Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 273 (2004) 373–389

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

A screen for downstream effectors of *Neurogenin2* in the embryonic neocortex

Pierre Mattar[a], Olivier Britz[b], Christine Johannes[a], Marta Nieto[c], Lin Ma[a], Angela Rebeyka[a], Natalia Klenin[a], Franck Polleux[d], François Guillemot[b], Carol Schuurmans[a,*]

[a]*University of Calgary, Calgary, Alberta, Canada T2N 4N1*
[b]*Division of Molecular Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK*
[c]*Beth Israel Deaconess Medical Center, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA*
[d]*Department of Pharmacology, University of North Carolina, Neuroscience Research Building, Chapel Hill, NC 27599-7250, USA*

Received for publication 14 April 2004, revised 18 June 2004, accepted 22 June 2004
Available online 27 July 2004

Abstract

Neurogenin (*Ngn*) *1* and *Ngn2* encode basic-helix-loop-helix transcription factors expressed in the developing neocortex. Like other proneural genes, *Ngns* participate in the specification of neural fates and neuronal identities, but downstream effectors remain poorly defined. We set out to identify *Ngn2* effectors in the cortex using a subtractive hybridization screen and identified several regionally expressed genes that were misregulated in *Ngn2* and *Ngn1;Ngn2* mutants. Included were genes down-regulated in germinal zone progenitors (e.g., *Nlgn1*, *Unc5H4*, and *Dcc*) and in postmitotic neurons in the cortical plate (e.g., *Bhlhb5* and *NFIB*) and subplate (e.g., *Mef2c*, *srGAP3*, and *protocadherin 9*). Further analysis revealed that *Ngn2* mutant subplate neurons were misspecified and that thalamocortical afferents (TCAs) that normally target this layer instead inappropriately projected towards the germinal zone. Strikingly, *EphA5* and *Sema3c*, which encode repulsive guidance cues, were down-regulated in the *Ngn2* and *Ngn1;Ngn2* mutant germinal zones, providing a possible molecular basis for axonal targeting defects. Thus, we identified several new components of the differentiation cascade(s) activated downstream of *Ngn1* and *Ngn2* and provided novel insights into a new developmental process controlled by these proneural genes. Further analysis of the genes isolated in our screen should provide a fertile basis for understanding the molecular mechanisms underlying corticogenesis.

Keywords: neocortex; development; *Neurogenin*; downstream effectors; thalamocortical; subplate; neuronal specification; axonal targeting

Introduction

The neocortex is subdivided into more than 40 tangential areas and six radial layers, each characterized by unique neuronal morphologies, axonal projections, molecular identities, and functions (Job and Tan, 2003). The striking degree of neuronal diversity in the neocortex is generated during development via progressive changes in the cellular output of an initially multipotent pool of cortical precursors, but the molecular mechanisms responsible for changes in precursor cell competence remain poorly understood (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell and Kaznowski, 1991).

Ngn1 and *Ngn2*, which encode basic-helix-loop-helix (bHLH) transcription factors with proneural activity (Fode et al., 1998, 2000; Ma et al., 1998), contribute to the specification of a neuronal versus glial identity in cortical progenitors (Nieto et al., 2001; Sun et al., 2001) and at the same time influence the type of neuron that is generated (Fode et al., 2000; Parras et al., 2002). In particular, *Ngn1* and *Ngn2* are together required to specify several characteristics of early born (i.e., deep layer) cortical neurons,

Abbreviations: CP, cortical plate; dig, digoxygenin; E, embryonic day; gz, germinal zone; IZ, intermediate zone; *Ngn*, Neurogenin; P, postnatal day; pp, preplate; sp, subplate; SVZ, subventricular zone; TCA, thalamocortical afferent; VZ, ventricular zone.

* Corresponding author. 2277 HSC, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1. Fax: +1 403 270 0737.
E-mail address: cschuurm@ucalgary.ca (C. Schuurmans).

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.06.013

including their regional identity, glutamatergic neurotransmission phenotype, and laminar-specific properties (Schuurmans et al., 2004). In addition to their role in activating cortical gene transcription, *Ngn1/2* are also required to repress expression of *Mash1*, a more distantly related bHLH factor, that specifies a GABAergic rather than glutamatergic neurotransmission phenotype when misexpressed in *Ngn2* and *Ngn1;Ngn2* mutant cortical progenitors (Fode et al., 2000; Parras et al., 2002). However, with the exception of *Mash1*, the expression of all other markers analyzed to date appears unaltered in *Ngn2* mutant cortical progenitors. This suggests either that proneural genes can act alone to redirect neuronal differentiation at a relatively late stage in neural lineage progression, or that key participants in this process remain to be identified. Moreover, despite the dramatic conversion in phenotype of cortical neurons in *Ngn2* and *Ngn1;Ngn2* mutants, from glutamatergic to GABAergic, only a handful of genes are known to be down-regulated in these mutant neurons, including a few cortical-specific transcription factors (e.g., *Math2*, *Nscl2*, *Tbr1/2*, and *Id2*), the signaling molecules *Slit1* and *Robo1*, and two vesicular glutamate transporters (i.e., *VGLUT1* and *VGLUT2*; Schuurmans et al., 2004), suggesting that additional components of the *Ngn2*-dependent differentiation cascade remain to be identified.

The changes in cell-fate exhibited following ectopic expression of *Mash1* in *Ngn1/2* expression domains are analogous to those obtained when the *Drosophila* proneural *achaete-scute* genes are ectopically expressed in precursors that normally express *atonal*, and vice versa (Jarman et al., 1993). In these experiments, ectopic expression of *achaete-scute* genes generates external sensory organs where *atonal* would normally generate chordotonal organs. Thus, proneural genes in both *Drosophila* and vertebrates specify neural lineage identities and influence the differentiation of mature cell types in the resultant lineage (Bertrand et al., 2002). Yet even for the well-characterized *Drosophila* proneural genes, extensive surveys for downstream effectors have not been reported. Consequently, we have an excellent understanding of the functional roles of proneural genes but know very little about the molecular mechanisms that mediate these functions.

In order to understand better the genetic program(s) executed by the proneural gene *Ngn2* in the neocortex, we performed a subtractive hybridization screen between wild-type and *Ngn2* mutant telencephalons. We identified 46 genes expressed in the embryonic neocortex, including a group of 19 that displayed highly regionalized patterns of expression. Expression analysis of the regionalized genes revealed that the vast majority (16/19) were misregulated in *Ngn2* single mutant and more strikingly in *Ngn1;Ngn2* double mutant cortices. These genes included transcription factors, but surprisingly also included genes involved in migration and axonal pathfinding. Further expression analyses of three of the identified genes (*Mef2c*, *srGAP3*, and *protocadherin 9*) revealed previously uncharacterized defects in the *Ngn2* mutant cortical subplate, a transient neuronal population with important pioneering roles for guiding afferent and efferent axonal projections in cortical development (McConnell et al., 1989, 1994; Super et al., 1998). Accordingly, thalamocortical afferent (TCA) axons followed aberrant trajectories in the *Ngn2* and *Ngn1;Ngn2* mutant cortices, forming disorganized axon bundles that disrupted the cortical germinal zone. Interestingly, several receptors and ligands that have previously been shown to provide repressive cues for axons at different locations along their migration pathway (e.g., *Sema3c* and *EphA5*) were down-regulated in the *Ngn2* mutant germinal zone, suggesting that the loss of these molecules may contribute to the observed axonal targeting defects (Bagnard et al., 2001; Dufour et al., 2003; Gao et al., 1998; Knoll and Drescher, 2002; Steup et al., 2000; Takahashi et al., 1998).

Materials and methods

Maintenance and genotyping of $Ngn2^{lacZ}$ and Ngn1 mutant mice

$Ngn2^{lacZ}$+/–;*Ngn1*+/– single and double heterozygous intercrosses were set up to obtain heterozygous and homozygous single and double mutant embryos. Embryos were staged using the day of the vaginal plug as E0.5 and genotyping was performed by PCR as described (Fode et al., 2000; Ma et al., 1998).

β-galactosidase detection and cell sorting

E12.5 dorsal telencephalons were dissected from embryos obtained from $Ngn2^{lacZ}$+/– heterozygous intercrosses. Cortical cells were dissociated and stained with fluorescein digalactopyranoside (FDG; Sigma) and sorted by fluorescence-activated cell sorting (FACS) as described previously (Nieto et al., 2001).

RNA extraction, cDNA synthesis, and subtraction

Cells (8000–10,000 lacZ+) were collected from each embryo and sorted directly into 200 μL of 4 M guanidinium isothiocyanate (EM Science). Approximately 1 μg of total RNA was extracted from the dorsal telencephalon of each embryo using a scaled-down Chomczynski method (Chomczynski and Sacchi, 1987). RNA was reversed transcribed using the SMART system (Clontech) and two rounds of subtraction for wild-type minus mutant and mutant minus wild-type were carried out with the PCR-Select cDNA subtraction kit (Clontech). Resultant cDNA were cloned into pBluescript and rescreened by gridding colonies on plates. Two colony lifts were made per plate, and paired lifts were hybridized with wild-type or *Ngn2* mutant cDNA. Only clones with a significant difference in signal intensity from the two probes were pursued. The

efficiency of the subtraction was assessed by Southern blot analysis. Briefly, equal amounts of plasmid DNA were digested to release *Pax6*, *Math2*, *Tbr1*, *Nscl2*, and *Dlx1* inserts that were separated on a 0.8% gel, transferred to a nylon membrane, and hybridized with random-primed, radiolabeled probes from unsubtracted *Ngn2*^{lacZ}+/− cDNA, WT-mutant subtracted cDNA, and mutant-WT subtracted cDNA.

Identification and isolation of full-length cDNAs

We sequenced 200 clones that came through our second round of subtractive screening of the wild-type minus *Ngn2* mutant telencephalon library. Sequenced clones were identified using the BLAST algorithm (NCBI) to screen nucleotide and EST databanks. Through our sequencing, we identified 132 unique sequences, of which 46 were analyzed further. GenBank accession numbers for cDNAs matching isolated clones are presented in Supplementary Table 1.

RNA in situ hybridization

RNA in situ hybridization was carried out on 10 μm cryostat sections as previously described (Cau et al., 1997). Digoxygenin (dig)-labeled RNA probes were generated using T3, T7, or SP6 RNA polymerases and a dig-RNA-labeling mix (Roche). In addition to antisense probes, we generated 44 sense probes, and in no case did we observe specific staining in the telencephalon (data not shown). Templates for dig-probes were as follows (accession numbers listed in parentheses): *Akt3* (AF124142), *Bhlhb5* (AF504925), *Dcc* (X85788), *EphA5* (NM_007937), *Mef2c* (L13171), *Neurotractin* (AJ487032), *Sema3c* (X85992). We also obtained full-length I.M.A.G.E. Consortium [LLNL] cDNA clones (Lennon et al., 1996) for selected genes from Open Biosystems (Huntsville, AL) as follows (GenBank accession numbers in parentheses): *Axotrophin* (BC025029), IMAGE:4935124; *Cml66* (BC031583), IMAGE:4952090; *CugBP2* (BC026856), IMAGE:4503295; *EDG-1 (BC049094), IMAGE:6415920; ELAVl4 (BC048159), IMAGE:5703005; Myotrophin (BC043084), IMAGE:6417343; Neuroligin1 (BC005523), IMAGE:3494913; Pegasus-like (BC048183), IMAGE:6826723; PHD6-like (BC043127), IMAGE:6410361; Ptbp2 (BC010255), IMAGE:3709255; srGAP3 (BC030457), IMAGE:5401611; and WWP2 (BC048184), IMAGE:6827381.*

Immunohistochemistry, histology, and birthdating

For histology, P0 brains were fixed in Bouin's fixative for 3 days, dehydrated in an ethanol–xylene series, embedded in paraffin, and 7 μm sections were cut and stained with hematoxylin–eosin as described previously (Rhinn et al., 1998). For birthdating, pregnant females were injected intraperitoneally with 100 μg/g bromodeoxyuridine (BrdU; Sigma) on E11.5, sacrificed at P0, processed for wax sectioning as above, and immunostained with anti-BrdU (Boehringer Mannheim) as described (Gradwohl et al., 1996). Antisomatostatin (Dako), MAP2 (Sigma), calretinin (Swant), TUJ1 (Neuronal class III β-tubulin; Covance), and L1 (Roche) immunostaining were performed on 10 μm cryostat sections as described (Nieto et al., 2001). Anterograde tracing using DiI (Molecular Probes) was performed as described (Seibt et al., 2003).

Results

Construction of a subtracted cDNA library enriched for neocortical genes dependent on Ngn2 function

Early born preplate and deep-layer cortical plate neurons are misspecified in *Ngn2* mutants, acquiring a GABAergic rather than glutamatergic identity (Fode et al., 2000; Schuurmans et al., 2004). To identify downstream components of the *Ngn1/2*-dependent differentiation cascade(s) that may underlie this change in neuronal phenotype, we devised a subtractive screen between the dorsal telencephalon of wild-type and *Ngn2* mutant embryos. For this purpose, cortical cells were isolated from individual E12.5 preplate-stage embryos that were heterozygous (i.e., "wild type") or homozygous (i.e., "mutant") for an *Ngn2*^{lacZ} replacement allele (Fode et al., 2000), taking advantage of β-galactosidase (βgal) to label expressing cells with FDG, a vital fluorescent substrate (Fig. 1B). At preplate stages, *Ngn2* transcripts were detected in cortical progenitors in the ventricular zone (Fig. 1A), whereas βgal was detected in both progenitor cells and postmitotic neurons of *Ngn2*^{lacZ} embyros, with staining significantly more intense in *Ngn2*^{lacZ} homozygous versus heterozygous cortices due to an additional copy of the gene (Fig. 1B). While *Ngn2* was not expressed in postmitotic neurons (Fig. 1A), βgal protein expressed from the *Ngn2* locus persisted in differentiated neurons due to its slow turnover (Fig. 1B). Thus, the sorting procedure employed isolated both cells that normally express *Ngn2*, as well as the neurons generated from these progenitors.

For each embryo, βgal (+) cortical cells were separated from βgal (−) cells by flow cytometry (FACS), yielding 8000–10,000 cells. RNA was extracted from βgal (+) cells and cDNA was synthesized and amplified by PCR. Two rounds of subtraction in both directions were then performed between cDNA derived from βgal (+) cells isolated from *Ngn2*^{lacZ}+/− and *Ngn2*^{lacZ}−/− cortices, producing pools of genes enriched either in the wild-type or *Ngn2* mutant telencephalon.

To assess the efficiency of subtraction, pools of subtracted cDNA were radiolabeled by random priming and hybridized to Southern blots on which equivalent quantities of known genes had been transferred (Fig. 1C). The wild-type minus *Ngn2* mutant subtraction yielded an

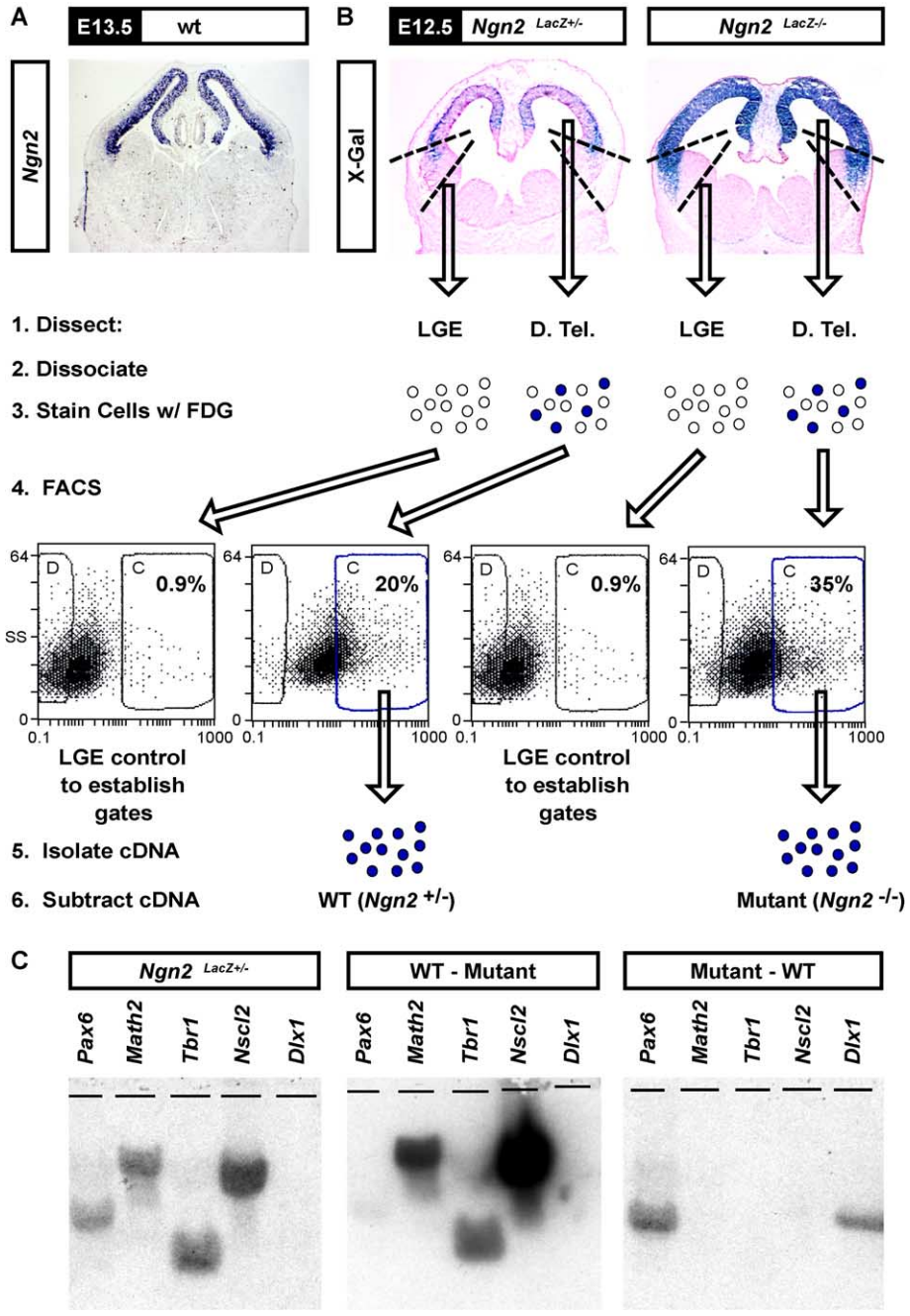


Fig. 1. Experimental design for subtracting expressed genes in $Ngn2^{lacZ}+/-$ ("wild-type") and $Ngn2^{lacZ}-/-$ ("mutant") cortical cells. (A) Frontal section of E13.5 telencephalon hybridized with a probe for *Ngn2*. (B) X-gal staining of frontal sections through the telencephalon of E12.5 $Ngn2^{lacZ}+/-$ and $Ngn2^{lacZ}-/-$ embryos revealed βgal activity in the cortical preplate and cortical progenitor cells, which were significantly higher in mutants due to two copies of the *lacZ* gene. βgal+ cells were FACS sorted, using the lateral ganglionic eminence (LGE) as negative tissue to set gates. mRNA extracted from βgal+ cells was reverse transcribed and PCR amplified and used in subtractions between WT-mutant and mutant-WT. (C) For quality control, unsubtracted wild-type and subtracted cDNA pools were radiolabeled and used to probe Southern blots on which equivalent quantities of known plasmids had been blotted. In the wild-type minus mutant subtraction, *Math2*, *Tbr1*, and *Nscl2* transcripts were enriched, and in mutant minus wild-type subtraction, *Pax6* was enriched and *Dlx1* was ectopically expressed. D. Tel, dorsal telencephalon; FACS, fluorescence-activated cell sorting; FDG, fluorescein digalactopyranoside; LGE, lateral ganglionic eminence; and WT, wild type.

expected increase in hybridization signal to *Math2*, *Tbr1*, and *Nscl2*, markers of a dorsal, cortical identity that had previously been shown to be down-regulated in *Ngn2* mutants (Fode et al., 2000). In contrast, in the *Ngn2* mutant minus wild-type subtraction, *Math2*, *Tbr1*, and *Nscl2* transcripts were depleted from the library, whereas ectopic *Dlx1* transcripts were detected and *Pax6* message was enriched (Fig. 1C). Both *Pax6* and *Dlx1* are ectopically expressed in misspecified cortical neurons in *Ngn2* mutants (Fode et al., 2000; C.S., F.G., unpublished observation],

confirming that the subtraction was successful and did enrich for the expected genes.

Sequence and expression analyses of telencephalic library clones

We hypothesized that the wild-type minus *Ngn2* mutant subtraction would yield genes important for the specification of a cortical, glutamatergic identity and thus focused on analyzing these clones. Subtracted cDNAs were cloned to generate a library; and as a further enrichment, the library was rescreened by hybridizing individual colonies with wild-type and *Ngn2* mutant cDNA probes, with only those clones that differentially hybridized pursued further (data not shown). To increase our chances of identifying novel regulators of cortical development, we used a random sequence approach, in the end sequencing a total of 132 clones (data not shown).

To identify cDNA sequences, BLAST searches were performed. The majority (76%) of the genes identified were represented only once in the library, whereas 24% appeared more than once. Fifty-eight percent of the unique genes had been previously characterized in mouse, 30% were putative orthologues of known genes from other species, and 12% matched ESTs whose encoded products were unknown. Four clones mapped to genomic regions with no matching ESTs and were not analyzed further. The identified genes were assigned into groups according to their reported biological roles and/or the presence of characteristic functional domains (Supplementary Table 1). We used our sequencing data to select 46 of the independent genes identified for further study, focusing primarily on transcription factors, receptor/signal transduction proteins, and a few miscellaneous and unknown genes (Supplementary Table 1).

Expression patterns of the 46 genes presented in Supplementary Table 1 were examined by RNA in situ hybridization on sections of E13.5 telencephalon, and all were shown to be expressed in the dorsal telencephalon, their patterns overlapping at least in part with X-gal stained $Ngn2^{lacZ}$ cortices (data not shown; Figs. 2 and 3). We chose E13.5 as the stage of analysis to survey gene expression in several cortical compartments at the same time. This was possible due to the lateral-to-medial gradient of neurogenesis, which results in a developmentally more advanced cortex in lateral versus medial regions. Specifically, in the medial aspect of E13.5 frontal sections, the cortex consists of a ventricular zone (VZ) and a preplate layer of postmitotic neurons, which are the first cortical neurons to differentiate (Figs. 2A and D; insets). In contrast, in more developmentally advanced lateral regions, progenitors are located in a VZ and subventricular zone (SVZ), and a cortical plate composed of a second wave of differentiating neurons has started to form.

Analysis of our subtracted clones revealed several expression patterns, including genes that were expressed in progenitors throughout the neural tube [i.e., pan-progenitor; e.g., G-protein coupled receptor *EDG1*, transcription factor *Pegasus-like* (data not shown)]. In contrast, other genes displayed pan-neuronal expression profiles, with transcripts detected in postmitotic neurons right through the embryonic neural tube [e.g., *doublecortin* (*Dcx*), a microtubule-associated protein (LoTurco, 2004), and *Zfp10-like*, a putative transcription factor (Figs. 2A, A′ , D, and D′)]. In the cortex, pan-neuronal genes were expressed at high level in postmitotic neurons in the preplate medially and in the cortical plate laterally, as well as displaying weaker expression in the SVZ and intermediate zone (Figs. 2A, A′ , D, and D′). Finally, several genes displayed complex regionalized patterns, including those predominantly (e.g., nuclear factor *Dach1* and *Fut9*, which synthesizes the LewisX epitope; (Nishihara et al., 2003); data not shown) or more abundantly (e.g., transcription factors *Id4* and *Zac1*; data not shown) expressed in dorsal versus ventral telencephalic progenitors. Regionalized neuronal markers were also identified, including those that were expressed at higher levels in dorsal versus ventral telencephalic neurons (e.g., transcription factor *NFIB*; data not shown). Finally, several genes had complex, regionalized patterns and were expressed in subpopulations of dorsal and ventral telencephalic progenitors and neurons, including the netrin receptor *Unc5H4* (Fig. 3M; Engelkamp, 2002); *neuroligin-1* (*Nlgn1*), which plays an important role in synaptogenesis (Fig. 3A; Scheiffele et al., 2000), and the guidance molecule *Sema3c* (Fig. 3V; Bagnard et al., 2000).

Expression analysis of pan-neuronal markers in Ngn2 and Ngn1;Ngn2 mutants

We next set out to determine whether the genes we identified were misregulated in the *Ngn2* single mutant cortex, as anticipated from the design of our screen. We first focused our analysis on the pan-neuronal genes, predicting that these genes would be down-regulated in dorsal and not ventral telencephalic neurons in *Ngn2* mutants. Indeed, transcript levels for both *Dcx* and *Zfp10-like* were reduced, albeit not completely lost, in the medial preplate of E13.5 *Ngn2* single mutants (Figs. 2B, B′ , E, E′), a region where *Ngn1* is not expressed and cannot compensate for the loss of *Ngn2* (Fode et al., 2000). In addition, expression of *Dcx* and *Zfp10-like* was greatly reduced in the dorsomedial SVZ of *Ngn2* mutants (Figs. 2B, B′ , E, and E′). To confirm that the reduced expression of *Dcx* and *Zfp10-like* reflected a true decrease in neurogenesis in the preplate of *Ngn2* mutants, we examined expression of neuronal β-tubulin (TUJ1), a bona fide pan-neuronal marker. In dorsomedial sections, TUJ1 expression was reduced in the *Ngn2* mutant preplate (Fig. 2H) as compared to wild-type (Fig. 2G), confirming that overall numbers of postmitotic neurons were reduced as a consequence of the loss of *Ngn2* expression.

We also examined *Zfp10-like* and *Dcx* expression in E13.5 *Ngn1;Ngn2* double mutants. In striking contrast to the reduced expression of *Zfp10-like* and *Dcx* in *Ngn2* single

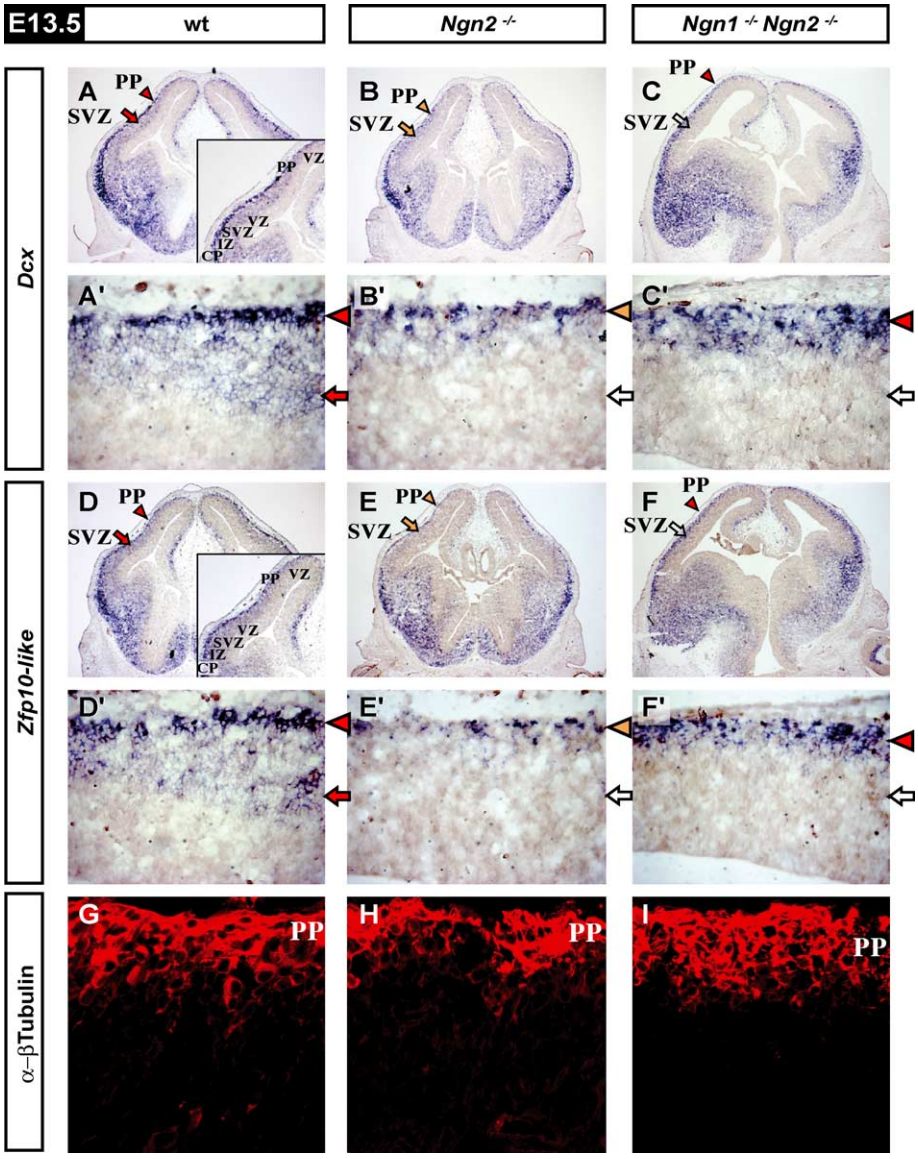
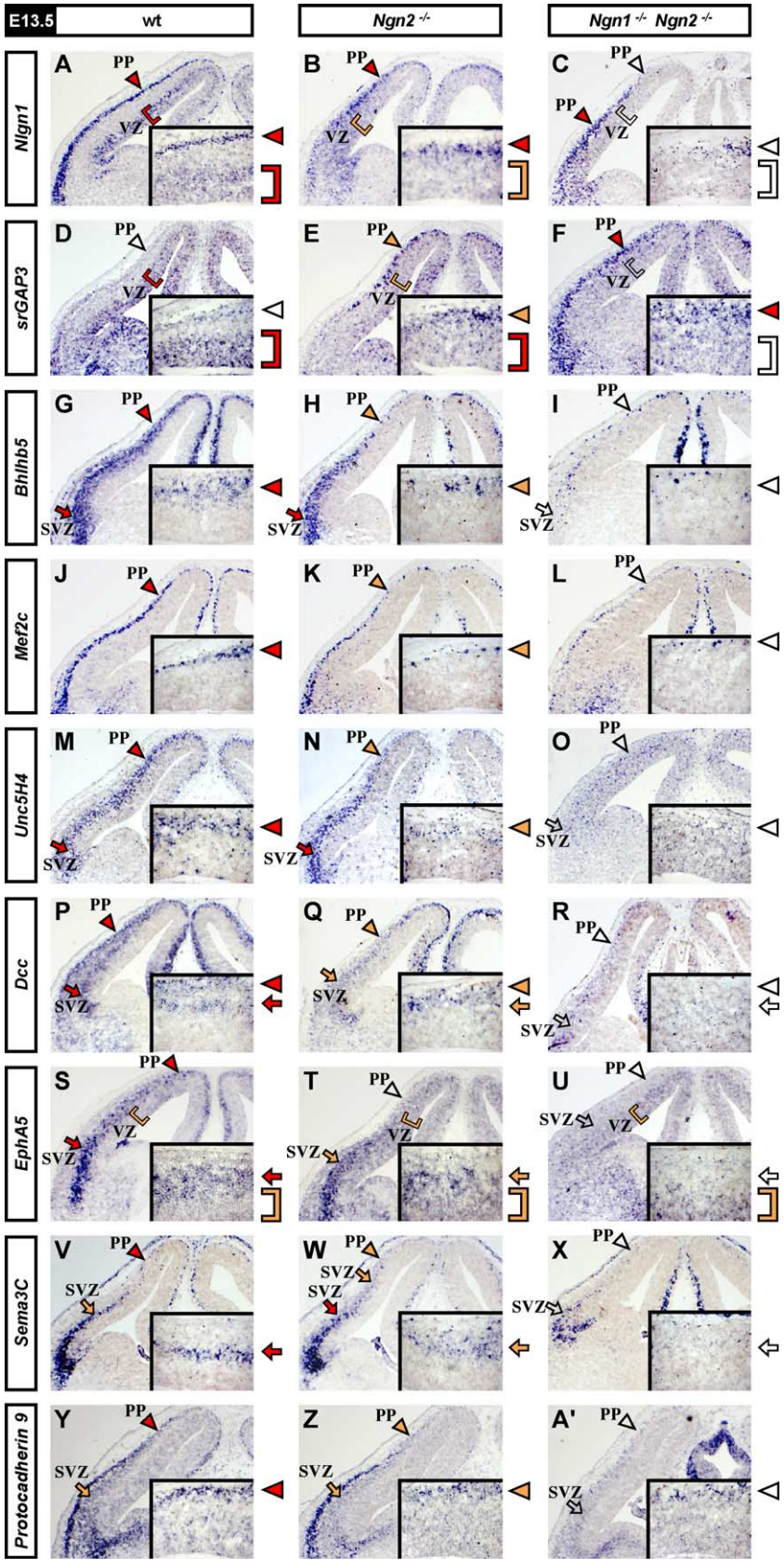


Fig. 2. Expression analysis of pan-neuronal markers in E13.5 *Ngn2* and *Ngn1;Ngn2* mutant cortices. Frontal sections of E13.5 wild-type (wt) telencephalon were hybridized in situ with dig-labeled RNA probes. Insets in A′ –C′ and D′ –F′ show higher magnifications of the dorsomedial neocortex. (A–C and A′ –C′) *Dcx* expression in wild-type (A and A′), *Ngn2* mutant (B and B′), and *Ngn1;Ngn2* mutant (C and C′) telencephalon. (D–F and D′ –F′) *Zfp10-like* expression in expression in wild-type (D and D′), *Ngn2* mutant (E and E′), and *Ngn1;Ngn2* mutant (F and F′) telencephalon. (G–I) TUJ1 expression in the preplate of E12.5 wild-type (G), *Ngn2* mutant (H), and *Ngn1;Ngn2* mutant (I) cortices. For *Dcx*, *Zfp10-like*, and TUJ1, expression was down-regulated in the PP (arrowheads) and dorsomedial SVZ (arrows) of *Ngn2* mutants. In *Ngn1;Ngn2* double mutants, expression was down-regulated in the medial and lateral SVZ (arrows) but up-regulated in the PP (arrowheads). Arrows and arrowheads are color-coded as follows: red, high expression; orange, medium expression; clear, low expression; all in relative, qualitative terms. VZ, ventricular zone; PP, preplate; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; and wt, wild type.

mutants, the pan-neuronal genes were up-regulated in the preplate of *Ngn1;Ngn2* double mutants (Figs. 2C, C′ , F, and F′). This result was not completely unexpected given that we had previously reported an up-regulation of the pan-neuronal marker *SCG10* in the preplate of E12.5 *Ngn1;Ngn2* mutants that was suggestive of an early, ectopic burst of neurogenesis in this mutant background (Fode et al., 2000). Indeed, our analysis of TUJ1 expression confirmed the expansion of the

Fig. 3. Expression analysis of regionalized clones in E13.5 *Ngn2* and *Ngn1;Ngn2* mutant cortices. Frontal sections of E13.5 wild-type (A, D, G, J, M, P, S, V, and Y), *Ngn2* mutant (B, E, H, K, N, Q, T, W, and Z), and *Ngn1;Ngn2* mutant (C, F, I, L, O, R, U, X, and A′) telencephalon hybridized in situ with dig-labeled RNA probes for regionalized genes isolated in our subtractive screen (A–C) *Neuroligin 1*; (D–F) *srGAP3*; (G–I) *Bhlhb5*; (J–L) *Mef2c*; (M–O) *Unc5H4*; (P–R) *Dcc*; (S–U) *EphA5*; (V–X) *Sema3c*; and (Y–A′) *protocadherin 9*. Insets show higher magnification of medial neocortex. Arrowheads indicate preplate; arrows indicate SVZ; brackets indicate VZ. Differences in gene expression are highlighted by changes in the color of the arrows, arrowheads, and brackets as follows: red, high expression; orange, medium expression; clear, strongly reduced expression; all in relative, qualitative terms. VZ, ventricular zone; SVZ, subventricular zone; PP, preplate; and wt, wild-type.


E13.5
wt
Ngn2 -/-
Ngn1 -/- Ngn2 -/-
Nlgn1
srGAP3
Bhlhb5
Mef2c
Unc5H4
Dcc
EphA5
Sema3C
Protocadherin 9
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
A'
PP
VZ
SVZ

neuronal layer in E13.5 double mutants (Fig. 2I). Finally, it should be noted that in contrast to the expression of *Zfp10-like* and *Dcx* in the preplate, these genes were both down-regulated in the SVZ of *Ngn1;Ngn2* double mutants, as observed in *Ngn2* single mutants, suggesting a different mechanism of gene regulation in these two territories (Figs. 2C, C′, F, and F′).

Expression analysis of regionalized markers in Ngn2 and Ngn1;Ngn2 mutants

Early born preplate and deep-layer cortical plate neurons are misspecified in *Ngn2* mutants, acquiring a GABAergic rather than a glutamatergic identity (Fode et al., 2000; Schuurmans et al., 2004). One of the goals of our subtractive screen was to identify new components of the specification and/or differentiation programs underlying these cell fate decisions. We speculated that the genes that regulate cell-type-specific processes in some neural populations and not others would be expressed in a regionalized manner in the neural tube, prompting us to focus on the regionalized genes isolated in our screen for the rest of the study.

Table 1
Alterations in expression patterns of regionalized genes isolated from subtractive screen in *Ngn2* and *Ngn1;Ngn2* mutants

Gene name(s)	Category	Expression (*Ngn2* and *Ngn1;Ngn2* mutants versus wild type)
Zac1 (Plagl1/LOT1)	Transcription factor	↓ VZ, PP neurons
Id4 (IDB4)	Transcription factor	No change
Dach1	Transcription factor	No change
Nuclear Factor IB	Transcription factor	↓ PP neurons
Bhlhb5 (Beta3)	Transcription factor	↓ SVZ, PP neurons
Mef2c	Transcription factor	↓ PP neurons
Dcc	Receptor or signal transduction	↓ SVZ, PP neurons
Unc5H4	Receptor or signal transduction	↓ SVZ, PP neurons
Sema3c (SemE)	Receptor or signal transduction	↓ SVZ, PP neurons
EphA5 (Bsk)	Receptor or signal transduction	↓ SVZ, PP neurons
Neurotractin (KILON)	Receptor or signal transduction	↑ PP neurons
srGAP3 (Fnbp2)	Receptor or signal transduction	↓ VZ, ↑ PP neurons
AKT3 (PKB gamma)	Receptor or signal transduction	↓ PP neurons
Ptbp2	Splicing or RNA binding	↓ VZ
ELAV-like 4 (HuD)	Splicing or RNA binding	↓ VZ, SVZ
Neuroligin 1 (Nlgn1)	Neurotransmission	↓ VZ, PP neurons
fucosyltransferase 9	Adhesion related	No change
protocadherin 9	Adhesion related	↓ PP neurons
NAP-22 (Basp1)	Miscellaneous	↓ PP neurons

Summary of genes that displayed regionalized patterns of expression in the E13.5 neural tube. Description of changes in the expression patterns of these regionalized genes in *Ngn2* and *Ngn1;Ngn2* mutants at E13.5 is noted. VZ, ventricular zone; SVZ, subventricular zone; and PP, preplate.

We compared the expression profiles of our regionalized genes in E13.5 wild-type, *Ngn2* single and *Ngn1*;*Ngn2* double mutant cortices. As summarized in Table 1, 16/19 regionalized genes tested were clearly misregulated in *Ngn2* and *Ngn1;Ngn2* mutants by RNA in situ hybridization. As previously demonstrated, gene expression defects in *Ngn2* mutants were restricted to the dorsomedial cortex, where *Ngn1* is not expressed and cannot compensate for the loss of *Ngn2* (Fode et al., 2000), whereas defects extended throughout the cortex in *Ngn1;Ngn2* double mutants (Fig. 3). Before this study, extensive analyses revealed no defects in neuronal specification in *Ngn1* single mutants and they were therefore not examined (Fode et al., 2000; Schuurmans et al., 2004).

Of the regionalized genes expressed in cortical progenitors, *Nlgn1*, which is a postsynaptically localized protein in neurons (Scheiffele et al., 2000), and *srGAP3*, an intracellular mediator of *slit*/*robo* signaling (Wong et al., 2001), were clearly down-regulated in cortical progenitors in the VZ of *Ngn2* and *Ngn1;Ngn2* mutants (Figs. 3A–F). To date, *srGAP3* and *Nlgn1* are the only two genes known to be down-regulated in *Ngn2* and *Ngn1;Ngn2* mutant cortical VZ progenitors, raising the possibility that they may contribute to the misspecification of progenitor cells. Finally, as further evidence of misregulated expression, *srGAP3*, which is also normally expressed in ventral telencephalic neurons, was ectopically expressed in preplate neurons in *Ngn2* and *Ngn1;Ngn2* mutants (Figs. 3E and F), as are several other genes normally expressed in the ventral telencephalon at this stage, including *Dlx1* and *GAD1* (Fode et al., 2000).

The largest group of misregulated genes included those whose expression was disrupted in cortical preplate neurons. Included in this category were *Bhlhb5* (Figs. 3G–I), which encodes a bHLH transcription factor; *Mef2c* (Figs. 3J–L), which encodes a MADS-box transcription factor; and *NFIB* (data not shown), a member of the Nuclear Factor I family of transcription factors. In addition, transcript levels of the signal transduction molecules *Unc5H4* (Figs. 3M–O), *Dcc* (Figs. 3P–R), *EphA5* (Figs. 3S–U), and *Sema3c* (Figs. 3V–X) and for miscellaneous molecules such as *protocadherin 9* (Figs. 3Y–A′) and *NAP–22* (data not shown; Table 1) were clearly down-regulated in the preplate of *Ngn2* and *Ngn1;Ngn2* mutants. Given that these genes were all down-regulated in preplate neurons in *Ngn2* single and *Ngn1;Ngn2* double mutants, even though neuronal numbers were increased in the double mutant preplate, suggested to us that their down-regulation was likely linked to neuronal specification defects observed in both mutant genotypes, and not the neurogenesis deficits that were only apparent in *Ngn2* single mutants at E13.5 (see above).

Finally, we observed that several of our misregulated genes were aberrantly expressed in the SVZ. Genes disrupted in the *Ngn1;Ngn2* mutant SVZ included *Bhlhb5* (Figs. 3G–I), *Unc5H4* (Figs. 3M–O), *Dcc* (Figs. 3P–R), *EphA5* (Figs. 3S–U), and *Sema3c* (Figs. 3V–X). Defects in the SVZ were most evident in *Ngn1;Ngn2* double mutants as the SVZ had only developed in the very lateral cortex by E13.5, and lateral

domains were not severely affected in *Ngn2* mutants due to compensation by *Ngn1* (Fode et al., 2000). The disruption of gene expression in the SVZ may reflect a change in gene expression in SVZ progenitors, or in differentiating neurons that are migrating through the SVZ into the cortical plate.

Defects in the differentiation of Ngn2 mutant cortical plate and subplate neurons

To investigate further *Ngn1/2*-dependent defects in regionalized gene expression in postmitotic neurons, we analyzed *Ngn2* single mutants at E15.5, a stage when deep-layer cortical plate neurons, which display aberrant molecular identities in *Ngn2* and *Ngn1;Ngn2* mutants, have differentiated (Schuurmans et al., 2004). At E15.5, *Ngn2* continued to be expressed in cortical progenitor cells in the germinal zone, and not in postmitotic neurons (Figs. 4A and B). In our analysis, we noted that *Bhlhb5* (Figs. 4C and D), *srGAP3* (Figs. 4E and F), *Mef2c* (Figs. 4G and H), and *Nlgn1* (Figs. 4I and J), which were all expressed in neurons in the wild-type cortical plate, displayed a disorganized and characteristic patchy pattern of expression in the lower cortical plate/intermediate zone in *Ngn2* mutants. Notably, the patchy expression pattern of these genes was reminiscent of that previously observed for *Math2*, *Id2*, *GluR2*, *Robo1*, and *Slit1* in *Ngn2* mutants (Schuurmans et al., 2004), and in this previous study it was shown that cortical neurons not expressing these genes instead acquired an aberrant GABAergic phenotype (Schuurmans et al., 2004). We therefore suggest that the loss of *Bhlhb5*, *srGAP3*, *Mef2c*, and *Nlgn1* suggests that these genes are components of the normal cortical differentiation program, a process that is deregulated as a consequence of the loss of *Ngn1/2* function.

As differentiation proceeds, cortical plate neurons migrate into the middle of the preplate, splitting this layer into an overlying marginal zone and underlying subplate layer. At E15.5, we noted that *Mef2c* was expressed in the subplate of wild-type embryos and that this expression was absent in *Ngn2* mutants (Figs. 4G, G′, H, and H′). *Mef2c* has been reported to be expressed in mature cortical neurons, including subplate neurons, where it acts as a differentiation or survival factor (Leifer et al., 1997; Lyons et al., 1995; Mao et al., 1999), and the loss of *Mef2c* expression in the *Ngn2* mutant subplate suggested that these neurons were either missing or misspecified. To distinguish between these possibilities, we further characterized subplate defects at P0, when this layer is more clearly discernable. At P0, *Ngn2* continued to be expressed in germinal zone progenitors but was not detected in postmitotic neuronal populations (Figs. 5A and B). Of our subtracted clones, *Mef2c* (Figs. 5C and D), *protocadherin 9* (Figs. 5E and F), and *srGAP3* (Figs. 5G and H) all clearly demarcated the subplate in P0 wild-type cortices, but not in *Ngn2* mutants. To determine whether subplate neurons were present but misspecified in *Ngn2* mutants, we labeled P0 cortices with the neuronal marker *SCG10* (Figs. 5I and J),

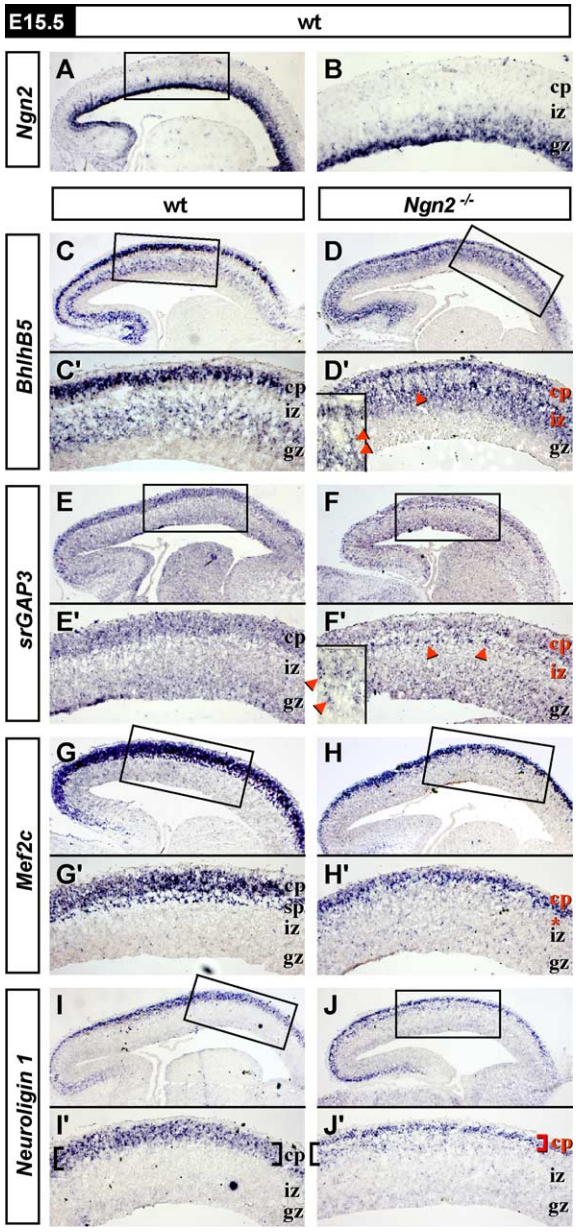
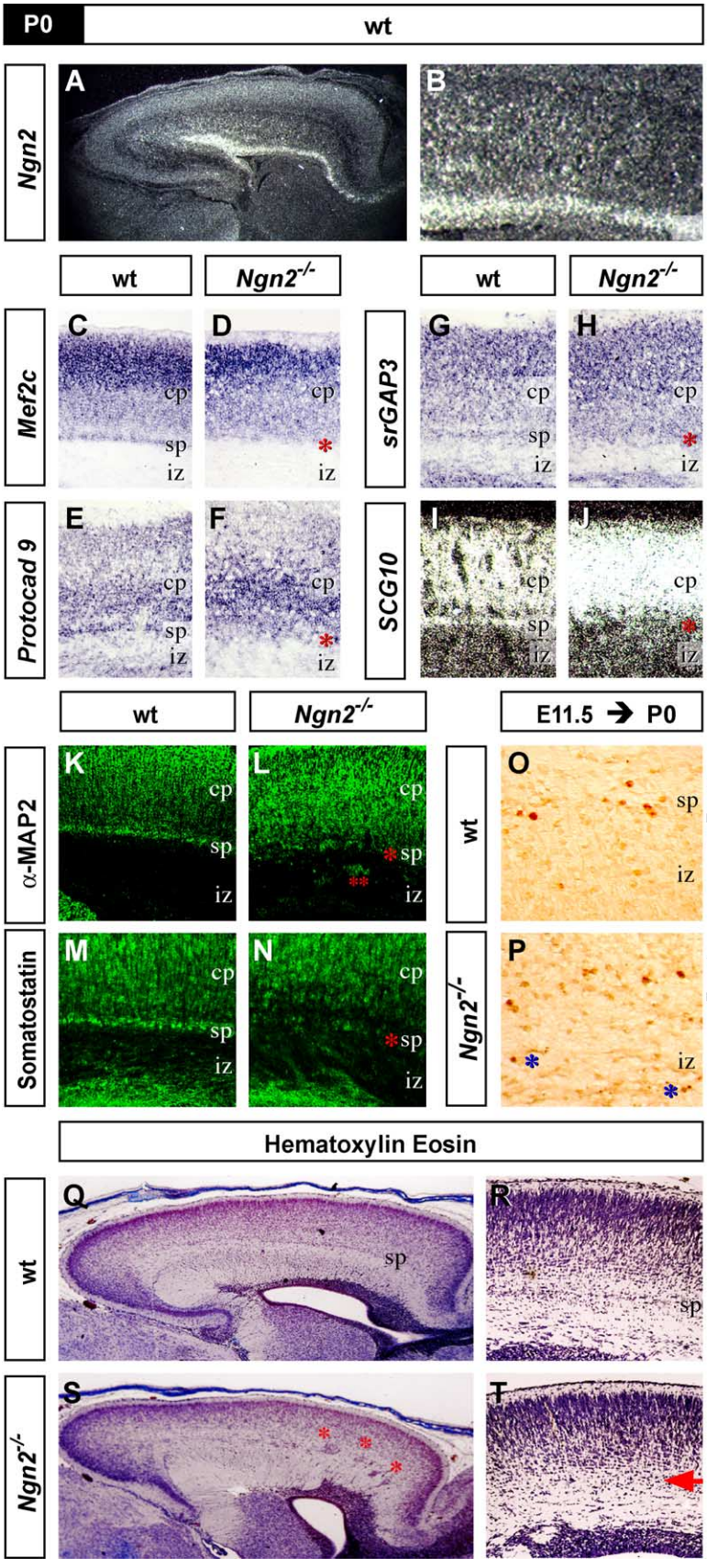


Fig. 4. Defects in gene expression in subplate and early born cortical plate neurons in E15.5 *Ngn2* mutants. Expression analysis of sagittally sectioned E15.5 wild-type and *Ngn2* mutant telencephalon. (A) Expression of *Ngn2* in wild-type telencephalon. (B) Higher magnification of boxed region in (A). (C, C′, D, and D′) *Bhlhb5* expression in wild-type (C and C′) and *Ngn2* mutants (D and D′). Large gaps in CP expression were observed in *Ngn2* mutants, along with ectopic expression in the IZ (D′; arrowheads). (E, E′, F, and F′) *srGAP3* expression in wild-type (E and E′) and *Ngn2* mutants (F and F′). Gaps in expression were observed in CP neurons in *Ngn2* mutants (F′; arrowheads). (G, G′, H, and H′) *Mef2c* expression in wild-type (G and G′) and *Ngn2* mutants (H and H′). Expression was reduced in the CP and lost in the SP (asterisk) in *Ngn2* mutants (H′). (I, I′, J, and J′) *Nlgn1* expression in wild-type (I and I′) and *Ngn2* mutants (J and J′). While caudal expression was similar between wild-type and *Ngn2* mutants (compare left brackets; I′ and J′), rostral expression levels were reduced in the *Ngn2* CP (compare right brackets; I′ and J′). GZ, germinal zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; and wt, wild-type.


P0
wt
Ngn2
A
B
wt
Ngn2-/-
wt
Ngn2-/-
Mef2c
C
D
srGAP3
G
H
cp
sp
iz
Protocad 9
E
F
SCG10
I
J
wt
Ngn2-/-
E11.5 → P0
α-MAP2
K
L
wt
O
Somatostatin
M
N
Ngn2-/-
P
Hematoxylin Eosin
wt
Q
R
Ngn2-/-
S
T

α-MAP2 (Figs. 5K and L), and α-somatostatin (Figs. 5M and N), all of which delineated a clearly visible row of subplate cells in wild-type cortices, and instead labeled unorganized, ectopic neuronal clusters within the intermediate zone of *Ngn2* mutants. Similarly, in histological sections of P0 cortices, the subplate appeared as a clearly visible row of cells beneath the cortical plate in wild-type cortices (Figs. 5Q and R), but a distinct subplate layer was not discernable in the rostromedial cortex of *Ngn2* mutants, and instead, ectopic cellular aggregates formed in the intermediate zone (Figs. 5S and T). Finally, to show definitively that early born subplate neurons were generated but failed to segregate to their normal positions in *Ngn2* mutants, we used birthdating. BrdU was injected into pregnant dams at E11.5, when subplate neurons are generated (Smart and Smart, 1977), followed by immunohistochemical labeling at P0. In P0 *Ngn2* mutants, neurons born at E11.5 migrated correctly beneath the cortical plate in the vicinity of the subplate but were also found in ectopic sites within and beneath the intermediate zone (Figs. 5O and P). Thus, subplate neurons are generated but are misspecified and disorganized in *Ngn2* mutants.

Aberrant thalamocortical and corticothalamic axonal trajectories in Ngn1/2 mutants

Previous studies have demonstrated that the subplate is critical for TCA axonal pathfinding (Ghosh and Shatz, 1993; McConnell et al., 1989, 1994; Super et al., 1998), suggesting that disruption and misspecification of this layer in *Ngn2* and *Ngn1;Ngn2* mutants may interrupt axonal guidance in the cortex. Moreover, we were struck by the observation that several of the *Ngn1/2*-regulated genes identified in our screen had been implicated in axon guidance (Bagnard et al., 2001; Braisted et al., 2000; Dufour et al., 2003; Gao et al., 1998; Skaliora et al., 1998; Yun et al., 2003). In particular, we noted that *EphA5*, which provides guidance cues for afferent (i.e., TCA) and/or efferent (e.g., corticothalamic afferent) connections in the cortex (Dufour et al., 2003; Gao et al., 1998), was expressed at reduced levels in the SVZ of *Ngn2* and *Ngn1;Ngn2* mutants. As well, *Sema3c*, which can act as an axonal chemorepellent (Mark et al., 1997; Steup et al., 2000), was down-regulated in the SVZ, raising the possibility that loss of these cues might also disrupt TCA pathfinding. Although defects in TCAs in *Ngn2* mutants have been previously documented, they were attributed to intrinsic defects in the dorsal thalamus (Seibt et al., 2003), and the underlying molecular defects had not been identified. We thus set out to determine whether defects intrinsic to the cortex may also interfere with TCA pathfinding in the *Ngn2* and *Ngn1;Ngn2* mutant neocortices.

We examined the distribution of TCAs first by immunostaining with an anti-L1 antibody (Figs. 6A–F), which revealed an aberrant organization and fasciculation of thalamic axonal tracts that disrupted the cortical germinal zone in E18.5 *Ngn2* mutants. We also examined the distribution of TCAs in E18.5 cortices with an antibody to calretinin, a calcium binding protein expressed by thalamic neurons that innervate the cortex (Frassoni et al., 1998). In *Ngn2* single and *Ngn1;Ngn2* double mutants, a reduced number of calretinin+ fibers exited the internal capsule and entered the neocortex (data not shown), as expected from the previous demonstration of defects intrinsic to the dorsal thalamus (Seibt et al., 2003). However, of the thalamic fibers that did enter, many took abnormally deep trajectories instead projecting beneath the intermediate zone, towards the ventricular surface (Figs. 6G–I). To confirm the abnormal targeting of TCAs, thalamic projections were traced by implanting a DiI crystal into the dorsal thalamus, with anterograde transport of DiI labeling a trajectory along the subplate layer (Figs. 6J). In *Ngn2* single and *Ngn1;Ngn2* double mutants, the majority of DiI-labeled thalamic axons that reached the cortex inappropriately targeted the germinal layers beneath the white matter (Figs. 6K and L). We suggest that the aberrant trajectory of TCAs in the neocortex is due to the loss of attractive cues from subplate neurons, and possibly the loss of inhibitory cues in the SVZ.

Discussion

The primary objective of this study was to gain new insights into the transcriptional program(s) executed downstream of the proneural transcription factor *Ngn2*. In this regard, our subtractive screen was successful; leading to the identification of two pan-neuronal and 16 regionalized genes whose expression had not previously been known to be misregulated in *Ngn2* mutants. Moreover, through expression analyses of our subtracted clones, we identified the cortical subplate as an additional population of neurons displaying specification defects in the *Ngn2* and *Ngn1;Ngn2* mutant cortices. This led to the demonstration that *Ngn1/2* function is required for pathfinding by thalamocortical

Fig. 5. Subplate neurons are generated but misspecified in *Ngn2* mutants. (A) Expression of *Ngn2* in sagittally sectioned perinatal (E18.5) wild-type telencephalon. (B) Higher magnification of germinal zone from (A). (C–F) P0 expression analysis revealed *Mef2c* (C and D), *protocadherin 9* (E and F), and *srGAP3* (G and H) were expressed in the CP and SP in wild-type cortices (C, E, and G) and a lack of SP staining in *Ngn2* mutants (D, F, and H; *). (I–N) Neuronal markers *SCG10* (I and J), anti-MAP2 (K and L), and antisomatostatin (M and N) revealed a clear SP in wild-type cortices (I, K, and M) and a disrupted SP layer in *Ngn2* mutants (J, L, and N; *), with ectopic neurons in the IZ (L; **). (O and P) BrdU birthdating at E11.5 followed by immunostaining at P0 revealed that neurons born at E11.5 were located in the correct region beneath the CP (bracket) in wild-type (O) and *Ngn2* mutant (P) cortices, and ectopic BrdU+ cells were also within the *Ngn2* mutant IZ (P; *). Histological analysis of P0 wild-type (Q and R) and *Ngn2* mutant (S and T) cortices revealed a disorganization of the subplate in *Ngn2* mutants (T; arrow), which was instead replaced by neuronal aggregates (S; *). cp, cortical plate; gz, germinal zone; sp, subplate; iz, intermediate zone; and wt, wild-type.

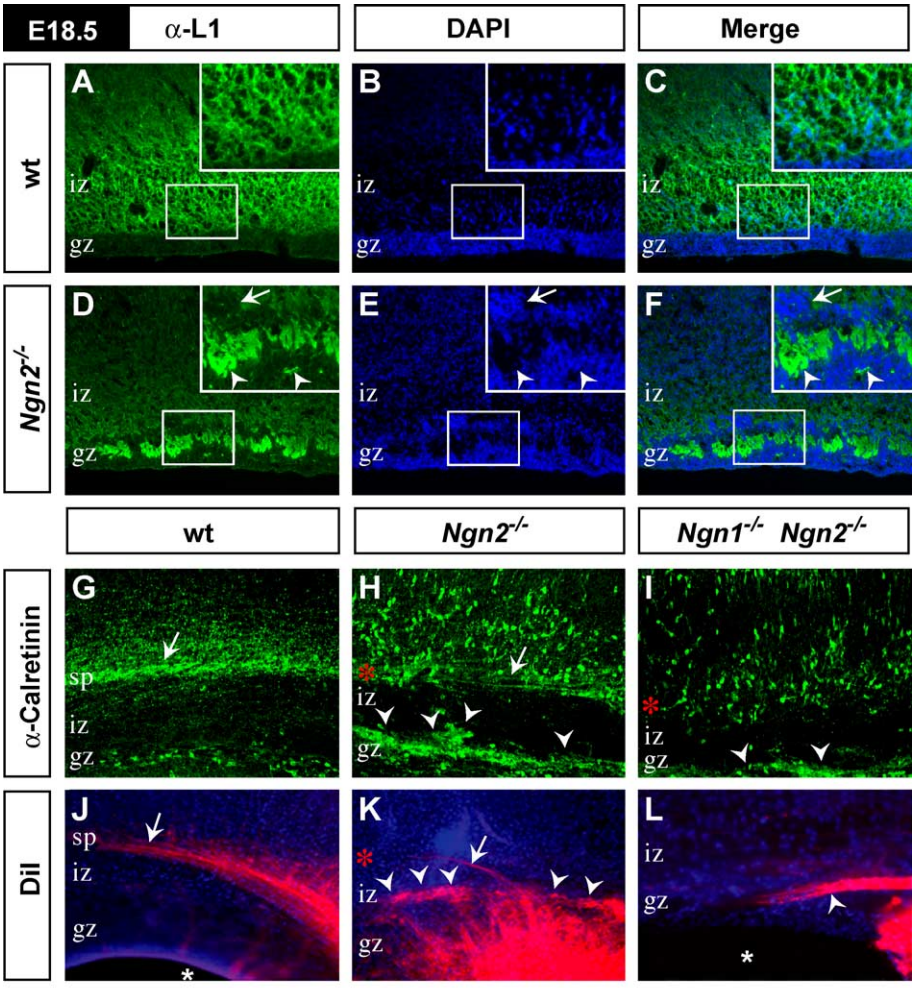


Fig. 6. Thalamocortical and corticofugal projections are perturbed in *Ngn2* and *Ngn1;Ngn2* mutants. (A–F) L1 immunostaining of E18.5 wild-type (A–C) and *Ngn2* mutant (D–F) sagittal sections through the cortex revealed aberrant axonal bundles (arrowheads) that projected into the germinal zone (germinal zone identified histologically with DAPI), causing it to be superficially displaced (arrows) in the *Ngn2* mutant cortex. (G–I) Calretinin immunostaining of frontal sections through the cortex labeling cortical afferent and efferent projections revealed normal trajectories above the intermediate zone (arrows) in wild-type cortices (G), partial misrouting to the ventricular surface (arrowheads) in *Ngn2* mutants (H), and complete misrouting in *Ngn1;Ngn2* mutants (I). (J–L) E18.5 anterograde-labeled, frontally sectioned TCA projections targeted the subplate layer in wild-type cortices (J; arrow), but only a few axons correctly targeted the subplate layer (K; *) in *Ngn2* mutants (arrow), with the majority instead projecting towards the ventricular surface (arrowheads). In *Ngn1;Ngn2* mutants, all TCAs projected to the ventricular surface (L; arrowheads). cp, cortical plate; gz, germinal zone; sp, subplate; iz, intermediate zone; TCA, thalamocortical afferent; and wt, wild-type.

axons that reach the neocortex, a cohort of axons that normally pathfind by targeting the subplate layer.

Identification of differentiation cascades activated downstream of Ngn2

Before this screen, we knew that *Ngn1* and *Ngn2* activities were absolutely required to induce a dorsal, glutamatergic-specific differentiation program in early born cortical neurons, but known components of the downstream pathway included only HLH (i.e., *Math2*, *Nscl1*, *NeuroD*, *NeuroD2*, and *Id2*) and T-box (*Tbr1* and *Tbr2*) transcription factors, *Robo1* and *Slit1* signaling molecules, and *VGLUT1/2* transporters (Schuurmans et al., 2004). In this study, we have identified an additional 16 regionalized genes that are misregulated in *Ngn2* and *Ngn1;Ngn2* mutant neocortices. Strikingly, among these genes are those that are regulated in the VZ, SVZ, preplate, and cortical plate, suggesting that *Ngn1/2* are required to activate a downstream genetic cascade that initiates in the VZ. However, at the same time, it is important to note that the genes isolated in our screen may not be direct targets of *Ngn1/2*, but rather the genes we isolated may be deregulated in *Ngn2* and *Ngn1;Ngn2* mutant cortices as a consequence of the loss of neurons or the loss of a cortical glutamatergic identity. Regardless, the identification of these 16 *Ngn2*-regulated genes greatly expands our knowledge of the genetic cascade that composes the cortical differentiation program. It is also important to note that to date, our screen is only the second survey designed to identify downstream effectors of

proneural bHLH transcription factors; and in the first study, gene profiling was performed on transformed neuroendocrine cells, which may not accurately reflect gene expression in vivo (Hu et al., 2004), making our screen the first to search for bona fide, in vivo components of the regulatory cascade(s) activated downstream of a proneural gene.

Although genomic analyses for proneural-regulated genes have not been conducted in most neural lineages, analyses of expression profiles of candidate target genes in proneural mutants have led to the identification of several downstream genes that typically fall into four categories. Firstly, bHLH transcription factors are known to act in regulatory cascades, activating bHLH and other types of transcription factors. For example, the bHLH genes *Math2* and *NeuroD* are known to be activated downstream of *Ngn1* and *Ngn2* in the cortex (Fode et al., 2000). In other systems, *achaete-scute* and *atonal* genes in *Drosophila* have been shown to be upstream of transcription factors that include *hunchback*, *prospero*, and *cut* (Cabrera and Alonso, 1991; zur Lage et al., 2003), and *Brn3*, *Ebf-1*, *Gfi-1*, *Hox11L2*, *Islet-1*, *Islet-2*, *Krox24*, *Lhx2*, *MyT1*, and *Phox2* have been identified as proneural-dependent genes in vertebrates (Cau et al., 2002; Hirsch et al., 1998; Kury et al., 2002; Lo et al., 2002; Perron et al., 1999; Yang et al., 2003). These studies thus suggest that proneural genes mediate their functions to a great extent by activating transcription factor regulatory cascades. Here, we have identified three new transcription factors, namely, *Mef2c*, *Bhlhb5*, and *NFIB*, that are activated downstream of *Ngn2* in differentiated cortical neurons (Fig. 7). The expression of the transcription factor *Zac1* in progenitors also appears to be partly regulated by *Ngn2*.

A second broad category of proneural-regulated genes includes markers of a mature neuronal phenotype, such as *NCAM*, *neurofilament*, and the *ELAVL4* homologs *ELAV* and *HuC/HuD* (Ferreiro et al., 1994; Park et al., 2003; zur Lage et al., 2003). Here we have shown that *Ngn2* regulates the expression of two pan-neuronal markers, *Zfp10-like* and *Dcx*. The demonstration that the expression of pan-neuronal markers, and hence neurogenesis, is regulated by proneural genes is not surprising given the known requirement for proneural genes to function as neural determination genes (Bertrand et al., 2002; Schuurmans and Guillemot, 2002). At the same time, several neuronal subtype markers, including *β3-AChR*, *L7*, *mGluR6*, *VGLUT1/2*, *GluR2*, *GAD1/2*, and *Xomp2*, have been shown to be regulated by proneural activity in different systems (Burns and Vetter, 2002; Fode et al., 2000; Lo et al., 1998; Matter-Sadzinski et al., 2001; Schuurmans et al., 2004; Tomita et al., 2000). This is consistent with the known role of proneural genes in specifying neuronal subtype identities in both fly and mammals (Fode et al., 2000; Jarman et al., 1993; Lo et al., 2002; Parras et al., 2002). Interestingly, two *Ngn1/2*-regulated genes isolated in our screen may also participate in neuronal subtype specification, including *Nlgn1* and the glutamate receptor gene *GluR6*.

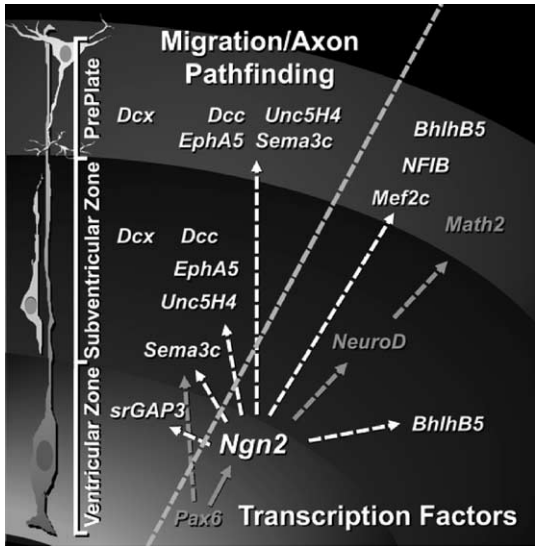


Fig. 7. Model depicting *Ngn2*-dependent genetic cascades in the cortex. *Pax6* directly regulates *Ngn2* (solid arrow; Scardigli et al., 2003), but whether *Ngn2* directly regulates any of the targets identified in our screen remains to be determined (broken lines). For example, we showed that *Sema3c* is deregulated in *Ngn2* mutants and others demonstrated that *Sema3c* is dependent on *Pax6* (Jones et al., 2002). Given that *Pax6* directly regulates *Ngn2*, *Pax6* may regulate *Sema3c* indirectly via *Ngn2*. It also remains to be determined whether *Ngn2* activates a single or multiple genetic cascades as depicted in the diagram. For example, *Ngn2* may activate different pathways that separately influence events such as neuronal specification, differentiation, migration, survival, and axonal guidance.

Proneural transcription factors are known to be required to activate genes involved in cell–cell communication, such as *rhomboid*, *scabrous*, *delta*, *c-RET*, and the neuropeptide receptor *NKD* (Heitzler et al., 1996; Lo et al., 1998; Okabe and Okano, 1997; Rosay et al., 1995; Singson et al., 1994), as well as genes involved in migration or pathfinding, including *Slit1*, the slit receptors *Robo1* and *Robo3*, and *CXCR4* (Kury et al., 2002; Schuurmans et al., 2004; Zlatic et al., 2003). This suggests that proneural genes control more diverse cellular processes than previously anticipated, possibly including cell adhesion, neuronal migration, and axonal pathfinding. Consistent with this idea, several of the *Ngn2*-regulated genes we identified in this screen could potentially be involved in cell–cell signalling and adhesion (i.e., *Dcc*, *Unc5H4*, *Sema3c*, *EphA5*, *protocadherin 9*, and *srGAP3*; Fig. 7). Further analyses of these molecules may provide a molecular basis for the neuronal migration defects (Schuurmans et al., 2004) and axonal guidance defects (see below) that we have observed in *Ngn2* and *Ngn1;Ngn2* mutant cortices. Finally, in our screen we also identified several genes involved in RNA processing and ubiquitination, suggesting that proneural genes may regulate an even more varied range of processes, including alternative splicing and ubiquitin-mediated turnover. Indeed, a role for proneural genes in RNA processing is supported by studies in *Drosophila*

(Kovalick and Beckingham, 1992; Parkhurst et al., 1993), as well as the genes isolated in this screen involved in RNA dynamics.

In summary, the wide range of *Ngn1*- and *Ngn2*-regulated genes uncovered in our screen suggests that these proneural transcription factors regulate a multitude of cellular processes (Fig. 7). This is highly analogous to myogenic bHLH transcription factors, which induce both transcriptional cascades and the expression of differentiated muscle-specific genes, including myosin and muscle creatine kinase (Edmondson and Olson, 1989; Molkentin and Olson, 1996). We can now begin to decipher whether these genes fall into a single linear differentiation program, or if *Ngn1/2* activate several downstream programs in parallel. For example, although we know that *Ngn1* and *Ngn2* cooperate to specify a cortical regional identity, a glutamatergic neurotransmitter phenotype, and deep-layer phenotypes in early born cortical neurons (Schuurmans et al., 2004), the extent to which these pathways are independent or overlap remains to be determined.

Identification of a new biological function for Ngn2 in guiding TCAs trajectories in the neocortex

We have shown here that subplate neurons, which normally provide attractive cues for TCAs, are disorganized in *Ngn2* and *Ngn1;Ngn2* mutants. Moreover, *Ngn2* mutant subplate neurons are misspecified and fail to express genes like *Mef2c*, *protocadherin 9*, and *srGAP3*, while still expressing the pan-neuronal markers MAP-2 and *SCG10*. Previously, we had shown that cortical preplate neurons were misspecified in *Ngn2* and *Ngn1;Ngn2* mutants (Fode et al., 2000), but we had not examined which of the two populations derived from this structure, namely, the cortical subplate and marginal zone, were affected.

The abnormal differentiation of the subplate layer in *Ngn2* and *Ngn1;Ngn2* mutants initially prompted us to examine the projection patterns of TCAs in mutant cortices given the known role of the subplate in guiding these axons. Indeed, we found that TCAs that did reach the cortex in *Ngn2* and *Ngn1;Ngn2* mutants projected aberrantly towards the germinal zone. Moreover, L1-immunostaining revealed that the *Ngn2* mutant TCAs formed abnormal axonal bundles that disrupted the germinal zone of the cortex. Before this study, it was known that defects intrinsic to the *Ngn2* mutant dorsal thalamus result in a shift in the areas of the cortex invaded by TCAs (Seibt et al., 2003). We suspect that the abnormal subplate also contributes to area shifts in TCA trajectories in *Ngn2* and *Ngn1;Ngn2* mutants (Seibt et al., 2003). In particular, it was shown previously that when the subplate was chemically ablated, TCAs were able to enter the cortex, as we observed in *Ngn2* and *Ngn1;Ngn2* mutants, but grew past their targets and were unable to invade the cortical plate (Ghosh and Shatz, 1993; Ghosh et al., 1990). Thus, *Ngn2* and *Ngn1;Ngn2* mutant thalamic defects may not completely account for abnormal TCA trajectories in these mutant backgrounds.

Another abnormal aspect of the TCA trajectories in *Ngn2* and *Ngn1;Ngn2* mutants was that the thalamic axons aberrantly projected towards and disrupted the cortical germinal zone. This phenotype is likely independent of the subplate defects given that chemical ablation of the subplate only rarely results in TCAs projecting to the germinal zone (Ghosh and Shatz, 1993). Moreover, although a reduced number of corticofugal axons exit the cortex in *Ngn2* mutants (Schuurmans et al., 2004), and these axon tracts are known to provide important guidance cues for TCAs, we suggest that corticofugal defects cannot entirely explain TCA defects in *Ngn2* mutants. For example, *Tbr1* mutants do not have a subplate and display defects in corticofugal projections, but the end result is arrest of TCAs in the ventral telencephalon, and not aberrant projections within the cortex itself (Hevner et al., 2001). We thus suggest that the abnormal trajectory of TCAs towards the germinal zone in *Ngn2* and *Ngn1;Ngn2* mutants may be due to the loss of repulsive cues in this layer. In this regard, the reduced expression of *EphA5* and *Sema3c* in the SVZ of *Ngn2* and *Ngn1;Ngn2* mutants is interesting. *EphA* receptors are known to act as pathfinding cues for vomeronasal axons (Knoll and Drescher, 2002). Furthermore, interactions between *EphA5* and *ephrin-A5* can mediate repulsive interactions that exclude limbic thalamic afferents from innervating the somatosensory cortex (Dufour et al., 2003; Gao et al., 1998). Similarly, semaphorins can operate as chemorepellants (Mark et al., 1997; Steup et al., 2000) and have been suggested to prevent the extension of TCA axons to the ventricular surface of the cortex (Skaliora et al., 1998), and *Neuropilin 2*, a high affinity receptor for *Sema3c*, is expressed in the thalamus (Chen et al., 1997; Giger et al., 1998). Similar defects in thalamocortical axon pathfinding and fasciculation have been previously observed in $Pax6^{-/-}$ mutants, and these defects were suggested to be in part attributable to a loss of *Sema3c* expression (Jones et al., 2002). Indeed, since *Pax6* has been shown to directly, transcriptionally regulate the expression of *Ngn2* (Scardigli et al., 2003), it is possible that *Pax6* regulates *Sema3c* indirectly through *Ngn2* (Fig. 7). We thus propose that the normal targeting of TCAs to the subplate is negatively regulated by chemorepulsive factors in the germinal zone (e.g., *EphA5* and *Sema3c*) that may be under the transcriptional control of *Ngn2*. Future investigations will test this possibility directly.

In summary, through the expression analysis of our subtractive clones, we have identified a number of genes that are regulated by *Ngn1* and *Ngn2* in the developing neocortex, including not only transcription factors, but also genes potentially involved in cell–cell communication, adhesion, migration, and other functions. We also describe a novel biological role for *Ngn2* in the specification of subplate neurons and in providing appropriate guidance cues for TCAs in the neocortex. It is anticipated that

further analysis of the genes isolated in our screen will reveal additional roles for the *Ngn1/2* in cortical development.

Acknowledgments

We are very grateful to Helen Cooper, Isabel Hanson, Brian Hemmings, Hisashi Narimatsu, Eric Olson, Andreas Püschel, Albert Reynolds, Fritz Rathjens, Iljona Skerjanc, Marc Tessier-Lavigne, Pierre Vanderhaeghen, and Zheng-Ping Xu for kindly providing cDNAs. We also thank Raffaella Scardigli for advice on cDNA subtraction, and Sarah McFarlane for helpful comments on the manuscript. This work was supported by Canadian Institutes of Health Research (CIHR) operating grant MOP44094 to C.S. P.M. is supported by a CIHR Institute of Genetics Doctoral Research Award and L.M. is supported by a CIHR training grant studentship. C.J. was supported by AHFMR and CIHR training grant summer studentships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.06.013.

References

Bagnard, D., Thomasset, N., Lohrum, M., Puschel, A.W., Bolz, J., 2000. Spatial distributions of guidance molecules regulate chemorepulsion and chemoattraction of growth cones. J. Neurosci. 20, 1030–1035.

Bagnard, D., Chounlamountri, N., Puschel, A.W., Bolz, J., 2001. Axonal surface molecules act in combination with semaphorin 3a during the establishment of corticothalamic projections. Cereb. Cortex 11, 278–285.

Bertrand, N., Castro, D.S., Guillemot, F., 2002. Proneural genes and the specification of neural cell types. Nat. Rev., Neurosci. 3, 517–630.

Braisted, J.E., Catalano, S.M., Stimac, R., Kennedy, T.E., Tessier-Lavigne, M., Shatz, C.J., O'Leary, D.D., 2000. Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. J. Neurosci. 20, 5792–5801.

Burns, C.J., Vetter, M.L., 2002. Xath5 regulates neurogenesis in the Xenopus olfactory placode. Dev. Dyn. 225, 536–543.

Cabrera, C.V., Alonso, M.C., 1991. Transcriptional activation by heterodimers of the *achaete-scute* and daughterless gene products of Drosophila. EMBO J. 10, 2965–2973.

Cau, E., Gradwohl, G., Fode, C., Guillemot, F., 1997. *Mash1* activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124, 1611–1621.

Cau, E., Casarosa, S., Guillemot, S., 2002. *Mash1* and *Ngn1* control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129, 1871–1880.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19, 547–559.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Desai, A.R., McConnell, S.K., 2000. Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development 127, 2863–2872.

Dufour, A., Seibt, J., Passante, L., Depaepe, V., Ciossek, T., Frisen, J., Kullander, K., Flanagan, J.G., Polleux, F., Vanderhaeghen, P., 2003. Area specificity and topography of thalamocortical projections are controlled by ephrin/*Eph* genes. Neuron 39, 453–465.

Edmondson, D.G., Olson, E.N., 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. 3, 628–640.

Engelkamp, D., 2002. Cloning of three mouse Unc5 genes and their expression patterns at mid-gestation. Mech. Dev. 118, 191–197.

Ferreiro, B., Kintner, C., Zimmerman, K., Anderson, D., Harris, W.A., 1994. XASH genes promote neurogenesis in Xenopus embryos. Development 120, 3649–3655.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C., Guillemot, F., 1998. The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron 20, 483–494.

Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J., Guillemot, F., 2000. A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev. 14, 67–80.

Frantz, G.D., McConnell, S.K., 1996. Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron 17, 55–61.

Frassoni, C., Arcelli, P., Selvaggio, M., Spreafico, R., 1998. Calretinin immunoreactivity in the developing thalamus of the rat: a marker of early generated thalamic cells. Neuroscience 83, 1203–1214.

Gao, P.P., Yue, Y., Zhang, J.H., Cerretti, D.P., Levitt, P., Zhou, R., 1998. Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections. Proc. Natl. Acad. Sci. U. S. A. 95, 5329–5334.

Ghosh, A., Shatz, C.J., 1993. A role for subplate neurons in the patterning of connections from thalamus to neocortex. Development 117, 1031–1047.

Ghosh, A., Antonini, A., McConnell, S.K., Shatz, C.J., 1990. Requirement for subplate neurons in the formation of thalamocortical connections. Nature 347, 179–181.

Giger, R.J., Urquhart, E.R., Gillespie, S.K., Levengood, D.V., Ginty, D.D., Kolodkin, A.L., 1998. Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 21, 1079–1092.

Gradwohl, G., Fode, C., Guillemot, F., 1996. Restricted expression of a novel murine *atonal*-related bHLH protein in undifferentiated neural precursors. Dev. Biol. 180, 227–241.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., Simpson, P., 1996. Genes of the Enhancer of split and *achaete-scute* complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development 122, 161–171.

Hevner, R.F., Shi, L., Justice, N., Hsueh, Y., Sheng, M., Smiga, S., Bulfone, A., Goffinet, A.M., Campagnoni, A.T., Rubenstein, J.L., 2001. Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29, 353–366.

Hirsch, M.R., Tiveron, M.C., Guillemot, F., Brunet, J.F., Goridis, C., 1998. Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development 125, 599–608.

Hu, Y., Wang, T., Stormo, G.D., Gordon, J.I., 2004. RNA interference of *achaete-scute* homolog 1 in mouse prostate neuroendocrine cells reveals its gene targets and DNA binding sites. Proc. Natl. Acad. Sci. U. S. A. 101, 5559–5564.

Jarman, A.P., Grau, Y., Jan, L.Y., Jan, Y.N., 1993. *atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell 73, 1307–1321.

Job, C., Tan, S.S., 2003. Constructing the mammalian neocortex: the role of intrinsic factors. Dev. Biol. 257, 221–232.

Jones, L., Lopez-Bendito, G., Gruss, P., Stoykova, A., Molnar, Z., 2002. Pax6 is required for the normal development of the forebrain axonal connections. Development 129, 5041–5052.

Knoll, B., Drescher, U., 2002. Ephrin-As as receptors in topographic projections. Trends Neurosci. 25, 145–149.

Kovalick, G.E., Beckingham, K., 1992. Calmodulin transcription is limited to the nervous system during Drosophila embryogenesis. Dev. Biol. 150, 33–46.

Kury, P., Greiner-Petter, R., Cornely, C., Jurgens, T., Muller, H.W., 2002. Mammalian *achaete-scute* homolog 2 is expressed in the adult sciatic nerve and regulates the expression of Krox24, Mob-1, CXCR4, and p57kip2 in Schwann cells. J. Neurosci. 22, 7586–7595.

Leifer, D., Li, Y.L., Wehr, K., 1997. Myocyte-specific enhancer binding factor 2C expression in fetal mouse brain development. J. Mol. Neurosci. 8, 131–143.

Lennon, G., Auffray, C., Polymeropoulos, M., Soares, M.B., 1996. The I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression. Genomics 33, 151–152.

Lo, L., Tiveron, M.C., Anderson, D.J., 1998. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development 125, 609–620.

Lo, L., Dormand, E., Greenwood, A., Anderson, D.J., 2002. Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells. Development 129, 1553–1567.

LoTurco, J., 2004. Doublecortin and a tale of two serines. Neuron 41, 175–177.

Lyons, G.E., Micales, B.K., Schwarz, J., Martin, J.F., Olson, E.N., 1995. Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. J. Neurosci. 15, 5727–5738.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J.L., Anderson, D.J., 1998. Neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20, 469–482.

Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M., Greenberg, M.E., 1999. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science 286, 785–790.

Mark, M.D., Lohrum, M., Puschel, A.W., 1997. Patterning neuronal connections by chemorepulsion: the semaphorins. Cell Tissue Res. 290, 299–306.

Matter-Sadzinski, L., Matter, J.M., Ong, M.T., Hernandez, J., Ballivet, M., 2001. Specification of neurotransmitter receptor identity in developing retina: the chick ATH5 promoter integrates the positive and negative effects of several bHLH proteins. Development 128, 217–231.

McConnell, S.K., Kaznowski, C.E., 1991. Cell cycle dependence of laminar determination in developing neocortex. Science 254, 282–285.

McConnell, S.K., Ghosh, A., Shatz, C.J., 1989. Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science 245, 978–982.

McConnell, S.K., Ghosh, A., Shatz, C.J., 1994. Subplate pioneers and the formation of descending connections from cerebral cortex. J. Neurosci. 14, 1892–1907.

Molkentin, J.D., Olson, E.N., 1996. Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. 6, 445–453.

Nieto, M., Schuurmans, C., Britz, O., Guillemot, F., 2001. Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron 29, 401–413.

Nishihara, S., Iwasaki, H., Nakajima, K., Togayachi, A., Ikehara, Y., Kudo, T., Kushi, Y., Furuya, A., Shitara, K., Narimatsu, H., 2003. Alpha1,3-fucosyltransferase IX (Fut9) determines Lewis X expression in brain. Glycobiology 13, 445–455.

Okabe, M., Okano, H., 1997. Two-step induction of chordotonal organ precursors in Drosophila embryogenesis. Development 124, 1045–1053.

Park, S.H., Yeo, S.Y., Yoo, K.W., Hong, S.K., Lee, S., Rhee, M., Chitnis, A.B., Kim, C.H., 2003. Zath3, a neural basic helix-loop-helix gene, regulates early neurogenesis in the zebrafish. Biochem. Biophys. Res. Commun. 308, 184–190.

Parkhurst, S.M., Lipshitz, H.D., Ish-Horowicz, D., 1993. achaete-scute feminizing activities and Drosophila sex determination. Development 117, 737–749.

Parras, C.M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D.J., Guillemot, F., 2002. Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity. Genes Dev. 16, 324–338.

Perron, M., Opdecamp, K., Butler, K., Harris, W.A., Bellefroid, E.J., 1999. X-ngnr-1 and Xath3 promote ectopic expression of sensory neuron markers in the neurula ectoderm and have distinct inducing properties in the retina. Proc. Natl. Acad. Sci. U. S. A. 96, 14996–15001.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R.R., Le Meur, M., Ang, S.L., 1998. Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. Development 125, 845–856.

Rosay, P., Colas, J.F., Maroteaux, L., 1995. Dual organisation of the Drosophila neuropeptide receptor NKD gene promoter. Mech. Dev. 51, 329–339.

Scardigli, R., Baumer, N., Gruss, P., Guillemot, F., Le Roux, I., 2003. Direct and concentration-dependent regulation of the proneural gene *Neurogenin2* by Pax6. Development 130, 3269–3281.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., Serafini, T., 2000. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell 101, 657–669.

Schuurmans, C., Guillemot, F., 2002. Molecular mechanisms underlying cell fate specification in the developing telencephalon. Curr. Opin. Neurobiol. 12, 26–34.

Schuurmans, C.A.O., Nieto, M., Stenman, J.M., Britz, O., Klenin, N., Seibt, J., Brown, C., Tang, H., Cunningham, J.M., Dyck, R., Walsh, C., Campbell, K., Polleux, F., Guillemot, F., 2004. Sequential Phases of Neocortical Fate Specification Involve Neurogenin-Dependent and -Independent Pathways. Embo. J. online publication, July 1, 2004.

Seibt, J., Schuurmans, C., Gradwhol, G., Dehay, C., Vanderhaeghen, P., Guillemot, F., Polleux, F., 2003. *Neurogenin2* specifies the connectivity of thalamic neurons by controlling axon responsiveness to intermediate target cues. Neuron 39, 439–452.

Singson, A., Leviten, M.W., Bang, A.G., Hua, X.H., Posakony, J.W., 1994. Direct downstream targets of proneural activators in the imaginal disc include genes involved in lateral inhibitory signaling. Genes Dev. 8, 2058–2071.

Skaliora, I., Singer, W., Betz, H., Puschel, A.W., 1998. Differential patterns of semaphorin expression in the developing rat brain. Eur. J. Neurosci. 10, 1215–1229.

Smart, I.H., Smart, M., 1977. The location of nuclei of different labelling intensities in autoradiographs of the anterior forebrain of postnatial mice injected with [3H]thymidine on the eleventh and twelfth days postconception. J. Anat. 123, 515–525.

Steup, A., Lohrum, M., Hamscho, N., Savaskan, N.E., Ninnemann, O., Nitsch, R., Fujisawa, H., Puschel, A.W., Skutella, T., 2000. *Sema3c* and netrin-1 differentially affect axon growth in the hippocampal formation. Mol. Cell. Neurosci. 15, 141–155.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M.Z., Zubiaga, A., Hua, X., Fan, G., Greenberg, M.E., 2001. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 104, 365–376.

Super, H., Soriano, E., Uylings, H.B., 1998. The functions of the preplate in development and evolution of the neocortex and hippocampus. Brain Res. Brain Res. Rev. 27, 40–64.

Takahashi, T., Nakamura, F., Jin, Z., Kalb, R.G., Strittmatter, S.M., 1998. Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat. Neurosci. 1, 487–493.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., Kageyama, R., 2000. Mammalian *achaete-scute* and *atonal* homologs regulate neuro-

nal versus glial fate determination in the central nervous system. EMBO J. 19, 5460–5472.

Wong, K., Ren, X.R., Huang, Y.Z., Xie, Y., Liu, G., Saito, H., Tang, H., Wen, L., Brady-Kalnay, S.M., Mei, L., Wu, J.Y., Xiong, W.C., Rao, Y., 2001. Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell 107, 209–221.

Yang, Z., Ding, K., Pan, L., Deng, M., Gan, L., 2003. *Math5* determines the competence state of retinal ganglion cell progenitors. Dev. Biol. 264, 240–254.

Yun, K., Garel, S., Fischman, S., Rubenstein, J.L., 2003. Patterning of the lateral ganglionic eminence by the Gsh1 and Gsh2 homeobox genes regulates striatal and olfactory bulb histogenesis and the growth of axons through the basal ganglia. J. Comp. Neurol. 461, 151–165.

Zlatic, M., Landgraf, M., Bate, M., 2003. Genetic specification of axonal arbors: *atonal* regulates robo3 to position terminal branches in the Drosophila nervous system. Neuron 37, 41–51.

zur Lage, P.I., Prentice, D.R., Holohan, E.E., Jarman, A.P., 2003. The Drosophila proneural gene amos promotes olfactory sensillum formation and suppresses bristle formation. Development 130, 4683–4693.