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Cortical Neurons Require Otx1 for the Refinement of Exuberant Axonal Projections to Subcortical Targets

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Summary

Information processing in the nervous system depends on the creation of specific synaptic connections between neurons and targets during development. The homeodomain transcription factor Otx1 is expressed in early-generated neurons of the developing cerebral cortex. Within layer 5, Otx1 is expressed by neurons with subcortical axonal projections to the midbrain and spinal cord. Otx1 is also expressed in the precursors of these neurons, but is localized to the cytoplasm. Nuclear translocation of Otx1 occurs when layer 5 neurons enter a period of axonal refinement and eliminate a subset of their long-distance projections. *Otx1* mutant mice are defective in the refinement of these exuberant projections, suggesting that Otx1 is required for the development of normal axonal connectivity and the generation of coordinated motor behavior.

Introduction

The ability of neural circuits to integrate sensory information from the environment and generate appropriate motor movements and behaviors requires that neurons form specific sets of synaptic connections during development. The mammalian cerebral cortex, which is responsible for high-level perceptual analysis and executive functions, is subdivided into a number of functionally distinct areas that process different sensory modalities and motor outputs. Information flows through each area in a highly systematic manner, facilitated by the organization of the cortex into layers (Gilbert and Kelly, 1975; Lund et al., 1975). In primary sensory areas, for example, sensory inputs are conveyed via the thalamus into the middle layer of cortex, layer 4, which in turn relays inputs in an orderly fashion through the different cortical layers (Gilbert and Wiesel, 1979; Gilbert, 1983; Martin and Whitteridge, 1984; Katz, 1987). Long-distance axonal outputs are also organized by layer, with descending connections to subcortical targets arising from the deep layers. Layer 5 neurons convey signals controlling motor behavior via their projections to the colliculi, pons, and spinal cord (Gilbert and Kelly, 1975; Wise and Jones, 1977; Bates and Killackey, 1984; O'Leary and Koester, 1993). In contrast, axons innervating other cortical areas, either within the same hemisphere or contralaterally, arise from several layers but primarily from layers 2/3 and 5 (Wise and Jones, 1976; Segraves and Rosenquist, 1982; Segraves and Innocenti, 1985).

While it is clear that axonal connections in the adult cortex are highly organized by layer, the mechanisms that enable neurons to form layer-specific patterns of connectivity during development are not well understood. It has long been appreciated that neurons in different layers are generated at different times during development: neurons born at early times migrate away from the ventricular zone (where they are generated) and form the deepest layers of the cortical plate (layers 6 and 5). Later-generated neurons migrate past the deep layers into more superficial positions (layers 4 through 2) (reviewed in McConnell, 1988). Transplantation studies have revealed that neurons acquire a laminar identity, which specifies the layer to which the cell will migrate, by the time of the cell's terminal mitotic division (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996).

Genes that control laminar identity might be expected to show layer-specific patterns of expression during development. One such gene is the homeodomain transcription factor *Otx1*, one of two mammalian homologs of the fly gene *orthodenticle* (Simeone et al., 1992, 1993). At early stages of neural development, *Otx1* is expressed in the forebrain and midbrain, where it shows a "nested" pattern with three other homeobox genes (Simeone et al., 1992). Analysis of *Otx1* mutant mice has revealed that *Otx1* plays a role in regulating the size of the cortex and mesencephalon, the differentiation of sense organs, and, in collaboration with *Otx2*, the specification of anterior brain regions (Suda et al., 1996; Acampora and Simeone, 1999). At later stages during corticogenesis of normal animals, *Otx1* is expressed at high levels in layers 5 and 6; expression is maintained in these layers into adulthood (Frantz et al., 1994b). *Otx1* is also expressed in progenitor cells at early times in the ventricular zone, when deep-layer neurons are being generated. By the time ventricular cells produce upper-layer neurons, *Otx1* expression is markedly decreased (Frantz et al., 1994b). The expression pattern of *Otx1* led us to speculate that this gene might be involved in the specification of laminar identity within the ventricular zone (Frantz et al., 1994b; McConnell, 1995).

Here we explore the role of *Otx1* in cortical development by characterizing further the neurons that express Otx1 protein, describing the subcellular localization of Otx1 at different stages of development, and examining the differentiation of cortical neurons in *Otx1* mutant mice (Acampora et al., 1996). To our surprise, *Otx1* has no obvious role in laminar specification, but rather plays an important role in later stages of differentiation. Our analysis focuses on layer 5 neurons, which express *Otx1*

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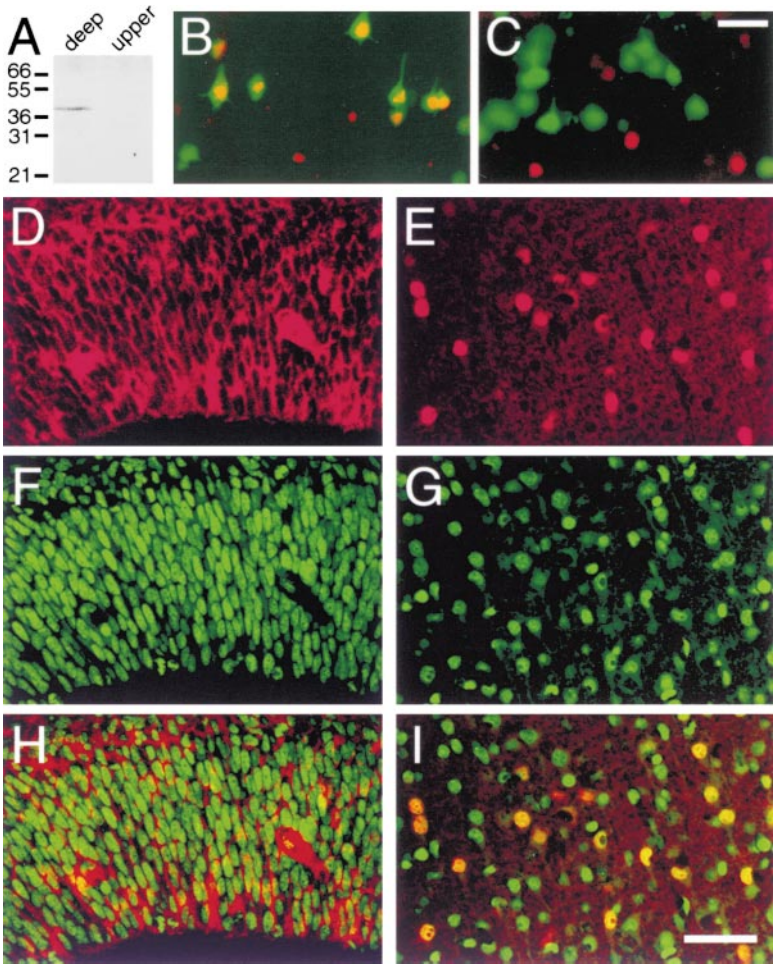

Figure 1. Otx1 Is Found in the Nucleus of Subcortical Projection Neurons and in the Cytoplasm of Ventricular Cells

(A) In Western analysis of brain extracts prepared from the deep layers (4–6) of rat cortex (left lane), the anti-Otx1 antibody recognizes a single band at the predicted molecular weight of Otx1 (~40 kDa). This band is absent from extracts prepared from the upper layers 1–4 (right lane).

(B and C) Layer 5 neurons were retrogradely labeled with FluoroGold (green) on P16 and immunostained with Otx1 antibodies (red) on P24. (B) Significant overlap is observed between Otx1 staining and retrograde labeling from a subcortical target, the superior colliculus. (C) No overlap is found between Otx1 staining and retrograde labeling from the contralateral cortex. Scale bar, 50 μm.

(D–I) Otx1 protein is localized in the cytoplasm of progenitor cells in the rat ventricular zone, but is translocated to the nucleus after neurons migrate to the cortical plate. (D, F, and H) E15 ventricular zone. (E, G, and I) Layer 5 in a P6 rat. Sections were labeled with (D and E) anti-Otx1 antibody (red) and (F and G) the dye Syto11 (green), which stains cell nuclei. (H and I) Superimposed images of Otx1 and Syto11 staining. (D and H) Otx1 is found in the cytoplasm of ventricular cells. (E and I) By P6, Otx1 has translocated to the nuclei of a subset of layer 5 neurons. The pial surface is up in all images. Scale bar, 50 μm.

at the highest levels (Frantz et al., 1994b). *Otx1* is expressed in a subset of neurons in layer 5, which is known to contain projection neurons with two discrete patterns of axonal connectivity: one set projects subcortically to targets that include the superior colliculus and pons, while the other extends axons across the corpus callosum to the contralateral hemisphere (O'Leary and Stanfield, 1985; Koester and O'Leary, 1993; Kasper et al., 1994). Our studies reveal that Otx1 is expressed selectively in layer 5 neurons with subcortical axonal projections and that *Otx1* is required for the emergence during development of their normal pattern of connectivity.

Results

Otx1 Protein Is Expressed by Subcortically but Not Cortically Projecting Neurons in Layer 5

To ascertain whether Otx1 is expressed selectively by layer 5 neurons that extend subcortical versus callosal axons, we combined retrograde labeling techniques with antibody staining for Otx1 protein. A monoclonal antibody was generated against a bacterially expressed, truncated Otx1 protein that lacked the conserved N-terminal homeodomain. This antibody recognized a single band on a Western blot corresponding to the 40 kDa Otx1 protein (Figure 1A). Injections of the retrograde tracer FluoroGold were placed into the superior colliculus, pons, or contralateral cortex of postnatal day (P) 16 rats. At P24, injections into the colliculus or pons labeled projection neurons exclusively in layer 5, whereas cortical injections labeled neurons predominantly in layers 2/3 and 5, as described previously (O'Leary and Stanfield, 1985). Immunohistochemistry for Otx1 overlapped extensively with FluoroGold staining in animals that received subcortical tracer injections (Figure 1B), but in no case did a callosally projecting neuron express Otx1 (Figure 1C). After injections into colliculus or pons, 38%–50% of retrogradely labeled neurons were immunopositive for Otx1 (Table 1). In contrast, cells labeled from the contralateral hemisphere never showed Otx1 staining, even after tracer injections at much earlier ages (E19 or P2; Table 1). Otx1 is thus expressed selectively by subcortical projection neurons in layer 5.

Regulated Translocation of Otx1 Protein from Cytoplasm to Nucleus during Development

The presence of Otx1 in a subset of neurons defined by their projection pattern suggests several possible functions for Otx1 in development. Otx1 might play a role in the initial determination of a deep-layer fate, the choice to form a subcortical axonal projection, axonal pathfinding, the recognition of subcortical targets, or the

Table 1. Summary of Retrograde Labeling and Otx1 Expression

Age at Injection	Age at Perfusion	Retrograde Tracer	Target Structure Injected	No. Dye-Labeled Cells	No. Otx1-Labeled Cells	No. Double-Labeled Cells	% Dye-Labeled Cells that Were Otx1$^+$
P16	P24	FG	Sup. colliculus	181	103	68	37.6
P16	P24	FG	Pons	249	170	112	45.0
P16	P24	FG	Contra. cortex	175	54	0	0
P2	P11	FG	Contra. cortex	236	179	0	0
P1	P5	FG	Sup. colliculus	202	96	49	51.0
E19	P5	FB	Contra. cortex	116	142	0	0
E19	P1	FB	Contra. cortex	161	100	0	0

Abbreviations: Sup. colliculus, superior colliculus; Contra. cortex, contralateral cortex; FG, FluoroGold; FB, Fast Blue.

refinement of axonal projections. Because the activity of a transcription factor can be mediated by translocation between the cytoplasm and cell nucleus (e.g., Liou and Baltimore, 1993), we examined the subcellular localization of Otx1 at times that correlate with the events listed above. Laminar determination occurs in the ventricular zone (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Antibody staining of the E15 rat ventricular zone (during layer 5 neurogenesis: Frantz et al., 1994a) revealed abundant expression of Otx1 by progenitor cells (Figures 1D and 1H), as predicted by in situ hybridization (Frantz et al., 1994b). However, double labeling with the nuclear stain Syto-11 (Figures 1F and 1H) showed that Otx1 is localized predominantly to the cytoplasm and is largely excluded from cell nuclei, suggesting that Otx1 does not play an important role in laminar determination by regulating transcription in progenitor cells.

Because *Otx1* is not expressed by callosally projecting neurons labeled during embryonic life, we wondered whether Otx1 defines the fates of subcortical versus callosal projection neurons in layer 5. The distinction between these two cell types is apparent by E19, when layer 5 neurons initially extend axons (Koester and O'Leary, 1993). However, Otx1 is still localized to the cytoplasm in the large majority of neurons at E19 (data not shown). Antibody staining reveals that Otx1 protein translocates into cell nuclei during the first week of postnatal life (Figures 1E, 1G, and 1I), when the axons of deep-layer neurons are reaching their long-distance targets. Although we cannot rule out the possibility that Otx1 is present in nuclei at sufficient levels to alter transcription at earlier times, the visible accumulation of Otx1 in nuclei during postnatal life suggests that the protein is likely to function during late stages of differentiation such as axonal pathfinding, target selection, or axonal refinement.

Analysis of Otx1 Expression in Mutant Mice

To assess the role of *Otx1* in cortical development, we examined the laminar architecture and axonal projections of *Otx1* mutant mice. The mutation was generated by the targeted replacement of the first and second exons of *Otx1*, including most of the homeodomain, with a *lacZ-neo* marker (Acampora et al., 1996). Although this mutation was thought to result in a null phenotype (Acampora et al., 1995, 1996, 1998), Western analysis revealed the presence of a 33 kDa protein in heterozygous and homozygous mice (Figure 2A). The design of the *Otx1* targeting construct should have precluded transcription of the third exon of *Otx1*, but in situ hybridization using probes to this exon revealed the presence of transcript in the mutant cortex (Figure 2C). The expression pattern of the mutant transcript differs markedly from that in wild-type mice (Figure 2B), raising the possibility that expression is influenced by the upstream targeting cassette.

The presence of the 33 kDa protein in mutant mice raises the question of whether this protein might retain partial Otx1 function or even exert a dominant effect. Several lines of evidence suggest strongly that neither is the case. First, although Otx1 immunoreactivity is evident in layer 5 and 6 neurons in wild-type mice (Figure 2D), immunoreactivity is not detectable in brain sections from homozygous mutant mice (Figure 2E), suggesting that little of the 33 kDa protein is expressed on a per-cell basis. Second, while homozygous mutant mice have smaller cortices, body size, and eyes than normal mice and are prone to epileptic seizures (Acampora et al., 1996), heterozygous mice are phenotypically wild type (Acampora et al., 1996, and Figures 4–7), demonstrating that the 33 kDa protein does not have a dominant function. Third, an independent disruption of the *Otx1* locus has produced a putative null allele with an early cortical phenotype similar to that described by Acampora et al. (1996); however, this mutation also results in neonatal lethality (Suda et al., 1996). Homozygous mutants in the present study can grow to adulthood, but viability depends on their genetic background: when these mice are crossed into a C57/Bl6 background, comparable to the mutants generated by Suda et al. (1996), fewer than 1.5% of the homozygous mutant mice survive the perinatal period (data not shown). Fourth, any Otx1 protein that is expressed in mutant animals clearly lacks a functional homeodomain, incapacitating the protein from binding DNA or regulating transcription directly. Collectively these data suggest that the *Otx1* mutation used here represents a severe or complete loss of function.

The Production and Migration of Layer 5 Neurons Is Normal in *Otx1* Mutant Mice

The cytoplasmic localization of Otx1 in cortical progenitor cells suggests that Otx1 is unlikely to regulate the initial determination of deep-layer fates, which are established in the ventricular zone (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Nevertheless, it is important to ascertain directly whether laminar

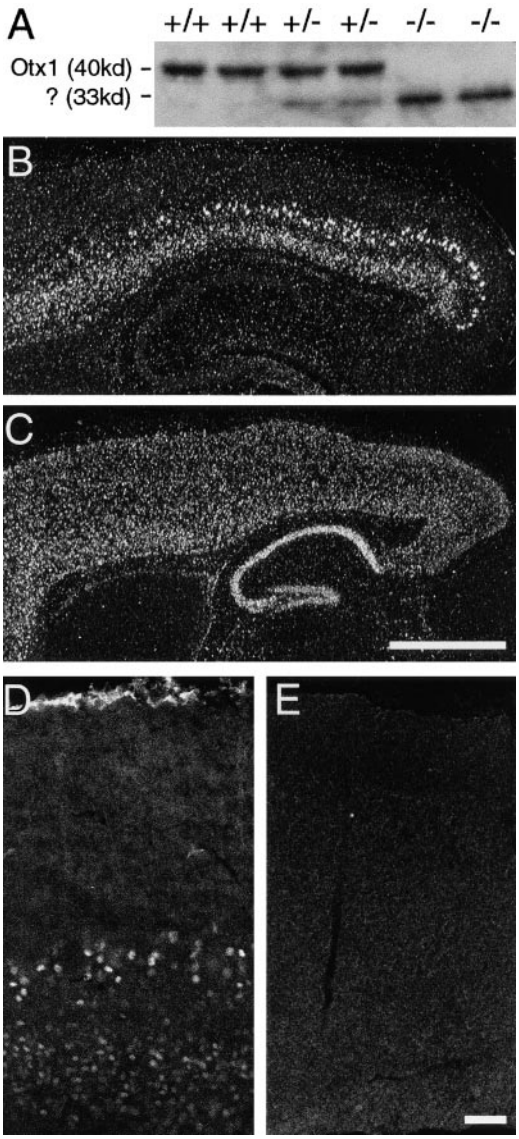

Figure 2. Disruption of *Otx1* Expression in Mutant Mice

(A) Western analysis showing that the anti-Otx1 antibody recognizes full-length Otx1 protein (40 kDa) from +/+ and +/− mice. In *Otx1* −/− mutant mice, the full-length Otx1 protein is absent but a 33 kDa protein is present. This smaller protein is also present in +/− mice, but at lower levels.

(B and C) In situ hybridization of (B) control and (C) *Otx1* −/− mutant cortex, using probes to the third exon of *Otx1*. (B) *Otx1* is normally expressed in layers 5 and 6 of the cortex, and is absent from the hippocampus. (C) In *Otx1* −/− mice, the third exon of *Otx1* is expressed, but is present in an aberrant pattern: mRNA is distributed diffusely across layers 2–6 of cortex and in the hippocampus. Darkfield views of sagittal sections; anterior is to the left. Scale bar, 1 mm.

(D and E) Immunohistochemistry on (D) control and (E) *Otx1* −/− mutant cortex, using antibodies generated against the C-terminal domain of Otx1. The pial surface is at the top, and layer 6 is at the bottom. (D) Nuclear staining of neurons in layers 5 and 6 is apparent in the adult wild-type mouse. (E) Despite the presence of *Otx1* mRNA in layers 2–6, Otx1 protein is barely detectable. Scale bar, 100 μm.

histogenesis proceeds normally in *Otx1* mutant mice, particularly given the reduced size of the cerebral cortex in these animals (Acampora et al., 1996). Unfortunately, expression of the *lacZ* reporter gene is not maintained postnatally in mutant animals (Acampora et al., 1996). Instead, several molecular markers were used to assess the formation of layer 5 in control and mutant mice. Two genes, *protease nexin1* (Figure 3E) and the POU homeobox gene *SCIP* (Figure 3C), are expressed by many subcortical projection neurons in layer 5 (Frantz et al., 1994a; Reinhard et al., 1994; T. Kan and S. K. M., unpublished), and the third, the ETS transcription factor *er81* (Lin et al., 1998), is expressed strongly in layer 5 (Figure 3A). All showed normal patterns of expression in both control and −/− mice (Figures 3A–3F), suggesting that the formation and migration of layer 5 neurons is unimpeded in *Otx1* mutants. Markers for other layers (the orphan nuclear receptor *RORβ* in layer 4: Schaeren-Wiemers et al., 1997; the protein tyrosine phosphatase *PTPλ* in layers 5 and 6: Cheng et al., 1997; and *calbindin*, predominantly in layer 2/3: Kawaguchi and Kubota, 1993) also showed normal distributions in mutant mice (not shown). Thus, Otx1 does not appear to play a role in the initial determination of laminar fates in cortex, nor in the migration of neurons to their normal laminar destinations.

Callosal and Thalamic Projections Are Normal in *Otx1* Mutant Mice

Otx1 protein translocates into the nuclei of layer 5 neurons during the first week of postnatal life when layer 5 neurons form long-distance axonal projections. To assess the role of Otx1 in regulating the development of axonal connectivity, small injections of the anterograde tracers ^{3}H-leucine or rhodamine dextran were placed into the visual cortex of adult +/+ and +/− controls and of *Otx1* −/− mutant mice. If Otx1 acts cell autonomously, the axonal connections of neurons that do not express Otx1 should be normal, whereas those of layer 5 subcortical projection neurons might be altered specifically. Consistent with this prediction, the patterning of neocortical callosal projections was similar in control (Figure 4A) and mutant mice (Figures 4B and 4C). In both, anterogradely labeled axons formed columns extending through all layers of the contralateral cortex. Injections of rhodamine dextran also resulted in a normal pattern of retrograde labeling, including both the deep and superficial layers, in the mutant cortex (Figure 4C). Thus, Otx1 does not play a major role in the pathfinding or targeting of callosal axons, consistent with the finding that Otx1 is not expressed by these neurons.

Somewhat surprisingly, projections to thalamic targets also appeared normal in mutant mice. *Otx1* is expressed by neurons of cortical layer 6, many of which form thalamic projections (Gilbert and Kelly, 1975), although Otx1 expression levels are lower than in layer 5 (Frantz et al., 1994b, and Figure 2D). Injections of ^{3}H-leucine into visual cortex labeled axon terminals in appropriate thalamorecipient nuclei in both control (Figure 4D) and mutant mice (Figures 4E and 4F), including the dorsal and ventral lateral geniculate nuclei, the reticular nucleus magnocellular, and the zona inserta (dorsal and ventral). Little if any label was visible in somatosensory nuclei (the ventrobasal complex) or in other nonvisual regions. Thus, despite the presence of *Otx1* in many layer 6 neurons (Frantz et al., 1994b), we observed no

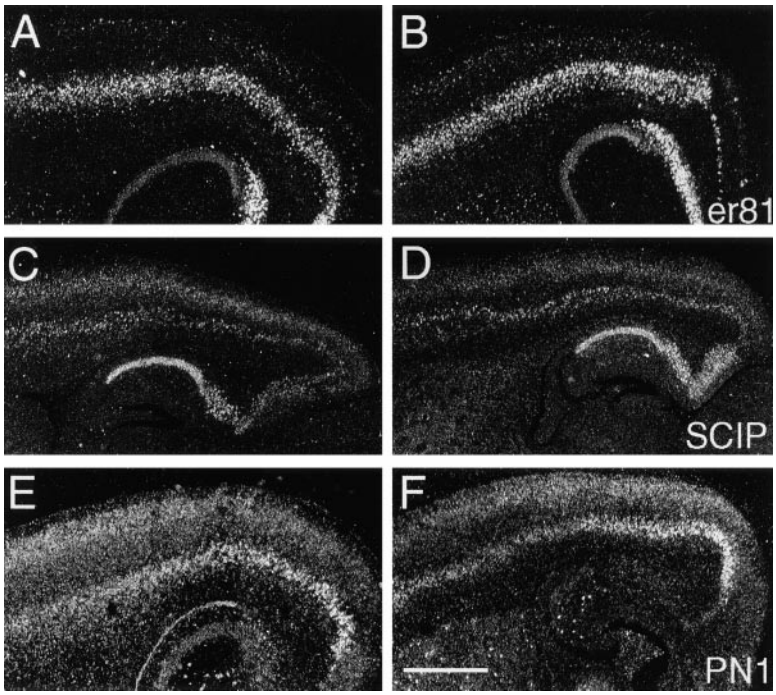

Figure 3. Layer Formation Appears Normal in *Otx1* Mutant Mice

In situ hybridization on (A, C, and E) control and (B, D, and F) *Otx1* −/− mutant cortex, using probes to: (A and B) *er81*; (C and D) *SCIP*; (E and F) *Protease nexin1* (PN1). Dark-field views of sagittal sections; anterior is to the left.

(A and B) The ETS transcription factor *er81* is expressed in layer 5 in both control and mutant mice.

(C and D) The POU homeodomain gene *SCIP* is expressed in layers 2/3 and 5 in both control and mutant mice.

(E and F) PN1 is expressed in layer 5 in both control and mutant mice.

Scale bar, 1 mm.

obvious consequence of the mutation on projections arising from this layer.

Subcortical Axonal Projections of Layer 5 Neurons Are Aberrant in *Otx1* Mutant Mice

In contrast, subcortical projections originating in layer 5 were markedly aberrant in *Otx1* mutant mice. In normal animals, layer 5 neurons in each area of the cortex project to a distinctive subset of subcortical targets. In adult visual cortex, layer 5 neurons normally project axons to the pons and superior colliculus, whereas layer 5 neurons in motor cortex innervate the pons and spinal cord (Gilbert and Kelly, 1975; Wise and Jones, 1977; Stanfield et al., 1982; Bates and Killackey, 1984; Stanfield and O'Leary, 1985; O'Leary and Koester, 1993). In the following sections, we discuss each of these targets in turn, presenting evidence that the pattern of subcortical axon terminations is disrupted in *Otx1* mutant mice.

The pons relays information from the cerebral cortex to the cerebellum to aid in the control of movement and posture (Kandel et al., 1991). Normal visual cortical axons project to the rostral region of the basilar pons (Heffner et al., 1990; O'Leary et al., 1991) (Figure 4G). Axons in *Otx1* mutant animals, in contrast, terminate not only in the rostral pons but also in the caudal pons (Figures 4H and 4I), a region that normally receives input from motor cortex.

Even more strikingly abnormal are the projections from layer 5 to the dorsal midbrain. Projections from visual cortex to the superior colliculus mediate visual orienting behaviors and the generation of eye and head movements, whereas neurons in auditory cortex normally innervate the inferior colliculus, which mediates the startle reflex to loud sounds (Kandel et al., 1991). Injections of ^{3}H-leucine into visual cortex of control mice revealed normal projections of layer 5 neurons to the superficial gray and optic nerve layers of the superior colliculus (Figure 4J) (Harvey and Worthington, 1990). In all mutant mice examined, the projections from visual cortex showed two striking differences. First, although labeled axons were visible in the superficial gray and optic nerve layers of the superior colliculus, label was also distributed in abnormally deep positions (intermediate gray and white layers, Figures 4K and 4L). Second, axons consistently failed to observe the boundary between the superior and inferior colliculi (Figures 4K and 4L, arrows), and invaded the inferior colliculus, which is not a normal target of mature visual cortical axons. Retrograde labeling with Fast Blue from the inferior colliculus confirmed that layer 5 neurons in visual cortex gave rise to these projections (not shown). Collectively these data suggest that layer 5 neurons in *Otx1* mutant mice are impaired in their ability to select or maintain connections with appropriate subcortical targets.

The Identities of the Superior and Inferior Colliculi Are Specified Normally in *Otx1* Mutant Mice

While it is possible that *Otx1* plays an important role in targeting layer 5 axons to appropriate terminal destinations, it is also possible that *Otx1* is required for the normal differentiation of the target structures, the superior and inferior colliculi. *Otx1* is normally expressed throughout the tectal anlage during embryonic life and is maintained into adulthood in the inferior colliculus (Simeone et al., 1992; Frantz et al., 1994b). Abnormalities of midbrain development have been observed in about 20% of B6/D2-derived *Otx1* mutant mice (but not in 129/Sv-derived mice, the strain used here) (Acampora et al., 1996). To ascertain whether the identities of the superior and inferior colliculi are established normally in mutant mice, we examined the expression of several genes that distinguish these two structures in adults. The Eph ligand *ephrin-A5* is normally expressed in a rostral-caudal

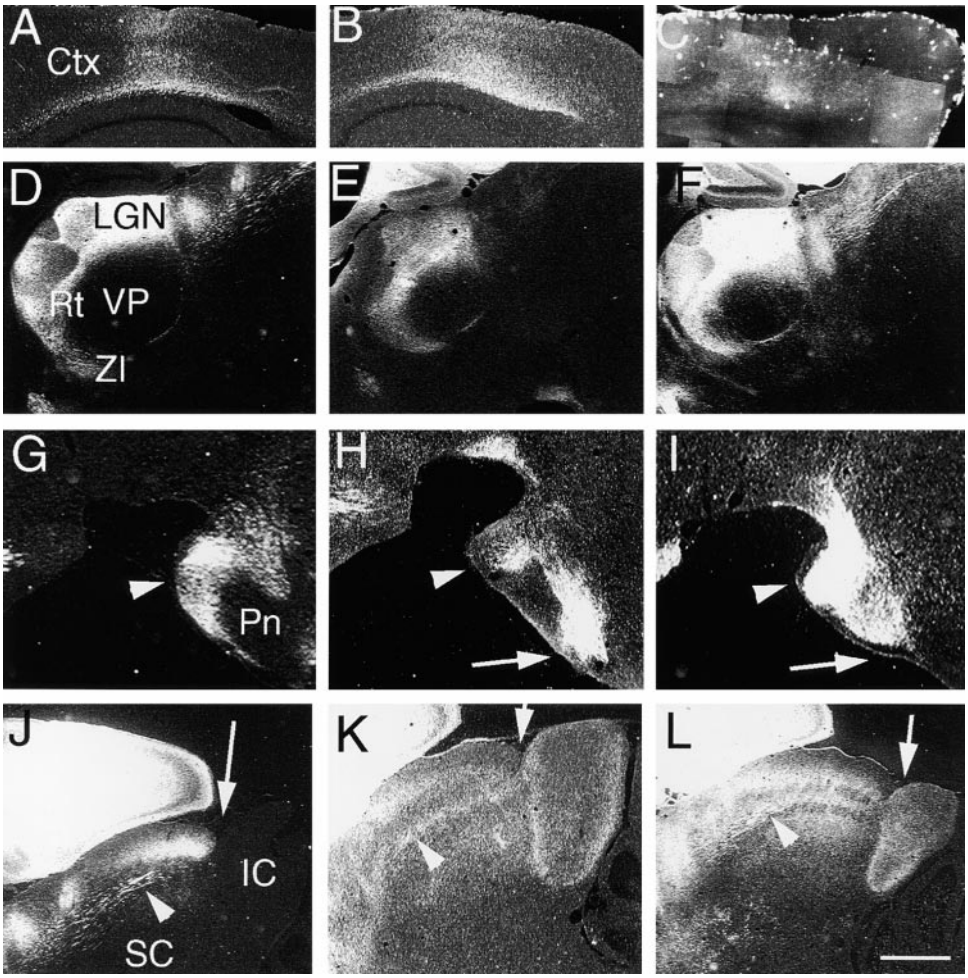

Figure 4. Axonal Projections in *Otx1* Mutant Mice

Visualization of axonal projections following tracer injections into the visual cortex of (A, D, G, and J) control or (B, C, E, F, H, I, K, and L) *Otx1* −/− mutant adult mice. All panels except (C) show dark-field views of sagittal sections processed for autoradiography to reveal ^{3}H-leucine labeling. (C) Fluorescence micrograph of rhodamine dextran labeling. Dorsal is up, anterior is to the left.

(A–C) Projections to contralateral cortex: Both (A) control and (B and C) *Otx1* mutant mice show a patchy distribution of axon terminals within the homotopic (occipital) region. (A and B) Axon terminals are labeled with ^{3}H-leucine. (C) Rhodamine dextran injections result in both anterograde transport to axon terminals and retrograde labeling of neurons in both deep and superficial layers.

(D–F) Projections to thalamus: (D) In control mice, axons from occipital cortex innervate the lateral geniculate nucleus (LGN), a visuorecipient target, but avoid the somatosensory region (ventrobasal complex, VP). (E and F) In *Otx1* mutants, projections to thalamus are similar to those in controls.

(G–I) Projections to pons: (G) In control mice, projections to the basilar pons (Pn) terminate primarily in the anterior region (arrow). (H and I) In *Otx1* mutants, the projection to the pons enters both anterior and posterior regions (arrows).

(J–L) Projections to dorsal midbrain: (J) In control mice, visual axons enter the superior colliculus (SC) in the intermediate gray layer (arrowhead) and terminate in the superficial gray and optic nerve layers. The axons do not enter the inferior colliculus (IC), observing a sharp boundary between these two regions (arrow). (K and L) In *Otx1* mutants, axons are distributed unusually deeply within the superior colliculus (below arrowhead), similar to the distribution of axons prior to the period of refinement (Bruce, 1993; Khachab and Bruce, 1999). Even more strikingly, all *Otx1* mutants showed visual axons in an inappropriate target, the inferior colliculus.

Scale bar for (A)–(F) and (J)–(L) is 1 mm; scale bar for (G)–(I) is 1.6 mm.

gradient in the superior colliculus (Cheng et al., 1995), but is weak or absent in the inferior colliculus (Figure 5A). The pattern of *ephrin-A5* expression was indistinguishable in control and mutant mice; both showed a strong boundary of gene expression at the caudal limit of the superior colliculus (Figures 5A and 5B). In addition, the expression of *er81* and *RORβ*, which are also expressed in distinct patterns in the two colliculi, were similar in controls and mutants (not shown).

To ascertain whether the midbrain can pattern other axonal inputs appropriately, we examined retinal projections that normally terminate within the superior but not the inferior colliculus (Edwards et al., 1986). Injections of ^{3}H-leucine were made into both eyes of adult control and mutant mice. In all mice examined, labeled terminals were strictly confined to the superficial layers of the superior colliculus (Figures 5C and 5D). Retinal axons did not mistakenly innervate the inferior colliculi of mutant mice as might be expected were this structure abnormally specified. Collectively these data suggest that

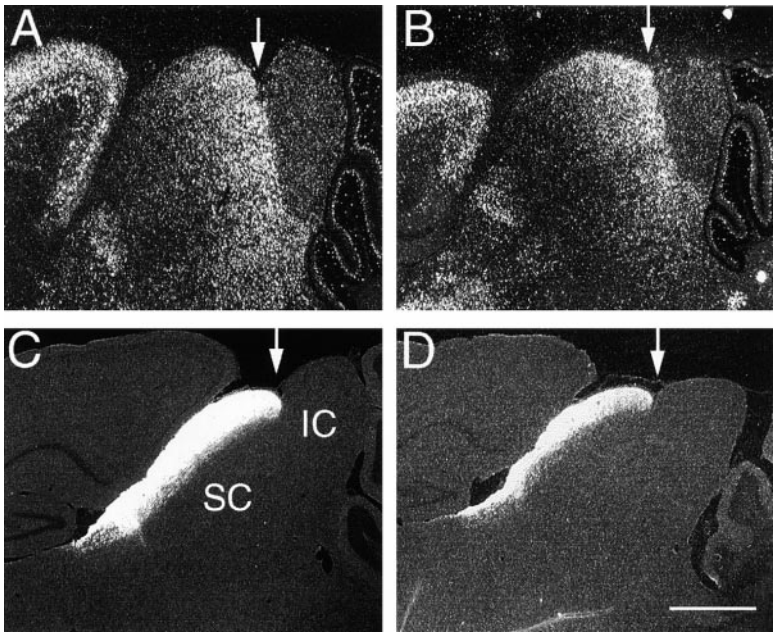

Figure 5. Normal Patterning of the Midbrain in *Otx1* Mutant Mice

(A and B) In situ hybridization for the Eph ligand *ephrin-A5*, which is normally expressed in a rostral-caudal gradient in the superior colliculus but is weak or absent in the inferior colliculus. Expression of *ephrin-A5* is nearly identical in (A) control and (B) *Otx1* mutant mice. Each arrow marks the boundary between the superior colliculus and inferior colliculus.

(C and D) Results of injections of ^{3}H-leucine made into both eyes of adult (C) control and (D) *Otx1* mutant mice. In both cases, axons were strictly confined to the superficial-most layers of the superior colliculus (SC) and absent from the inferior colliculus (IC).

Sagittal sections; dorsal is up, anterior is to the left.

Scale bar, 1 mm.

the identities of the superior and inferior colliculi are established normally in *Otx1* mutant mice, and that the ability of layer 5 axons to target appropriately to these structures requires *Otx1*. Direct affirmation of this hypothesis would require a cortex-specific knockout of the *Otx1* gene.

Otx1 Mutant Mice Retain Their Normally Transient Projections from Visual Cortex to Spinal Cord

In mouse and cat, layer 5 neurons in visual cortex project axons to their normal target, the superior colliculus, and also extend transient axonal projections into the inferior colliculus at early postnatal ages (Bruce, 1993; M. Y. Khachab and L. L. Bruce, personal communication). Indeed, layer 5 neurons throughout the cortex normally undergo a period of exuberant axonal connectivity during which axons are extended to many possible subcortical targets, including those typical of different cortical areas (Stanfield et al., 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). During the first two weeks of postnatal life in the rat, these exuberant projections are eliminated in an area-specific pattern (Figure 8). One of the most striking examples of transient axonal projections are the axons that extend from visual cortex to the spinal cord (Stanfield et al., 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). Whereas neurons in somatomotor cortex retain their spinal axons, layer 5 neurons in rodent occipital cortex eliminate these projections during the first two weeks of postnatal life. The presence of visual cortical projections to the inferior colliculus in *Otx1* mutant mice raised the possibility that layer 5 neurons had failed to refine their exuberant projections, and led us to ask whether spinal axons from visual cortex are eliminated in *Otx1* mutants.

Injections of ^{3}H-leucine into visual cortex of adult +/+ or +/− mice produced little if any labeling in the region of the pyramidal decussation (Figure 6A), where the axons of layer 5 neurons cross contralaterally and extend dorsally to form the corticospinal tract. In *Otx1* mutant mice, however, similar injections resulted in the striking and robust labeling of visual cortical axons descending past the pons, through the pyramidal decussation, and into the spinal cord (Figures 6B and 6C), suggesting that layer 5 neurons in visual cortex have failed to eliminate their normally transient spinal axons.

To identify directly the cells that extend these axons, injections of the retrograde tracer Fast Blue were placed at the pyramidal decussation of both control and mutant adult mice. The pattern of retrogradely labeled neurons in control mice (Figure 7A) resembles that published previously for other rodents (Wise and Jones, 1977; Stanfield et al., 1982; Bates and Killackey, 1984; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). Labeled neurons were present exclusively in layer 5 in frontal and somatomotor regions, with a posterior boundary of expression overlying the anterior third of the hippocampus. This boundary corresponds to the anterior limit of occipital cortex in mouse. In normal mice, labeled layer 5 neurons were distributed across roughly 62% of the anterior-posterior extent of cortex in sections from midhemisphere (Figures 7A and 7B). In *Otx1* mutant mice, retrogradely labeled neurons also occupied layer 5; however, the spatial extent of labeling was expanded into distinctly more posterior regions (Figures 7B and 7C), with roughly 84% of the anterior-posterior extent of cortex containing labeled neurons in comparable sections. The area of expanded label extended into occipital cortex, but did not appear to impinge on the primary visual cortex (area 17), a region that may not normally extend transient spinal axons (Miller, 1988). These results show definitively that layer 5 neurons in occipital cortex have failed to retract their exuberant axonal connections to the spinal cord in *Otx1* mutant animals.

Discussion

The production of precisely wired neuronal circuits during development entails the guidance of axons to appropriate targets and the formation of specific synaptic

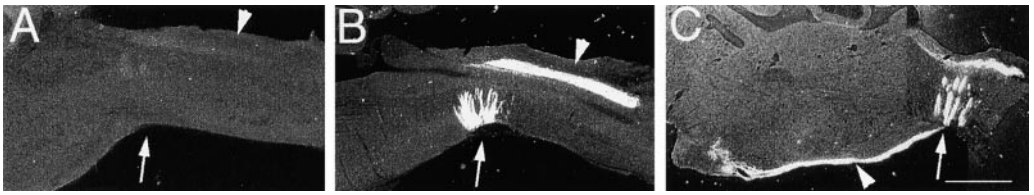

Figure 6. *Otx1* Mutant Mice Fail to Eliminate Axonal Projections from Visual Cortex into the Spinal Cord

Projections from visual cortex to the spinal cord of adult mice revealed by anterograde labeling with ^{3}H-leucine. Sagittal sections; dorsal is up, anterior is to the left.

(A) In control mice, little or no labeling is observed in the ventral midbrain past the level of the pons.

(B and C) In *Otx1* mutants, labeled axonal projections are visible within the pyramidal decussation (arrow), where axons cross to the contralateral side and ascend to form the corticospinal tract (arrowhead in B). The photomontage in (C) also shows labeling of corticospinal axons in the pons and ventral midbrain (arrowhead).

Scale bar, 1 mm.

connections. In many animals, a period of exuberant, transient connectivity precedes the establishment of an adult pattern. Layer 5 neurons of the cerebral cortex provide a striking example of exuberance, establishing connections to a number of subcortical targets, then eliminating some projections during early postnatal life (Stanfield et al., 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). Despite the widespread nature of this phenomenon, little if anything is known about the molecules that control large-scale axonal remodeling. Our evidence implicates the homeodomain transcription factor Otx1 in this process. Otx1 is expressed specifically in layer 5 neurons that form subcortical connections, and Otx1 protein is conveyed to cell nuclei when these connections undergo refinement. In mice lacking functional Otx1 protein, layer 5 neurons in visual cortex fail to prune their normally transient axon collaterals in the inferior colliculus and the spinal cord, revealing that Otx1 is required for axon remodeling. This retention of axons in inappropriate target structures may contribute to the abnormal behaviors of *Otx1* mutant mice, which include spinning and epileptic seizures (Acampora et al., 1996).

The Formation and Elimination of Exuberant Connections

The establishment of an adult pattern of subcortical axonal projections by layer 5 neurons is divisible into three discrete phases (O'Leary and Koester, 1993). First, layer 5 neurons throughout the cortex extend simple, unbranched axons into the spinal cord (Figure 8A). Second, branches emerge from the trunks of the parental axons to invade other targets, including the pons, superior and inferior colliculi, and deep cerebellar nuclei (Figure 8B). Third, a subset of branches is maintained depending on the cortical area in which each neuron resides. Neurons in visual cortex eliminate their projections to the spinal cord, whereas motor neurons retain this projection but eliminate their tectal collaterals (Figure 8C). Although layer 5 neurons initially form connections with many subcortical targets, each is appropriate for some subset of neurons in layer 5, depending on their area of origin. Only later do layer 5 neurons employ area-specific information to eliminate certain connections selectively.

Axonal pathfinding and initial target selection by layer 5 neurons in *Otx1* mutant mice appear to be normal, as

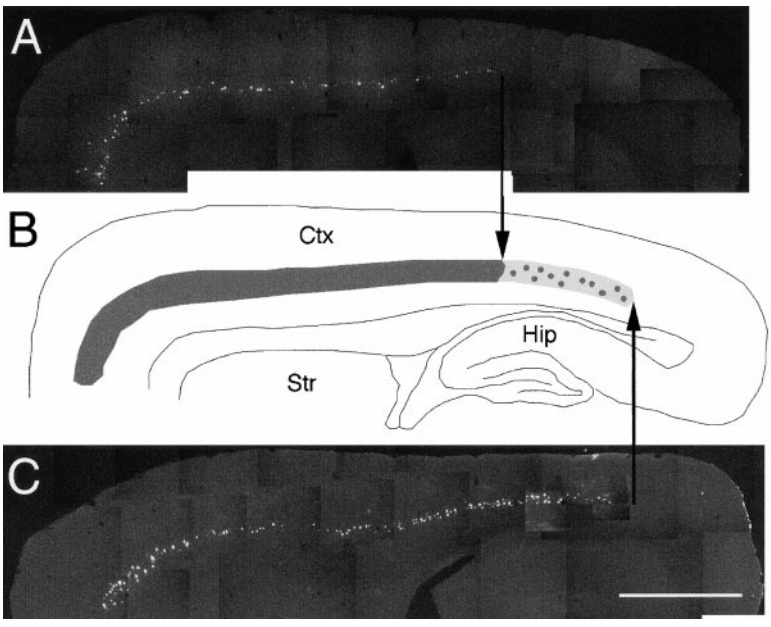

Figure 7. Retrograde Labeling of Spinal Projection Neurons in Adult *Otx1* Mutant Mice

Retrograde labeling resulting from injections of the fluorescent retrograde tracer Fast Blue placed at the pyramidal decussation of control and *Otx1* −/− mutant mice. Sagittal sections; dorsal is up, anterior is to the left.

(A) In control mice, labeled neurons (white dots) were present exclusively in layer 5 in frontal and somatomotor regions, with a posterior boundary of expression overlying the anterior third of the hippocampus.

(B) Summary diagram of pattern of retrograde labeling in layer 5 of control (dark gray) and *Otx1* mutant (light gray) mice. Abbreviations: Ctx, cortex; Str, striatum: Hip, hippocampus.

(C) The pattern of retrograde labeling extends posteriorly into the occipital cortex in *Otx1* mutants.

Scale bar, 1 mm.

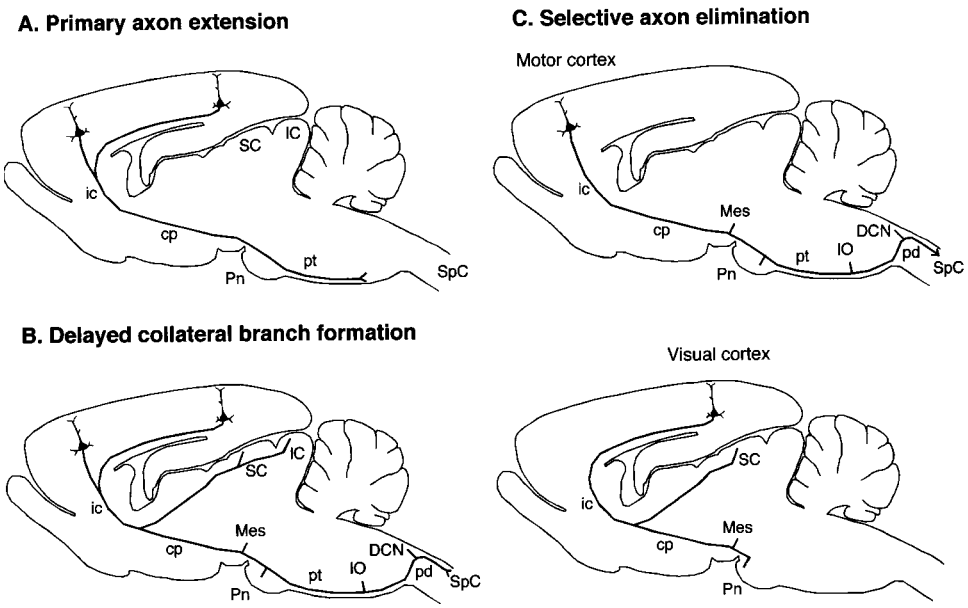

Figure 8. Three Phases of Development of Layer 5 Subcortical Axon Projections

Three phases of development of layer 5 subcortical axon projections, based on a figure published in O'Leary and Koester (1993).

(A) Primary axon extension: layer 5 neurons throughout the cortex extend simple, unbranched axons into the spinal cord.

(B) Delayed collateral branch formation: branches emerge from trunks of parental axons to invade other target regions, including the pons and tectum.

(C) Selective axon elimination: subsets of these branches are maintained selectively by layer 5 neurons depending on the cortical area in which they reside. Neurons in visual cortex eliminate projections to the spinal cord (bottom), whereas motor neurons eliminate collaterals to the tectum (top).

Abbreviations: ic, internal capsule; cp, cerebral peduncle; Mes, mesencephalon; SC, superior colliculus; IC, inferior colliculus; Pn, pons; pt, pyramidal tract; IO, inferior olivary nucleus; DCN, deep cerebellar nuclei; pd, pyramidal decussation; SpC, spinal cord.

no projections to targets outside of the normal repertoire were detected (although the extent of projections in the inferior colliculus and pons may be greater than that observed during normal development). Otx1 is required for the last step of subcortical axon development, in which exuberant connections undergo extensive refinement. This may explain why the projections of layer 6 neurons, which also express Otx1, appear normal in mutant mice, since corticothalamic axons appear not to undergo a period of exuberant axonal connectivity (McConnell et al., 1994). One reason that occipital cortical neurons in *Otx1* mutant mice fail to prune their projections to the inferior colliculus and spinal cord might be that the visual cortical identities of these neurons were not specified properly. We believe this is not the case because callosal and thalamic projections in mutant animals were appropriate for visual cortex, indicating that areal determination has proceeded normally. These results suggest that Otx1 regulates axonal refinement through a mechanism directly related to the process of pruning.

Although our study was confined to the distribution of visual subcortical projections, we predict that the refinement of layer 5 connections from other regions of cortex is also affected in *Otx1* mutant mice. These projections, however, are difficult to study in mouse because the motor areas of cortex, which eliminate their tectal axon branches (O'Leary and Koester, 1993), are spatially intermingled with somatosensory areas, which retain their projections to the superior colliculus (Dräger, 1981). It is difficult to envision how Otx1 might regulate axon refinement in an area-specific pattern when *Otx1* itself is expressed throughout the cortex, and the translocation of Otx1 protein into cell nuclei lacks area specificity. One possibility is that Otx1 may partner with as yet undiscovered area-specific transcription factors to direct the machinery for axonal refinement to specific branches of an axon. It is not clear whether Otx1 is permissive or instructive in such a process.

The above hypothesis raises the issue of whether Otx1 acts cell autonomously during axon refinement. Our retrograde labeling experiments in rats suggest that only a subset (38%–50%) of subcortical projection neurons in layer 5 express Otx1 (Table 1). If Otx1 acts cell autonomously, only Otx1-expressing neurons would be expected to show defects in axon pruning. Indeed, in *Otx1* mutant mice, the density of neurons with spinal axons differed between areas that normally eliminate their spinal projection and those that do not. In somatomotor cortex, which normally maintains a spinal projection (O'Leary and Koester, 1993), retrogradely labeled neurons were large and densely packed (Figure 7A). In occipital cortex of *Otx1* mutants, in contrast, the density of labeled neurons was about a third of that in somatomotor regions, and these were noticeably smaller in size (Figure 7C). We thus hypothesize that only those layer 5 neurons in occipital cortex that normally express Otx1 were unable to eliminate their spinal axons, resulting in a lower density of retrogradely labeled neurons compared to more rostral regions. Unfortunately we were

unable to test this hypothesis directly because the *lacZ* marker gene targeted to the *Otx1* locus is not expressed postnatally (Acampora et al., 1996, 1998).

Mechanism of Otx1 Action

Otx1 encodes a homeodomain transcription factor known to bind DNA and regulate gene transcription (Furukawa et al., 1997), suggesting that the requirement for Otx1 in axon refinement is mediated by the transcriptional control of target genes involved in axon elimination. Transcription factors have been implicated in early stages of axonal growth and pathfinding (e.g., Giniger et al., 1994; Bossing et al., 1996; Mastick et al., 1997; Prasad et al., 1998); however, the nature of their targets remains largely a mystery. Indeed, surprisingly little is known at the cellular level about the mechanisms that underlie large-scale axonal refinement. The bulk of pruning is due to the elimination of axon branches rather than to cell death (O'Leary and Stanfield, 1985), but we do not know whether these axon branches degenerate, are suddenly severed, or gradually withdraw and collapse backward to the parental axon. For visual axons that extend transiently into the rat spinal cord, many millimeters of axon are eliminated between P4 and P14 (Stanfield et al., 1982; O'Leary and Stanfield, 1985; Stanfield and O'Leary, 1985). In comparison, the remodeling of axons at the neuromuscular junction, which has been observed directly using time-lapse confocal microscopy, involves changes in axon length on the order of micrometers and is mediated primarily by the regression of unsuccessful branches (Nguyen and Lichtman, 1996).

A better understanding of axon refinement at the cellular level might speed the identification of putative Otx1 targets. If spinal axons from visual cortex were, for example, to regress slowly back to the pons during the period of elimination, one might hypothesize that the axons had acquired responsiveness to a repulsive activity present in the spinal cord. Several ligands that are repulsive to growth cones, including semaphorins and ephrins, are expressed in the developing spinal cord (Messersmith et al., 1995; Bergemann et al., 1998). One possibility is that during their initial growth, layer 5 axons are unresponsive to inhibitory ligands in the spinal cord, perhaps due to the absence of appropriate receptors. During the first week of postnatal life, as Otx1 is translocated into the nucleus, layer 5 neurons in occipital cortex might begin to express receptors that subsequently mediate axon withdrawal from the repellant region. We have compared the expression of the EphA class of ephrin receptors between wild-type and *Otx1* mutant mice on P6 and P10, but observed no differences in receptor expression by in situ hybridization (not shown), suggesting that the EphA receptors are not critical for subcortical axon refinement. EphB family members and receptors for semaphorins remain viable candidates for regulating this process.

These hypotheses focus on a role for Otx1 in regulating the transcription of target genes involved directly in the process of axon elimination. There are, however, other possibilities. Neuronal activity plays an important role in refining exuberant connections between the two cerebral hemispheres. The final pattern of callosal connectivity is altered in Siamese cats, which have an abnormal cortical representation of the vertical midline (Shatz, 1977), and in strabismic animals in which the eyes are crossed (Berman and Payne, 1983). Callosal refinement is also disrupted by epileptic seizure activity (Morin et al., 1994). It is plausible that Otx1 might regulate neuronal activity in a manner that alters the patterning of subcortical axonal connections. Indeed, *Otx1* $-/-$ mutant animals have a higher incidence of epilepsy than do their $+/-$ littermates (Acampora et al., 1996). However, no role for patterned neuronal activity has yet been demonstrated for the pruning of exuberant subcortical connections. Another possibility is that Otx1 is required indirectly for axon refinement by controlling the production of a hormone or modulator essential for this process. Interestingly, hypothyroid rats fail to eliminate their exuberant projections from visual cortex to the spinal cord (Li et al., 1995); however, thyroid hormone levels have been measured in *Otx1* mutant animals, and the levels are normal (Acampora et al., 1998).

Regulation of Translocation of Otx1 from Cytoplasm to Nucleus

Otx1 protein is sequestered in the cytoplasm of cortical progenitor cells and migrating neurons. The protein visibly enters the nucleus only during the first week of postnatal life, at the onset of axon refinement. The regulated translocation of transcription factors from the cytoplasm into the nucleus is an increasingly common theme in developmental biology. The localization of a transcription factor to the nucleus can be regulated by several mechanisms. (1) Transcription factors may be anchored in the cytoplasm by another protein, as for the DNA-binding protein NF-κB, which is sequestered in the cytoplasm of immune cells by I-κB. Following stimulation, I-κB is phosphorylated and subsequently degraded, releasing NF-κB to enter the nucleus and regulate the transcription of immunologically important target genes (Liou and Baltimore, 1993). (2) "Chaperone" proteins can enhance or stimulate nuclear import. For example, the homeodomain protein extradenticle is present in most cells of the *Drosophila* embryo but is translocated into the nucleus of only a subset (Mann and Abu-Shaar, 1996; Aspland and White, 1997). Translocation requires another homeobox protein, homothorax, which binds extradenticle and enables nuclear entry (Rieckhof et al., 1997). (3) Posttranslational modifications can affect interactions with the machinery for nuclear import or export. The yeast transcription factor Pho4 can be phosphorylated by a cyclin-dependent kinase, which triggers Pho4 export from the nucleus and its accumulation in the cytoplasm (Kaffman et al., 1998).

Although we do not know how Otx1 is triggered to accumulate the nucleus of layer 5 neurons, two lines of evidence suggest that Otx1 is sequestered actively in the cytoplasm prior to nuclear import. First, the sea urchin homolog of Otx (SpOtx) is found in the cytoplasm during early development and only later is translocated into cell nuclei (Chuang et al., 1996). Two-hybrid analysis has revealed that SpOtx can bind to α-actinin, suggesting that the protein may be anchored to the cytoskeleton prior to nuclear import (Chuang et al., 1996). Preliminary studies suggest that this mechanism may be similar in mammals: Otx1-GFP fusion proteins localize efficiently to nuclei in nonneuronal and neuronal cell

lines, but remain in the cytoplasm of cortical ventricular cells (Y. A. Z. and S. K. M., unpublished.) Further elucidation of the processes that trigger the nuclear import of Otx1 may shed light on the mechanisms by which neurons initiate axon refinement during development.

Experimental Procedures

Animals

Long-Evans rats were obtained from Simonsen Labs. 129/Sv-derived *Otx1* +/− mice were bred in our colony and genotyped by PCR using published methods (Acampora et al., 1996). For this study, +/+ and +/− mice were used as controls, and −/− mice are referred to as *Otx1* mutants.

Antibody Generation

A monoclonal anti-Otx1 antibody was generated by immunizing mice with a fusion protein in which the C terminus (amino acids 118–269) of rat Otx1 was fused to maltose-binding protein; this construct lacks the Otx1 homeodomain and N-terminal sequence. Hybridoma cell lines were generated and screened by immunostaining rat brain sections with supernatant and a Texas Red–conjugated secondary antibody. The specificity of the Otx1 antibody was confirmed by Western analysis (Figure 1A).

Injections of Retrograde Tracers and Double Labeling with Otx1 Antibodies

Rats were anesthetized by intramuscular injection of ketamine (75 mg/kg), xylazine (6 mg/kg), and atropine (0.04 mg/kg). The retrograde dyes FluoroGold or Fast Blue were injected into the superior colliculus, pons, or contralateral cortex of rat brains, with 4–8 animals used for each of four ages (P16, P2, P1, E19; Table 1). Some animals were also used for labeling with Otx1 antibodies (Table 1; see below). Injection sites were confirmed by fluorescence microscopy. To retrogradely label corticotectal neurons, a small hole was drilled through the skull overlying the superior colliculus. A glass micropipette filled with dye (FluoroGold, 2%–4% in saline, or Fast Blue, 2%–4% in saline) was lowered 300–500 μm below the dura and 0.2–0.5 μl was injected. For pons injections, P16 rats were anesthetized and the micropipette was targeted to the pons using surface landmarks (Stanfield and O'Leary, 1985). To retrogradely label callosal neurons in postnatal rats, six holes were drilled through the skull overlying visual and somatomotor cortices, and injections were made at depths of 0.5–1 mm below the dura. For fetal injections, pregnant rats were anesthetized on E19 and the uterine horns were exposed. Fast Blue was injected with a hand-held micropipette inserted through the uterine musculature and into the cortex.

Animals were sacrificed 4–9 days after injection (Table 1) with an overdose of Nembutal and perfused with 4% paraformaldehyde. Brains were sectioned on a freezing microtome at 25 μm. Sections were mounted onto slides and viewed using epifluorescence microscopy. Some sections were also processed for immunohistochemistry using the Otx1 antibody (1:10) and a Texas Red–conjugated secondary antibody. For quantitative analysis of double labeling, the number of fluorescently labeled cells was counted in regions of layer 5 where both retrograde label and Otx1 staining were apparent. Images of labeled cells were acquired with a BIORAD confocal microscope and processed with Adobe Photoshop.

To retrogradely label corticospinal projection neurons in *Otx1* mutant and control mice, injections of Fast Blue were made into the pyramidal decussation at P28 or P79 (+/+, n = 2; +/−, n = 5; −/−, n = 3). Mice were anesthetized with Avertin (20 μl/g body weight). 0.2–0.5 μl of 3% Fast Blue in saline was injected bilaterally at a depth of 500 μm below the dura. Animals survived 7 days and brains were processed as described above.

Injections of Anterograde Tracers

Mice were anesthetized with Avertin. For cortical injections, a small flap of bone was removed over occipital cortex and tracer (^{3}H-leucine: 0.5 μl, 100 μCi/μl; or 3 kDa rhodamine dextran: 0.5 μl, 3%–5% in saline) was injected through a glass micropipette at a depth of 300 μm below the dura. Animals received ^{3}H-leucine injections at ages between P25 and P92 (+/+, n = 6; +/−, n = 10; −/−, n = 7) and fluorescent dextran injections on P33 (+/+, n = 4; −/−, n = 1). Mice were sacrificed 5–7 days after injection and perfused as described above. Brain sections were processed for autoradiography (see below) or fluoresence microscopy. Images were acquired with a CCD camera using OpenLabs (Improvision) and processed using Adobe Photoshop.

To visualize the projections of retinal ganglion cells to the superior colliculus, adult mice (>6 months) were anesthetized and injections of ^{3}H-leucine (1 μl, 100 μCi/μl) were made into the vitreous humor of each eye (+/+, n = 2; +/−, n = 3; −/−, n = 2). Mice were sacrificed 9 days later and their brains processed for autoradiography (see below).

In Situ Hybridization

Methods for in situ hybridization are described in Frantz et al. (1994a). Brains were placed in OCT embedding compound, frozen on dry ice, and stored at −80°C until use. 15 μm cryostat sections were mounted on polylysine-coated slides, postfixed in 4% paraformaldehyde, washed in PBS, dehydrated in ethanol, and stored at −80°C. Sections were pretreated with proteinase K (0.9 μg/ml) and acetic anhydride, and dehydrated in a series of ethanols. Hybridization occurred overnight at 65°C. Following hybridization the sections were treated with ribonuclease A (50 μm/ml) at 37°C for 30 min then washed at high stringency in 0.1× SSC at 60°C. Probes included *Otx1* (A. Simeone: Simeone et al., 1992; Frantz et al., 1994b), *SCIP* (G. Lemke: Frantz et al., 1994a), *PN1* (D. Monard: Reinhard et al., 1994), *er81* (T. Jessell: Lin et al., 1998), and *ephrin-A5* (J. Frisen: Frisen et al., 1998). None of the sense control probes generated a signal above background levels (not shown).

Autoradiography and Photography

Sections containing radioactive label were dehydrated in a series of alcohols and xylenes, air-dried, being dipped into Kodak NTB2 nuclear track emulsion, and stored for 3 weeks in the dark at 4°C. Sections were then developed with Kodak D-19, fixed with Kodak Rapid Fix, and counterstained with cresyl violet. Images were captured using a CCD camera and processed using Adobe Photoshop.

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