A screen for downstream effectors of Neurogenin2 in the embryonic neocortex

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Abstract

Neurogenin (Ngn) 1 and Ngn2 encode basic-helix-loop-helix transcription factors expressed in the developing neocortex. Like other proneural genes, Ngn1 and Ngn2 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.
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, Ncl2, Tbr1/2, and Id2), the signaling molecules Slit1 and Robol, 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 proneuralachaete-scute genes are ectopically expressed in precursors that normally express atonal, and vice versa (Jarman et al., 1993). In these experiments, ectopic expression ofachaete-scute genes generates external sensory organs where atonal would normally generate chordotonal organs. Thus, proneural genes in both Drosophila and vertebrates specify neuronal 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 repulsive 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 Ngn2lacZ+ and Ngn1 mutant mice

Ngn2lacZ+;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 Ngn2lacZ+/- 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 PCRSel ect 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, Nbc12, and Dlx1 inserts that were separated on a 0.8% gel, transferred to a nylon membrane, and hybridized with random-primer, radiolabeled probes from unsubtracted Ngn2lacZ+/− 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). Digoxigenin (dig)-labeled RNA probes were generated using T3, T7, or SP6 RNA polymerases and a dig-RNA-labelling 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 in parentheses): Akt3 (AF124142), Bhlhb5 (AF504925), Dcc (X85788), EphA5 (NM_007937), Mef2c (L13171), Neurotratin (AJ487032), Sema3e (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; CagBP2 (BC026856), IMAGE:4503295; EDG-1 (BC049094), IMAGE:6415920; ELAV14 (BC048159), IMAGE:5703005; Myotrophin (BC043084), IMAGE:6417343; Neurogln1 (BC005523), IMAGE:3494913; Pegasus-like (BC048183), IMAGE:6826723; PHD6-like (BC043127), IMAGE:6410361; Pthb2 (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 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 Ngn2lacZ− 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 Ngn2lacZ− embryos, with staining significantly more intense in Ngn2lacZ− 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 Ngn2lacZ+/− and Ngn2lacZ−/− 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
expected increase in hybridization signal to Math2, Tbr1, and Nscr2, 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 Nscr2 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 Ngn2lacZ 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 Zip10-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 Fur9, 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); neurologin-1 (Ngn1), 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 Zip10-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 Zip10-like was greatly reduced in the dorsomedial SVZ of Ngn2 mutants (Figs. 2B, B’, E, E’). To confirm that the reduced expression of Dcx and Zip10-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 Zip10-like and Dcx expression in E13.5 Ngn1;Ngn2 double mutants. In striking contrast to the reduced expression of Zip10-like and Dcx in Ngn2 single
mutants, the pan-neuronal genes were up-regulated in the preplate of $\text{Ngn1;}\text{Ngn2}$ double mutants (Figs. 2C, C, and F). This result was not completely unexpected given that we had previously reported an up-regulation of the pan-neuronal marker $\text{SCG10}$ in the preplate of E12.5 $\text{Ngn1;}\text{Ngn2}$ 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 preplate in $\text{Ngn1;}\text{Ngn2}$ double mutants, with expression 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.

**Fig. 2.** Expression analysis of pan-neuronal markers in E13.5 $\text{Ngn2}$ and $\text{Ngn1;}\text{Ngn2}$ 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’) $\text{Dcx}$ expression in wild-type (A and A’), $\text{Ngn2}$ mutant (B and B’), and $\text{Ngn1;}\text{Ngn2}$ mutant (C and C’) telencephalon. (D–F and D’–F’) Zfp10-like expression in expression in wild-type (D and D’), $\text{Ngn2}$ mutant (E and E’), and $\text{Ngn1;}\text{Ngn2}$ mutant (F and F’) telencephalon. (G–I) TUJ1 expression in the preplate of E12.5 wild-type (G), $\text{Ngn2}$ mutant (H), and $\text{Ngn1;}\text{Ngn2}$ mutant (I) cortices. For $\text{Dcx}$, Zfp10-like, and TUJ1, expression was down-regulated in the PP (arrowheads) and dorsomedial SVZ (arrows) of $\text{Ngn2}$ mutants. In $\text{Ngn1;}\text{Ngn2}$ 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.

**Fig. 3.** Expression analysis of regionalized clones in E13.5 $\text{Ngn2}$ and $\text{Ngn1;}\text{Ngn2}$ mutant cortices. Frontal sections of E13.5 wild-type (A, D, G, J, M, P, S, V, and Y), $\text{Ngn2}$ mutant (B, E, H, K, N, Q, T, W, and Z), and $\text{Ngn1;}\text{Ngn2}$ 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) Neurologin 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’V) 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.
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

<table>
<thead>
<tr>
<th>Gene name(s)</th>
<th>Category</th>
<th>Expression (Ngn2 and Ngn1;Ngn2 mutants versus wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zac1 (Plagl1/LOT1)</td>
<td>Transcription factor</td>
<td>↓ VZ, PP neurons</td>
</tr>
<tr>
<td>Id4 (IDB4)</td>
<td>Transcription factor</td>
<td>No change</td>
</tr>
<tr>
<td>Dach1</td>
<td>Transcription factor</td>
<td>No change</td>
</tr>
<tr>
<td>Nuclear Factor IB</td>
<td>Transcription factor</td>
<td>↓ PP neurons</td>
</tr>
<tr>
<td>Bhlhb5 (Beta3)</td>
<td>Transcription factor</td>
<td>↓ SVZ, PP neurons</td>
</tr>
<tr>
<td>Me2c</td>
<td>Transcription factor</td>
<td>↓ PP neurons</td>
</tr>
<tr>
<td>Dcc</td>
<td>Receptor or signal transduction</td>
<td>↓ SVZ, PP neurons</td>
</tr>
<tr>
<td>Unc5H4</td>
<td>Receptor or signal transduction</td>
<td>↓ SVZ, PP neurons</td>
</tr>
<tr>
<td>Sema3c (SemE)</td>
<td>Receptor or signal transduction</td>
<td>↓ SVZ, PP neurons</td>
</tr>
<tr>
<td>EphA5 (Bsk)</td>
<td>Receptor or signal transduction</td>
<td>↓ SVZ, PP neurons</td>
</tr>
<tr>
<td>Neurotractin (KILON)</td>
<td>Receptor or signal transduction</td>
<td>↑ PP neurons</td>
</tr>
<tr>
<td>srGAP3 (Fnb2p)</td>
<td>Receptor or signal transduction</td>
<td>↓ VZ, ↑ PP neurons</td>
</tr>
<tr>
<td>AKT3 (PKB gamma)</td>
<td>Receptor or signal transduction</td>
<td>↓ PP neurons</td>
</tr>
<tr>
<td>Ptb2</td>
<td>Splicing or RNA binding</td>
<td>↓ VZ</td>
</tr>
<tr>
<td>ELAV-like 4 (HuD)</td>
<td>Splicing or RNA binding</td>
<td>↓ VZ, SVZ</td>
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<td>Neurologin 1 (Ngln1)</td>
<td>Neurotransmission</td>
<td>↓ VZ, PP neurons</td>
</tr>
<tr>
<td>fasciculytransferase 9</td>
<td>Adhesion related</td>
<td>No change</td>
</tr>
<tr>
<td>protocadherin 9</td>
<td>Adhesion related</td>
<td>↓ PP neurons</td>
</tr>
<tr>
<td>NAP-22 (Basp1)</td>
<td>Miscellaneous</td>
<td>↓ PP neurons</td>
</tr>
</tbody>
</table>

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, Ngn1, 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 Ngn1 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; Me2c (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),
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. 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 germinial 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
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, N scl1, 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, acheta-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 Bm3, Eth-1, Gfi-1, Hox11L2, Iset-1, Iset-2, Kroxo24, Lhx2, MyT1, and Phox2 have been identified as proneural-dependent genes in vertebrates (Cauf 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 Nf1b, that are activated downstream of Ngn2 in differentiated cortical neurons (Fig. 7). The expression of the transcription factor Zac1 in progenitor cells 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 ELAV4 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, Zip10-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 Ngn1 and the glutamate receptor gene GluR6.

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 Slt1, 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 training grant studentship. C.J. is supported by a CIHR training grant studentship. L.M. is supported by a CIHR operating grant MOP44094 to C.S. P.M. is supported by a CIHR training grant studentship. C.J. was supported by AHFMR and CIHR training grant.

Appendix A. Supplementary data


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