

Role of the Atypical Cadherin Celsr3 during Development of the Internal Capsule

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The development of axonal tracts requires interactions between growth cones and the environment. Major bundles, particularly in the internal capsule, are completely defective in mice with constitutive mutation of Celsr3. In order to understand better how Celsr3 controls axonal tract formation, we generated a conditional allele that allowed inactivation of Celsr3 in different sectors of the forebrain. Effects of Celsr3 inactivation specifically in the telencephalon, in the ventral forebrain, or in the cortex, demonstrate essential roles for the gene, both in the neurons that project their axons to subcerebral targets such as the spinal cord, as well as in cells that guide projecting axons through the ventral forebrain. These observations provide unequivocal in vivo evidence that heterotypic interactions between axons and guidepost cells govern axonal path formation in mammals, and that Celsr3 plays a key role in this process. In absence of cortico-subcortical connections, mice can survive up to P20, allowing studies of behavior and cortical maturation. Mutant mice with defective corticospinal tracts survive normally and provide a model to evaluate in vivo the role of this tract in motor function in rodents.

Keywords: axonal guidance, Celsr3, guidepost cells, internal capsule

Introduction

During development, axonal growth cones are guided to their target by attractive and repulsive signals (Tessier-Lavigne 2002) that can act at a distance, such as those provided by diffusible molecules (Serafini et al. 1994; Lyuksyutova et al. 2003), or more locally, such as cues provided with guiding cells (Bentley and Caudy 1983), by extracellular matrix components, or guidance by fiber to fiber interactions (Molnar and Blakemore 1991). Like some other tracts, those that travel through the internal capsule (IC), are fully defective in constitutive Celsr3 mutant mice (Tissir et al. 2005), which thus provide a tool to probe their development.

The mature IC is formed by three main tracts, namely thalamocortical fibers that run from dorsal thalamus to neo-cortex, reciprocal corticothalamic projections, and subcerebral projections such as the corticospinal tract—terminology proposed by Molyneaux et al. (2007). During development, the anlage of the IC forms in the basal forebrain and pioneer axons from cortical subplate and thalamus are thought to be guided in their progression by a variety of mechanisms that remain poorly defined. Among guidance factors, the role of early local cells scattered in the subplate—subplate scaffold (McConnell et al. 1989; Ghosh et al. 1990)—in the basal telencephalon—“corridor cells” (Lopez-Bendito et al. 2006)—and the region of the thalamic eminence—“perireticular thalamic nuclei” (Mitrofanis and Guillery 1993)—has been suspected long ago (Molnar and

Blakemore 1995; Metin and Godement 1996; Molnar et al. 2003). Early cells that assist in axon guidance have been designated by different names according to location and phenotypic markers (Hanashima et al. 2006). Inasmuch as our work is primarily concerned with the role of Celsr3 expression rather than with the precise identity of such cells, we use the term “guidepost cells,” borrowed from studies in *Drosophila* (Bentley and Caudy 1983) in a loose sense, without reference to their specific phenotypic features.

Together with Celsr1 and Celsr2, Celsr3 is a seven pass cadherins similar to *Drosophila Flamingo/Starry night (Fmi/Stan)* (Chae et al. 1999; Usui et al. 1999). In the fly, together with genes such as *Frizzled (Fz)* and *Van Gogh (Vang)*, *Fmi/Stan* regulates planar cell polarity and neurite development (Chae et al. 1999; Usui et al. 1999; Gao et al. 2000). Axonal anomalies of *Celsr3* deficient mice are similar to those in *Fzd3* mutant mice (Wang et al. 2002), suggesting that Celsr and Fzd proteins work together to regulate axonal tract formation, like their orthologs do in flies (Klein and Mlodzik 2005). The observation that all fiber tracts that travel in the IC are defective in Celsr3-deficient mice indicates that Celsr3 expression is clearly necessary for their progression, but constitutive Celsr3 mutant mice do not allow us to test whether expression is required in growing axons, guidepost cells or both.

To inactivate Celsr3 in a cell or region-specific manner, we produced a conditional mutant allele (“floxed” allele, *Celsr3^f*). Crosses were made with mice that express Cre in 1) the forebrain in a pattern similar to expression of *Foxg1* (Hebert and McConnell, 2000); 2) cortical and olfactory structures like *Emx1* (Gorski et al. 2002); 3) Sectors of the ventral telencephalon like *Nkx2.1*, *Gsh2*, (Kessaris et al. 2006); 4) Ventral telencephalon plus ventral diencephalon under the *Dlx5/6* enhancer (Stenman et al. 2003). In order to simplify reading, we use the shorthand “|” for mice with conditional inactivation of *Celsr3*. For example, *Celsr3^f|Foxg1* is short for [*Celsr3^f/-;Foxg1-Cre/+*]. Observations of these mutant mice indicate that Celsr3 is required intrinsically in neurons of origin of subcerebral projections such as the corticospinal tract. In contrast to subcerebral tracts, Celsr3-defective corticothalamic axons develop normally, suggesting that normal thalamocortical fibers might guide them to the thalamus as posited by the “handshake hypothesis” (Molnar and Blakemore 1991, 1995). Both thalamic and all subcortical fibers fail to progress through the ventral telencephalon when Celsr3 is absent from a stream of cells in that territory, demonstrating for the first time the role of guidepost cells in thalamocortical wiring in vivo. A zone critical for axonal navigation in the region of the IC extends from the thalamic eminence through the ventral telencephalon. In

ventral telencephalon, this zone contains early cells that express high levels of *Celsr3*, *Dlx5/6*, and *Islet1*, but not *Gsh2* and *Nkx2.1*, and may be similar to the “corridor” defined previously (Lopez-Bendito et al. 2006). Altogether, these observations suggest that *Celsr3* regulates axonal tract formation by mediating heterotypic interactions between growing axons and guidepost cells, presumably via *Celsr3*-mediated homophilic adhesion (Zhou et al. 2008). Among the mutants produced, three are also interesting as neurobiological models. *Celsr3*|*Foxg1* and *Celsr3*|*Dlx5,6* mice survive postnatally until about P18–P20. These animals have neither corticopetal nor corticofugal projections (“neocortex isole”). Although they are small and weak, they display a surprisingly complex and elaborate behavioral repertoire. Another useful model is the *Celsr3*|*Emx1* mouse: these animals have no corticospinal tract, yet they survive normally and do not display any obvious, gross motor deficit.

Results and Discussion

Deletion of exons 19–27 from the *Celsr3* gene generates a null allele (Tissir et al. 2005). A conditional *Celsr3* allele was produced by flanking exons 19–27 with *loxP* sites (“floxed” allele abbreviated “*Celsr3* *f*”). Homozygous *Celsr3**f/f* and heterozygous *Celsr3**+/-* mice are phenotypically and histologically normal, and fertile. Mice with regional inactivation of *Celsr3* were generated by crossing double heterozygous males [*Cre**+*; *Celsr3**+/-*] with homozygous floxed *Celsr3**f/f* females. In such crosses, inactivation requires Cre-mediated modification of one floxed allele only, thereby increasing efficiency.

Progression of all Axonal Tracts in the IC Requires *Celsr3* Expression in Guidepost Cells

In *Celsr3*|*Foxg1* mice, *Celsr3* is still expressed in dorsal thalamus, yet all three fiber components of the IC, namely thalamocortical (TCA) and corticothalamic afferents (CTAs), as

well as subcerebral projections are fully defective, like in null *Celsr3* mutants. Instead of turning towards the striatum, thalamic fibers follow the same abnormal pathway as in constitutive *Celsr3* mutants (Tissir et al. 2005). Following DiI injection in cortex, no retrograde labeling of thalamic neurons is observed. Injection of DiI in thalamus does not label cortical neurons, but stains thalamic axons that run into the basal forebrain, and others that cross the midline ventrally, close to the optic chiasm, and invade the contralateral thalamus, like in constitutive *Celsr3* and *Fzd3* mutants (Wang et al. 2002). Thus, expression of *Celsr3* in thalamic axons is not sufficient to direct them to the cortex.

Celsr3 was deleted in the ventral telencephalon using *Dlx5/6-Cre* mice that express Cre in the ventral telencephalon as well as in the ventrobasal diencephalon (Fig. 1) (Zerucha et al. 2000; Stenman et al. 2003). In *Celsr3*|*Dlx5/6* mice, the tracts in the IC are fully defective. Some thalamic axons make an incomplete turn at the diencephalon–telencephalon boundary. However, they fail to progress through the ventral telencephalon and instead run obliquely through the pallidum and amygdala. Cortical efferent fibers cross the pallial–subpallial boundary and enter the lateral part of the basal forebrain where they stall and fail to progress in the corridor (Lopez-Bendito et al. 2006). Instead, they form an abnormal mass of fibers that protrudes in the lateral ventricle. Following DiI injections in cortex and thalamus, no labeling of thalamic or cortical cell can be observed, confirming the absence of both CTA and TCA. Similarly, no subcerebral projections form in that mutant, as indicated by the complete absence of corticospinal tract. Thus, *Celsr3* expression by *Dlx5/6*-positive cells is required for progression of thalamic and all subcortical (subcerebral as well as corticothalamic) axons through the ventral telencephalon.

To define better this population of *Dlx5/6* forebrain guidepost cells, we compared expression of *Celsr3* (using *in situ* hybridization), *Nkx2.1* and *Gsh2* (using crosses with ROSA26R mice and immunohistochemistry), *Dlx5/6* (using the

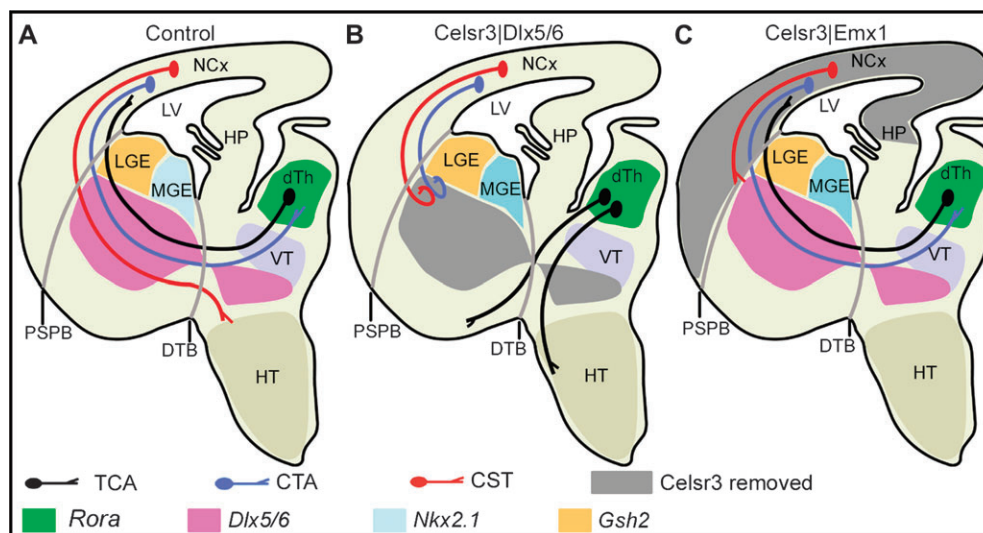


Figure 1. Region-specific *Celsr3* inactivation affects development of tracts in the IC. Schemas of IC development in normal (A), *Celsr3*|*Dlx5,6* (B), and *Celsr3*|*Emx1* mice at E15.5. Three tract components of the IC, namely TCAs (black), CTA fibers (blue), and subcerebral projections (CST, red) are present at this stage in normal animals. In *Celsr3*|*Dlx5,6*, *Celsr3* is inactivated in the intermediate region of the ventral telencephalon and diencephalons (gray areas in B). Thalamic fibers fail to turn normally at the diencephalon–telencephalon boundary (DTB), whereas cortical axons are blocked at the external aspect of the basal telencephalon. In *Celsr3*|*Emx1* mice, reciprocal thalamocortical projections form normally, but subcerebral projections fail to cross the pallial subpallial border (PSPB). NCx: neocortex; LV: lateral ventricle; LGE and MGE: lateral and medial ganglionic eminences; dTh: dorsal thalamus; VT: ventral thalamus; HT: hypothalamus; HP: hippocampus. Expression patterns of some markers are shaded in color. Drawings adapted from Lopez-Bendito and Molnar (2003).

EGFP reporter inserted in the locus), and Islet1 (immunohistochemistry) at E12.5, prior to growth of fibers in the ventral telencephalon, and at E14.5, when the first tracts form in the IC. At E12.5, the pattern of *Celsr3* expression is rather similar to that of *Dlx5/6*, with maximal signal in the vicinity of the pallidal anlage, defining a zone along the ventricular and subventricular zones of ganglionic eminences, where Islet1 is also present. At the level of the ganglionic eminences and striatal anlage, *Nkx2.1* and *Gsh2* are strongly expressed in large foci of the ventricular zone and in mantle regions, but much lower levels are found in the intermediate region where expression of *Celsr3* mRNA and *Dlx5/6*-EGFP is maximal. At E14.5, zones of *Gsh2* and *Nkx2.1* expression border axonal bundles of the incipient IC, whereas Islet1 expressing cells are consistently seen surrounding and in close contact with axonal tracts, in the region of highest *Dlx5/6* signal and minimal *Gsh2* and *Nkx2.1* signal.

To try and identify which precursors generate *Dlx5/6* positive guidepost cells, we used *Gsh2-Cre* and *Nkx2.1-Cre* mice that express the Cre recombinase in proliferative zones of the lateral and medial ganglionic eminences, respectively. In *Celsr3*^{fl}/*Gsh2*, *Celsr3*^{fl}/*Nkx2.1*, and double *Celsr3*^{fl}/*Gsh2**Nkx2.1* mice, the different fiber components of the IC develop normally. This shows that expression of *Celsr3* in *Gsh2*- and *Nkx2.1*-expressing cells in the ventral telencephalon is dispensable for growth of thalamic and cortical efferent axons, or that putative *Celsr3* expressing guidepost cells along the IC express neither *Nkx2.1* nor *Gsh2*, and derive from *Nkx2.1* and *Gsh2* negative precursors. Alternatively, the expression of the *Gsh2-Cre* from the bacterial artificial chromosome transgene may not recapitulate endogenous expression perfectly.

Altogether, our observations suggest that *Celsr3* is required in basal forebrain guidepost cells which are *Nkx2.1* and *Gsh2*-negative, but positive for *Dlx5/6* and most probably for Islet1. It should be possible to investigate this further using Islet1-Cre mice (Lin et al. 2006) and other Cre-expressing transgenic animals when they become available.

Several studies have previously suggested a role for guidepost cells in thalamocortical wiring (Mitrofanis and Guillery 1993; Metin and Godement 1996; Hevner et al. 2002; Lopez-Bendito and Molnar 2003). Our data provide the first unequivocal demonstration that such guidepost cells indeed play a key role in vivo and that *Celsr3* is an integral part of the guidance mechanism. The molecular identity of these cells is not fully defined, and guidepost cells are probably heterogeneous, cells from different origins expressing similar guidance cues. The observation that corticofugal fibers progress beyond the pallial-subpial boundary in *Celsr3*^{fl}/*Dlx5/6* mice, whereas they fail to do so in constitutive mutant or *Celsr3*^{fl}/*Foxg1* mice, suggests that some *Dlx5/6*-negative guidepost cells might be implicated at that key developmental carrefour, as proposed to explain the phenotype of mice deficient in the highwire ubiquitin ligase homolog Phr1 (Bloom et al. 2007). Similarly, *Celsr3*-positive cells in the diencephalon probably assist in the turning of thalamic axons towards the basal forebrain, and they likely differ from those that are generated in the ganglionic eminence and provide guidance to corticofugal and thalamic axons through the ventral telencephalon. Although they may share markers with reticular thalamic neurons, diencephalic guidepost cells are probably a different population, forming the so-called perireticular nucleus (Mitrofanis and Guillery 1993; Deng and Elberger 2003). The identity and role of *Celsr3* in such diencephalic guidepost cells remains to be studied further.

Thalamocortical and corticothalamic fibers progress in the basal forebrain along a corridor defined by cells that migrate tangentially from the ventricular zone of the lateral ganglionic eminence (LGE) where they are generated, and express LGE markers such as Islet1 (Lopez-Bendito et al. 2006). Since corridor cells are *Nkx2.1*-negative, and no defect in thalamocortical mapping was detected in *Celsr3*^{fl}/*Nkx2.1* mice, they could be similar to the *Celsr3*- and Islet1-positive cells that we find intermingled with IC axons. In addition to *Celsr3* and *Nrg1-Erb4* (Lopez-Bendito et al. 2006), other factors in the ventral thalamic region and/or the ganglionic eminences are involved in guiding axons through the IC. Besides corridor cells, so-called “IC” cells in ventral telencephalon (positive for *Nkx2.1*), send axons to dorsal thalamus, thereby guiding early thalamocortical axons across the diencephalon-telencephalon boundary. These IC cells are defective in *Lhx2* mutant mice, providing an explanation for the aberrant thalamocortical axon development in these mice (Molnar and Cordery 1999; Lakhina et al. 2007). Cells in the mantle zone of the ganglionic eminence secrete netrin-1, which has attractive activity for early corticofugal axons (Metin et al. 1997). Netrin-1 acts in vitro as an attractant for dorsal thalamic axons, and deleted in colon cancer (DCC) and neogenin, two receptors implicated in this effect of netrin-1, are expressed in dorsal thalamus. In addition, thalamocortical projections through the ventral telencephalon are disorganized in *netrin-1*^{-/-} mice (Braisted et al. 1999; Yee et al. 1999; Braisted et al. 2000). Other molecular mechanisms implicated in development of the IC include Slit/Robo interactions (Andrews et al. 2006; Lopez-Bendito et al. 2007), homophilic interactions mediated by OL-cadherin (Uemura et al. 2007), and Eph/ephrinA signaling, although the latter appear more concerned with the accuracy of reciprocal thalamocortical mapping than with early axonal guidance (Cang et al. 2005; Dufour et al. 2003; Torii and Levitt 2005). Thus far, none of these systems has been dissected out in detail in vivo.

Expression of *Celsr3* in Cortex is Necessary for Development of Subcerebral but not Corticothalamic Projections

To test whether *Celsr3* is required for normal development of cortical efferent axons, we produced *Celsr3*^{fl}/*Emx1* mice, in which *Celsr3* is inactivated early, from E10.5, in the cortical anlage (Gorski et al. 2002) (Fig. 1C). In these mice, subcerebral projections such as the corticospinal tract (CST) are fully defective, showing that *Celsr3* is indeed required cell autonomously in the neurons of origin of subcerebral axons, in addition to being required in *Dlx5/6* positive guidepost cells in the ventral telencephalon. Furthermore, a proportion of subcerebral axons, visualized by their expression of the Thy1-YFP transgene (Feng et al. 2000), are routed to the thalamus instead of projecting to the cerebral peduncle. Somewhat unexpectedly, and in contrast to subcerebral projections, the corticothalamic and thalamocortical components of the IC develop normally in *Celsr3*^{fl}/*Emx1* mice. Both in *Celsr3*^{fl}/*Emx1* and control mice, injections of Dil in the cortex at P0 result in labeling of thalamic neurons, whereas administration of Dil in thalamus result in filling of cortical cells. Furthermore, whereas cortical barrels predictably fail to develop in mice with no thalamocortical afferents, such as *Celsr3*^{fl}/*Foxg1* and *Celsr3*^{fl}/*Dlx5/6* mice, they form normally in *Celsr3*^{fl}/*Emx1* mice, confirming that thalamocortical afferents reach and map

normally to the cortex. Thus, inactivation of *Celsr3* in corticothalamic axons does not prevent them from passing beyond the striatum and reaching the thalamus, and does not influence the reciprocal growth and mapping of thalamic fibers to the cortex.

Concerning factors that affect subcerebral projections, they have been more recently and less closely examined than those that regulate development of thalamocortical connections. Studies of cortical lamina-specific markers have identified genes that are expressed rather specifically in layer 5 neurons (Molyneaux et al. 2007). Among them, the transcription factor *Fezf2* is crucial to their differentiation and formation of subcerebral projections (Hirata et al. 2004; Chen, Rasin, et al. 2005; Chen, Schaevitz, et al. 2005; Molyneaux et al. 2005), whereas *Ctip2* regulates subcerebral axon extension and refinement of their collaterals during maturation (Arlotta et al. 2005), and *Otx1* is required for the development of corticotectal projections, presumably by regulating pruning of axonal branches (Weimann et al. 1999). In contrast to the factors that regulate formation of layer 5 neurons, relatively little is known about the cues that guide their axons to their targets. Insulin-like growth factor-1 (IGF-I), acting via the IGF-I receptor and downstream signaling pathways, enhances the extent and rate of cerebrosplinal axon outgrowth (Ozdinler and Macklis 2006), and guidance of CST axons is defective in L1 mutant mice (Cohen et al. 1998). Wnt proteins repel CST axons, an effect attributed to binding to Ryk receptors expressed on these axons (Liu et al. 2005). The CST is very diminutive in mice with inactivation of the OL-protocadherin (Uemura et al. 2007), indicating that this molecule plays an important role during development of all fiber systems in the IC. In most of the mutants mentioned above, the CST is misrouted after crossing the ventral telencephalon. On the other hand, the CST is unable to cross the ventral telencephalon in *Celsr3|Emx1* mice and our observations demonstrate for the first time that guidepost cells are implicated at the pallial subpallial border or in the lateral basal forebrain. Furthermore, *Celsr3|Emx1* mice provide a unique model to study how subcerebral axons segregate from their corticothalamic counterparts when they reach the medial aspect of the IC, en route to the cerebral peduncles, an important developmental event that hitherto received little attention apart from studies in *Nkx2.1* mutant mice (Marin et al. 2002).

Another question raised by our observations in *Celsr3|Emx1* mice is why is *Celsr3* expression required in subcerebral, but not in corticothalamic projection neurons? An explanation could be that other *Celsr* proteins, particularly *Celsr2*, may act redundantly with *Celsr3*. We recently obtained *Celsr2* mutant mice that will be used to test this possibility. Another, not exclusive, explanation could be the normal development of thalamocortical afferent axons in *Celsr3|Emx1* mice. In those mice, *Celsr3* expression is preserved in dorsal thalamic neurons and in the basal forebrain, allowing normal progression of thalamocortical fibers to the subcortex where they can encounter *Celsr3*-deficient corticothalamic axons and may help them travel to the thalamus, as predicted by the "handshake hypothesis" (Molnar and Blakemore 1991, 1995; Hevner et al. 2002). Our observations are definitely compatible with such a mechanism. Like constitutive inactivation, *Celsr3* inactivation in the ventral telencephalon and ventral diencephalon prevents normal growth of all IC fiber systems. On the other hand, selective inactivation of *Celsr3* in cortical neurons perturbs development of subcerebral projections such as the

corticospinal tract, while leaving corticothalamic fibers untouched. This might indicate that normal development of subcerebral projections require *Celsr3* expression both in axons and guidepost cells, whereas expression in guidepost cells only would be sufficient for normal development of corticothalamic fibers. Alternatively, *Celsr3* may be required in all axonal IC tracts, including corticothalamic fibers. However, corticothalamic fibers could develop normally despite inactivation of *Celsr3* in cortical neurons when thalamic axons provide an alternative guidance cue, as proposed by the handshake. The handshake model predicts that, although *Celsr3* is involved in turning of thalamic axons to the IC, selective inactivation of *Celsr3* in thalamic neurons should not prevent their normal development, provided corticothalamic fibers are able to contact them somewhere on the pathway. The hypothesis also predicts that simultaneous inactivation of *Celsr3* in cortex (e.g., *Celsr3|Emx1*) and in thalamus (*Celsr3|thalamic Cre*) would disturb formation of both corticothalamic and thalamocortical projections. Thus far, however, no mouse line is currently available that expresses Cre sufficiently early in dorsal thalamus to allow us to test that hypothesis.

Cortical Maturation in Absence of Extracortical Connections and Subcerebral Connections

As described above, *Celsr3|Dlx5/6* and *Celsr3|Foxg1* mice have a neocortex with no connections with the rest of the brain. Only corticocortical connections are present, a malformation that we refer to as "neocortex isolé". We were surprised to find that some of these animals do survive up to P21, when cortical maturation is considered complete, thus providing a model to study for the first time maturation of an isolated cortex. These mice are small and ataxic, yet able to run, bite, eat, drink or swim. A first question is to what extent cortical areas do form

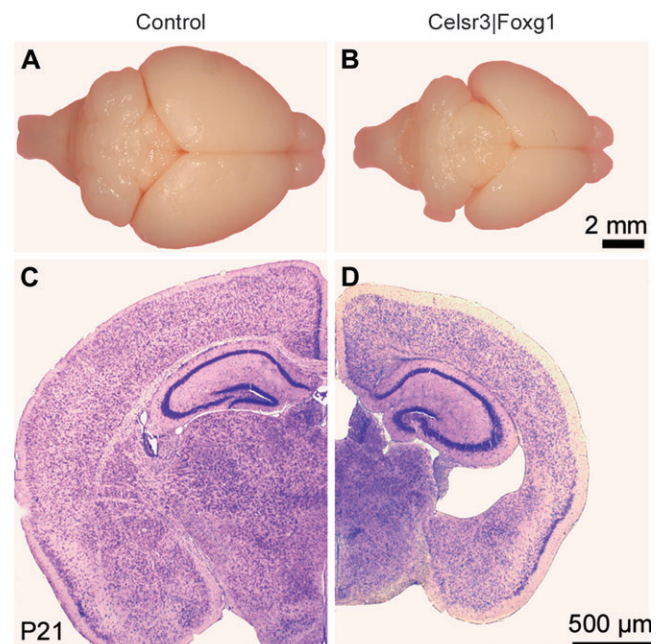


Figure 2. Cortical maturation without extrinsic connections. (A, B) Macroscopic view of normal and *Celsr3|Foxg1* brains at P21, showing the dramatic cortical atrophy in the mutant. (B, C) Coronal, Nissl-stained sections confirm atrophy of neocortical fields, whereas the pyriform cortex and hippocampal formation are relatively normal. Note also the atrophy of thalamic nuclei.

in that situation. To answer that question, we carried out *in situ* hybridization experiments using area-specific markers that are independent of thalamic fibers, namely *Cad8*, *EphA7*, and *Id2*. This preliminary study suggests that the main cortical areas do develop normally in the absence of any input or cortical output. At the end of maturation, there is a prominent atrophy of all neocortical areas, whereas pyriform cortex and hippocampus are relatively unaffected—although olfaction and hippocampus-related functions have not been tested (Fig. 2). The cortical neurons that survive assume an almost normal maturation, although some dendrite atrophy is measured with the Sholl method. Interestingly, the mutant cortex is normally populated with interneurons that are able to migrate from ganglionic eminences in the absence of cortical efferent axons. At this stage, no electrophysiological study has yet been carried out, but it should be interesting to study the spontaneous and evoked electrophysiological properties of cortical cells, as well as the rhythmic activity of such an isolated neocortex.

Celsr3^{Emx1} mice have no subcerebral projections, particularly no corticospinal tract, yet they have no evident gross motor anomalies. Their growth is similar to that of normal mice, and they are able to mate, deliver and nurse their babies. This confirms that most motor control in rodents is extrapyramidal and subcortical. The role of the corticospinal tract could be in the fine tuning of motor performances and it will thus be informative to evaluate motor coordination in these mice.

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Notes

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