were sacrificed and brains or embryos were processed for hybridization on tissue sections. After the NBT-BCIP color reaction the sections were processed for BrdU immunohistochemistry with anti-BrdU antibody (Sigma). For anti-BrdU antibody detection we used Alexa Red or Alexa Green secondary antibody (Molecular Probes). For the BrdU labeling of E13.5 embryos color reactions were performed using the ABC kit (Vector Laboratories).

RESULTS

Cloning of *Svet1* cDNA by cDNA subtraction

In order to isolate molecular markers of the cortical SVZ we performed cDNA subtraction of two cDNA libraries from E15.5 and E18.5 mouse cortical tissue. At E15.5 the thickness of the SVZ is at its maximum and it is the peak neurogenetic day for neurons destined for upper layers (Smart and Smart, 1982). By E18.5 the SVZ is much thinner than at E15.5 and cortical neurogenesis is almost over. One of the clones identified was more abundant in E15.5 cDNA than in E18.5 cDNA and showed an interesting pattern in SVZ. We referred to it as *Svet1* (subventricular tag). The original clone we obtained from our subtracted library was 370 bp long. In order to isolate more cDNA sequence for the *Svet1* gene we constructed a cDNA library from E15.5 telencephalon tissue. Screening of 10^6 clones revealed two positives which comprised 3934 bp sequence (GeneBank accession number AF323987). This sequence did not contain any open reading frame longer than 213 bp. We found no homologous gene in any public database, including the EST database. Northern analysis revealed that the size of the *Svet1* mRNA is more than 10 kb (not shown). We concluded therefore that this sequence is probably a part of either the 3′ or 5′ non coding region of the gene. We are currently performing experiments in order to isolate the full cDNA copy of the gene.

Expression of *Otx1* and *Svet1* during development of the cortex

We performed comparative analysis of *Otx1* and *Svet1* expression during development of the cortex. It has been shown (Frantz et al., 1994) that *Otx1* is expressed in both mature layer V-VI neurons and in the precursors of these cells while they are still in the ventricular zone. Therefore, *Otx1* serves as a marker for the cortical cells committed to deep layer fate. For all experiments with *Svet1*, RNA synthesized from the antisense strand was used as a negative control. In these negative control experiments no signal was detected above background (data not shown).

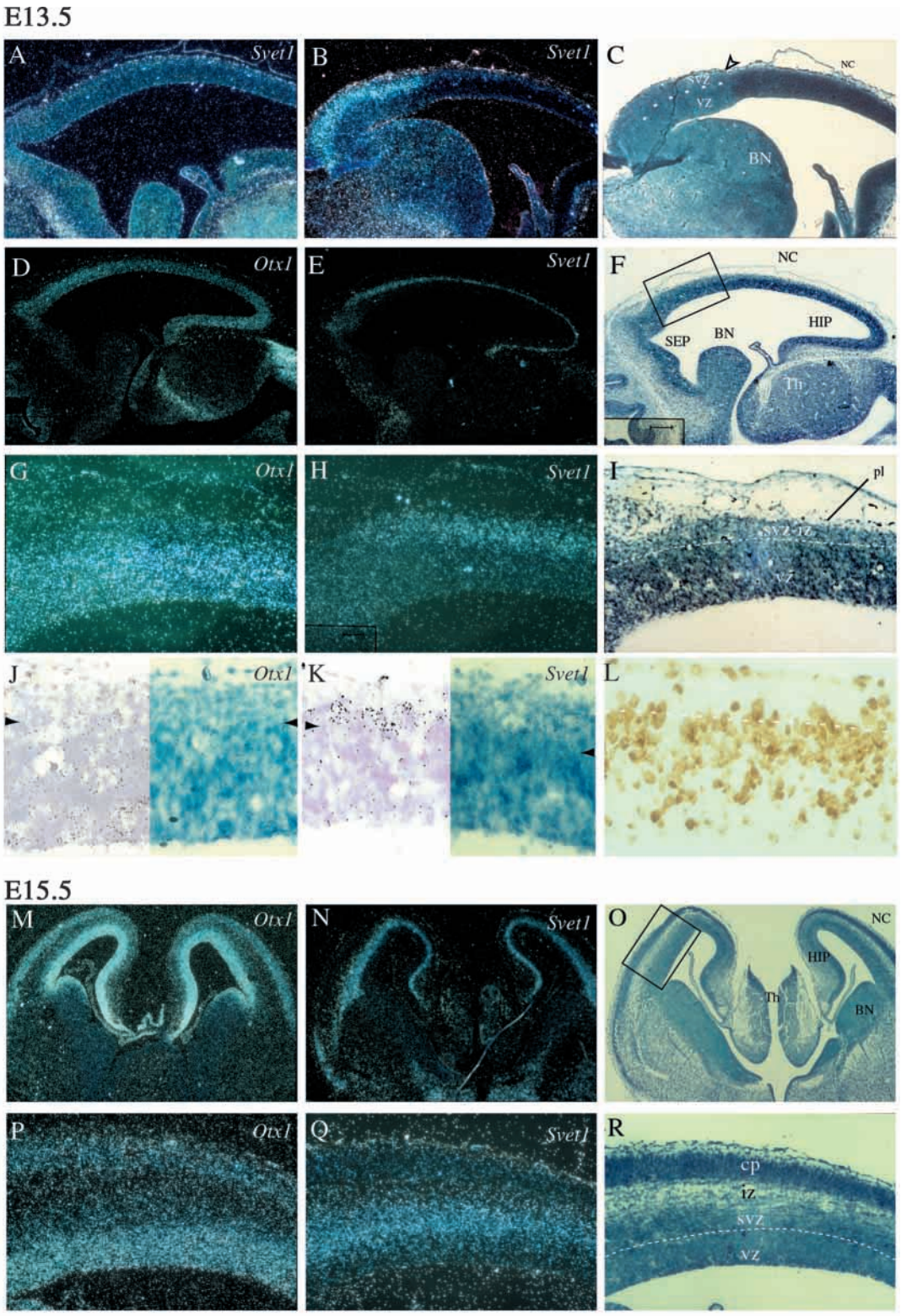

Fig. 1. *Svet1* RNA distribution in developing cortex. (A,B,D,E,G,H,M,N,P,Q) dark field; (C,F,I.O,R) bright field. *Svet1* expression begins in the SVZ immediately after its first appearance in the rostrolateral cortex (B). The arrowhead in C indicates the transition between the rostral part of the cortex where the SVZ has just begun to form and the caudal part where there is no SVZ yet. The dashed line in C denotes the boundary between SVZ and VZ, which coincides with that of *Svet1* expression. (D-R) Comparison of *Otx1* and *Svet1* expression in the E13.5 (D-L) and E15.5 (M-R) cortex. *Otx1* is expressed in the cells of the cortical ventricular zone of both E13.5 (D,G,J) and E15.5 (M,P). In the cortex of E15.5 embryos, *Otx1* is expressed in the cortical plate and intermediate zone. Note that in P and Q the expression of *Otx1* is at similar levels in the SVZ and IZ, while *Svet1* is confined to the SVZ only. I and R are high magnification views of the regions boxed in F and O. The dashed line in R demarcates the boundary between SVZ and VZ, which coincides with the boundary between *Svet1* and *Otx1* expression. (J,K) High power view of the region boxed in F. Arrowheads show the lower limit of *Svet1* (K) and upper limit of *Otx1* (J) expression. Left sides of J and K have been digitally contrasted by means of Adobe Photoshope software in order to demonstrate silver grain density more clearly. (L) Immunohistochemistry with anti-BrdU antibody on an adjacent section. The dashed line denotes the approximate boundary between the VZ an SVZ (svz + iz according to Altman and Bayer). The cortical region is the same as indicated by the box in F. BN, basal nuclei; cp, cortical plate; HIP, hippocampus; iz, intermediate zone; NC, neocortex; pl, primordial plexiform layer; SEP, septum; Th, thalamus. Bar in F, 100 μm.

In the neocortex *Svet1* starts to be expressed at E13.5. Fig. 1 shows the distribution of the *Svet1* transcript in the E13.5 mouse embryonic cortex: a signal detected in the SVZ of the most anterolateral part of the neocortex (Fig. 1B), but is still absent in the more medial parts at this stage (Fig. 1A). In the more posterior part of the cortex there is no *Svet1* expression (Fig. 1B). The sharp boundary of *Svet1* expression corresponds exactly with a boundary between the developmentally more advanced anterior

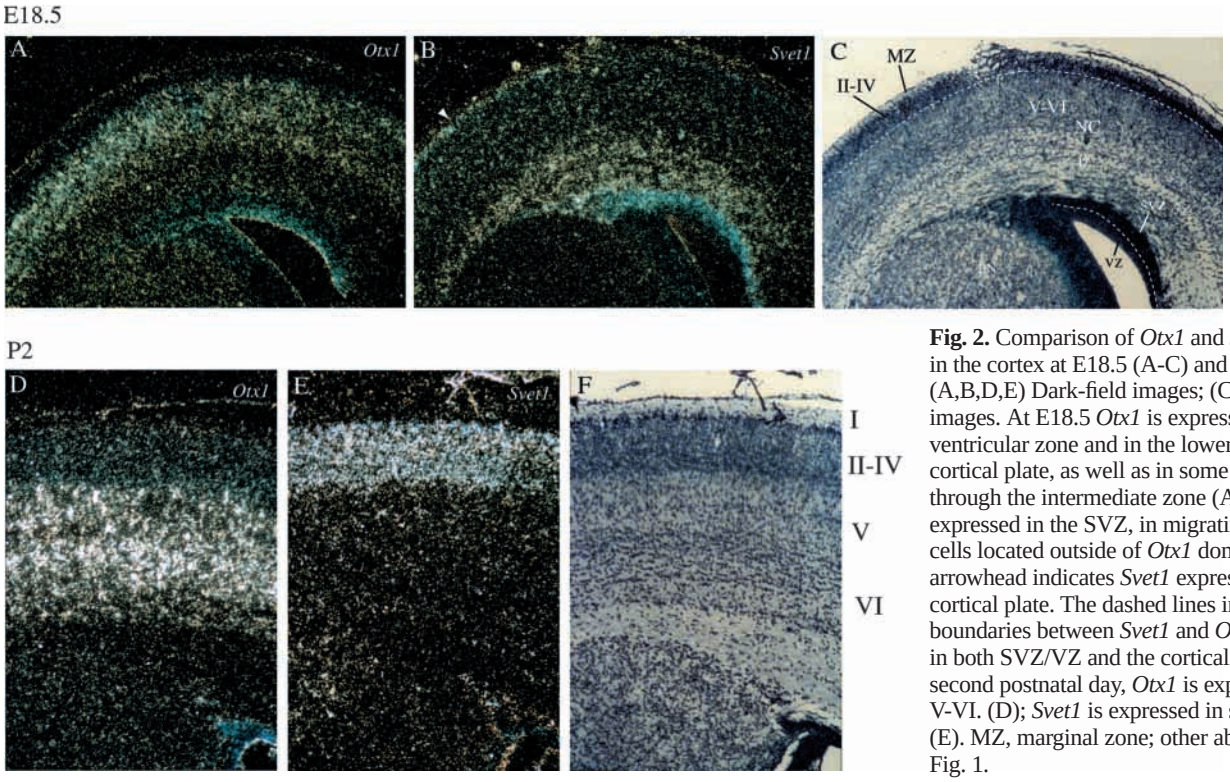

Fig. 2. Comparison of *Otx1* and *Svet1* expression in the cortex at E18.5 (A-C) and P2 (D-F). (A,B,D,E) Dark-field images; (C-F) bright-field images. At E18.5 *Otx1* is expressed in the ventricular zone and in the lower part of the cortical plate, as well as in some cells migrating through the intermediate zone (A). *Svet1* is expressed in the SVZ, in migrating cells and in cells located outside of *Otx1* domain (B). The arrowhead indicates *Svet1* expression in the cortical plate. The dashed lines in C denote the boundaries between *Svet1* and *Otx1* expression in both SVZ/VZ and the cortical plate. On the second postnatal day, *Otx1* is expressed in layers V-VI. (D); *Svet1* is expressed in superficial layers (E). MZ, marginal zone; other abbreviations as in Fig. 1.

part of the cortex, which already has the SVZ and the less advanced posterior part, where the SVZ has not yet begun to form (Fig. 1C). Further laterally, we did not observe any *Svet1* expression in the cortex (Fig.1 A). Therefore *Svet1* expression can be detected in the SVZ immediately after its appearance. Hence, the order of appearance of the first *Svet1*-positive cells in the cortex follows both the anterior-to-posterior and lateral-to-medial gradients of the cortical neurogenesis (Bayer and Altman 1991). In the developmentally more advanced littermates at E13.5, the *Svet1* expression appears to be evenly distributed throughout the whole neocortical SVZ (Fig. 1E). Labeling with mitotic marker BrdU revealed some proliferating cells in the layer of *Svet1*-positive cells (Fig. 1L,K), allowing identification of this layer as SVZ as opposed to cortical plate (which characteristically lacks any mitotic cells). This nomenclature agrees with Altman and Bayer (1991, figs 2-12) who refer to the cell layer between the VZ and primordial plexiform layer as emerging subventricular and intermediate zone (sv+iz).

The cortical expression of *Svet1* remains essentially identical to this pattern at E14.5-E15.5. It is still expressed in the SVZ (Fig. 1N,Q). We did not observe any *Svet1*-positive cells in the neocortex outside of the SVZ at this stage. However, we found *Svet1*-positive cells in the piriform plate of the piriform cortex (Fig. 1N). At E18.5 *Svet1* expression was found in the SVZ as well as within other regions of the cerebral wall (Fig. 2B). Hybridization is particularly strong in the intermediate zone, which consists of migrating neurons, most of which are fated for the upper layers. The majority of *Svet1*-positive migrating cells are found in the intermediate zone, although there are some *Svet1*-expressing cells within the cortical plate (Fig. 2B). Those cells begin to accumulate in the outermost part of the cortical plate outside of the *Otx1* domain (Fig. 2A,B). This *Otx1*-negative domain in the cortical plate is presumably made up by upper layer neurons just beginning to arrive. At this stage, *Svet1*-positive cells arrive first at the most lateral parts of the neocortex respecting the lateral-to-medial gradient of neurogenesis (Fig. 2B). Along the anteroposterior axis, the wave of *Svet1*-positive neurons reaches the outermost part of the cortical plate, following the anteroposterior gradient of corticogenesis (anterior first, posterior last; not shown). This 'posterior delay' takes about 1 day.

Svet1 has a complex expression pattern outside the cerebral cortex. Within the developing CNS it is expressed in some differentiating fields of the septum (Figs 3H, 1F). Outside the forebrain it is expressed in some postmitotic cells of the tegmentum and tectum. It is expressed in the ventricular zone and adjacent region of the inferior colliculus. It is also expressed in some neurons within the ventral horn of the spinal cord (Fig. 3C).

At E13.5 the cortical domains of *Otx1* and *Svet1* expression do not overlap. *Otx1*, as reported previously (Frantz et al., 1994; Simeone et al., 1993), is expressed within the ventricular zone (Fig. 1D,G,J). We did not detect any hybridization signal above background for *Otx1* in the SVZ (Fig. 1G,J). At the same time, *Svet1* is expressed in the SVZ with no expression in the ventricular zone (Fig. 1H). At E15.5 *Otx1* expression is also apparent, albeit at a more moderate level, in the cortical plate (Fig. 1M,P). Interestingly, while *Otx1* shows expression at the same level in the intermediate and SV zones, *Svet1* is detected in the SVZ only (Fig. 1M-Q). At this stage *Otx1*, expression in the SVZ and IZ presumably labels young neurons fated for the deep layers. It has been shown that cells of the cortical plate, which are positive for *Otx1* at this stage are young neurons of layers V and VI (Frantz et al., 1994). At E18.5 *Otx1* expression is prominent in the lower two thirds of the cortical plate (Fig. 2A). The expression domain is expanded compared to that at E15.5. This domain contains *Otx1*-positive deep layer

neurons as well as younger upper layer neurons (*Otx1*-negative cells) migrating upwards. Reduced *Otx1* expression persists in the ventricular zone (Fig. 2A). At this stage we still detect *Otx1* signal in the IZ and SVZ, which has been attributed to the migrating deep layer neurons (Frantz et al., 1994).

Expression of *Svet1* in the mitotically active cells of the cortical SVZ

In order to determine whether *Svet1*-positive cells undergo their last mitotic division in the SVZ, the correlation of *Svet1* expression with the cell cycle was investigated by analyzing it in cortices labeled with BrdU. E15.5 or E14.5 pregnant mice were injected with BrdU and sacrificed 2 hours later. This time period, according to cell cycle kinetic studies (Takahashi et al., 1995), is still short enough to claim that all BrdU-positive cells are mitotically active. However, under these conditions not all mitotic cells are labeled, but only those that are either in the S or G_2/M phase of the mitotic cycle. In our double labeling experiments we found four types of cells within the SVZ: (1) *Svet1*/BrdU negative; (2) *Svet1* positive/BrdU negative; (3) *Svet1* negative/ BrdU positive and (4) *Svet1* positive/BrdU positive (Fig. 4A-C). The last subpopulation represented approximately 10-15% of all BrdU-positive cells in the SVZ. This demonstrates that *Svet1* starts to be expressed in the dividing precursors of the SVZ but not in postmitotic cells migrating through it. It is remarkable that, according to the results of non-isotopic in situ hybridization, *Svet1* mRNA is localized to the cell nucleus (Fig. 4C,E).

Postnatal expression of *Svet1* and *Otx1*

In the P2 cortex, *Svet1*-positive cells are situated in the upper layers (II-IV), which are easily distinguishable from the deep layers by their morphologies (Fig. 2E-F). *Svet1*-positive cells are most abundant in the somatosensory cortex, and almost absent in the cingulate area (not shown).

At P14, upper cortical layers are individually distinguishable. *Svet1*-positive cells are found as a subpopulation of layers IV, III and II (not shown). No *Svet1* expression is detectable in the cortex beyond P60.

Otx1 continues to be expressed at P2 by some layer V and VI neurons (Fig. 2D), as reported for the rat (Frantz, 1994). From P2 onwards the *Otx1* expression domain does not overlap with that of *Svet1*.

Svet1 expression in the cortex of '*reeler*' mutant

Reeler is a spontaneous mouse mutant in which the normal layered structure of the cortex is inverted (see Rakic and Caviness, 1995 for review). We examined whether *Svet1* maintains its expression, specifically confined to the SVZ and upper layers in their new inverted location.

The earliest stage we examined was P0. We detected the same level of *Svet1* expression within the SVZ in mutant and wild-type newborns (Fig. 5A,B). *Svet1* was also detected in the intermediate zone and in the lower part of the cortical plate (Fig. 5B). However, in contrast to what happens in wild type at this stage, there were no *Svet1*-positive cells in the outermost part of the *reeler* cortex (Fig. 5A,B).

In the wild-type cortex of P3, *Svet1* expression was detected within the upper third of the cortical plate, where layer II-IV neurons are situated (Fig. 5E). In the mutant cortex *Svet1*-positive cells occupy the lower part of the cortical plate (Fig. 5F). At this time there was no *Svet1* signal in the upper part of the cortex. Therefore, the position of *Svet1*-positive cells in the *reeler* cortex was inverted. Interestingly, regional differences in the representation of *Svet1*-positive cells were preserved in the mutant (Fig. 5E,F). In both the mutant and in the wild-type cortex the *Svet1*-positive cells were most abundant in somatosensory cortex. These results show that *Svet1* specifically delineates the SVZ as well as a subpopulation of upper layer neurons.

Expression of *Svet1* and *Otx1* in the *Sey, Emx2* and *Otx1* mutants

The expression patterns of *Otx1* and *Svet1* in the cerebral cortex suggest that the SVZ of the neocortex plays an important role in the generation of superficial layer neurons. To further

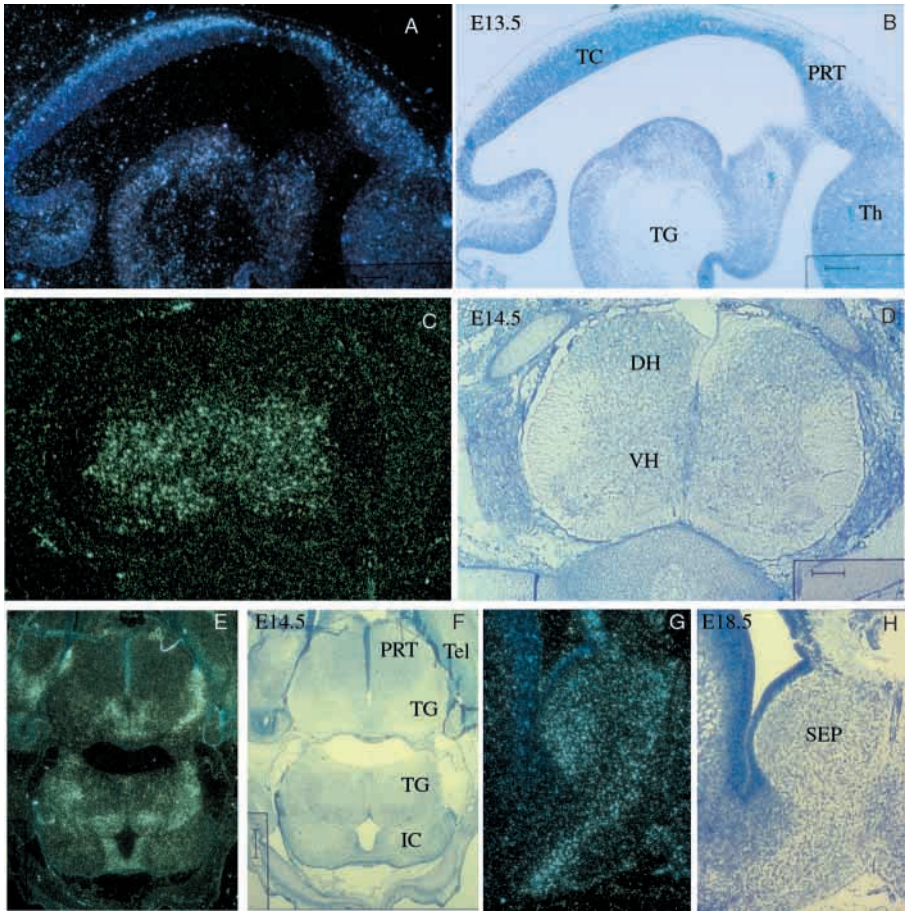

Fig. 3. Localization of *Svet1* RNA in the E13.5 (A,B) and E14.5 (E,F) midbrain region; E14.5 spinal cord (C,D) and E18.5 septum (G,H). DH, dorsal horn of the spinal cord; IC, inferior colliculus; PRT, pretectum; TC, tectum; TG, tegmentum; VH, ventral horn of the spinal cord. Bars in B and F, 100 μm; in D, 50 μm.

test this hypothesis we asked whether a perturbation in the development of the SVZ results in an abnormal differentiation of upper layer neurons. We examined the distribution of *Svet1* and *Otx1* expression in the cortex of mouse mutants with phenotypes which indicated that the differentiation of the cortical SVZ might be altered.

No obvious differences in the *Svet1* expression were observed in the SVZ of the E15.5 and E18.5 cortex of *Otx1*$^{-/-}$ (Acampora et al., 1996) or *Emx2*$^{-/-}$ (Pellegrini et al., 1996) embryos. Neither the expression of *Svet1* in the SVZ, nor the position and amount of *Svet1*-expressing cells were affected (not shown) in these mutants. Weimann et al. (Weimann et al., 1999) have also reported normal lamination in *Otx1* homozygous mutants.

Svet1 and *Otx1* expression in the *Pax6* mutant E15.5 cortex

The cortical plate of *Pax6* homozygous mutant (*Sey*) embryos is underdeveloped, while the germinative neuroepithelium (VZ/SVZ) is enlarged (Schmahl et al., 1993; Caric et al., 1997; Götz et al., 1998; Bishop et al., 2000). *Svet1* expression was greatly affected in the neocortical SVZ, but preserved in the SVZ of the cingulate cortex (Fig. 6E). There is a mediolateral gradient of severity, so that *Svet1*-positive cells are absent in the lateral cortex, but still present, although in a reduced amount in the SVZ of the most medial neocortex (Fig. 6E). The absence of *Svet1*-expressing cells in the *Sey* neocortical SVZ is accompanied by an increase in size of this compartment, particularly noticeable in the frontal cortex and decreasing gradually towards occipital regions (Schmahl et al., 1993). Therefore, *Svet1* expression is most severely affected in those cortical areas showing the most drastic anatomical defects.

At E15.5 *Otx1* expression in the neocortical ventricular zone of *Sey*/*Sey* embryos was not affected (Fig. 6D). There was no observed decrease or increase in the thickness of the *Otx1*-positive domain within the ventricular zone. Nor were any differences in the size of the *Otx1* domain in the cortical plate between *Sey* embryos and their littermates detected.

Otx1 and *Svet1* expression in the E18.5 frontal cortex of *Sey* mutant

By E18.5 the degree of dysgenesis of the neocortical SVZ of *Sey* had increased compared to E15.5 (Fig. 6). No *Svet1*-positive cells were seen in the frontal cortex except for a small strip of *Svet1*-positive cells within the cingulate cortex (Fig. 6K). In contrast *Otx1* expression within the VZ was not disturbed. The expression domain of *Otx1* in the CP was of comparable size in the mutant and wild-type brains (Fig. 6G,J). This indicates that the genesis of the deep layer neurons (at least those, that express *Otx1*) is not disrupted in the *Sey* mutant. Moreover, the vast majority of the cells in the mutant cortical plate were located within the *Otx1* domain (Fig. 6J,L). In contrast to the wild type cortical plate (Fig. 6J,I), there were almost no cells in the outer most *Otx1* domain of the cortical plate. In the wild type at this stage this outer part of the cortical plate consists of at least three cell populations. (1) The first and oldest cell population is formed by layer I cells (Marin-Padilla, 1978), most of which are reelin positive (D'Arcangelo et al., 1995). They arrive in the cortical plate before the *Otx1*-positive cells as judged by their radial position according to 'inside-out' gradient of corticogenesis (Bayer and Altman, 1991). The expression of reelin in the layer I of the mutant was not reduced (not shown, see also Stoykova et al., 2000), indicating that the position of these cells in *Sey* mutants was not affected. (2) The second population consists of darker stained cells negative for both *Svet1* and *Otx1*. This cell population is a population of cells of upper layers that arrive in the cortical plate after the *Otx1*-positive cells. They are located between MZ (*reelin*-positive cells) and *Otx1*-positive cells. These cells are also negative for RoRβ (not shown), a gene expressed in some cells of layers V-IV (Schaeren-Wiemers et al., 1997). This subpopulation of cells cannot be more precisely characterized for the moment because of the lack of molecular markers. These cells are not found in the cortical plate of the *Sey* mutant (Fig. 6J,L). (3) The third cell population is the latest generated of the three, and expresses *Svet1*. Most of the *Svet1*-positive cells are still migrating in the wild-type cortex at this stage (Fig. 6H). *Svet1*-positive cells are missing, and indeed other kinds of cells are greatly reduced in the mutant intermediate zone (Fig. 6L). The SVZ of the mutant is expanded even more than at E15.5 (Fig. 6L) and still does not contain (except in the cingulate cortex) *Svet1*-positive cells (Fig. 6K). The absence of a *Svet1* signal in the mutant SVZ indicates that cells in this zone are abnormal.

Expression of *Otx1* and *Svet1* in the E18.5 occipital cortex of *Sey* mutant

In the *Sey*/*Sey* mutant cortex the expression of *Otx1* does not

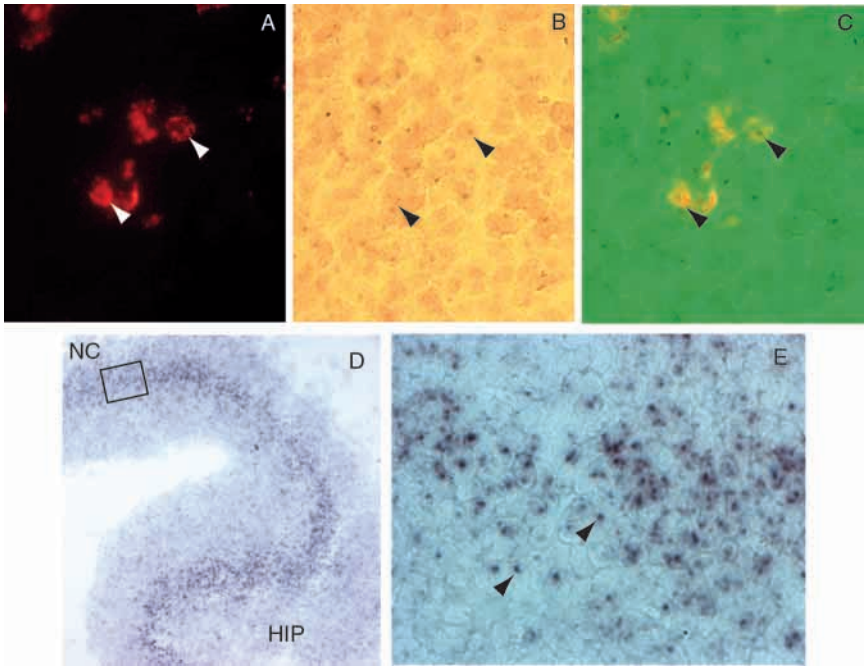

Fig. 4. (A-C) BrdU labeling (A) and digoxigenin *Svet1* hybridization (B) at E15.5. (C) Superimposition of A and B. Cells positive for both labels indicated by arrowheads. (D) Digoxigenin hybridization with *Svet1* probe in E14.5 brains. (E) High magnification view of boxed region in D.

show differences along the A-P axis (Fig. 7D,J). In contrast, the *Svet1* expression in the mutant SVZ is much more affected in the anterior than in the posterior part. In contrast to the frontal *Sey* cortex, we observed *Svet1*-positive cells within the SVZ of occipital cortex, although there were fewer of them (Fig. 7H,K). This correlates with the severity of the morphological abnormalities in the mutant cortex (Fig. 7C,F,I,L; also Schmahl et al., 1993). Precisely in the parts of the cortex where both morphology and *Svet1* expression were less disturbed, we observed cells in the cortical plate outside of the *Otx1* expression domain. Also cells moving through the intermediate zone were observed. Cortical plate of the wild-type occipital cortex at this stage does not contain *Svet1*-positive cells in contrast to frontal cortex, because of developmental delay of this region (Fig. 7C,I).

DISCUSSION

We have isolated a new molecular marker, *Svet1*, which is specifically expressed in the embryonic SVZ and the upper layers of the mature cortex. We carefully analysed an extensive collection of cortex sections of all embryonic ages hybridized them with antisense *Svet1* mRNA. Then we correlated these observations with the corresponding developmental events as revealed in parallel sections stained with Giemsa, as well as with the known neurogenetic gradients of cortical cells; finally, we performed BrdU-*Svet1*-double labeling. Taken together, our data suggest that *Svet1* labels a certain SVZ mitotic subpopulation and its progeny. This view is fully supported by independent neurogenetic studies, as well as our own analysis of developmental expression of *Svet1* in several cortical mutants. Using *Svet1* RNA as a molecular marker in conjunction with *Otx1* (which labels a subpopulation of deep layer neurons), we examined layer formation in wild type cortex and in the cortex of four mutants (*reeler*, Small eye, $Emx2^{-/-}$ and $Otx1^{-/-}$) known to have abnormalities in corticogenesis. The data obtained strongly support the view that some cortical progenitors in the SVZ become fated to become upper cortical layer cells. This hypothesis was first suggested by Smart and McSherry (Smart and McSherry, 1982) on the basis of tritiated thymidine experiments. The contrasting, widely accepted view is that all neocortical neurons are born in the ventricular zone, the SVZ producing only glial cells (Bayer and Altman, 1991). We propose a model of cortical lamination where the specification of deep layers occurs in the ventricular zone, while the SVZ is important for the generation and proper specification of the upper layers.

Molecular aspects of *Svet1*

We isolated 3934 bp of the cDNA sequence of *Svet1*. This part of the gene does not contain any extensive ORF. As indicated by northern hybridization the transcript is rather large – more than 10 kb. Therefore, we are still missing more than 6 kb of sequence. The *Svet1* transcript is localised in the cell nucleus. Xist is the only other example of a large RNA localised in the nucleus. Xist is a gene encoding a large (17 kb in humans, 15

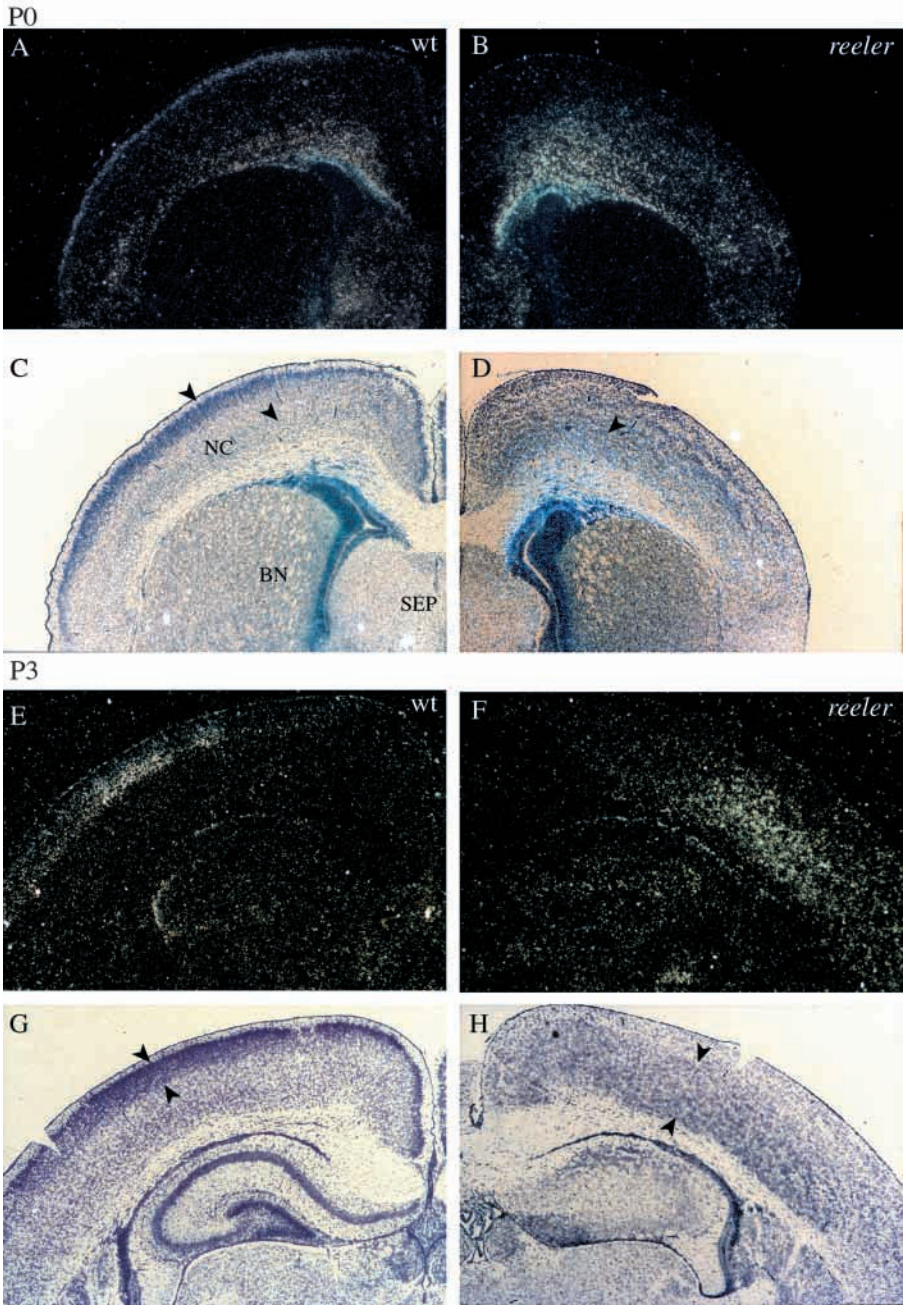

Fig. 5. Localization of *Svet1* RNA in the E18.5 and P3 wild-type and '*reeler*' brains. (A,B,E,F) dark-field, (C,D,G,H) bright-field views. In C,D,G,H the *Svet1* expression domain is indicated by arrowheads. Wt, wild type.

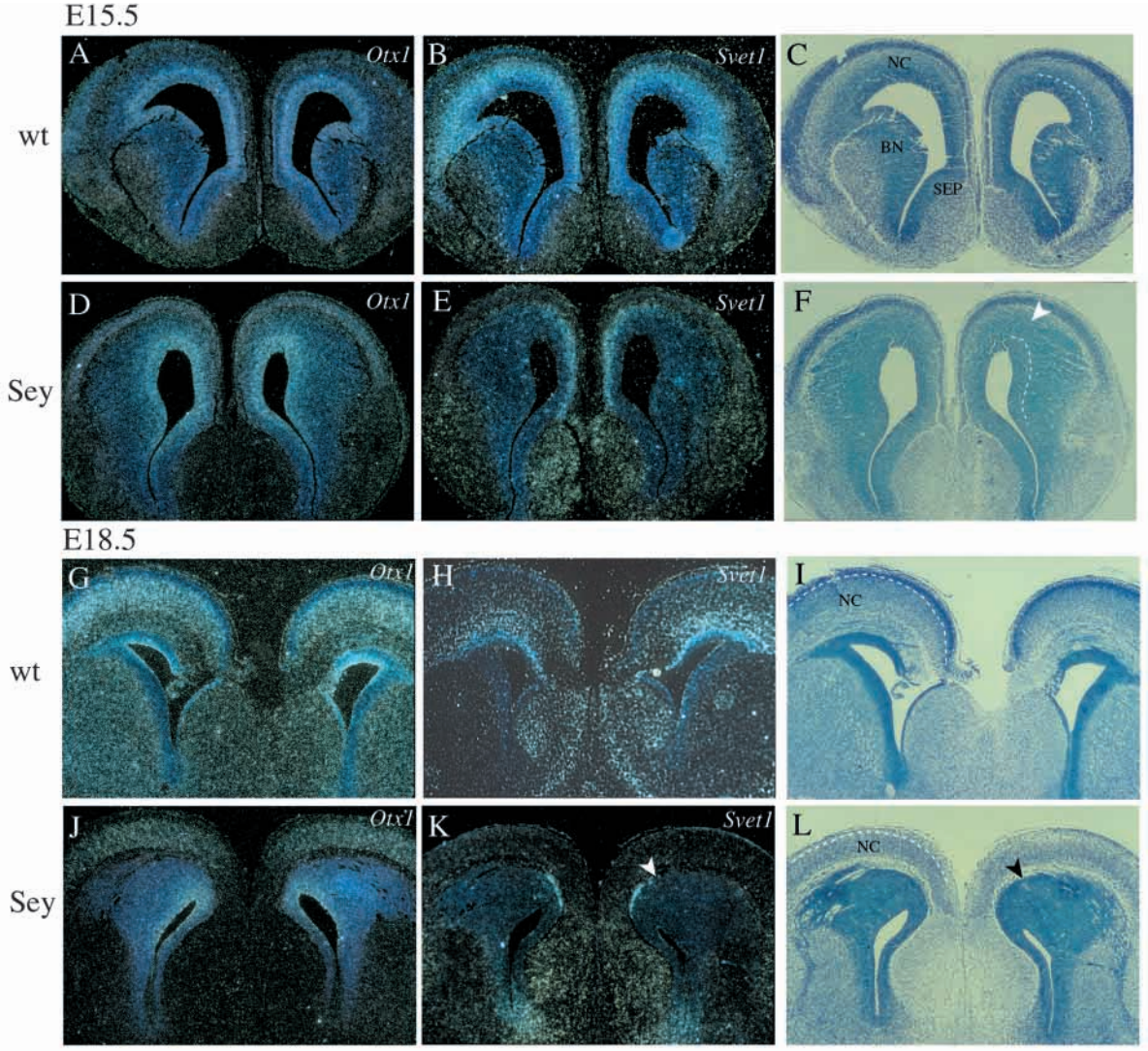

Fig. 6. (A-F) Comparison of the *Otx1* and *Svet1* cortical expression at the stage E15.5 in wild type (A,B) and homozygous *Sey* mutant (D-E). The size of the ventricular zone in the *Sey* mutant is not affected, as revealed by *Otx1* expression (D). The SVZ is enlarged, its morphology is altered and it does not express *Svet1* (F), which can be seen only in the cingulate cortex (E). (G-L) Comparison of the *Otx1* and *Svet1* cortical expression at the stage E18.5 of wild-type (G-I) and *Sey* mutant (J-L). Deep layer neurons (*Otx1*-positive cells) are present in the *Sey* cortical plate (J). Superficial layer neurons (*Svet1*-positive cells; *Otx1*/*Svet1*-negative cells of the cortical plate) are absent in the *Sey* cortex. *Svet1* is not expressed in the SVZ of the *Sey* cortex (K). Arrowheads in F, K and L mark the lateral limit of *Svet1* expression.

kb in mice) non-translated RNA localized within the nucleus and essential for X chromosome inactivation in mammalian females (Brockdorff et al., 1992; Brown et al., 1992). It is conceivable that *Svet1* RNA might also be a non-translated RNA with regulatory functions in the nucleus. To test this hypothesis, experiments are in progress in order to clone the full *Svet1* cDNA and to generate a null mutant.

Otx1 and *Svet1* label two different cortical progenitor subpopulations

At the peak of deep layer neurogenesis (E13.5), *Otx1*-positive cells were found in the VZ only (see also Frantz et al., 1994). At the peak of upper layer neurogenesis (E15.5) *Otx1*-positive cells are still accumulating within the VZ and some are still migrating through the SVZ and IZ. However, a large number of them has already arrived in the cortical plate. The expression level of *Otx1* within the SVZ and IZ was comparable, suggesting that there is no accumulation of *Otx1*-positive cells within the SVZ. *Svet1*-positive cells start to appear in the SVZ at E13.5 and accumulate within the SVZ for at least 3 days. This observation is consistent with data (Bayer and Altman, 1991) showing that some cells stop in the SVZ before initiating their migration. They emerge from the SVZ at around E17.5 and the most intense migration takes place at E18.5. From these non-overlapping spatiotemporal patterns of expression we conclude therefore that *Otx1* and *Svet1* expression labels two non-overlapping precursor subpopulations for the deep and the upper layers, respectively.

Svet1 is expressed by dividing SVZ cells and their progeny

After 2 hours in vivo labeling with BrdU, we found that a fraction

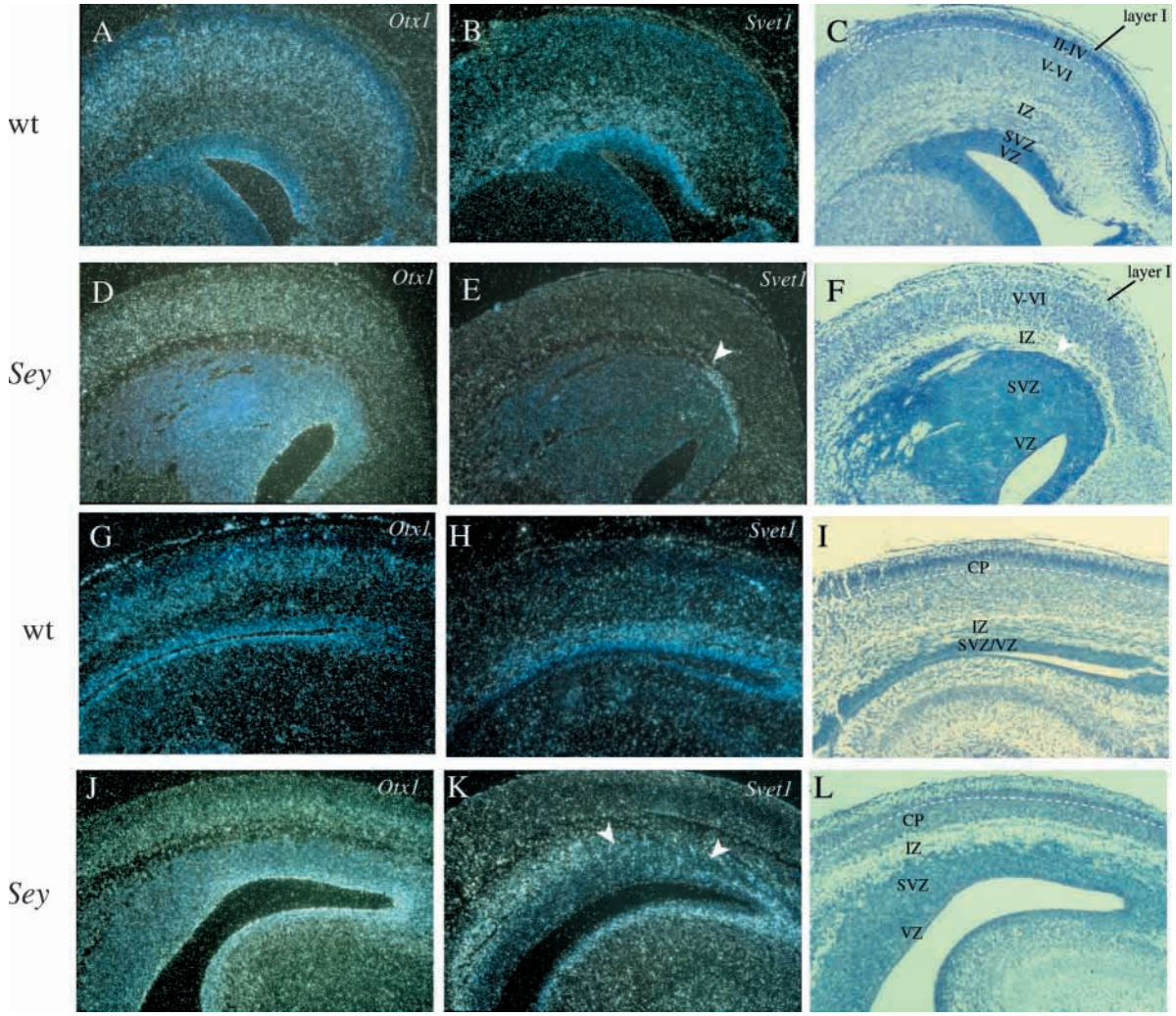

Fig. 7. Anteroposterior differences in the cortical lamination of the E18.5 *Sey* mutant. Transverse sections through the wild type (A-C) and *Sey* (D-F) frontal and the wild type (G-I) and *Sey* (J-L) occipital cortex. The dashed line on C,F,I and L demarcates the upper boundary of deep layers. Note the absence of granular cells of upper layers above the dashed line in F. Arrowheads in E and F mark the lateral limit of *Svet1* expression. *Svet1* cells are reduced in number but not completely missing in the SVZ of the mutant occipital cortex. Arrowheads in K mark the altered SVZ region. Note the presence of upper layer cells above the dashed line on L.

of the progenitors in the SVZ (approximately 10-15% of the all BrdU-positive cells) was expressing *Svet1*, a fact suggesting that a portion of the SVZ progenitors express *Svet1* before their exit from the mitotic cycle. Under the conditions used (analysis 2 hours after BrdU injection) all BrdU-positive cells are still mitotically active (Takahashi et al., 1995) and only a small portion of all cycling cells will be BrdU positive. Labeling for a more extended period would mark more cycling cells but also some postmitotic cells. These data indicate that *Svet1* starts to be expressed in a subpopulation of SVZ cortical progenitors and can therefore be used as marker of this subpopulation of cortical precursors. The fact that *Svet1* expression appears in the SVZ and gradually moves outwards through the IZ to end up confined to the upper layers, suggests a role of the SVZ in the specification

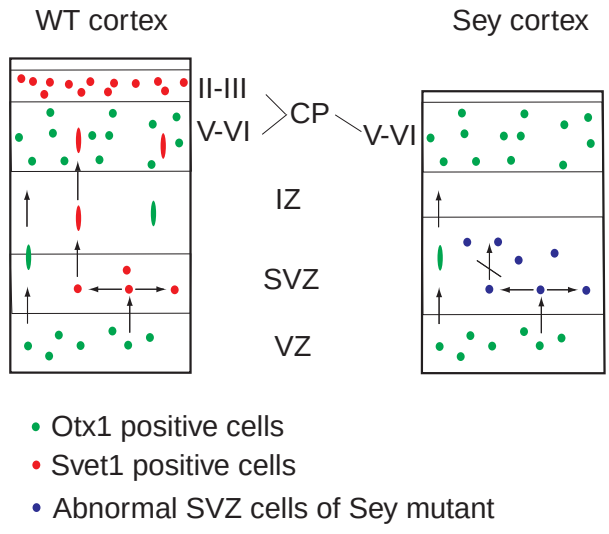

Fig. 8. A hypothesis for the role of SVZ in cortical lamination (see Discussion for the details)

of upper layer fate. The lineage relation between *Svet1*-positive cells in SVZ and upper layers is emphasized by analysis of *Svet1* expression in the cortex of the *reeler* mutant (where layers II to VI are inverted); in *reeler*, the expression of *Svet1* labels 'upper' layer cells in their inverted arrangement.

Sey mutant cortex supports a lineage relation between SVZ and upper layers

Sey is a null mutation within the *Pax6* gene (Hill et al., 1991). The homozygotes, which die at birth (Hogan et al., 1988), lack eyes and have severe abnormalities in different CNS regions (Osumi et al., 1997; Stoykova et al., 1996; Warren and Price, 1997; Stoykova et al., 1997; Götz et al., 1998; Warren et al., 1999, Bishop et al., 2000). The cortical plate in *Sey*/*Sey* is hypocellular, while the germinative neuroepithelium (VZ and SVZ), is hypercellular (Schmal et al., 1993; Caric et al., 1997). The accumulated cells in the enlarged VZ/SVZ are mitotically active (Stoykova et al., 1997; Brunjes et al., 1998) and generate neurons (Caric et al., 1997) with an abnormal differentiation profile (Warren et al., 1999). Using the two markers (*Otx1* and *Svet1*), we examined whether the sizes of the two proliferative compartments, VZ and SVZ, are equally affected by the *Pax6* deficiency. The *Otx1* domain in the VZ was preserved in the mutant cortex, indicating that the thickness and at least part of the molecular determinants of this compartment are normal in *Sey*. The size and appearance of the mutant SVZ, however, are largely distorted as revealed by Giemsa staining, and *Svet1* expression is completely abolished, suggesting that the main defect of the mutant proliferative compartment is confined to the SVZ. Consistently, late-born neurons accumulate in the SVZ of *Sey* homozygotes as revealed by BrdU injection experiments (Caric et al., 1997). One reason for this disappearance of *Svet1* expression in the *Sey* brain could be the ventralization reported in this mutant (Stoykova et al., 2000; Toresson et al., 2000; Yun, 2001).

In the cortical plate of *Sey*/*Sey*, the size of the *Otx1* domain was comparable to that of the wild-type embryos. This suggests that the number of the *Otx1*-positive neurons (i.e., the presumptive deep layers) is not affected (see also Caric et al., 1997), and is in agreement with the normal appearance of the mutant VZ. The few *Svet1*-positive cells that have reached the CP in wild type at this stage are absent in the mutant. In fact, the complete upper part of the CP (*Otx1* negative, partially *Svet1* positive in wild type) is absent in these mutants, so that the marginal zone is now in direct contact with the *Otx1* domain (which represents presumably the presumptive deep layers). The marginal zone itself seems unaffected to judge by the expression of specific marker reelin (not shown, see also Stoykova et al., 2000). Since the morphological defects in the *Sey*/*Sey* cortex are much more pronounced in the frontoparietal as compared to the occipital region (Schmahl, 1993; Caric et al., 1997; Bishop et al., 2000), *Svet1* expression could still exist in caudal regions of the mutant cortex. Consistent with this prediction, *Svet1*-positive cells were detectable in the occipital SVZ, whose CP also shows presumptive upper cortical layers. Together, these observations support the hypotheses that SVZ generates upper layers, and that *Svet1* is a lineage marker of SVZ.

Neurogenetic support for a lineage relation between SVZ and upper layers

Further support for the idea that the SVZ plays a role in the production of the upper layers comes from neurogenetic studies. At E14.5 the proliferative compartment of the SVZ represents 11% of the total proliferative population of the neocortex, reaching the maximal value of 35% at E15.5 (Takahashi et al., 1994; Takahashi et al., 1995), which is also the peak of *Svet1* expression intensity in SVZ. The proliferative activity of the SVZ begins to decline at E16.5 coinciding with the beginning of the migration of *Svet1* cells out of the SVZ. In addition, during peak neurogenesis of upper layers in rat (E17-E20), a band of cells heavily labeled by tritiated thymidine appears in the SVZ ('sojourn zone'; Bayer and Altman, 1991). Finally, if the SVZ is a primary source of upper layers neurons and the VZ is a source of deep layers neurons, it would be expected that their relative sizes would be different in areas of the neocortex with a different 'upper layers-to-deep layers' ratio. In the cortex of rodents where this ratio is relatively uniform, the sizes of the SVZ and VZ are comparable. In the monkey however the SVZ is five times larger than the VZ in the parieto-occiptal junction at E75 when the neurogenesis of the upper layers has just started. Accordingly, the upper layers in the monkey occipital cortex occupy twice as much of the whole cortical thickness as compared with the rodent cortex (Rakic, 1972). Therefore, independent neurogenetic data correlate very well with the hypothesis of SVZ origin of the upper layers.

A model of cortical lamination

We propose the following model (Fig. 8) of cortical lamination. Deep layer fate is generated in the VZ and carried by *Otx1* positive, multipotent cells. Some of these cells could migrate through the SVZ after the last mitosis, reach the cortical plate and become deep layer neurons. Another portion of these cells, after leaving the VZ, settle in the SVZ for some days, and change their phenotype from '*Otx1* positive' to '*Svet1* positive'. This hypothesis is in agreement with the transplantation studies of McConnell and coauthors (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996) showing different developmental potentials of the early and lately born cortical progenitors. Signals in the SVZ environment could confer the *Svet1* cells, still mitotically active, to an upper layer fate. *Pax6* appears to be involved in the generation of this environment, most probably through a non cell-autonomous mechanism. This is suggested by the fact that, when transplanted to wild-type cortex, late born (E16*) Sey/Sey* neurons are able to find their final location (Caric et al., 1997).

We thank Dr G. Alvarez-Bolado and Dr T. Thomas for fruitful discussion and helpful suggestions on the manuscript. We thank Dr A. Simeone for providing of *Otx1* embryos. Thanks are due to S. Eckert for a technical assistance, R. Altschäffel for the photography and M. Schuster for help in preparing the manuscript. We acknowledge R. Libal and BTL crew for caring for the mice. This work was supported by the Max-Plank Society. V. T. was a recipient of an EMBO fellowship.

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