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A Novel Role for p75NTR in Subplate Growth Cone Complexity and Visual Thalamocortical Innervation

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In cortical development, subplate axons pioneer the pathway from neocortex to the internal capsule, leading to the proposal that they are required for subsequent area-specific innervation of cortex by thalamic axons. A role for p75 neutrophin receptor (NTR) in area-specific thalamic innervation of cortex is suggested by the observation that p75NTR expression is restricted to subplate neurons in a low-rostral to high-caudal gradient throughout the period of thalamocortical innervation. *In vitro*, neurotrophin 3 binding to p75NTR increases neurite length and filopodial formation of immunopurified subplate neurons, suggesting a role for p75NTR in subplate growth cone morphology and function *in vivo*. Consistent with this idea, subplate growth cones have markedly fewer filopodia in mice lacking p75NTR than in wild type mice. Despite this gross morphologic defect, many subplate axons in knock-out mice pioneer the projection to the internal capsule as they do in wild-type mice. However a few subplate axons in the knock-out mice make ectopic projections rostral in the intermediate zone and frontal cortex. Concomitant with the altered morphology of subplate growth cones, mice lacking p75NTR have diminished innervation of visual cortex from the lateral geniculate nucleus, with markedly reduced or absent connections in 48% of knock-out mice. Thalamic projections to auditory and somatosensory cortex are normal, consistent with the gradient of p75NTR expression. Our present results are unusual in that they argue that p75NTR functions in a novel way in subplate neurons, that is, in growth cone morphology and function rather than in axon extension or neuronal survival.

Key words: filopodia; development; outgrowth; NT3; p75NTR expression; cell death

Distinct areas within adult mammalian neocortex receive precisely restricted innervation from individual thalamic nuclei (Sherman and Guillery, 2001). Although several molecules involved in pathfinding of thalamic axons into the internal capsule have been described recently (Kawano et al., 1999; Miyashita-Lin et al., 1999; Tuttle et al., 1999), very little is known about the cellular mechanisms and molecular cues guiding thalamic and neocortical axons to their targets within neocortex.

In developing neocortex, a population of the first postmitotic neurons, subplate neurons, are the first to extend axons toward the internal capsule, pioneering what will become a robust pathway between cerebral cortex and thalamus (McConnell et al., 1989; De Carlos and O'Leary, 1992). Growing thalamocortical axons encounter subplate axons in the internal capsule (Molnar et al., 1998a; Auladell et al., 2000) and then turn to grow tangentially in the intermediate zone until they come to reside beneath their cortical targets. Ablation of subplate neurons beneath presumptive primary visual cortex before the arrival of axons from the lateral geniculate nucleus (LGN) of thalamus causes these axons to fail to recognize their target and to grow past, occasionally innervating inappropriate areas (Ghosh et al., 1990). Although thalamocortical and corticothalamic pathways are spatially (Miller et al., 1993) and phenotypically (Bicknese et al., 1994) separate in mature brain, it is proposed that early thalamic axons use subplate axons as a scaffold on their way to cortex (Molnár and Blakemore, 1995). Studies of thalamocortical axons in reeler mice are consistent with this conclusion (Caviness, 1976; Molnar et al., 1998b). Taken together, these observations have led to the proposal that subplate neurons are required for formation of thalamocortical connections. However, subplate neuron ablations have only been performed after subplate neurons have established their pioneer projection to the internal capsule (Ghosh et al., 1990). Therefore, the functional necessity of this pioneer projection, as well as its role in determining area-specific innervation, remains unproven. Furthermore, the molecular mechanisms involved in subplate neuron guidance of thalamic axons to their targets remain unknown.

One molecule that could play a role is the p75 neurotrophin receptor (p75NTR). Within developing neocortex, subplate neurons uniquely express p75NTR (Allendoerfer et al., 1990). Near birth in rodent, this expression takes the form of a low-rostral to high-caudal gradient (Mackarehtschian et al., 1999), suggesting a possible role in regional specificity of neocortex. Mice lacking p75NTR display innervation defects in the peripheral nervous system (Lee et al., 1992, 1994).

To understand the role of p75NTR expression by subplate neurons in the precise and selective innervation of neocortex by sensory thalamus, we analyzed subplate growth cones and thalamocortical connections in p75NTR knock-out and wild-type mice. We found significantly fewer filopodia on subplate growth

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cones in p75NTR knock-out mice. Although subplate axons in p75NTR knock-out mice make occasional ectopic projections, most of the subplate axons reach the internal capsule. Subsequently, however, there is reduced thalamic innervation of visual cortex in knock-out mice, but normal innervation of auditory and somatosensory cortex. This study provides the first evidence that subplate neurons and p75NTR play a role in area-specific innervation of neocortex.

MATERIALS AND METHODS

Mice and genotype analysis. Unless indicated otherwise, mice used in this study were littermates generated from intercross of parents heterozygous for targeted inactivation of full-length p75NTR expression (Lee et al., 1992) using a breeding strategy identical to that described by Yeo et al. (1997). Mice were housed using standard conditions; their care and use conformed to guidelines from the Animal Care and Use Committee at University of California, Berkeley. Littermates were genotyped by PCR of tail genomic DNA as described (Yeo et al., 1997).

Acquisition and analysis of digital images. Digital images were acquired using either a Nikon Optiphot or Eclipse 800 microscope (Nikon Instruments Inc., Melville, NY) with a cooled CCD camera (Spot2, Diagnostic Instruments, Sterling Heights, MI). Digital images were analyzed on an Apple G4 computer (Apple, Cupertino, CA) using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

p75NTR in situ *hybridization*. *In situ* hybridization probe templates were obtained by RT-PCR of total RNA made from embryonic mouse spinal cord. PCR primers used for amplification of mouse fragments were designed from published sequences (Radeke et al., 1987) and were as follows: p75NTR intracellular domain: 5′-TGACCACTGTGATGGGCAG-3′/5′-GCCTCGTGGGTAAAGGAGTC-3′; extracellular domain: 5′-GAGAGTGCTGCAAAGCCTG-3′/5′-AATGAGGTTGTCGGTGGTG-3′. A cDNA template specific for exon III in the extracellular domain was generated by subcloning the *Bgl*I to *Hin*dIII fragment of the extracellular cDNA. Amplified fragments were gel purified and cloned into pBSII-KS vector. Plasmids were sequenced to verify the product. *In situ* hybridization was performed as described (Lein et al., 1999). In light of reports of a naturally occurring splice variant of p75NTR (Dechant and Barde, 1997), we analyzed p75NTR expression using all three probes. No differences in the expression patterns or levels were observed with the three probes, and so only the expression pattern of the exon III-specific probe is shown.

Subplate neuron culture. Subplate neurons were isolated and immunopurified from embryonic day (E) 17 rat brains as described (DeFreitas et al., 2001). Cultures were maintained for 48–96 hr with the following conditions: no exogenous neurotrophin, 3 ng/ml recombinant human BDNF (Alomone, Jerusalem, Israel), recombinant human neurotrophin 3 (NT3) (Alomone), or neurotrophin + 200 μg/ml of FAb fragments of a polyclonal antibody (Rex) to p75NTR (Weskamp and Reichardt, 1991). The Rex antibody was the generous gift of Lou Reichardt (University of California, San Francisco). Live subplate neurons were labeled with calcein-AM (Molecular Probes, Eugene, OR). Digital images of live neurons and their processes were acquired, and total process area, neurite number, length, and filopodial number were quantitated in NIH Image.

Carbocyanine dye labeling. Mice were fixed by transcardial perfusion with 0.1 M sodium phosphate buffered 4% paraformaldehyde or immersion-fixed in the same fixative (embryonic ages). Brains were removed and stored in fixative with 0.02% sodium azide. Small (~100 μm) similar-sized crystals of 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate (DiI) (D-282, Molecular Probes) or 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine perchlorate (DiD) (D-307, Molecular Probes) were placed into visual or auditory cortex (for *n*, see Table 1). The dye was allowed to transport at room temperature (embryonic ages) or 37°C for 3–6 weeks adjusted for postconceptual age. Coronal sections were cut at 50–100 μm on a vibratome. Sections were counterstained with 0.001% bisbenzamide, mounted, and imaged on a Nikon Optiphot epifluorescence microscope.

Subplate neuron growth cone analysis. To visualize subplate neuron growth cones, brains were removed from E13.5 mice (wild-type, $n = 3$; p75NTR knock-out, $n = 4$) produced from time-bred heterozygote matings (see Mice and genotype analysis). Subplate neuron axons were selectively labeled with DiI (see Carbocyanine dye labeling) placed in the preplate/developing visual cortex at E13.5. To control for gestational age, littermates were analyzed. High-resolution confocal images were acquired with a plan apo 100× oil objective (numerical aperture 1.40) in 1 μm step intervals in the *z* dimension, spanning the complete extent of the growth cone to ensure that the fiber terminated within the section and that the entire growth cone was imaged. Two-dimensional projections were reconstructed from the image stacks using NIH image brightest point projection; individual filopodia were counted and length was measured using the "neurite-labeling" macro (V1.1, ftp://rsbweb.nih.gov/pub/nih-image/user-macros/). Total filopodia number and individual filopodial length were measured for each growth cone (wild-type, $n = 24$; p75NTR knock-out, $n = 22$). In addition, the distance from the most distal filopodial tip to the base of the growth cone was measured in the wild-type mice. To measure growth cone area in the projection, a circle with a diameter equal to the mean growth cone to distal filopodial tip distance plus 1 SD was superimposed over the growth cone. Using a known microscope scale, NIH image was calibrated to measure true area in micrometers squared after a standardized threshold function performed on the selected growth cone area.

Transneuronal transport. Intraocular injection of ^{3}H-proline was used to label LGN axon terminals in layer 4 of visual cortex as described by Drager (1974). ^{3}H-proline (200 μCi; Amersham Biosciences, Piscataway, NJ) was injected into the posterior chamber of the left eye; 25 μm sagittal sections were dipped in NTB-2 autoradiographic emulsion (Eastman Kodak, Rochester, NY) and exposed in the dark for 4–6 weeks. After developing, the slides were counterstained with 0.001% bisbenzamide and coverslipped. After acquisition of digital images, silver grains representing ^{3}H-proline-labeled LGN terminals in visual cortex were identified by their gray scale values. A complete medial-to-lateral series of regularly spaced images encompassing all of the primary visual cortex was analyzed. Thresholding was used to define the cortical area innervated by LGN axons. This area was multiplied by the normalized mean gray scale density (255− mean density/255), a measurement of silver grain density, to obtain a weighted measure of innervation area/section. This value was averaged and multiplied by the total number of sections in the series and the section thickness to obtain the weighted innervation volume (innervation index).

Serotonin immunohistochemistry. Serotonin immunohistochemistry was performed as described by Lebrand et al. (1996).

Subplate neuron bromodeoxyuridine birth dating. Timed-pregnant mice received an intraperitoneal injection of 50 mg/kg of bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) at E12.5 (plug date = E0.5) to label subplate neurons in neocortex (Price et al., 1997).

BrdU staining. BrdU-labeled subplate neurons were visualized as described previously (Lein et al., 1999). Brains were rapidly removed from the cranium and flash-frozen in OCT mounting media (TissueTek, Sakura Finetek, Torrance CA) in a dry ice–95% ethanol bath. Briefly, coronal cryostat sections (10 μm thick) were fixed in 0.1 M sodium phosphate buffered 4% paraformaldehyde, extracted with 0.6% Triton X-100, acetylated, quenched in 3% hydrogen peroxide, and dehydrated through graded alcohols. To expose incorporated BrdU, the sections were microwaved for 10 min in 0.1 M sodium citrate, pH 5.0. Anti-BrdU antibody (IU4, Caltag, Burlingame, CA) was applied at 1:20,000 with 100 U/μl of exonuclease III (ExoIII) (Roche Molecular Biochemicals, Indianapolis, IN) in ExoIII buffer plus 100 mM NaCl with 1% bovine serum albumin, at 37°C for 1 hr. After washing, horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) was applied at 1:200 in blocking solution (supplied by manufacturer) for 30 min, followed by tyramide signal amplification (TSA) (Direct-green; PerkinElmer Life Sciences, Boston, MA). The sections were counterstained with 0.001% bisbenzamide. Subplate neuron density (cells per section) was counted in coronal sections at three selected locations corresponding to Figures 22, 48, and 65 of Franklin and Paxinos (1997). The subplate zone was identified using accepted criteria (Boulder Committee, 1970) and cytoarchitectonic features of neocortex (Bayer and Altman, 1990). Specifically, in the radial domain the subplate (layer 6B) was localized at the base of the cortical plate, immediately below layer 6 neurons, and contained characteristic pyramidal neurons. The subplate zone extended to the subventricular zone at early ages [E16.5 to postnatal day (P) 7] and into the myelinated white matter at later ages (>P14). The borders in the coronal plane were determined by the characteristic six-layered neocortex and extended to cingulate cortex in the medial direction and entorhinal cortex laterally. Only heavily BrdU-labeled cells falling into this region were counted. Heavily labeled cells were defined, as in previous studies (Price et al.,

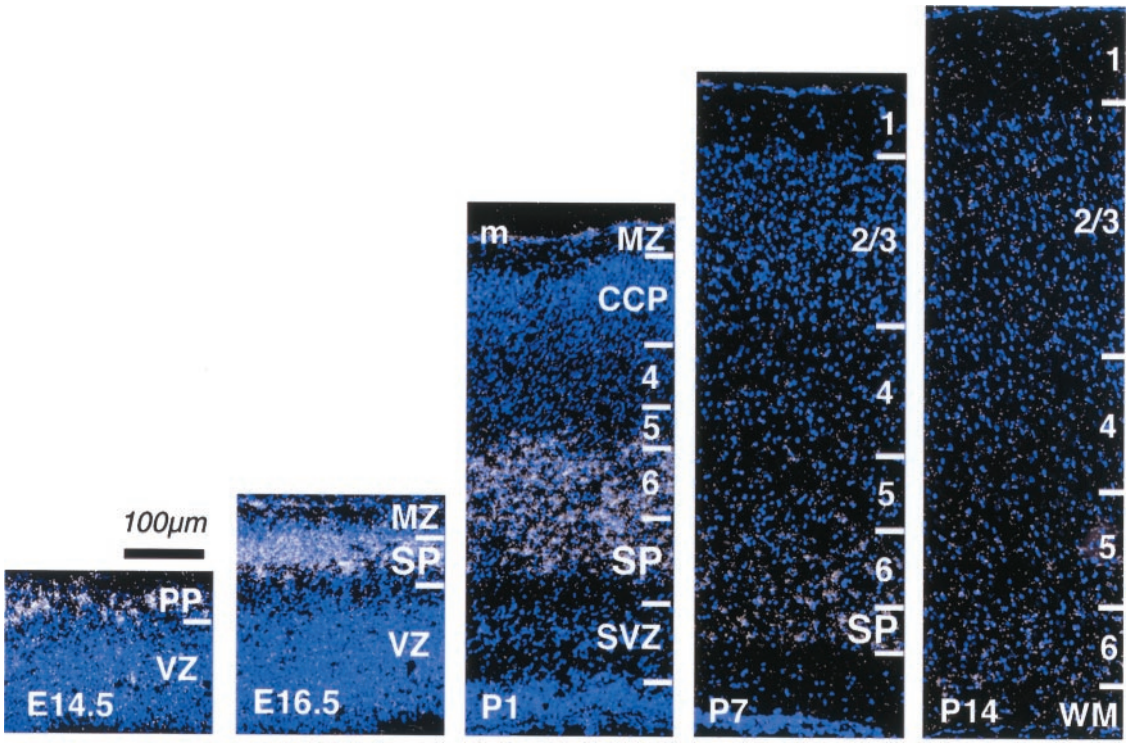

Figure 1. p75NTR expression in subplate during development. An *in situ* probe specific for exon III of p75NTR was used to survey p75NTR expression. High-magnification view of subplate p75NTR expression in cross section of cortex at ages from E14.5 through P14. *PP*, Preplate; *VZ*, ventricular zone; *SP*, subplate; *m*, meninges; *MZ*, marginal zone; *SVZ*, subventricular zone; *CCP*, condensed cortical plate; *WM*, white matter.

1997), as cells in which more than half the nucleus was labeled. Homozygous knock-out mice (c129ngfr; Jackson Laboratory, Bar Harbor, ME) ($n = 18$) were compared with BALB/c ($n = 6$) and 129sv ($n = 12$) wild-type control mice maintained in our colony. Both hemispheres were counted, and the section counts averaged across the three levels were analyzed. Counts were binned for the following ages: E18.5 and P0 ($n = 8$) and P3 and P7 ($n = 4$). Counts for these ages, as well as for P14 and P21, are reported as average cell number per section ± SD.

In situ *end labeling staining*. To visualize dying cells, we used a modified version of the *in situ* end labeling (ISEL+) method (Blaschke et al., 1996). Sections adjacent to those analyzed for BrdU were selected and prepared similarly and used for counts. A reaction mixture containing 1 μM biotin-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), 0.15 U/ml of terminal transferase (Invitrogen, Rockville, MD), 1× terminal transferase buffer, and 1% bovine serum albumin was applied, and the sections were incubated for 1 hr at 37°C. Sections were washed, then incubated with NeutraLite avidin–horseradish peroxidase (Molecular Probes) at 1:1000 in blocking solution (supplied with TSA-direct kit) for 30 min. The sections were washed and developed with TSA-Direct Cy3 (PerkinElmer Life Sciences). Labeled cells were counted only if they were located within the subplate as defined above. Data were binned for the following ages: E18.5 and P0 ($n = 16$) and P3 and P7 ($n = 4$). These ages as well as P14 and P21 are reported as average cell number per section ± SD. Double-labeling was performed at selected ages (P2–7), with the ISEL reaction performed before microwave treatment and BrdU primary antibody incubation. Visualization with direct-TSA was performed sequentially, with inactivation of peroxidase between development of ISEL and BrdU immunohistochemistry.

RESULTS

Subplate neurons express p75NTR mRNA throughout thalamocortical development

Subplate neurons have previously been shown to express p75NTR protein by immunohistochemistry during early cortical development (Allendoerfer et al., 1990). p75NTR is known to affect axon outgrowth (Yamashita et al., 1999; Davies, 2000), and mice lacking p75NTR have reduced sensory and sympathetic innervation in the peripheral nervous system (Lee et al., 1992, 1994). Subplate axons pioneer the intracortical pathway to the internal capsule (McConnell et al., 1989). These observations raise the question of whether p75NTR plays a role in early subplate neuron axon outgrowth and the establishment of subsequent connections between cortex and thalamus. p75NTR expression, however, has only been studied after development of thalamocortical projections (Allendoerfer et al., 1990, 1994; Koh and Higgins, 1991). To determine whether p75NTR is expressed at early ages when subplate axons pioneer the intracortical pathway to internal capsule and to elucidate fully the pattern of p75NTR expression in neocortex, we examined mRNA expression by *in situ* hybridization throughout development of the thalamocortical projection in mice. Subplate neurons are the first neocortical cells to express p75NTR soon after they migrate to form the preplate at E14.5 (Fig. 1). At every stage of development from E14.5 to P14, p75NTR is expressed by neurons in the subplate zone at the base of the developing cortical plate (Fig. 1), corresponding precisely to the location of immunostained subplate neurons observed by others (Allendoerfer et al., 1990). We found similar patterns of early expression in ferret, at earlier ages (E30) (P. S. McQuillen, M. F. DeFreitas, and C. J. Shatz, unpublished observations) than seen previously (Allendoerfer et al., 1994). This discrepancy likely results from higher sensitivity of *in situ* hybridization compared with the antibodies used in previous studies. Thus this early expression is not specific to mice and likely plays a similar role in other species. In mice, the level of p75NTR mRNA expression peaks in neocortex by P1 (Fig. 1). By P7, after the peak of cell death in the subplate, expression declines in cortex. By P14 only scattered cells at the base of the cortical plate express p75NTR (Fig. 1). This pattern of expression

persists in the adult (data not shown). Thus, subplate neurons are the first neocortical neurons to express p75NTR, and at early ages (E14.5–18.5), during formation of thalamocortical connections, they are the only cells in neocortex that express this receptor.

p75NTR modulates neurite outgrowth of subplate neurons in culture

Neurotrophin signaling via p75NTR stimulates axon outgrowth (Davies, 2000) and growth cone turning (Gundersen and Barrett, 1979) and may provide a stop signal to growing axons (Kohn et al., 1999). All of these effects are important for axon pathfinding, target selection, and innervation. To determine whether p75NTR signaling affects subplate neuron process outgrowth, we first used an *in vitro* assay to examine the morphology of immunopurified subplate neurons in response to neurotrophin binding by p75NTR. Rat subplate neurons were immunopurified with an anti-p75NTR monoclonal antibody as described previously (DeFreitas et al., 2001). Subplate neurons were purified from rat rather than mouse because function-blocking p75NTR antibodies are readily available for rat but not mouse, and subplate neurons cannot be immunopurified from p75NTR knock-out mice with a p75NTR antibody. Rat subplate neurons cultured on fibronectin in serum-free medium develop highly branched neurites with multiple filopodia (DeFreitas et al., 2001). Addition of NT3 significantly increases neurite length (Fig. 2*A*). This increase is dependent on NT3 binding to p75NTR, because anti-p75NTR FAb fragments, which are known to block binding of neurotrophin to p75NTR (Weskamp and Reichardt, 1991), block the NT3-dependent increase in neurite length completely (Fig. 2*A*). Surprisingly, addition of BDNF has no effect on neurite length (Fig. 2*A*), although both NT3 and BDNF stimulate the formation of broad lamellipodia extensions on neurites (data not shown) and can support survival via p75NTR (DeFreitas et al., 2001). NT3 also increases the number of filopodia (Fig. 2*B*), an effect that is also blocked by addition of anti-p75NTR FAb fragments (Fig. 2*B*). The fact that different neurotrophins can bind p75NTR but cause different responses in terms of cell signaling is not surprising in light of observations by many other groups (Kaplan and Miller, 2000). These differences in response are consistent with developmental changes in the expression of neurotrophins. Early in cortical development, during the period of subplate axon outgrowth, NT3 is the predominant neurotrophin expressed, whereas BDNF does not increase to similarly high levels until later ages (Lein et al., 2000). These observations indicate that *in vitro* p75NTR regulates subplate neuron process outgrowth. They also suggest that p75NTR signaling may contribute to normal subplate growth cone morphology *in vivo*.

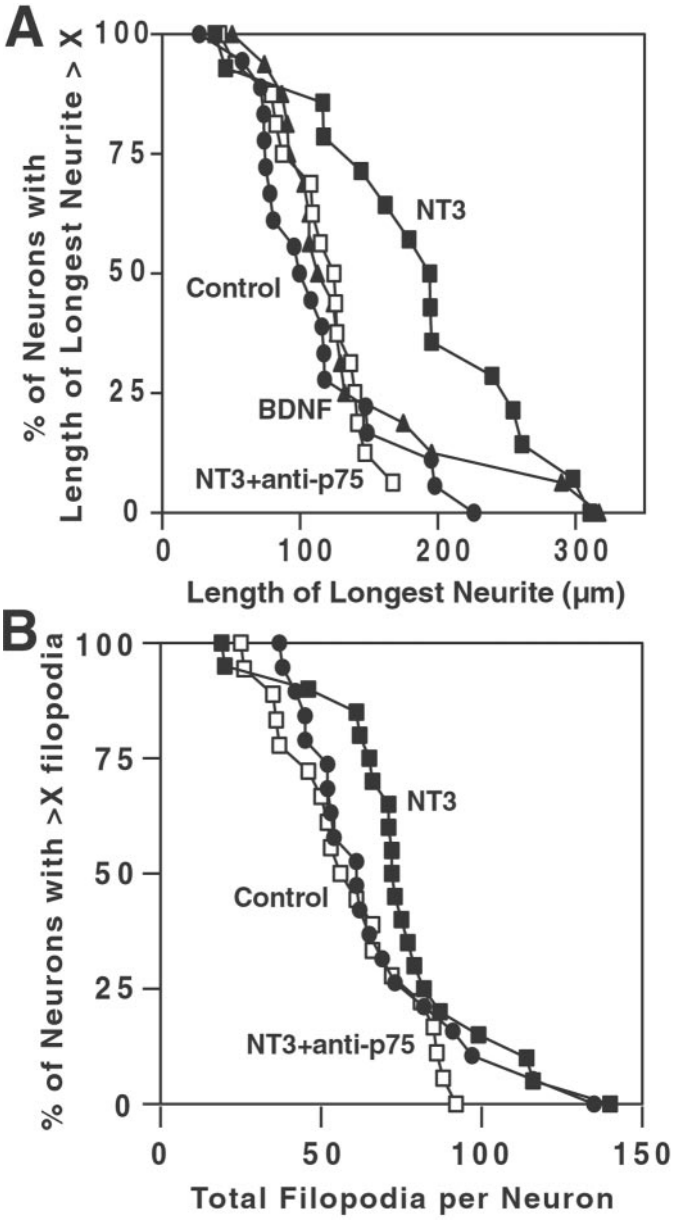

Figure 2. Blockade of NT3 binding to p75NTR reduces neurite length and filopodial number in cultured subplate neurons. Immunopurified subplate neurons were cultured on fibronectin in serum-free media with addition of BDNF (▲), NT3 (■), NT3 + anti-p75NTR FAb (□), or control (no neurotrophin) (●). *A*, After 5 d *in vitro*, the length of the longest neurite was measured and plotted against percentage of cells with length $> X$. *B*, After 2 d *in vitro*, the total number of filopodia per neuron was measured and plotted versus percentage of neurons with $> X$ total filopodia.

Absence of p75NTR reduces filopodial formation in p75NTR knock-out mice

To assess the role of p75NTR *in vivo*, we examined subplate growth cone morphology in wild-type and p75NTR knock-out mice. Subplate neuron growth cones are known to be larger and more complex than the growth cones of later-generated neurons, consistent with their role as pioneer neurons (Kim et al., 1991). To examine subplate growth cone morphology, a crystal of DiI was placed in the presumptive visual cortex of E14 wild-type and knock-out littermates. At E14, thalamic axons have not yet grown all the way to visual cortex (Auladell et al., 2000), and subplate axons are the only descending projection from cortex; thus subplate axons are selectively labeled. Individual growth cones at the leading edge of the labeled projection were selected for analysis so that the entire growth cone could be visualized with no part being obscured by neighboring fibers. Growth cones were analyzed in both knock-out and wild-type mice at the internal capsule (Fig. 3*A*). Examination of subplate neuron growth cones reveals a striking decrease in complexity in p75NTR knock-out mice when compared with wild-type (Fig. 3*B–D*). Growth cones in wild-type mice have twice as many filopodia as in knock-out (Fig. 3*B*) (mean 8.3 vs 4.1 filopodia/growth cone; $p < 0.0001$; unpaired t test). Total growth cone size (see Materials and Methods) is also diminished in the knock-outs (mean 47.5 vs 38.1 μm^2; $p = 0.0262$; unpaired t

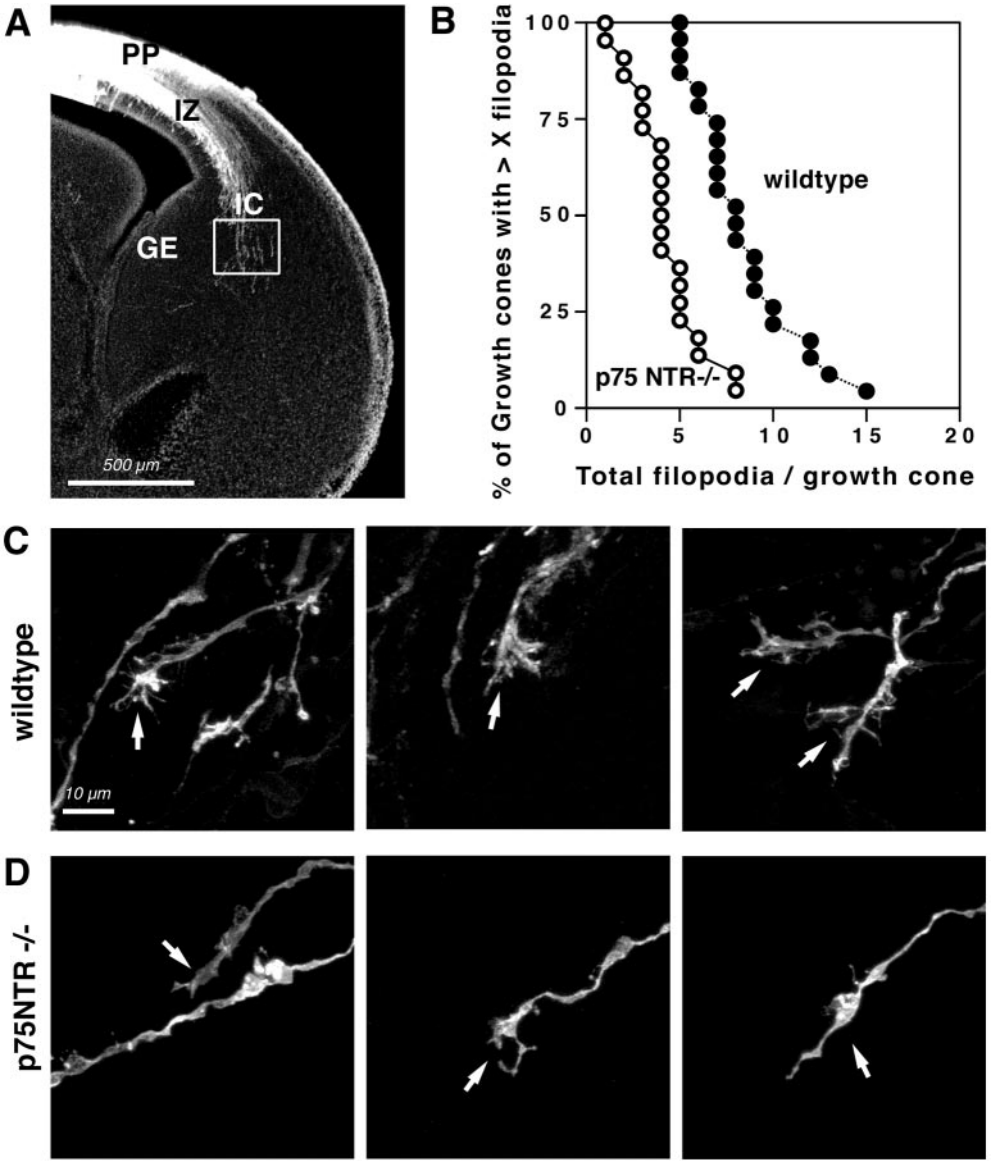

Figure 3. Abnormal subplate neuron growth cones in p75NTR knock-out mice. Subplate neuron growth cones were labeled at E13.5 with crystals of DiI inserted into developing visual cortex. *A*, Individual growth cones are imaged in both genotypes in the internal capsule, at the leading edge of labeled subplate projection (*boxed area*). *B*, Filopodia per growth cone measured for wild-type (●) and knock-out (○) mice. *C*, *D*, Representative examples of growth cone morphology from wild-type (*C*) or knock-out mice (*D*) are marked with *arrows*.

test). However, average filopodial length is unchanged (4.03 vs 4.04 μm). Filopodia in knock-out mice, unlike in wild-type mice, are often thick and occasionally contained ball-like extensions at the tips (Fig. 3*D*). Although there is a range in the number of filopodia per subplate growth cone in knock-out mice, there is a shift in the entire population of filopodia per subplate growth cone in knock-out mice, leading to smaller, more simple growth cones compared with wild-type littermates (Fig. 3*B*).

Subplate axons make occasional pathfinding errors after reaching the internal capsule

To determine whether the severe alteration in subplate growth cone morphology affects subplate axon extension and pathfinding, we labeled descending subplate axons (and some layer 6 axons) at E15.5 with small crystals of DiI placed into developing primary visual cortex. At this age, thalamic axons are just reaching subplate beneath visual cortex (Catalano et al., 1996; Auladell et al., 2000); thus the dye retrogradely labels very few thalamic axons. In both genotypes, at E15.5 there is a robust projection from primary visual cortex that traverses the intermediate zone and arrives at the internal capsule (Fig. 4*A*,*B*). Thus the ability of subplate neurons to read the long-range directional cues responsible for guidance rostral to the internal capsule does not appear to be altered by loss of p75NTR expression. Also, subplate axon extension is not noticeably reduced. At the internal capsule, all subplate axons in wild-type mice, and most in p75NTR knock-out mice, turn and enter the internal capsule (Fig. 4*A*,*B*). In two of six knock-out hemispheres, however, a few subplate axons failed to turn into the internal capsule and continued growing rostrally in the intermediate zone, occasionally turning lateral to innervate entorhinal cortex (Fig. 4*D*). This ectopic projection was never seen in wild-type mice (Fig. 4*C*) ($n = 6$ hemispheres). These observations indicate that despite a clear abnormality in filopodia in the p75NTR knock-out mice, subplate axon pathfinding is mostly normal. However, p75NTR may be needed for subplate growth cones to respond to local cues or interact with other cell types at the internal capsule.

p75NTR is expressed in a low-rostral, high-caudal gradient throughout thalamocortical development

p75NTR mRNA expression has been observed in a low-rostral to high-caudal gradient in rat cortex at E19 and P1 (Mack-

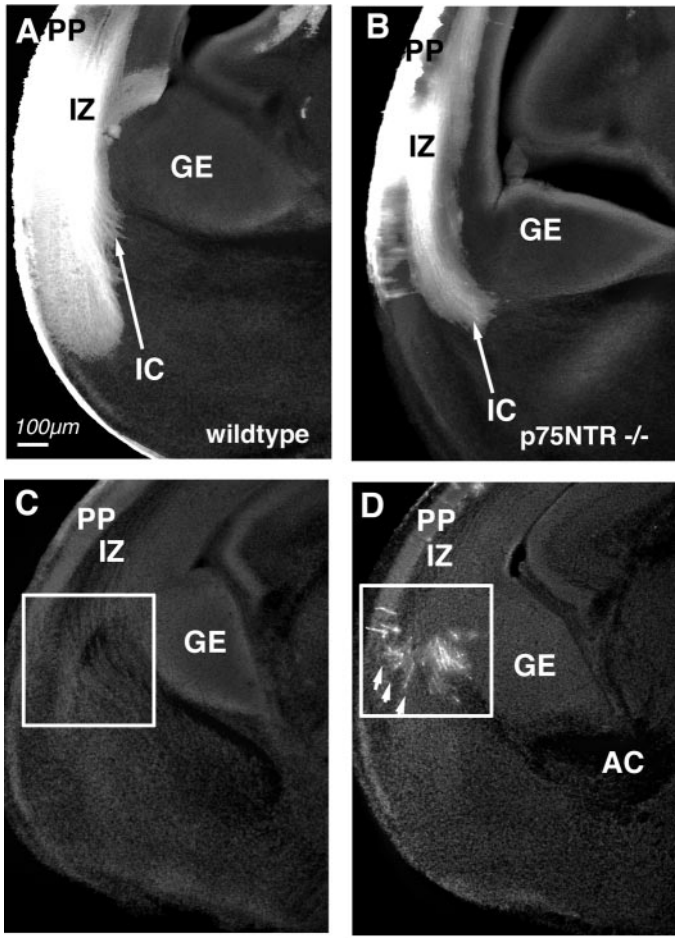

Figure 4. Subplate axons make pathfinding errors in p75NTR knock-out mice. Crystals of DiI were placed in presumptive visual cortex at E15.5, before LGN axons have arrived underneath caudal cortex, to label subplate neurons selectively. *A*, *B*, Coronal sections show DiI-labeled subplate axons reaching the internal capsule (*IC*) in wild-type mice (*A*) or p75NTR knock-out mice (*B*). *C*, *D*, In slightly more rostral sections, ectopic subplate axons were found in the p75NTR knock-out mice (*D*) but not wild-type mice (*C*), some of which turn lateral (*arrowheads* in *boxed area*) to enter the preplate (*PP*). *GE*, Ganglionic eminence; *IZ*, intermediate zone; *AC*, anterior commissure.

arehtschian et al., 1999). This suggests the possibility that p75NTR may function in some aspect of area-specific development of cortex. Although this expression gradient was noted earlier using immunohistochemistry, it was thought to be a gradient of maturation, with neurons in rostral cortex downregulating expression earlier than those more caudal (Koh and Loy, 1989). To determine whether the gradient observed at birth is a maturational or a spatial gradient, we examined expression in parasagittal sections throughout the lifespan of subplate neurons. From the earliest time at which p75NTR mRNA can be detected in neocortex (E14.5) until expression begins to diminish (>P7), p75NTR is expressed in a low-rostral to high-caudal gradient (Fig. 5*A–D*). Throughout this period, p75NTR is expressed uniformly in the subplate across the medial to lateral aspect of neocortex (data not shown). This expression pattern is not unique to rodent; similar patterns can be found in ferret, where expression persists in a rostrocaudal gradient in subplate neurons until at least P21 (McQuillen, DeFreitas, and Shatz, unpublished observations). At P21, expression is limited to subplate and layer 6 neurons in the posterolateral gyrus containing visual cortex. Thus, the low-rostral to high-caudal gradient of p75NTR expression is not caused by a maturational gradient and is present in caudal subplate throughout the period of development of thalamocortical projections [rodent: E14.5–P7 (Catalano et al., 1996; Auladell et al., 2000)]. The low-rostral–high-caudal gradient of p75NTR expression by subplate neurons, taken together with the known participation of subplate neurons in thalamocortical development, suggests that p75NTR might function in some aspect of cortical arealization and area-specific thalamocortical innervation.

p75NTR is also expressed in other brain structures at these times. Beginning as early as E14.5, cells in the diencephalon transiently express p75NTR at low levels. At E14, cells in reticular thalamus express p75NTR (Fig. 5*A*, *arrow*). Expression in reticular thalamus continues throughout development (Fig. 5*A–D*, *arrows*) and can be identified in the adult (data not shown). Although there is expression of p75NTR in posterior (Fig. 5*A*,*B*, *arrows*) and dorsal thalamus (data not shown) at very early ages (E14–16.5) (Fig. 5*A*,*B*), this expression disappears by E18.5. As has been reported (Koh and Higgins, 1991), we find no expression of p75NTR mRNA in the LGN, or any of lateral thalamus, at any age (data not shown). Other cell populations known to express p75NTR (Koh and Higgins, 1991) are also detected, including the basal forebrain complex (e.g., septal nuclei, diagonal band, and nucleus basalis of Meynert) (Fig. 5*B–D*, *asterisk*), the developing cerebellar anlage (Fig. 5*C*,*D*), and the meninges (Fig. 1). Expression in the basal forebrain and Purkinje cells of cerebellum increases throughout development, and a high level of expression is maintained in adulthood (data not shown). Importantly, expression in these brain structures is not in a gradient and does not correlate with positions of cortical projections.

Decreased visual thalamocortical innervation in p75NTR knock-out mice

Subplate neurons pioneer the projection from neocortex to internal capsule, and it has been proposed that subplate neurons are required for thalamocortical innervation (Ghosh et al., 1990). Subplate neurons beneath visual cortex express high levels of p75NTR mRNA during the period of thalamocortical development. In contrast, subplate neurons located in more anterior regions express comparatively lower levels of p75NTR mRNA. This observation suggests that p75NTR may function in establishing area-specific thalamic innervation of cortex. To consider whether p75NTR expression by subplate neurons is required for the area-specific formation of thalamocortical connections, thalamocortical axons were traced by retrograde transport of carbocyanine dyes between E17.5 and P10. At these ages, dye placement in cortex labels thalamus by a combination of retrograde labeling of thalamic neurons and anterograde labeling of the descending corticothalamic projections. Placement of DiD into the visual cortex (Fig. 6*A*,*B*) robustly labels neurons and fibers in the LGN of wild-type mice (Fig. 6*C*). Similar results are seen in heterozygous mice (data not shown).

In marked contrast, when similar dye injections are made into the visual cortex of p75NTR knock-out mice (Fig. 6*B*), there is a severe reduction (<than 5% of LGN area labeled) or complete absence of LGN labeling in 46% of the cases (Fig. 6*D*, Table 1). Although we observed a 10% rate of decreased label in LGN in wild-type mice caused by technical failure (Table 1, see footnote), the frequency of decreased label in LGN is significantly greater in p75NTR knock-out mice compared with their wild-type litter-

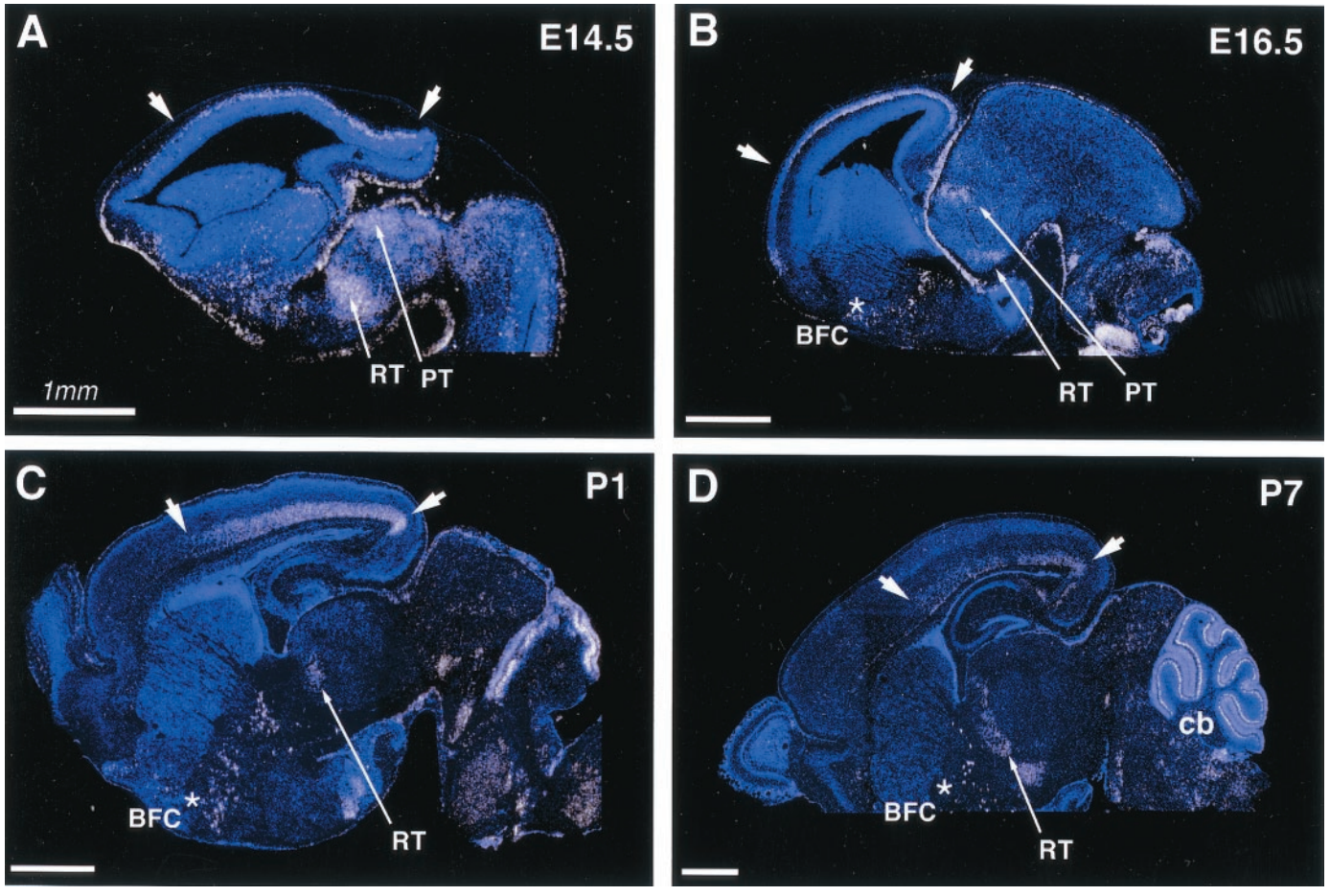

Figure 5. Gradient of p75NTR expression in subplate during development. *A–D*, Mid-sagittal sections at E14.5 (*A*), E16.5 (*B*), P1 (*C*), and P7 (*D*) demonstrate a low-rostral high-caudal gradient of p75NTR expression. *Arrowheads* denote rostral–caudal extent of p75NTR expression. Other structures with p75NTR expression are labeled or marked with *arrow* or *asterisk*. *PT*, Posterior thalamus; *RT*, reticular thalamus; *cb*, cerebellum; *BFC*, basal forebrain complex.

mates ($p = 0.01$; Fisher's exact test). All experiments with diminished transport were included in the analysis in both knock-out and wild-type mice. The brains from wild-type and knock-out littermates were processed simultaneously, in an identical manner. Thus, this difference cannot be explained by failure of the technique.

To further examine the thalamocortical pathway abnormality in p75NTR knock-out mice, dye crystals were placed directly into lateral dorsal thalamus at P10 to label LGN axons anterogradely and label cortical neurons retrogradely. In every case, wild-type and knock-outs, a bundle of labeled fibers could be followed up through the internal capsule and into the intermediate zone below cortical plate. In wild-type mice, axons turned caudal and extended beneath visual cortex. In three of five knock-out mice, however, the projection of thalamocortical axons was reduced underneath visual cortex as compared with wild-type mice (Fig. 6*E*,*F*). In both genotypes, cells in cortical areas rostral to visual cortex are labeled because of the close proximity of somatosensory thalamus and fibers of passage from medial geniculate nucleus (MGN) to the area of dye crystal placement in LGN. No cases of aberrant or ectopic thalamic fibers were noted. Thus, by means of both anterograde and retrograde tracing, it is evident that the projection from LGN to visual cortex is significantly reduced in the absence of p75NTR.

To obtain a more selective means of labeling the thalamocortical projection exclusively from the LGN at a later age, transneuronal transport of ^{3}H-proline after an intraocular injection was used. This method allows for a complete assessment of the thalamocortical projection from the LGN in mice aged 1 month or older, which is not possible with DiI. Thus, we examined the projection in mature (>90 d) mice by autoradiography and densitometric scanning of silver grains representing LGN axon terminals in visual cortex (see Materials and Methods). This independent technique confirms and extends our observations that in the p75NTR knock-out mice there is a reduction in the projection from LGN to cortex, which in some cases can be quite severe (Fig. 7, compare *A*, *B*). This was not caused by differences in uptake of ^{3}H-proline because the LGN was heavily labeled in every animal in both groups. A quantitative measure of innervation density (see Materials and Methods) confirmed that there is an overall reduction in the projection from LGN to visual cortex in the knock-out mice (Fig. 7*C*) (mean wild-type = 0.20 mm^3 × mean pixel density vs mean p75NTR knock-out = 0.12 mm^3 × mean pixel density; $p = 0.074$; unpaired t test). Innervation volume was slightly but not significantly smaller in knock-out mice (mean wild-type = 0.75 mm^3 vs mean p75NTR knock-out = 0.60; $p = 0.259$; unpaired t test). In all cases in which label could be detected in cortex, the rostral–caudal boundaries of primary visual cortex in the knock-out mice were unchanged when compared with

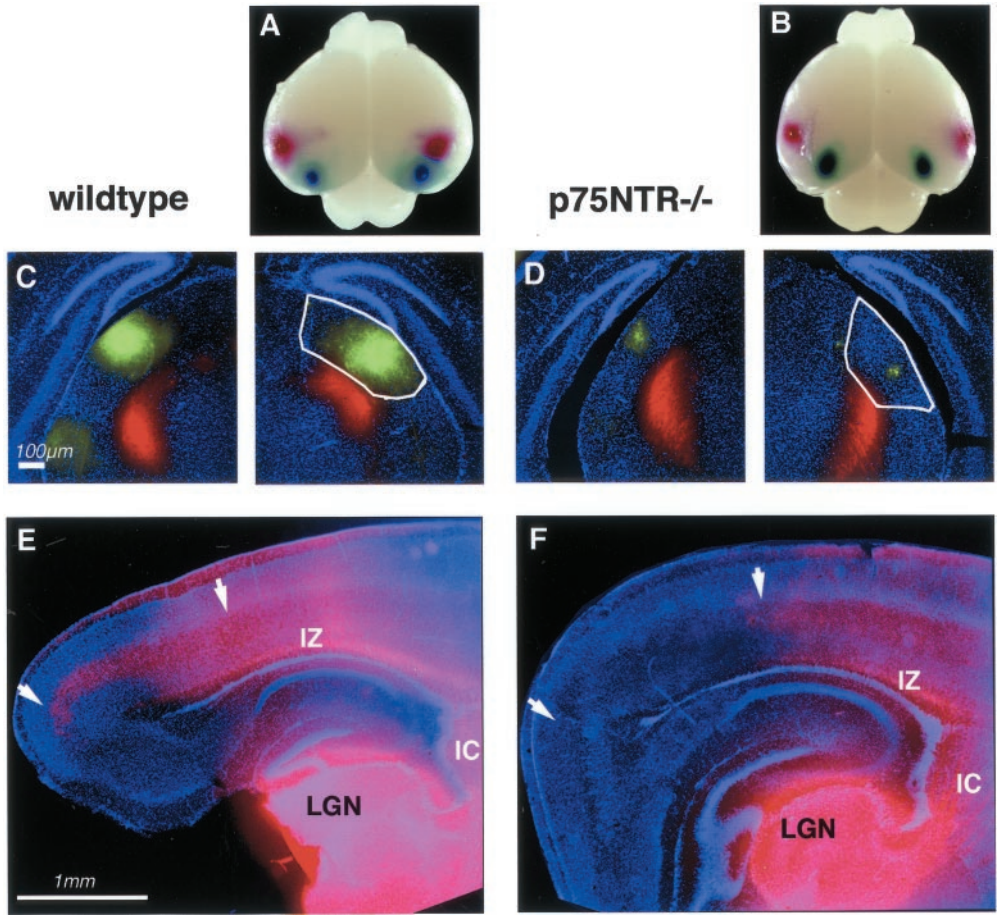

Figure 6. Reduced visual thalamocortical innervation in p75NTR knock-out mice. *A*, *B*, DiI (*red*) and DiD (*green*) crystal placement in auditory cortex and visual cortex, respectively, in wild-type (*A*) and knock-out (*B*) mice at P10. *C*, *D*, Retrograde labeling of cells and anterograde labeling of fibers in LGN and thalamus after cortical labeling shown in *A* and *B* in coronal sections. DiD (*green*) from visual cortex heavily labels the LGN (*outlined* in *white*) in wild-type (*C*) but not knock-out (*D*) mice. DiI (*red*) from auditory cortex labels fibers of passage heading to the MGN (shown in Fig. 8), as well as some cells of the ventrobasal nucleus of thalamus. *E*, *F*, Anterograde labeling of LGN axons and retrograde labeling of cortical cells after DiI (*red*) placement in the lateral dorsal thalamus. In these sagittal sections (caudal is to the *left*), labeled thalamic axons project from the LGN to the internal capsule (*IC*), then within the intermediate zone (*IZ*) toward visual cortex (denoted between *arrowheads*) in wild-type (*E*) but not knock-out (*F*) mice.

Table 1. Frequency of reduced thalamocortical innervation

		Visual		Auditory	Somatosensory
	E17.5–P10 Cortical DiI	P10 Thalamic DiI	P90 ^{3}H-Proline TNT	P2–10 Cortical DiD	P10 Serotonin IHC
p75NTR −/−	46% (13/28)	60% (3/5)	43% (3/7)	0% (0/8)	0% (0/4)
Wild type	10%[a] (2/20)	0% (0/2)	0% (0/8)	0% (0/4)	0% (0/4)

Data = % with reduced label (*n* = total with reduced label/total hemispheres); TNT, transneuronal transport; IHC, immunohistochemistry.

[a] Note: Failure of dye transport in normal animals occurs because of technical problems (e.g., depth of crystal placement, tissue preservation, and transport time) and gives an estimate of experimental error.

wild-type (Fig. 7*A*,*B*), indicating that primary visual cortex was not spatially contracted or located ectopically. Unlike with carbocyanine dye labeling at earlier ages, there were no cases with a complete absence of innervation. This presence of at least some innervation in all knock-out mice at these ages may be attributable to delayed innervation, although differences in the ability of the two techniques to visualize the entire projection from LGN to primary visual cortex cannot be excluded, because the transneuronal transport is performed at a much later age than the DiI labeling. In addition to the general decreased innervation in the knock-out mice (Fig. 7*C*), there were three cases of extreme decrease in innervation density in the knock-out mice, all with values outside of the range displayed by wild-type mice. These mice with values lower than 1 SD of the mean wild-type value were the only individuals scored as having abnormally reduced transport for the combined statistical contingency analysis. When all three techniques are considered together (placement of lipophilic dye in cortex, thalamus, and transneuronal transport), the frequency of abnormally reduced thalamocortical innervation is significantly greater ($p = 0.0002$; Fisher's exact test) in p75NTR knock-out mice (48%) when compared with wild-type mice (7%). This shift in the population behavior of LGN axons to generally lower innervation levels mirrors the decrease in filopodial number in subplate growth cones in the p75NTR knock-out mice.

Normal innervation of auditory and somatosensory cortex

The disruption of visual thalamocortical connections in p75NTR knock-out mice, taken together with the gradient of p75NTR expression with highest levels in visual cortex, implies a specific role for p75NTR in the formation of thalamocortical connections from the LGN. If so, then we would predict that the pathway from

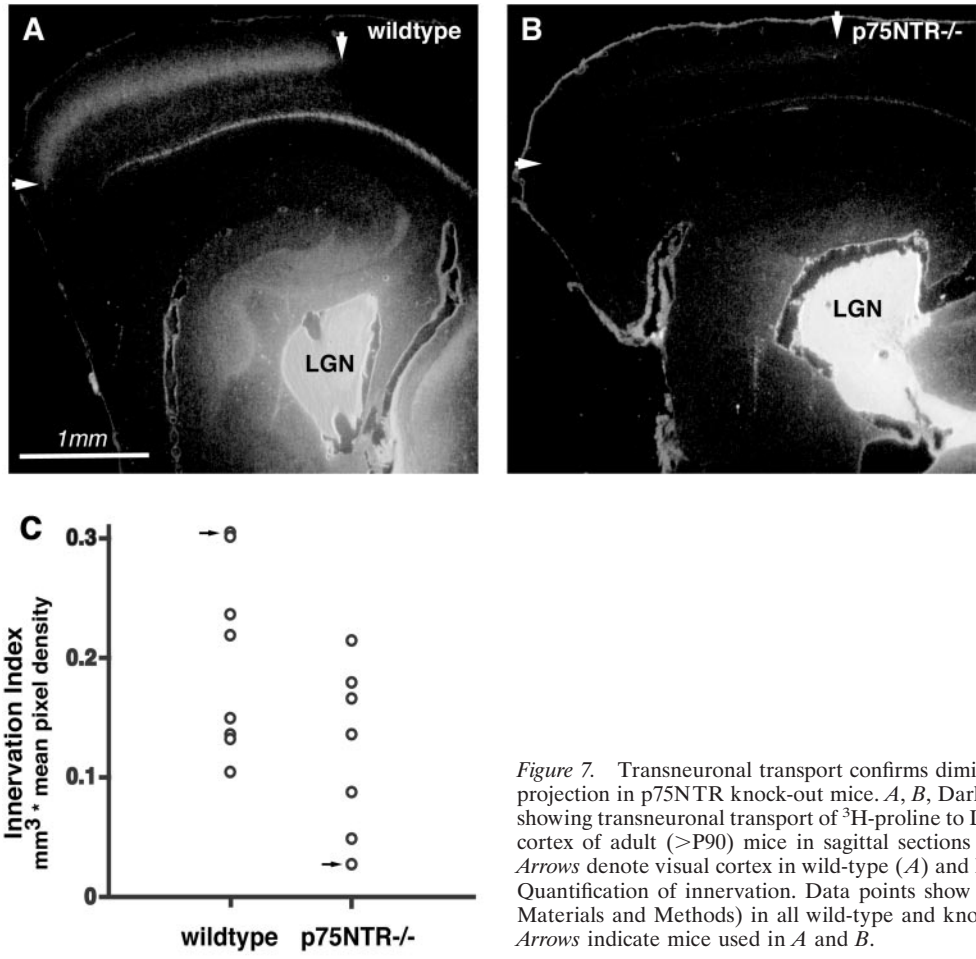

Figure 7. Transneuronal transport confirms diminished geniculocortical projection in p75NTR knock-out mice. *A*, *B*, Dark-field autoradiography showing transneuronal transport of ^{3}H-proline to LGN terminals in visual cortex of adult (>P90) mice in sagittal sections (caudal is to the *left*). *Arrows* denote visual cortex in wild-type (*A*) and knock-out (*B*) mice. *C*, Quantification of innervation. Data points show innervation index (see Materials and Methods) in all wild-type and knock-out mice examined. *Arrows* indicate mice used in *A* and *B*.

MGN to auditory cortex and from the lateral ventral posterior nucleus to somatosensory cortex to be less affected in p75NTR knock-out mice, given the relatively lower levels of p75NTR expression seen there normally. Indeed, this is exactly the case: after DiI injection into auditory cortex (dye crystal placement shown in Fig. 6*A*,*B*) in both wild-type and knock-out mice, there is robust label in MGN (Fig. 8*A*,*B*, Table 1) in all mice. To assess innervation of somatosensory cortex, we took advantage of the fact that sensory thalamic neurons take up, and become immunoreactive for, serotonin for a brief period of time early in development (Lebrand et al., 1996). Examination of somatosensory cortex demonstrates a normal patchy distribution of somatosensory axon terminals corresponding to the barrel representations of whisker vibrissae (Fig. 8*C*,*D*, Table 1) in all wild-type and knock-out mice. Therefore, loss of p75NTR expression selectively impairs visual thalamocortical innervation but does not affect thalamic innervation of more rostral auditory or somatosensory cortex.

Subplate neuron death is not altered in p75NTR knock-outs

Subplate neurons undergo pronounced cell death postnatally (Al-Ghoul and Miller, 1989; Bayer and Altman, 1990; Woo et al., 1991; Wood et al., 1992; Allendoerfer and Shatz, 1994; Price et al., 1997). This period of cell death coincides with decreased expression of p75NTR in neocortex (Allendoerfer et al., 1990). p75NTR signaling can support the survival of subplate neurons purified and maintained in culture (DeFreitas et al., 2001). Taken together, these observations suggest the possibility that loss of p75NTR expression may alter subplate neuron cell death. Early or increased subplate neuron cell death, rather than altered subplate growth cones, is an alternative explanation for the disruption of thalamocortical innervation that we have observed here. To rule out this possibility, subplate neurons in wild-type and p75NTR knock-out mice were labeled at their birth dates with a single pulse of BrdU (Price et al., 1997), and their numbers were quantitated at subsequent ages. After injections at E12.5, heavily BrdU-labeled cells are present at high density at the base of the cortical plate at P2 (Fig. 9*A*,*C*,*D*,*F*) in both wild-type and knock-out mice, indicating that subplate neurons do not undergo early cell death in the knock-out. By P28, only scattered, heavily labeled cells are present (data not shown) in both genotypes. When the groups are considered together, a single pulse of BrdU at E12.5 labels a mean of 79 (SD = 17) cells per section at E18.5. This number decreases to a mean of 28 cells per section (SD = 11) at P28. These observations are similar to previously reported values in other strains of mice (Price et al., 1997). Note that these values do not differ significantly between p75NTR knock-out and wild-type mice (Fig. 10), and in both genotypes a significant number of BrdU-labeled cells persist into adulthood. These results confirm that in the mouse there is a period of programmed cell death within the subplate during the immediate neonatal period (Price et al., 1997). Moreover, these observations demonstrate that the p75NTR

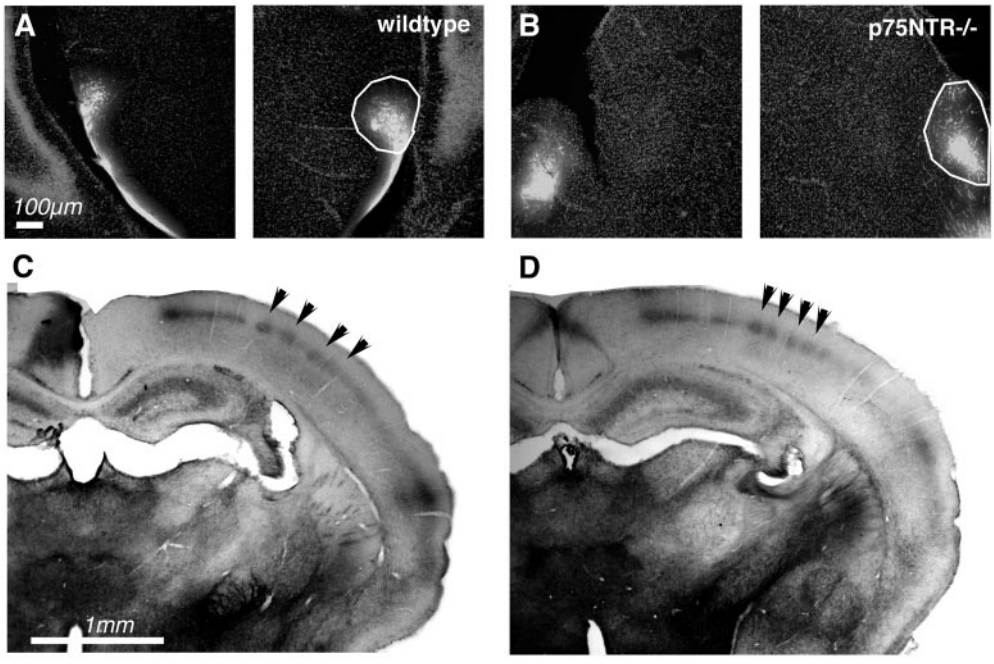

Figure 8. Normal auditory and somatosensory thalamocortical innervation. *A*, *B*, Retrograde labeling of cells in MGN (*outlined* in *white*) after DiI placement in auditory cortex (for location of DiI crystal placement, see Fig. 6*A*,*B*) in wild-type (*A*) and knock-out (*B*) mice at P10. Sections are coronal. *C*, *D*, Serotonin immunohistochemistry in coronal sections at the level of somatosensory cortex of wild-type (*C*) and knock-out (*D*) mice. Patches of staining indicated by *arrowheads* show thalamic axon terminals in the barrel representations of whisker vibrissae.

knock-out mice do not experience an earlier or significantly enhanced degree of subplate neuron death than their wild-type littermates.

It is conceivable that BrdU birth dating is not precise enough to detect small differences in subplate neuron death. In view of this concern, dying cells were also examined directly, by means of *in situ* end labeling of sections adjacent to those that were BrdU labeled, and with double labeling (Fig. 9*B*,*C*,*E*,*F*,*H*,*I*). ISEL-positive cells in the subplate can be detected initially at E18.5, and they reach a peak frequency during the first postnatal week (Fig. 10), when the number of BrdU-labeled cells per section has begun to decrease dramatically. Cells in the subplate, double labeled for ISEL and BrdU, could be found occasionally (Fig. 9*C*,*I*, *asterisk*), but as expected they are quite rare and therefore double-labeled cells were not quantified. The time course and numbers of ISEL-labeled cells in the p75NTR knock-out mice did not differ significantly from wild-type (Fig. 10), again confirming that subplate neuron death is not detectably altered in the p75NTR knock-out mice using this independent method. In retrospect, this conclusion is entirely consistent with the recent observation that subplate neurons *in vivo* are known to express the neurotrophin-selective receptors, TrkB and TrkC, in addition to p75NTR, and TrkC can also support the survival of purified subplate neurons *in vitro* in the absence of p75NTR signaling (DeFreitas et al., 2001; DeFreitas, McQuillen, and Shatz, unpublished observations). Thus, premature subplate neuron cell death does not explain the disruption of visual thalamocortical innervation in p75NTR knock-out mice.

DISCUSSION

Here we have considered the effects of p75NTR expression and signaling on pioneer subplate neurons, and consequently, in the formation of area-specific thalamocortical connections. Our observations point to a crucial early function for p75NTR in regulating morphology of subplate growth cones. Furthermore, our results argue that this early, selective subplate defect in p75NTR knock-out mice leads to impairment of subsequent formation of connections between LGN and visual cortex. We have confirmed that subplate neurons express p75NTR (Allendoerfer et al., 1990) in a gradient (Mackarehtschian et al., 1999) and extended these observations to demonstrate that a gradient of expression is maintained from the time that subplate neurons become postmitotic throughout the period of thalamocortical pathway formation and is not the result of a maturational gradient. Consistent with this p75NTR gradient, thalamocortical projections from LGN to primary visual cortex are reduced in mice lacking p75NTR expression. In 48% of p75NTR knock-out mice, the projection is severely reduced or absent, whereas connections to somatosensory and auditory cortex are normal. These results provide strong evidence in favor of an early requirement for subplate neurons in the formation of connections between thalamus and visual cortex.

Given recent results demonstrating a role for p75NTR in supporting the survival of subplate neurons *in vitro* (DeFreitas et al., 2001), we initially hypothesized that p75NTR regulates the survival of subplate neurons *in vivo*. However, no significant differences in the time course or amount of cell death could be detected between wild-type and mutant littermates, as estimated using two independent methods: BrdU birth dating and ISEL. In retrospect, this conclusion is consistent with the fact that subplate neurons also express mRNA for TrkB and TrkC (DeFreitas et al., 2001). *In vitro*, TrkC can support subplate neuron survival even in the absence of p75NTR signaling (DeFreitas, McQuillen, and Shatz, unpublished observations), and our results here suggest that the same is true *in vivo*. This analysis is important in that premature subplate neuron cell death cannot explain our observations of diminished thalamocortical innervation.

p75NTR regulates subplate growth cone morphology and pathfinding

Remarkably, p75NTR regulates subplate process formation *in vitro* and growth cone morphology *in vivo*. *In vitro*, NT3 binding to p75NTR stimulates neurite outgrowth and filopodial formation in immunopurified subplate neurons, implying a role for this signaling pathway in axon extension and pathfinding. *In vivo*, subplate growth cones derived from visual cortex in p75NTR knock-out mice are smaller and have significantly fewer filopodia

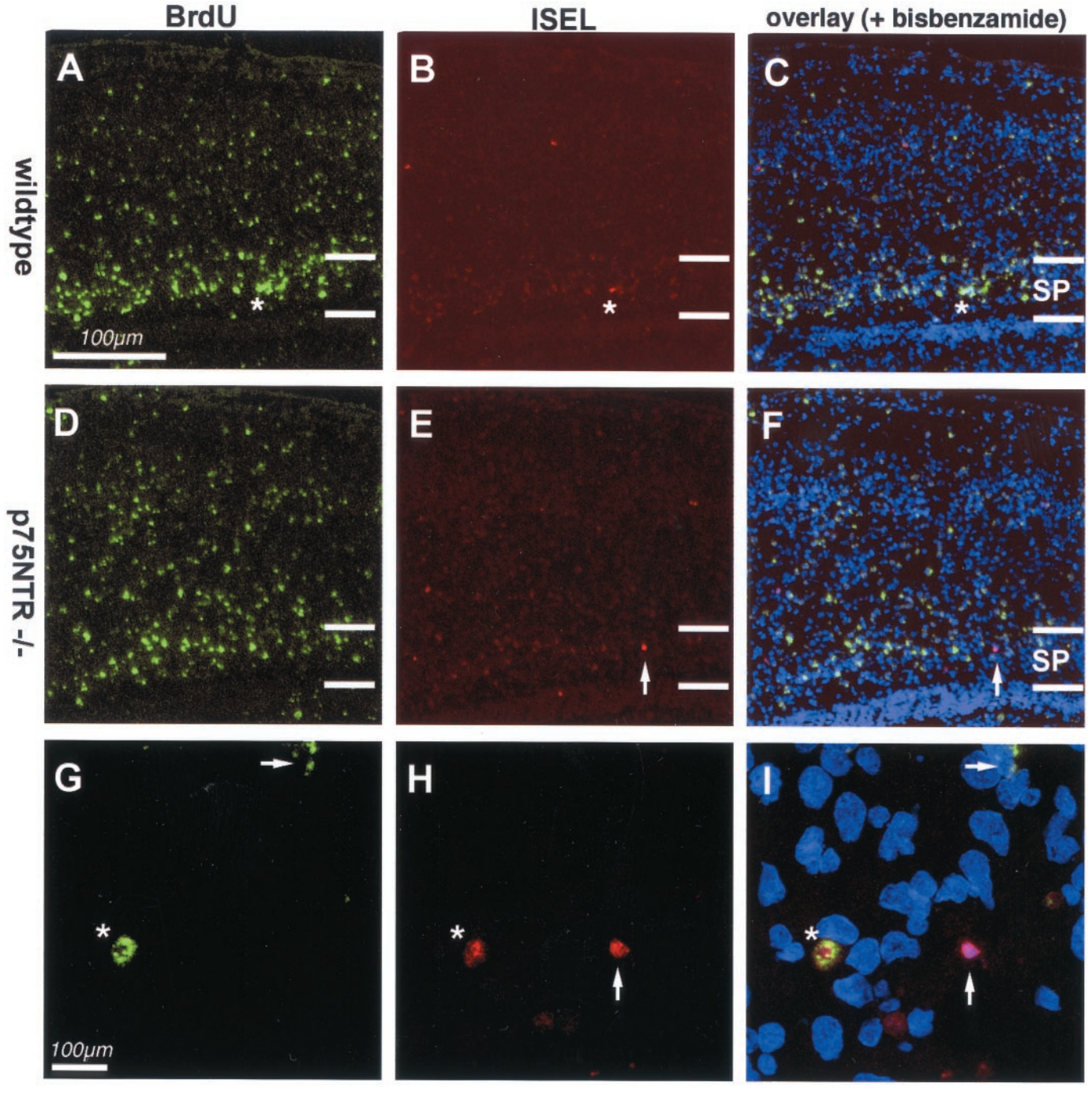

Figure 9. Subplate neuron generation and cell death are similar in p75NTR knock-out and wild-type mice. *A–I*, Coronal sections of P2 wild-type (*A–C*) and p75NTR knock-out mice (*D–I*) were double labeled with BrdU immunohistochemistry (*green*) and ISEL (*red*). *A*, *D*, Low-magnification views of BrdU immunohistochemistry performed in P2 wild-type (*A*) and knock-out mice (*D*) after injection of BrdU at E12.5 to label subplate neurons at their birth. *B*, *E*, ISEL staining of the same sections in wild-type (*B*) as compared with knock-out mice (*E*). Examples of dying cells in subplate (*asterisk* in *B*; *arrow* in *E*) can be seen in both genotypes. *C*, *F*, Overlay of BrdU labeling and ISEL confirms that subplate neurons undergo DNA fragmentation, consistent with programmed cell death (*blue label* is bisbenzamide nuclear counterstain). *G–I*, High-magnification image of a double-labeled cell (*asterisk* in *G–I*) along with single-labeled BrdU-positive (*horizontal arrow* in *G*, *I*) and ISEL-positive (*vertical arrow* in *H*, *I*) cell.

than those of wild-type mice. Despite stimulation of neurite outgrowth *in vitro*, the early subplate projection did not appear stunted or hypoplastic in p75NTR knock-out mice. This differs from observations in the peripheral nerves (Yamashita et al., 1999; Bentley and Lee, 2000), where sensory axon outgrowth is reduced. However, Schwann cell migration is also dependent on p75NTR signaling (Bentley and Lee, 2000); thus the reduced outgrowth of these peripheral nerves may be a secondary effect.

The data presented here suggest a novel role for p75NTR in subplate growth cone morphology and function. During development, subplate axons are the first to navigate the intracortical pathway to the internal capsule (McConnell et al., 1989; De

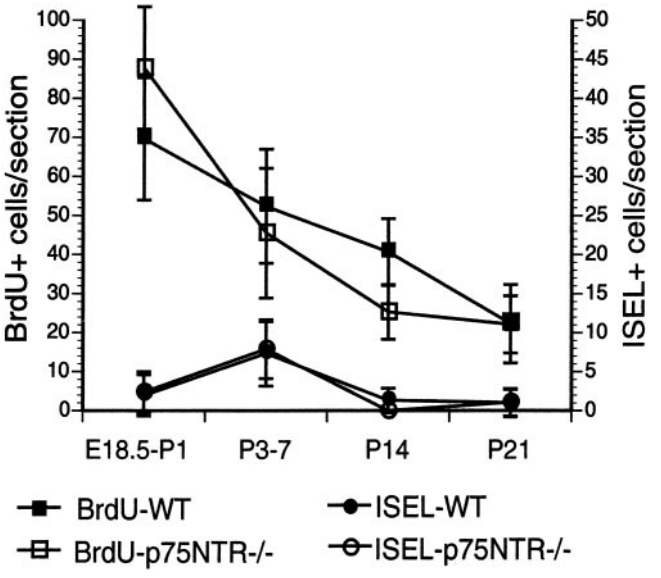

Figure 10. Developmental changes in subplate neuron cell counts and ISEL are similar in p75NTR knock-out and wild-type mice. Subplate neurons labeled at E12.5 with BrdU were visualized at E18.5, P1, P3, P7, P14, and P21 with BrdU immunohistochemistry (see Fig. 9). Dying cells with DNA strand breaks are detected with ISEL in adjacent sections. Mean cell counts from three coronal levels were binned at E18.5/P1 (onset of subplate neuron cell death) and P3/P7 (peak of cell death) time points (see Materials and Methods). Mean cells per section is plotted ±SD. BrdU-immunopositive subplate neuron number and ISEL-positive cell death are not significantly different between knock-out and wild-type mice at any age.

Carlos and O'Leary, 1992). As with pioneer neurons in other systems, early projecting subplate neurons manifest significantly larger and more complex growth cones than later projecting cortical neurons (Kim et al., 1991), implying that they are sampling the environment to make navigation decisions (Raper et al., 1983; Bovolenta and Mason, 1987). The smaller growth cones with fewer filopodia observed in p75NTR knock-out mice might be expected to decrease the fidelity of axon pathfinding and result in errors. Indeed this was observed in the p75NTR knock-out mice, with a fraction of subplate axons projecting ectopically into rostral neocortical regions. However, most subplate axons project normally to the internal capsule. *In vitro* analysis of filopodial formation and neurite outgrowth demonstrates that the entire population of p75NTR-expressing subplate neurons is affected by ligand binding to p75NTR. Immunopurified subplate neurons used for this analysis represent all neocortical cells expressing p75NTR, including subplate neurons rostral to visual cortex. However, in p75NTR knock-out mice, detectable pathfinding errors were observed in only a fraction of the neurons coming from caudal cortex, neurons that express the highest levels of p75NTR in wild-type mice. This partial effect is not surprising, because many examples of axon guidance, including guidance to and away from the ventral midline, can and almost always do involve redundancy (Tessier-Lavigne and Goodman, 1996). These axons may use multiple guidance cues, and loss of any one mechanism often has only a partial effect. It should be noted that because the large majority of subplate axons reach the internal capsule, they are still able to respond to long-range directional cues. Nevertheless, the diminished growth cone complexity in the p75NTR knock-out mice implies that subplate axons may have difficulty recognizing local cues at the internal capsule. This role for p75NTR in regulating growth cone morphology represents a novel function for p75NTR beyond its more familiar role in axon outgrowth (Yamashita et al., 1999; Bentley and Lee, 2000).

Possible mechanism of p75NTR regulation of subplate growth cones

It is well established that p75NTR can influence process formation in neurons. Ligand binding to p75NTR stimulates neurite outgrowth in cultured ciliary and hippocampal neurons (Brann et al., 1999; Yamashita et al., 1999). In contrast, p75NTR activation in cultured sympathetic neurons (Kohn et al., 1999) and adult sensory neurons (Kimpinski et al., 1999) inhibits neurite outgrowth. The mechanism of p75NTR modulation of neurite outgrowth appears to involve the Rho-family GTPase, RhoA (Yamashita et al., 1999). p75NTR expression activates RhoA in a ligand-independent manner, and ligand binding to p75NTR inhibits this activation (Yamashita et al., 1999). RhoA activation in turn decreases neurite outgrowth (Ruchhoeft et al., 1999; Yamashita et al., 1999; Shamah et al., 2001) through regulation of the actin cytoskeleton (Hall, 1998). Our results are consistent with this model because NT3 binding to p75NTR, which would be predicted to inactivate Rho, increases neurite outgrowth in cultured subplate neurons. In addition to these effects on neurite outgrowth *in vitro*, we observed abnormal growth cone morphology and ectopic projections of subplate axons in p75NTR knock-out mice. Similar changes in pathfinding and morphology, rather than axon elongation per se, have been observed after transfection of a dominant-negative isoform of RhoA (dn-RhoA) into *Xenopus* retinal ganglion cells (Ruchhoeft et al., 1999). This study noted "thickened filopodia with a balled appearance" of growth cones expressing dn-RhoA and pathfinding errors of retinal ganglion cells as they projected to tectum (Ruchhoeft et al., 1999). Growth cones that expressed dn-RhoA continued to grow along the surface of the tectum and beyond, rather than turning into their tectal target. This combination of abnormal growth cone morphology, with some axons growing beyond the point at which they should turn, is what we see in p75NTR knock-out mice. Because p75NTR activates Rho in the absence of ligand, these observations suggest the model that p75NTR regulates subplate axon pathfinding through ligand-independent activation of RhoA, rather than via ligand-dependent inactivation.

Consequences of altered subplate axon pathfinding for formation of thalamocortical projections

We show that lack of p75NTR leads to diminished thalamocortical innervation restricted to the LGN. How might lack of p75NTR expression result in such a restricted defect in the geniculocortical projection? Because neither LGN nor any other neurons belonging to the sensory thalamic nuclei of the lateral dorsal thalamus ever express detectable levels of mRNA for p75NTR, the deficit cannot be attributable to a direct effect on LGN neurons themselves. Other neurons that express p75NTR include the reticular thalamus, which makes early, transient projections both throughout cortex and to dorsal thalamus (Adams and Baker, 1995), and the basal forebrain, which sends axonal projections throughout the neocortex (Mechawar et al., 2000). However, given the uniform expression of p75NTR in these neurons and their homogeneous projections, it is extremely unlikely that they could account for the selective loss of projections from LGN but not those of other thalamic nuclei. Rather, it is most likely that the abnormality in growth cone morphology and pathfinding by caudal subplate neurons located underneath form-

ing visual cortex leads to the defect in the geniculocortical projection.

The question of what happens to missing LGN axons in knock-out animals is not definitively answered by our analysis. We know from early anterograde DiI labeling that LGN axons follow the normal trajectory to the internal capsule. We never observed misrouted LGN axons, nor were any LGN neurons retrogradely labeled with dye placed in auditory cortex. Thus we must conclude that LGN fibers project along their normal course, but a reduced number come to innervate visual cortex. Transneuronal transport of ^{3}H-proline at a later age demonstrates that visual cortex is not spatially contracted or ectopically located, again arguing that there are not major pathfinding errors of LGN axons. However, we cannot rule out the possibility that a minority of LGN axons makes a transient, early misprojection.

The low-rostral to high-caudal p75NTR expression gradient suggested the possibility that p75NTR itself functions as a gradient guidance cue for thalamic neurons analogous to ephrins in the tectum (Nakamoto et al., 1996; Tessier-Lavigne and Goodman, 1996). A related possibility is that p75NTR acts to specify cortical cell fate, in which case its absence would be manifested by alterations in the expression of area-specific genes. In both cases, loss of p75NTR expression would alter the spatial distribution of cortical areas. However, the absence of an ectopic geniculocortical projection as well as the normal size and position of visual cortex in knock-out mice discounts these possibilities.

Our data are most consistent with a model in which subplate neurons are required to pioneer the pathway from cortex into the internal capsule, where they must interact with growing thalamic axons, or some intermediate target, for the subsequent successful formation of the thalamocortical projection. Although all subplate neurons examined *in vitro* respond to p75NTR signaling, and all subplate growth cones from caudal cortex in the knock-out manifest striking abnormalities, many subplate axons project into the internal capsule. Subsequently, the entire projection from LGN to visual cortex is diminished as assessed by transneuronal transport. In younger mice, almost half of mice studied had greatly reduced (<5% of LGN area labeled) or absent visual thalamocortical innervation, indicating a profound disruption of visual cortical innervation. This observation suggests that the abnormal thalamocortical projection results from more than simply mistargeted subplate axons. Subplate growth cones in p75NTR knock-out mice must be functionally as well as morphologically defective. Many previous studies have shown that the growth cones of thalamic axons and those of subplate neurons are located adjacent to each other in the internal capsule (McConnell et al., 1989; De Carlos and O'Leary, 1992; Catalano et al., 1996; Molnar et al., 1998a; Auladell et al., 2000). It has been proposed that there are direct interactions between the two sets of growth cones in the internal capsule (Molnár and Blakemore, 1995). The deficit in geniculocortical projection to visual cortex could arise directly from an abnormality within subplate neurons, arguing strongly for a direct role for pioneer subplate neurons in controlling subsequent thalamic axon pathfinding.

A similar conclusion was reached recently in an analysis of Tbr1 knock-out mice, where thalamocortical and corticothalamic connections are completely absent (Hevner et al., 2001). Tbr1, like p75NTR, is expressed in subplate neurons; however, unlike p75NTR, Tbr1 is also expressed in all cortical layers, as well as in many thalamic regions (Hevner et al., 2001). Moreover, the cortical plate is severely diminished in the Tbr1 but not p75NTR knock-outs. Consequently the failure of thalamic axons to leave the diencephalon in the Tbr1 knock-out mice, although consistent with a requirement for subplate axons, could also be caused by loss of pathfinding cues in these Tbr1-expressing cells in the thalamus or by cues provided by the later-growing cortical plate axons. Mice lacking expression of the nuclear orphan receptor COUP-TF1 also fail to develop projections from thalamus to cortex (Zhou et al., 1999). However, the pioneer subplate neuron projection to the internal capsule appeared entirely normal in the COUP-TF1 mice (Zhou et al., 1999). Finally, mice lacking expression of sema6A develop a selective defect in thalamic innervation of caudal neocortex, with normal projection to rostral neocortex (Leighton et al., 2001), much like the selective defect in p75NTR knock-out mice. However in these mice, thalamocortical axons fail to turn up in the internal capsule and never reach subplate axons. Thus these studies do not provide conclusive evidence that subplate neurons are necessary for thalamocortical development.

In p75NTR knock-out mice, most aspects of early cortical development are normal, including corticogenesis, initial thalamic pathfinding, subplate generation, and cell death. Early subplate axon outgrowth appears normal. Thus with the exception of a selective defect in p75NTR-expressing subplate growth cones, the normal environment through which thalamic axons must navigate is preserved. Although the altered growth cone morphology is striking and fully penetrant, only a fraction of labeled subplate axons from caudal cortex appear to be mistargeted. Subsequently, we identify a significant and selective decrease in formation of connections from LGN to visual cortex, whereas innervation of auditory and somatosensory cortex appears normal. The coincidence of pathfinding abnormalities in visual subplate and diminished innervation of visual cortex by LGN axons suggests that normal subplate axons are required for the successful formation of area-specific thalamocortical connections. Our present results are unusual in that they argue that p75NTR functions in a novel way in subplate neurons, that is, in growth cone morphology and function rather than in axon extension or neuronal survival.

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