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Brn3a regulates the transition from neurogenesis to terminal differentiation and represses non-neural gene expression in the trigeminal ganglion

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Abstract

The POU-domain transcription factor Brn3a is expressed in developing sensory neurons at all levels of the neural axis, including the trigeminal ganglion, hindbrain sensory ganglia, and dorsal root ganglia. Changes in global gene expression in the trigeminal ganglion from E11.5 to E13.5 reflect the repression of early neurogenic genes, exit from the cell cycle, and initiation of the expression of definitive markers of sensory function. A majority of these developmental changes are perturbed in the trigeminal ganglia of Brn3a knockout mice. At E13.5, Brn3a$^{-/-}$ trigeminal neurons fail to repress a battery of developmental regulators which are highly expressed at E11.5 and are normally down-regulated as development progresses, and also fail to appropriately activate a set of definitive sensory genes. Remarkably, developing Brn3a$^{-/-}$ trigeminal neurons also ectopically express multiple regulatory genes associated with cardiac and/or cranial mesoderm development, although definitive myogenic programs are not activated. The majority of these genes are not ectopically expressed in the dorsal root ganglia of Brn3a null mice, perhaps due to redundant mechanisms of repression at spinal levels. These results underscore the importance of gene repression in regulating neuronal development, and the need for unbiased screens in the determination of developmental gene regulatory programs.

Keywords

Brn3a; POU domain; trigeminal ganglion; development; gene expression; transcription factor; homeodomain; sensory ganglion

INTRODUCTION

Information about the external world is conveyed to the vertebrate brain by special sense organs for vision, olfaction and hearing, and by the peripheral sensory nervous system mediating general somatic sensation. Somatic sensory neurons innervate the skin and muscles throughout the body, and produce specialized structures and molecular receptors for sensory modalities including pain, touch, temperature and position. At trunk levels, somatic sensory neurons reside in the dorsal root ganglia (DRG) associated with each spinal segment, and innervate the corresponding dermatomes and myotomes, while at cranial levels they reside primarily in the trigeminal ganglion (TG) and innervate the facial skin and musculature.

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Numerous studies have focused on the unique developmental origin of sensory neurons. At trunk levels the DRG are generated from neural crest precursors, while the sensory ganglia associated with the cranial nerves, such as the TG, include contributions from the neural crest and neurogenic placodes derived from surface ectoderm (Baker and Bronner-Fraser, 2001). The earliest-expressed genes known to have an essential role in specifying the sensory lineage are the bHLH factors neurogenin1 (Neurog1) and neurogenin2 (Neurog2), with Neurog1 playing a predominant role in the TG and DRG and Neurog2 the principal factor in sensory ganglia derived from the epibranchial placodes (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). In all sensory neurons, expression of the these factors is followed by the bHLH factors Neurod1 (BETA2) and Neurod4 (Math3), which are dependent on the neurogenins (Fode et al., 1998; Ma et al., 1998; Sommer et al., 1996).

Following the neurogenic phase of sensory development, coinciding approximately with the time of exit from the cell cycle, sensory neurons of the DRG and cranial ganglia express the “pan sensory” homeodomain transcription factors Brn3a (product of the *Pou4f1* gene) and Islet1 (Eng et al., 2001; Sun et al., 2008). Both factors are required to terminate the expression of the bHLH neurogenic factors (Eng et al., 2007; Eng et al., 2004; Sun et al., 2008). Brn3a has been shown to bind directly to enhancer regions within the Neurod1 and Neurod4 loci (Lanier et al., 2007), and a to negative autoregulatory region within the *Pou4f1* locus itself (Lanier et al., 2007; Trieu et al., 2003; Trieu et al., 1999). As sensory maturation progresses, both Brn3a$^{-/-}$ and Islet1$^{-/-}$sensory neurons fail to correctly express numerous markers of terminal sensory differentiation and subtype specification (Eng et al., 2004; Sun et al., 2008), demonstrating that these factors also have a direct or indirect positive regulatory role in determining sensory phenotypes.

In the present study we have examined changes in global gene expression in the trigeminal ganglion from E11.5 to E13.5, a critical period during which a majority of trigeminal neurons exit the cell cycle and initiate the expression of definitive sensory markers. In the absence of Brn3a, sensory neurons are delayed in executing the changes in gene expression characteristic of this time period. The expression of factors associated with cell proliferation is somewhat prolonged, but cell cycle exit is accomplished by E13.5 in both control and Brn3a$^{-/-}$ ganglia. At E13.5, Brn3a$^{-/-}$ TG neurons fail to repress a battery of developmental regulators which are highly expressed at E11.5 and are normally down-regulated as development progresses. Conversely, numerous genes associated with mature sensory phenotypes fail to be expressed at normal levels. Surprisingly, in the absence of Brn3a, developing TG neurons abnormally express several regulatory factors, including Nkx2.5 (tinman), Ankrd1 (CARP), Zfpm2 (Fog2), Lmcd1 (dyxin), Fgf10, Figf (VegfD), and Angpt1, which are normally associated with differentiating cardiac myocytes and/or cranial muscles rather than sensory neurons. This unexpected role for Brn3a in the repression of non-neural genes in sensory neurons illustrates that the gene expression programs regulated by transcriptional repressors may be occult and unexpected, and that unbiased screens for downstream targets are indispensible to understanding their functions.

MATERIALS AND METHODS

Animals, matings, tissue collection

Mice heterozygous for a Brn3a null allele (Eng et al., 2001; Xiang et al., 1996) were maintained in a C57bl/6 background. To generate tissue for microarray analysis, timed matings of Brn3a heterozygote animals were performed, and the embryos were harvested at E11.5 or E13.5. TG were removed by blunt dissection and carefully freed of adherent non-neural tissue with fine forceps. Dissected ganglia were placed in RNase inhibitor solution (RNAlater, Ambion, Austin TX), and RNA was prepared using the RNeasy system (Qiagen, Valencia, CA). Embryos were genotyped for Brn3a alleles using real-time qPCR from a

sample of tail or hind-limb tissue harvested at the time of ganglion dissection, using PCR primer pairs targeting the POU-domain of the Brn3a gene or the neomycin resistance cassette targeted to the Brn3a locus, as previously described. For analysis of Brn3a knockout and control ganglia, TG from five embryos were sufficient to provide approximately 5 μg of total RNA for a single microarray analysis. The generation of cDNA, production of labeled cRNA, and hybridization to Affymetrix 430v2 GeneChip arrays were all performed according to standard protocols provided by the manufacturer (Affymetrix, Santa Clara, CA).

Microarray and real-time PCR analysis

Microarray analysis of TG gene expression was performed on two developmental stages (E11.5, E13.5) and three genotypes (Brn3a +/+, +/− and −/−) in duplicate for a total of 12 analyses. Consistent with prior results (Eng et al., 2004), gene expression in heterozygotes correlated strongly with control ganglia and was not further analyzed. The primary analysis of microarray data, including determination of the absence/presence of the assayed transcripts, transcript expression levels, and the probability of change in transcript expression between samples ('change *p*') was performed with Microarray Suite 5.0 (MAS5, Affymetrix). Default MAS5 parameters were used for increase (I) and decrease (D) calls, which were $p < 0.002$ and $p > 0.998$ for I and D, respectively. All array values were scaled to a target value of 500 using global scaling. Microarray probe sets were related to the corresponding mouse transcripts using the NetAffx database (Affymetrix), based on the NCBI Build 36 annotation of the mouse genome.

For comparison of gene expression changes across developmental stages and between genotypes, we identified 17,351 probe sets from among the 45,037 probe sets on the array which were present in both samples of at least one stage/genotype, and had a mean expression value of at least 250 (0.5 times the scaled target value) in at least one stage/genotype. Transcripts listed in the ranked lists of changed genes (Table S1–S2, S4–S7) showed concordant I or D calls in both replicates for a given comparison (E11 WT x E13 WT, E11 WTxKO, E13 WTxKO). In cases of multiple probe sets identifying a single transcript, data are given for the probe set with the highest mean expression in any condition.

Classification of differentially expressed genes by gene ontology (GO) was performed using GeneSpring software version 9 (Silicon Genetics, Redwood City, CA). Four gene lists were created in GeneSpring with the "Arbitrary File Filter" based on significant expression differences between stages or genotypes (expression increased in development from E11.5 to E13.5, expression decreased in development from E11.5 to E13.5, expression increased in Brn3a knockout at E13.5, expression decreased in Brn3a knockout at E13.5). Each category consisted of all transcripts which exhibited concordant I or D calls in the replicate assays for a given comparison. Genes were organized into GO categories based on biological process using GeneSpring's GO browser, and GO categories enriched in our gene lists were then compiled.

Real time reverse transcription-PCR (RT-PCR) analysis was performed on samples of RNA from the trigeminal ganglia of E13.5 $Brn3a^{+/+}$ and $Brn3a^{-/-}$ embryos harvested as described above. Samples for RT-PCR analysis were harvested independently of those used for microarray analysis. First strand cDNA synthesis was performed with a Thermoscript RT-PCR system for first strand synthesis (Invitrogen) using 1μg of total RNA according to manufacturers instructions, and real-time PCR was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and an ABI 7300 real-time PCR system. Results were analyzed according to the cycle threshold difference method (Livak and Schmittgen, 2001) Oligonucleotide primer sets used for RT-PCR appear in Table S8.

In situ hybridization and immunofluorescence

Embryos for in situ hybridization and immunofluorescence studies were generated by timed matings as described above. Embryos were fixed by immersion in 4% paraformaldehyde for 30 minutes (E11.5) or 2 hours (E13.5). Tissue was embedded in Neg50 (Richard Allan Scientific) and 20μM sections were cut on a cryostat. Rabbit and guinea pig primary antibodies to Brn3a have been previously described (Fedtsova and Turner, 1995; Quina et al., 2005).

In situ hybridization was performed on cryostat sections using digoxigenin labeled cRNA probes. Sections were pre-treated with a 10 minute digestion in 10ug/ml proteinase K followed by 10 minutes in 0.5% acetic anhydride, 0.1M triethanolamine before being hybridized at 65C overnight using 1–2ug/ml of probe. Slides were washed at 65C under stringent conditions then blocked in 10% sheep serum. Signal was detected using an alkaline phosphatase conjugated sheep anti digoxigenin antibody (Roche).at 1:2000 followed by staining in BM Purple AP Substrate (Roche). Plasmids containing cDNAs were obtained from Open Biosystems. A list of in situ probes used in this study is provided in Table S8.

RESULTS

Gene expression changes in sensory maturation

Brn3a is expressed in sensory neurons at all axial levels around the time of their exit from the cell cycle, or “birthdate”, and its expression also coincides with the onset of expression of many genes which relate to specific neural functions. Thus the regulatory role of Brn3a must be understood in the context of normal developmental changes in gene expression taking place during this period. For this reason, we began the current studies by examining global gene expression in the developing trigeminal ganglion at E11.5 and E13.5, bracketing a period during which a large fraction of trigeminal neurons are born.

Microarray analysis of wildtype TG at E11.5 and E13.5 revealed extensive changes in gene expression (Figure 1). The changes were consistent with a transition from cell proliferation to terminal differentiation, and with the initiation of sensory subtype specification. Transcripts with the greatest increases in expression from E11.5 to E13.5 (Table S1) were heavily weighted toward neurotransmitters (Galanin, Th, Tac1/SP), neurotransmitter receptors (Htr3a, Htr3b, Npy5r, Grm3, Grm7), ion channels (Trpv1, Trpc4, Accn3, Scn7a), regulators of axonogenesis and synaptogenesis (Advillin, Sema3e, Syt4, Ncam2) and developmental regulators with roles in sensory specification (Ret, TrkA, Bnc1, Runx1, Hoxd1).

Genes which showed decreased expression from E11.5 to E13.5 included many factors associated with cell cycle progression, DNA replication, and mitosis (Table S2). Transcription factors associated with early steps in sensory neurogenesis were also markedly decreased in this developmental interval, including Neurog1, Neurod4, Pax3, Tcfap2b, Hes5 and Eya2, and the majority of the transcripts with the largest magnitude decreases could be assigned to one of these two classes.

We then performed a gene ontology (GO) biological process analysis to obtain a more comprehensive view of the changes in gene expression in this developmental interval. For this analysis, we defined sets of transcripts with replicated increases or decreases in expression between E11.5 and E13.5 ($p < 0.002$ and $p > 0.998$ for I and D, respectively). The gene ontology classification of the 2606 increased and 2451 decreased transcripts that met these criteria were then compared to the global set of 45037 transcripts represented on the array to determine over-represented categories. Transcripts increasing across developmental time clustered strongly in GO categories specific for neural function,

including synaptic transmission, ion transport, neural development and neurotransmitter secretion (Table 1, Table S3). For transcripts decreasing from E11.5 to E13.5, the major over-represented categories included DNA metabolism, cell cycle, and cell division. (Table 1, Table S3). Together these results give a comprehensive profile of the changes in gene expression that characterize the transition from sensory neurogenesis to terminal differentiation.

Regulatory targets of Brn3a

We next compared gene expression in Brn3a knockout and wildtype trigeminal ganglia at E11.5 (Tables S4, S5) and E13.5 (Tables 2, 3, S6, Table S7), revealing an extensive program of gene expression regulated by Brn3a. In general, the transcripts with altered expression at E11.5 were a subset of those changed at E13.5, and the direction of change was consistent at both stages. Some of these transcripts have been previously identified as downstream of Brn3a in the developing TG (Eng et al., 2004) or DRG (Eng et al., 2007), but many novel targets were identified due to improved genomic coverage by the arrays used in the present study and progress in annotation of the mouse genome.

A large fraction of the decreased transcripts in the Brn3a$^{-/-}$ TG represent developmental signaling molecules or transcription factors known to be associated with sensory subtype specification. Some of these changes, such as decreased expression of Runx3, TrkC, and Pou4f2/Brn3b, were evident beginning at E11.5. Other changes, including decreased expression of Bnc1, Hmx1, HoxD1 and Runx1, were not observed until E13.5, due to the later developmental expression of these factors. Loss of Brn3a also resulted in markedly decreased expression of a large number of neural-specific transcripts, including neurotransmitters (Gal, Adcyap1/Pacap, TH), receptors (Npy5r, Htr3b, Npy1r, Prokr1, Gpr149, Gpr64, Trpv1, Accn2), ion channels (Scn7a, Scn10a, Kcnab2, Kcna1) and cell surface molecules which mediate cell survival, migration and/or axon guidance (Ntrk3, Ntrk1, Rtn4rl2, Rtn4rl1). Many of these terminal differentiation genes are not significantly expressed in E11.5 control ganglia, and therefore the changes in Brn3a$^{-/-}$ ganglia were noted mainly at E13.5.

The set of genes with increased expression in E13.5 Brn3a$^{-/-}$ TG included the abnormal persistence of several transcription factors which are expressed in the early phases of sensory development, including Neurod1, Neurod4/Math3, Neurog1, Tcfap2b, Insm1, Six1, Eya1 and Eya2. Also increased are several cell cycle genes, including Cyclin B1, Cyclin D1, and Cdc20. However, the least expected finding is a marked increase in the expression of a set of genes normally associated with cardiac and cranial mesoderm development, which are examined in detail below.

An interaction between developmental stage and Brn3a genotype

The abnormal persistence of multiple classes of genes expressed in early development in the TG of Brn3a$^{-/-}$ mice led us to examine the global interaction between developmental changes in gene expression and Brn3a genotype. A comparative GO analysis of the transcripts altered in Brn3a$^{-/-}$ TG at E13.5 with changes in gene expression from E11.5 to E13.5 confirmed such a relationship (Table S3). Transcripts with decreased expression in the E13.5 Brn3a$^{-/-}$ TG were associated with GO categories including ion transport (including channels and receptors), cell communication (including signal transduction and neuropeptide signaling), and axonogenesis. Nearly all of these categories were also overrepresented in the GO analysis of transcripts increasing from E11.5 to E13.5 (Table 1, Table S3). Transcripts with increased expression in the E13.5 Brn3a$^{-/-}$ TG were associated with GO categories that included the cell cycle, cell division, DNA metabolism, and embryonic and neural development. The cell-cycle related categories were highly correlated

with the GO classification of transcripts decreasing from E11.5 to E13.5 (Table 1, Table S3).

We then performed a regression analysis of transcript expression for developmental stage x genotype to examine this relationship on a gene-by-gene basis (Figure 2A). As expected from the GO analysis, the direction and magnitude of differences in gene expression levels between E11.5 and E13.5 correlated with the effects of the loss of Brn3a expression at E13.5. A large cohort of transcripts which normally increase expression as development progresses failed to reach normal expression levels in the ganglia of E13.5 Brn3a$^{-/-}$ mice, and conversely, many transcripts which normally decrease with developmental time were increased at E13.5 in the knockout (sectors designated “developmental delay”, Figure 2A). The relationship of the fold change in transcript levels due to developmental stage versus the loss of Brn3a expression at E13.5 can be described by a linear regression with a slope of −0.30 and $R^2 = 0.26$. These results demonstrate a pervasive delay in the development of the Brn3a$^{-/-}$ TG at the transition from neurogenesis to neural differentiation. However, a large majority of the developmentally regulated genes exhibited less than two-fold changes in expression, suggesting that global developmental delay does not account for the large fold changes observed for the most strongly regulated transcripts.

We then examined the relationship between developmental changes in gene expression and Brn3a genotype for functional categories of genes, including regulators the cell cycle (including mitosis and DNA replication), mediators of axon growth and guidance, sequence specific transcription factors, and neurotransmitters/receptors. Factors mediating cell cycle progression and mitosis conformed closely to the regression line that describes the overall developmental delay in Brn3a$^{-/-}$ TG (Figure 2B), in that expression of genes mediating the cell cycle and DNA replication decreased from E11.5 to E13.5 and was increased in Brn3a$^{-/-}$ ganglia, while genes mediating cell cycle exit had the inverse relationship to both conditions. Most mediators of axon growth and guidance had increased expression from E11.5 to E13.5, as expected in maturing neurons, and also generally conformed to the linear relationship between developmental stage and Brn3a genotype.

In contrast, many genes encoding transcription factors and neurotransmitters do not conform to this linear relationship between developmental stage and Brn3a genotype. Thus many of the observed changes in these gene classes appear to represent “phenotype switching” by Brn3a$^{-/-}$ trigeminal neurons, which cannot be simply described as a failure to progress on schedule down the expected developmental pathway. A significant number of these transcripts (Trk receptors, Runx3, Ret, Trpa1, Hmx1) represent known markers of sensory subtypes, and the role of Brn3a in sensory subtype specification will be examined in a separate report (I. Dykes, in preparation). Finally, a subset of these transcripts encode factors most strongly associated with cardiac and cranial mesoderm development (highlighted in red).

In order to test the reliability of the gene expression changes detected by microarray, we examined the expression of 36 of the changed transcripts by quantitative RT-PCR using independent samples of E13.5 knockout and control TG tissue (Figure 3). In these assays all transcripts with >2-fold change on the microarray showed a similar direction and magnitude of change in the RT-PCR assay, including increased and decreased transcripts across all functional classes, with the exception of Mrg1, for which the change observed on the microarray was not replicated.

Persistent expression of cell-cycle associated genes raises the question of whether Brn3a$^{-/-}$ TG neurons exit the cell cycle at the expected time. Arguing against significantly prolonged cell division is the prior observation that Brn3a knockout TG do not have increased cell

number at any stage in development (Huang et al., 1999). Because the large majority of trigeminal neurons leave the cell cycle by E13.5 (E14.5 in rat, White et al., 1995), we employed BrdU labeling at E13.5, followed by analysis at E14.5, to assess directly whether neurons were delayed in cell cycle exit in Brn3a$^{-/-}$ganglia (Figure 4). Immunofluorescence for Islet1 was used to identify differentiating sensory neurons in the absence of Brn3a. Less than 2% of TG neurons incorporated BrdU at E13.5 in either control or Brn3a$^{-/-}$ knockout embryos, indicating that cell cycle exit is not significantly delayed in the absence of Brn3a.

De-repression of a cardiac gene expression program in the Brn3a$^{-/-}$ trigeminal ganglion

This global analysis of gene expression in E13.5 Brn3a$^{-/-}$ TG revealed that a significant number of the most increased transcripts are not expressed in control ganglia at either E11.5 or E13.5. Unexpectedly, several of these genes are known to be expressed in the developing heart, and in some cases structures derived from cranial mesoderm, including Ankrd1 (CARP), Lmcd1, Zfpm2 (Fog2), Figf (VegfD), Tcfap2d, Irx2, Msc (MyoR), Nkx2–5 (tinman), and Actc1 (cardiac muscle alpha actin 1). In addition, we have previously shown that expression of another regulator of cardiac development, Fgf10, is markedly increased in the TG of Brn3a$^{-/-}$ embryos. Fgf10 is not detected by the microarrays used to generate the current data set, due to a flaw in the design of the Fgf10 oligonucleotide probe set on the arrays used for these studies (Cox et al., 2006).

Increased expression of Irx2, Msc, and Fgf10 in the TG of Brn3a$^{-/-}$ mice has been previously verified by in situ hybridization (Cox et al., 2006; Eng et al., 2004). The novel changes in cardiac/mesoderm gene expression detected here were verified using in situ hybridization and RT-PCR with specific primer sets (Figure 5). In situ analysis of Lmcd1 and Prox2 revealed widely distributed expression in the TG of Brn3a$^{-/-}$ embryos, while Eya1 appeared restricted to a subset of neurons in the periphery of the ganglion. RT-PCR assays of these transcripts, as well as Ankrd1, Zfpm2, Tcfap2d, and Nkx2.5, generally revealed increases in expression at least as large the microarray analysis.

In order to understand the developmental timing and tissue specificity of the de-repression of genes associated with cardiac/mesodermal development in Brn3a$^{-/-}$embryos, we compared results for the E11.5 TG and E13.5 DRG for this set of genes (Figure 6, Table S6, S7). Tcfap2d, Irx1, Irx2, Figf, Zfpm2, and Actc1 were all abnormally expressed by E11.5 in the TG, and these changes constitute some of the largest magnitude changes observed at E11.5 (Table S5). However, increased expression of Ankrd1, Lmcd1 and Nkx2.5 was not observed until E13.5. In contrast, the expression of cardiac-related transcripts in the E13.5 Brn3a$^{-/-}$ DRG was minimal, suggesting that only the TG retains the potential to express this set of genes during sensory neurogenesis.

Recently, it has been reported that Brn3a is expressed from ~E10.5 onward in the developing mouse heart (Farooqui-Kabir et al., 2008). Cell-specific expression was reported from E13.0 in the atrio-ventricular (AV) valve cushions and leaflets, outflow tract, epicardium and cardiac ganglia, and at E14.5 in the intraventricular septa and ventricular myocardium. Because these results might relate to the observed regulation of cardiac genes by Brn3a in the TG, we examined the developing heart for expression of Brn3a protein (Figure S1, S2). The antibody used for Brn3a detection in these studies (Fedtsova and Turner, 1995) was generated to a region immediately N-terminal to the DNA binding POU domain, and should recognize both the reported “long” and “short” forms of Brn3a (Theil et al., 1993). The Brn3a antibody employed here gave a very strong signal in the DRG and spinal cord in the same sections used to examine heart expression (Figure S1D,H; Figure S2, B,D,F). However, in contrast to the previously reported results, we were unable to detect Brn3a protein in any of the structures examined in the previous study (Figure S1C,E,F; Figure S2A,C,E). We conclude that the dysregulation of cardiogenic genes in the trigeminal

ganglion of Brn3a$^{-/-}$ embryos does not reflect a role for Brn3a in normal heart development, but rather an ectopic program of gene expression which must be suppressed during trigeminal neurogenesis.

DISCUSSION

Delayed progression of sensory development in Brn3a$^{-/-}$ mice

Results reported here, together with earlier studies of the TG and DRG (Eng et al., 2007; Eng et al., 2004), together provide a comprehensive view of the regulatory role of Brn3a in sensory neurogenesis. First, Brn3a$^{-/-}$ knockout mice exhibit a pervasive delay in gene regulatory programs which characterize the transition from neurogenesis to definitive sensory differentiation. In the TG, a feature of this delay is the persistent expression of genes associated with cell division, but several factors argue against a key role for Brn3a in mediating cell cycle exit. First, expression of cell-cycle associated genes is only moderately increased in Brn3a$^{-/-}$ TG compared to controls. Second, significantly increased expression of cell cycle genes is not observed in Brn3a$^{-/-}$ DRG (Eng et al., 2007), arguing that it is not an essential mediator of cell cycle exit. Third, TG neurons are not increased in number at any stage in Brn3a$^{-/-}$ ganglia (Huang et al., 1999), and, as shown here, do not show persistent incorporation of BrdU beyond the usual time of cell cycle exit.

Loss of Brn3a also results in the abnormal persistence of neurogenic bHLH factors and other regulators associated with early neurogenesis. Brn3a has a well-established role in terminating the expression of Neurod1 and Neurod4 in the TG and DRG (Eng et al., 2004; Lanier et al., 2007). In the present report, profiling of gene expression across developmental time has shown that multiple transcription factors in several classes show a failure in their normal developmental down-regulation from E11.5 to E13.5 in the Brn3a$^{-/-}$ TG, including Tcfap2b (Ap2β), Eya1, Eya2, Insm1, Neurog1, Shox2, Six1, Zeb2 (Zfhx1b) and Nhlh2. The majority of these changes are also observed in the DRG, demonstrating a pan-sensory role for Brn3a in repressing genes associated with early sensory development.

Brn3a$^{-/-}$ TG exhibit decreased expression of a battery of genes associated with definitive sensory neurogenesis and subtype specification. Data from the current study confirm earlier studies showing that Brn3a is necessary for correct expression of the neurotrophin receptors TrkA and TrkC, with a particularly early and profound loss of TrkC expression (Huang et al., 1999; McEvilly et al., 1996; Xiang et al., 1996). These results also confirm markedly decreased expression of the runt-domain transcription factors Runx1 and Runx3 (Eng et al., 2007; Eng et al., 2004), which are required for the correct specification of nociceptors and proprioceptors, respectively (Chen et al., 2006; Kramer et al., 2006). These changes in gene expression at E11.5 and E13.5 are not likely to be attributable to selective death of trigeminal neurons, because significant reduction in trigeminal populations are not observed until E14.5 (Huang et al., 1999). The results presented here suggest that in the absence of Brn3a, trigeminal neurons fail to express a battery of channels and receptors associated with sensory function (Npy1r, Ntrk1/TrkA, Ntrk3/TrkC, Prokr1, Scn7a, Scn10a, Trpv1), but also exhibit increased expression of a number of markers (Htr3a, Ntrk2/TrkB, Slc6a1/Gat1, Sst/somatostatin, Trpa1,) suggesting a switch in phenotype, not simply failed maturation. A detailed analysis of phenotypic switching in Brn3a$^{-/-}$ sensory ganglia will be the subject of a separate report.

Brn3a is required to repress a cardiac/cranial mesoderm-related program of gene expression

An unexpected finding of this analysis of global gene expression in the Brn3a$^{-/-}$ TG is the derepression of a set of genes which are strongly associated with development of the heart,

although Brn3a$^{-/-}$ TG neurons retain their neuronal properties and in no sense differentiate into cardiac cells. Detailed studies over the last decade have elucidated the early morphological and molecular events of cardiac development that provide a context for these changes in gene expression. Bilateral cardiac progenitors arise within the splanchnic lateral mesoderm, condensing at the midline to form the heart tube. Early growth of the heart occurs by addition of progenitors to both anterior and posterior poles of the forming heart (van den Berg et al., 2009). The source of these progenitors is known as the second heart field which is located in pharyngeal mesoderm, including that of the pharyngeal arches (Abu-Issa and Kirby, 2007; Buckingham et al., 2005; Eisenberg and Markwald, 2004). Cardiac neural crest also migrates through the pharyngeal arches to contribute to septation of the outflow tract and aortic arch arteries (Hutson and Kirby, 2007).

Several of the genes exhibiting increased expression in the Brn3a$^{-/-}$ TG are associated with key events or cell populations in this developmental pathway. The homeodomain transcription factor Nkx2.5 (tinman), is first expressed in the lateral plate mesoderm, although loss of Nkx2.5 function does not arrest cardiac development until the looping stage (Biben and Harvey, 1997). Ankrd1 (originally described as CARP, for cardiac adriamycin responsive protein and also cardiac ankyrin repeat protein) is expressed in the heart tube at E8.5, and is downstream of Nkx2.5 (Jeyaseelan et al., 1997; Kuo et al., 1999). Ankrd1 is localized to the nucleus, and may be a transient repressor of some definitive cardiac muscle genes.

Zfpm2 (Fog2, friend of GATA 2) is a zinc-finger transcription factor expressed in the cardiac primordium from E8.5, and it is necessary for the development of both myocardium and cardiac vasculature (Tevosian et al., 2000). Zfpm2 interacts with Gata4 (Svensson et al., 1999), which itself is necessary for cardiac development (Kuo et al., 1997; Xin et al., 2006; Zeisberg et al., 2005). None of the Gata4/5/6 factors expressed in the developing heart exhibited increased expression in the Brn3a$^{-/-}$ TG, but a related factor, Gata3, was markedly increased. Because Zfpm2 can interact with Gata3 as well as Gata4 (Tevosian et al., 1999), dysregulation of Gata3 expression may intersect this pathway. Also potentially interacting with Gata signaling is Lmcd1 (LIM and cysteine-rich domains 1, dyxin), which is a LIM-domain containing protein expressed relatively late (E12.5) in the developing myocardium. Lmcd1 can bind to multiple GATA family proteins and is thought to repress GATA activity by an alternate mechanism to Fog2 (Rath et al., 2005).

In a prior study we have reported that Brn3a$^{-/-}$ TG exhibit markedly increased expression of another known regulator of cardiac development, Fgf10 (Cox et al., 2006). Fgf10 expression is not effectively assayed by the microarrays used in the present study (Affymetrix 430v2) due to a design flaw in the probe set for this transcript, but increased expression was detected on an earlier generation array and confirmed by in situ hybridization. Fgf10 is a marker of pharyngeal mesoderm cells which contribute to the outflow tract of the heart (Kelly et al., 2001), although the cardiac effects of Fgf10 ablation are subtle and may reflect redundancy of function in the Fgf family (Marguerie et al., 2006). We have previously concluded that a local effect of Fgf10 de-repression is not likely to account for the extensive program of altered gene expression in the trigeminal ganglion, because the necessary receptors are not expressed there (Cox et al., 2006).

Other cardiac transcripts are expressed in Brn3a$^{-/-}$ TG, but there is little data on their function. Irx1 and Irx2 have been identified in the ventricular septum (Christoffels et al., 2000), however, Irx2 knockouts do not exhibit a cardiac phenotype (Lebel et al., 2003), and Irx1 null mice have not been reported. Tcfap2d (Ap2δ) is expressed in the myocardial primordium at E9.5-E10.5 (Zhao et al., 2003), but its function is unknown. Figf (c-fos-induced growth factor; VegfD, Vascular endothelial growth factor D) is expressed in the

atrio-ventricular cushion at E12.5 (Avantaggiato et al., 1998). Loss of Figf alone has a minimal phenotype (Baldwin et al., 2005), and Figf, the closely related VegfC, and their principal receptor VEGFR-3 appear to be involved primarily in angiogenesis and lymphangiogenesis rather than cardiac development (Haiko et al., 2008).

Brn3a represses of regulators of mesoderm development

Some changes in gene expression in Brn3a$^{-/-}$ sensory ganglia may reflect the derepression of gene expression programs which are mesodermal or myogenic, but not specifically cardiac. This is consistent with recent work which has established close developmental and molecular relationships between the cranial paraxial mesoderm, which gives rise to the cranial musculature, and the splanchnic mesoderm which contributes to the heart (Tzahor, 2009). The bHLH transcription factor Msc (musculin, MyoR) is increased in the TG of Brn3a$^{-/-}$ mice, but unlike the majority of cardiac-associated genes, it is also de-repressed in the DRG (Eng et al., 2007). Consistent with this, Msc is normally expressed in cranial mesoderm and the anterior heart field (von Scheven et al., 2006), but is also expressed in paraxial mesoderm and developing muscles at trunk levels. Double knockouts of MyoR and the related bHLH factor capsulin result in specific loss of the muscles of mastication derived from the first branchial arch, but these mice survive until birth and cardiac abnormalities have not been reported (Lu et al., 2002). Another functionally interacting group of transcription factors which are increased in the Brn3a$^{-/-}$TG include Dach2, the *eyeless*-related genes Eya1, Eya2, and Eya4 and the *sine oculis* homologue Six1. These factors have known roles in somitogenesis and skeletal myogenesis, where they interact with each other and with Pax3 (Heanue et al., 1999). Together these results suggest that in addition to repressing canonical regulators of heart development, Brn3a represses a gene expression program which relates to general aspects of mesoderm development. However, we did not observe increased expression of any of the canonical regulators of definitive myogenesis, including Myod1, Myf5, Myf6 (MRF4), or Myog (myogenin), in the Brn3a$^{-/-}$ TG.

Given the extensive network of transcription factors regulated by Brn3a in sensory ganglia, it is difficult to predict whether any given target is regulated by direct Brn3a binding to cis-elements within that gene locus. In prior work we have used chromatin immunoprecipitation in embryonic sensory ganglia to demonstrate direct repression of Neurod1 and Neurod4 by Brn3a, but examination of the Msc locus from −20kb to +20kb relative to the start of transcription did not reveal Brn3a binding (Lanier et al., 2007). Similar examinations of the Gata3 and Fgf10 loci also did not reveal direct interactions in the E13.5 TG (J. Lanier, unpublished results). Although this suggests indirect regulation of these targets, in some cases the relevant enhancers may lie outside the regions examined. In principle, the direct targets of Brn3a in embryonic sensory neurons could be determined by genome-wide examination of immunoprecipitated chromatin using tiled arrays or high-throughput sequencing (ChIP-chip, ChIP-seq), but application of the current technologies is precluded by the small size of the available tissue samples.

The de-repression of cardiac gene expression in Brn3a$^{-/-}$ trigeminal neurons leads to the question of what underlying developmental process may link the developing TG and heart. One explanation is suggested by the observation that cells contributing to the TG and the heart have a common developmental origin in the cranial neural crest. Cardiac neural crest cells originate at hindbrain levels of the dorsal neural tube, contribute to the aorticopulmonary septum, and have an indirect role in the extension of the outflow tract by the secondary heart field (Hutson and Kirby, 2007). It is possible that Brn3a helps to discriminate the crest-derived sensory neural lineage from the cardiac lineage by repressing a battery of cardiac genes. However, the cardiac genes which are de-repressed in the Brn3a$^{-/-}$ TG are not restricted to the cardiac structures derived from neural crest, arguing for a more general role in the repression of cardiac/cranial mesoderm genes.

A second potential connection between Brn3a and cardiac development may relate to the activity transcription factor Islet1, which is co-expressed with Brn3a in sensory ganglia (Fedtsova et al., 2003). Islet1 is expressed extensively in the developing heart (Cai et al., 2003), where Brn3a is absent, and recent work has shown that Islet1^{+} precursors also contribute to a subset of cranial muscles (Nathan et al., 2008). The regulatory targets of Islet1 in heart development are largely unknown, but it is possible that the repressor activity of Brn3a opposes the activity of Islet1 on a set of cardiac/cranial muscle genes during trigeminal neurogenesis. However, if this is the case, there must be a redundant mechanism for repression in the DRG, since Islet1 is also expressed in DRG neurons, and loss of Brn3a is not sufficient to activate ectopic expression of most of the dysregulated cardiac genes at trunk levels.

Understanding occult targets of transcriptional repression

Perhaps the most important generalization derived from this study is that it provides striking evidence for the importance of transcriptional repression as a mechanism for generating correct neuronal phenotypes. Early models of cell-specific gene regulation, based largely on viral systems, emphasized transcriptional activation (Ptashne, 1988). Although such mechanisms are essential, it is now clear that to a large extent neuronal phenotypes are defined in development by overlapping patterns of transcriptional repression (Muhr et al., 2001), whose effects may then be stabilized by chromatin modifications (Allen, 2008). Thus to a large extent, neurons appear to be defined by what they are not, through progressive restriction of cell fate.

Targets of transcriptional repressors are occult by nature: they cannot necessarily be predicted from the normal program of gene expression for the target tissue, and are only revealed in the absence of the repressing factor. The results presented here for Brn3a in the TG illustrate the kind of unexpected programs of gene expression which may emerge when these repressor functions are disabled. Conceptually similar results have been recently obtained in the sensory ganglia of mouse embryos lacking Islet1, which unexpectedly mis-express of a set of spinal cord and hindbrain genes (Sun et al., 2008). Unbiased global assays of gene expression are thus essential to understanding repressor function, and are likely to reveal many more hidden relationships between developing cell types which may be important for applications such as the manipulation of stem cell fates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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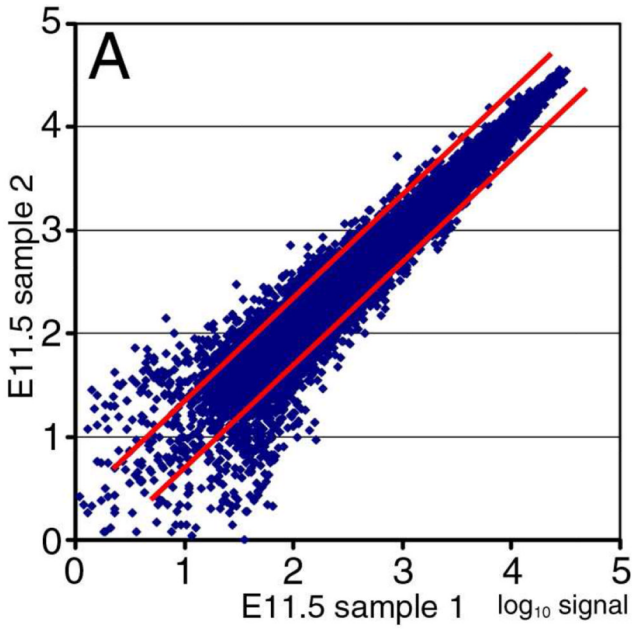

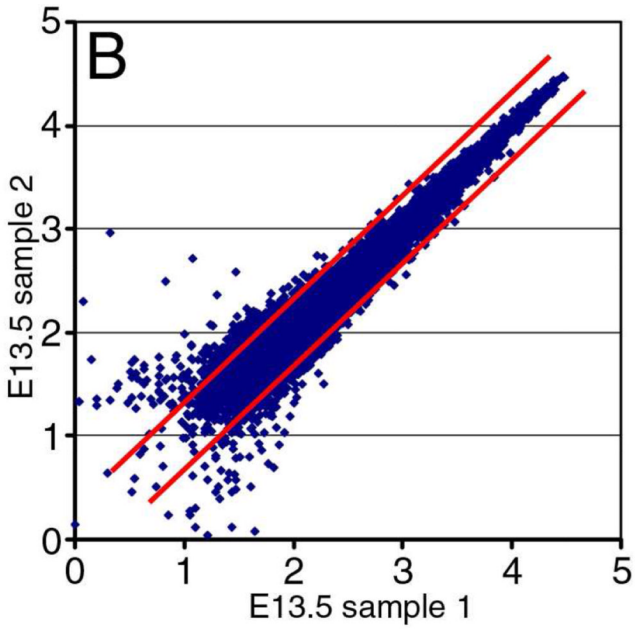

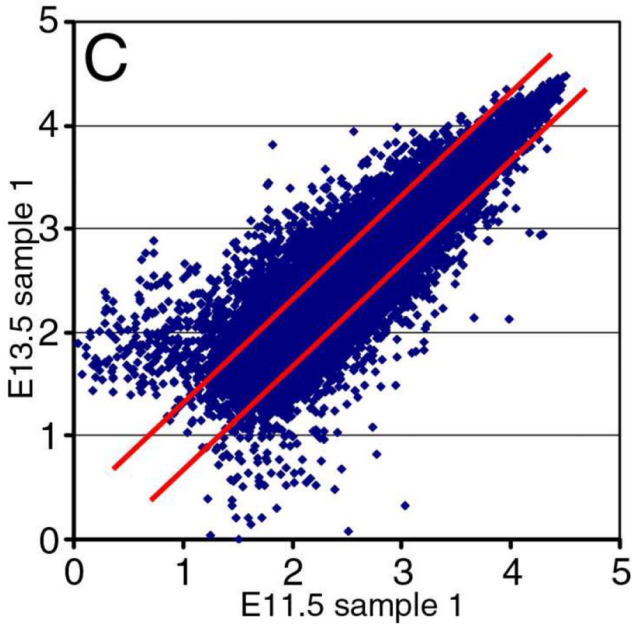

Probe sets:	45,037
Present in at least one condition:	17,351
E11.5 > E13.5	**E13.5 > E11.5**
2,979 probe sets	2,991 probe sets
2,100 unique genes	2,159 unique genes
686 >2 fold change	918 >2 fold change

Figure 1. Global developmental changes in TG gene expression

Microarray analysis was performed for E11.5 and E13.5 TG. For all comparisons, we defined a set of 17,251 probe sets, out of 45,037 the total probe sets on the array, which were reproducibly present in at least one of four conditions examined (E11.5 Brn3a$^{+/+}$, Brn3a$^{-/}$; E13.5 Brn3a$^{+/+}$, Brn3a$^{-/-}$).

(A,B) Comparison of global gene expression between replicates at E11.5 and E13.5. Repeat arrays for TG of a given stage and genotype were highly replicable.

(C) Comparison of global gene expression between E11.5 and E13.5. A significant fraction of transcripts were differently expressed at E11.5 and E13.5, with approximately 2,100 unique probe sets showing changes in each direction from E11.5 to E13.5. Diagonal lines represent 2-fold change. The most-changed transcripts appear in Table S1 and Table S2.

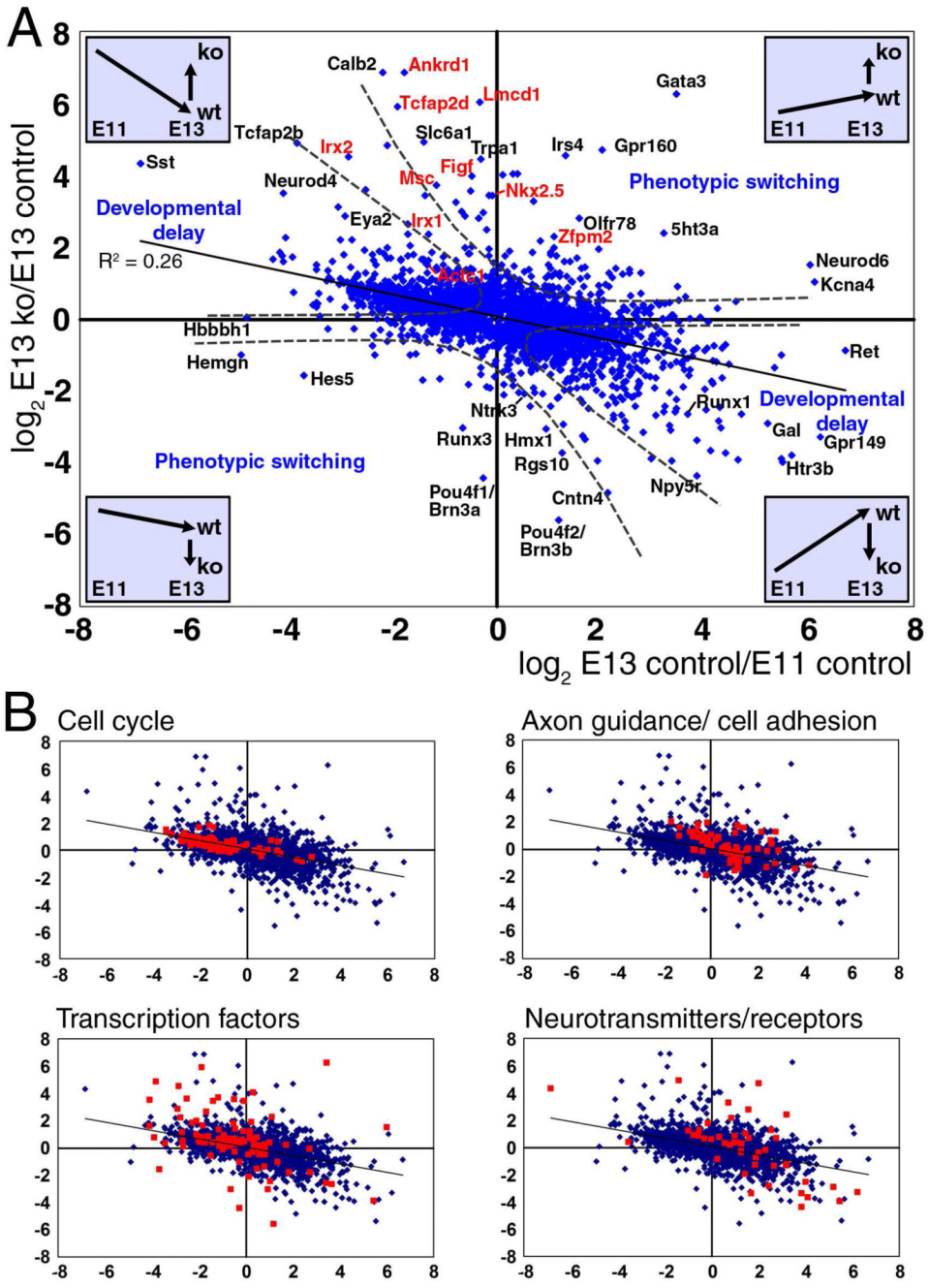

Figure 2. Interaction of developmental stage and Brn3a genotype

(A) Plot of the magnitude of developmental expression changes between E11.5 and E13.5 in control TG, versus changes resulting from the knockout of Brn3a at E13.5. Data are shown for probe sets representing 4194 identified, unique genes significantly changed (change $p < 0.002$, > 0.998) in one or both conditions. Changes in the Brn3a knockout TG are in most cases inversely correlated with developmental changes in gene expression, such that loss of Brn3a expression partially prevents or delays the changes in gene expression associated with sensory maturation. Dashed lines demarcate regions of the plot containing transcripts which tend to follow this trend ("developmental delay") and a much smaller number of transcripts for which loss of Brn3a exaggerates normal developmental change and which may represent

the expansion or loss of a specific sub-populations of differentiated neurons (“phenotypic switching”). The subset of transcripts highlighted in red have known roles in cardiac development but not the trigeminal ganglion.

(B) Distinct classes of transcripts are differentially expressed during development and in Brn3a knockout TG. Mapped transcripts include 123 cell cycle genes, 67 axon guidance/cell adhesion genes, 105 transcription factors, and 46 neurotransmitter system genes (including receptors and receptor-channels) derived from GO annotation and literature search. As expected, cell cycle genes are decreased in development, and increased in the Brn3a knockout, while neurotransmitter system genes exhibit the opposite behavior. Axon guidance/cell adhesion genes and transcription factors have variable roles with respect to developmental processes and show mixed patterns of expression changes.

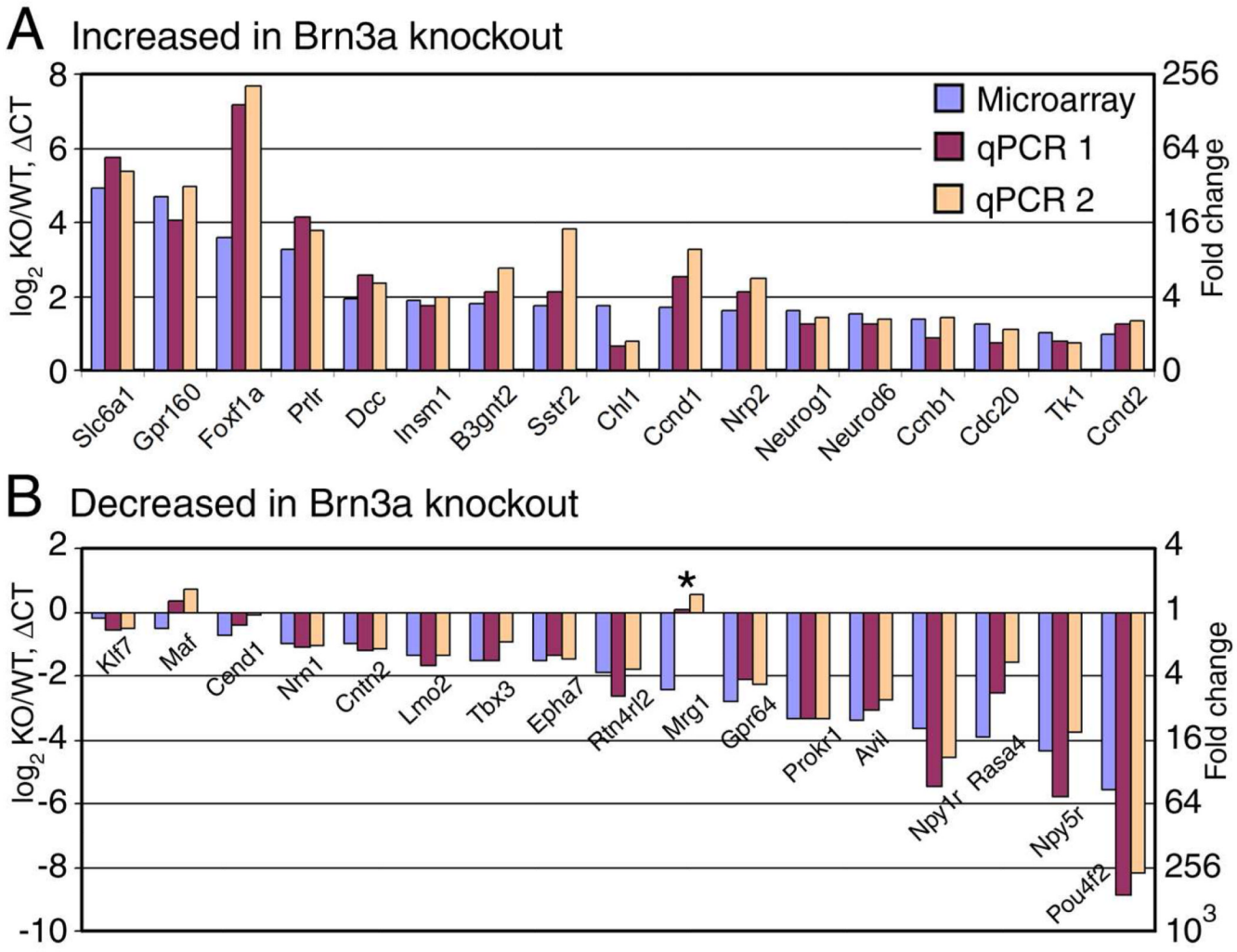

Figure 3. Quantitative PCR validation of increased and decreased transcripts in E13.5 Brn3a$^{-/-}$ trigeminal neurons

Changes are expressed as the $\log_2$ of the fold change in expression for Brn3a$^{-/-}$ ganglia relative to controls in the microarray assay, or as the comparable ΔCT value in quantitative RT-PCR assays.

(A) RT-PCR validation of transcripts increased in the Brn3a$^{-/-}$ TG.

(B) RT-PCR validation of transcripts decreased in the Brn3a$^{-/-}$ TG.

The direction and magnitude of changes determined by these two methods are similar for all transcripts exhibiting >2-fold change on the microarray, except for Mrg1 (asterisk). Findings were consistent across several functional categories, including cell cycle regulators (Ccnd1, Ccnb1, Cdc20, Tk1, Cend1), axon guidance/cell adhesion (DCC, B3gnt2, Chl1, Nrp2, Epha7, Rtn4rl2), transcription factors (Foxf1a, Insm1, Neurog1, Neurod6, Klf7, Lmo2, Tbx3, Pou4f2) and neurotransmitter systems (Slc6a1, GPR160, Sstr2, Prokr1, Npyr1, Npyr5).

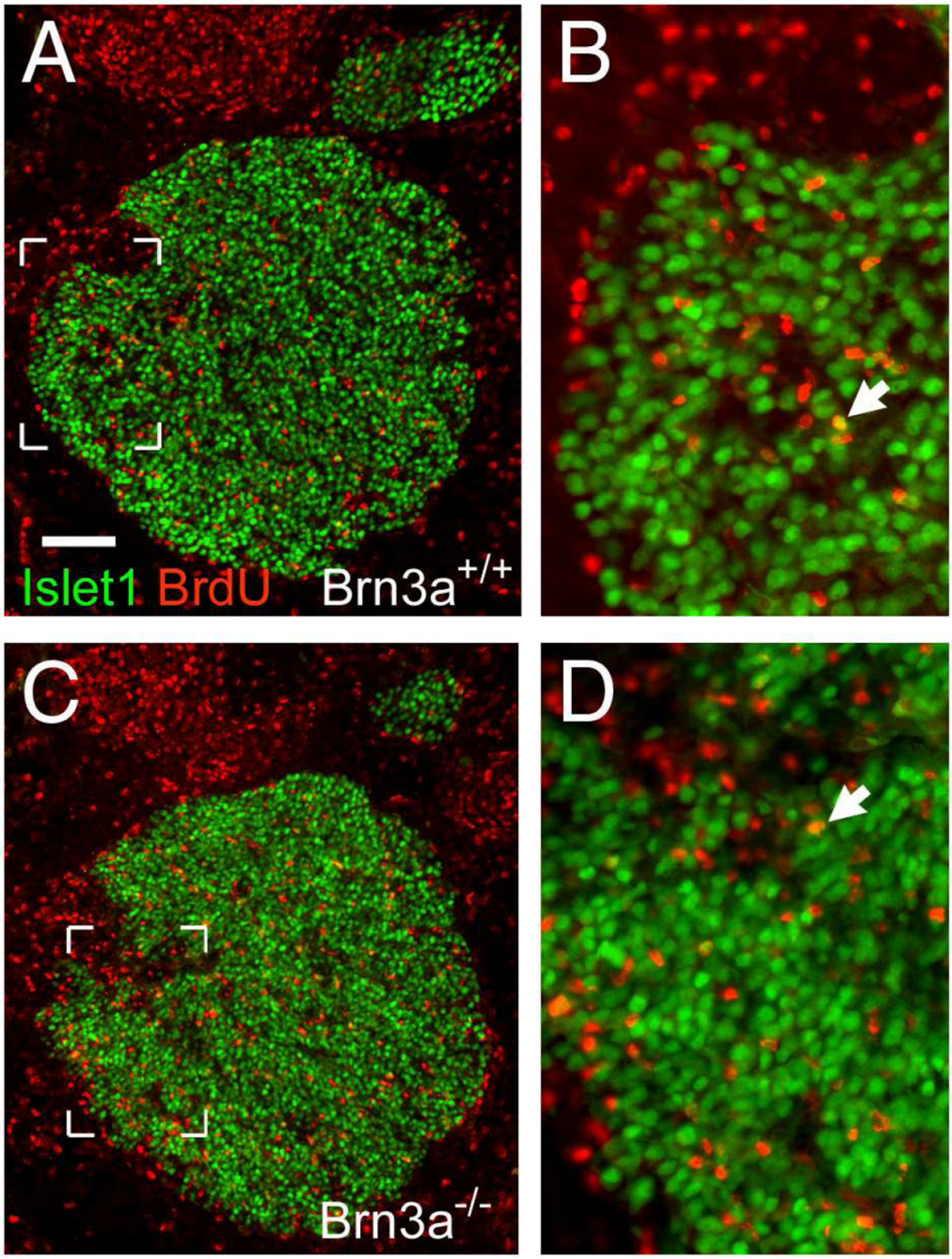

Figure 4. Confirmation of cell cycle exit in Brn3a$^{-/-}$ trigeminal neurons

Serial BrdU injections were performed at E13.5 to label dividing cells, and Brn3a$^{+/+}$ (A,B) and Brn3a$^{-/-}$ (C,D) embryos were harvested at E14.5 (Methods). Islet1 immunofluorescence was employed as marker of sensory neurons. BrdU incorporation in differentiating neurons is indicated by arrows in (B,D), and was rarely observed at this stage (<2% of Islet1^{+} cells) in either genotype. Scale: 50μM.

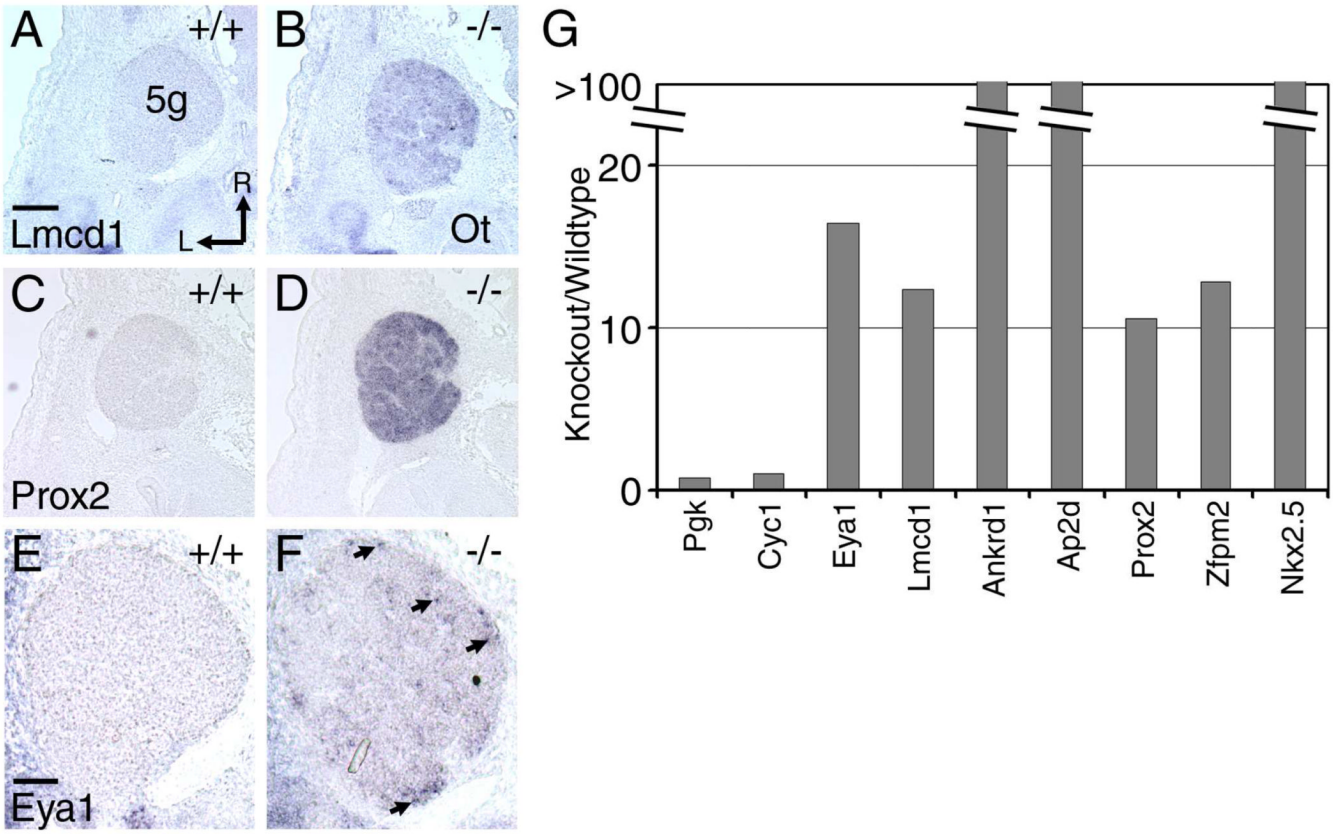

Figure 5. Altered expression of genes associated with cardiac/mesodermal development in developing Brn3a$^{-/-}$ trigeminal neurons

Embryos were harvested at E13.5 for analysis by in situ hybridization and quantitative RT-PCR.

(A–F) Expression of Lmcd1, Prox2 and Eya1 in the TG of Brn3a$^{+/+}$ (A,C,E) and Brn3a$^{-/-}$(B,D,F) embryos. Sections are in the horizontal plane. Increased expression of Lmcd1 and Prox2 was noted throughout the TG. Increased expression of Eya1 was observed predominantly in select neurons near the periphery of the ganglion (arrows, F).

(G) Quantitative RT-PCR assays of mRNA levels in the TG of Brn3a$^{+/+}$ and Brn3a$^{-/-}$ embryos. Phosphoglycerate kinase (Pgk) and Cytochrome C1 (Cyc1) were used as controls. Fold change was calculated by the cycle threshold difference method. Values are means of at least two determinations. Ankrd1, Ap2d and Nkx2.5 mRNA was not detectable in wildtype ganglia after 40 cycles of amplification, and a specific fold-increase in expression could not be calculated, but exceeded 100-fold. 5g, trigeminal ganglion; Ot, otic region; R, rostral, L, lateral. Scale: A–D, 200μM; E,F, 100μM.

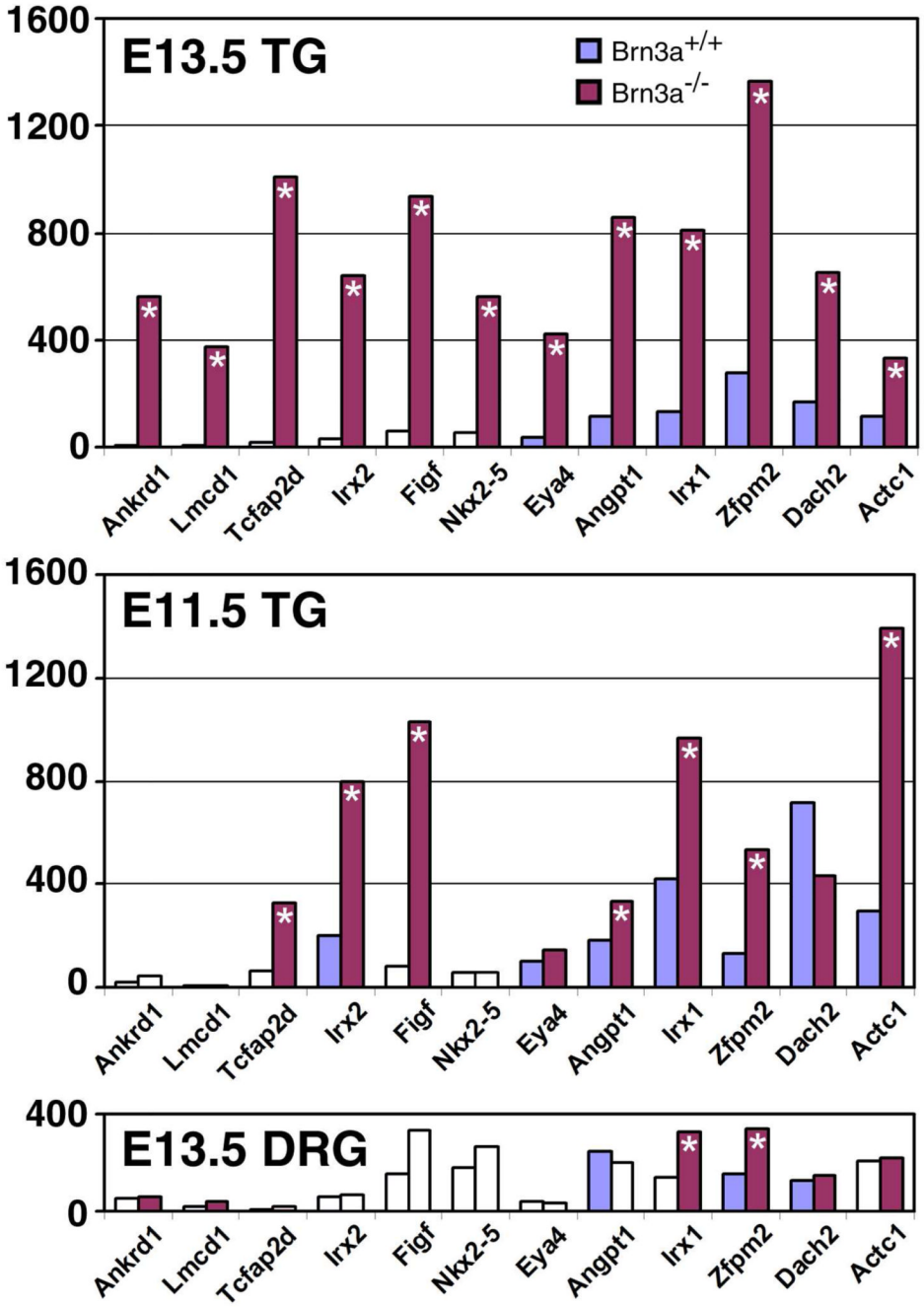

Figure 6. Timing and specificity of de-repression of cardiac genes in developing sensory neurons

Microarray data for Brn3a$^{+/+}$ and Brn3a$^{-/-}$ were obtained as described in Methods. Data for the E13.5 DRG are derived from a previously described data set (Eng, et al., 2007). Concordant increased (I) calls in replicate assays for a given transcript are indicated by an asterisk. Uncolored bars indicate expression below the threshold of reliable detection for the probe set and condition shown (absent calls).

Table 1

Gene ontology analysis of transcripts increasing and decreasing between E11.5 and E13.5 in the developing trigeminal ganglion

Of 45037 probe sets on the array, 2606 were reproducibly increased from E11.5 to E13.5 and 2451 were decreased. Selected GO categories are shown, complete data appear in Supplementary Table S3.

	Total genes in category		Changed genes in category		
Category	**Number**	**Percent**	**Number**	**Percent**	**p-Value**
Increasing in development:					
Transmission of nerve impulse	341	1.4	57	3.8	4.39E-12
Synaptic transmission	289	1.2	51	3.4	7.46E-12
Cation transport	950	3.9	107	7.2	6.89E-10
Nervous system development	1089	4.5	117	7.9	1.81E-09
Cell-cell signaling	485	2.0	65	4.4	2.39E-09
Synaptic vesicle transport	51	0.2	16	1.1	3.38E-08
Cell adhesion	1209	5.0	120	8.1	1.02E-07
Regulation of neurotransmitter levels	123	0.5	24	1.6	3.85E-07
Potassium ion transport	322	1.3	44	3.0	5.22E-07
Neurite morphogenesis	326	1.3	44	3.0	7.37E-07
Cell development	639	2.6	70	4.7	1.77E-06
Neurotransmitter secretion	90	0.4	19	1.3	1.78E-06
Exocytosis	144	0.6	25	1.7	2.16E-06
Cellular morphogenesis during differentiation	364	1.5	46	3.1	2.64E-06
Axonogenesis	273	1.1	37	2.5	4.95E-06
Synaptic vesicle exocytosis	19	0.1	8	0.5	8.02E-06
Neurophysiological process	772	3.2	76	5.1	3.08E-05
Calcium ion transport	203	0.8	28	1.9	0.000049
Decreasing in development:					
RNA metabolism	939	3.9	170	10.5	9.21E-34
DNA metabolism	1227	5.1	189	11.7	4.23E-28
Cell cycle	1424	5.9	194	12.0	3.16E-22

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	Total genes in category		Changed genes in category		
Category	**Number**	**Percent**	**Number**	**Percent**	**p-Value**
Ribosome biogenesis and assembly	249	1.0	63	3.9	1.27E-20
Cytoplasm organization and biogenesis	286	1.2	64	4.0	5.55E-18
M phase	377	1.6	75	4.6	9.59E-18
Cell division	430	1.8	79	4.9	1.55E-16
DNA replication	299	1.2	62	3.8	8.78E-16
DNA repair	392	1.6	73	4.5	1.07E-15
Mitotic cell cycle	438	1.8	74	4.6	1.29E-13

Table 2

Selected transcripts decreased in E13.5 TG of Brn3a knockout embryos

Microarray analysis was performed on duplicate samples for E13.5 TG, E11.5 TG, and E13.5 DRG from Brn3a WT and KO embryos. Complete data for the TG analysis appear in the Supplementary Tables S4–S7. DRG data are derived from a previously published data set (Eng 2007). Expression values associated with absent calls appear in parentheses. Transcripts are ranked by the fold decrease in E13.5 KO TG compared to WT controls. For the E13.5 TG WT to KO comparison, all transcripts shown exhibited a significant D call (p value >0.998) in both replicates. For all other comparisons, transcripts with replicated I or D change calls are indicated with an underscore. For all comparisons, fold change values for assays with an absent call in all conditions are not meaningful and are indicated by “abs”.

Changes for the E11.5 TG WT to KO comparison are generally concordant with the E13.5 results. However, several genes associated with sensory function were not yet expressed at E11.5 and are thus unchanged in the knockout. Underscored values indicate an D call in both replicates, with the exception of TrkA, which showed increased expression in the KO at E11.5. Changes for the E11.5 TG WT to E13.5 WT comparison are expressed such that values represent the fold increase in expression from E11.5 to E13.5. Increasing developmental expression is highly correlated with decreased expression in E13.5 KO ganglia (Figure 2). Changes for the DRG WT to KO comparison are frequently concordant with changes observed in the TG.

			Expression value							
			TG replicate 1		TG replicate 2					
Gene Symbol	**Gene Title**	**Class**	**E13 WT**	**E13 KO**	**E13 WT**	**E13 KO**	**E13 WT/ KO**	**E11 WT/ KO**	**E13 WT/ E13 WT/**	**E13 DRG WT/ KO**
Pou4f2	POU domain, class 4, factor 2; Brn3b	tx	891	(11)	910	(27)	**48.0**	23.5	2.3	4.5
Pou4f1	POU domain, class 4, factor 1; **Brn3a**	tx	5789	304	5847	241	**21.4**	27.5	0.8	5.4
Npy5r	Neuropeptide Y receptor Y5	nt	433	(20)	454	(23)	**20.5**	1.7	14.3	abs
Htr3b	5-hydroxytryptamine receptor 3B	nt	363	(27)	266	(13)	**15.6**	abs	44.6	abs
Pla2g7	Phospholipase A2, group VII	st	2479	172	2515	149	**15.5**	1.1	3.8	4.0
Bnc1	Basonuclin 1	tx	2638	205	2809	166	**14.7**	1.1	43.7	4.1
Rgs10	Regulator of G-protein signalling 10	st	5164	382	5931	458	**13.2**	7.1	2.4	3.7
Npy1r	Neuropeptide Y receptor Y1	nt	915	(80)	1073	(76)	**12.7**	abs	17.1	abs
Avil	advillin	nt	5647	538	6384	621	**10.4**	7.5	14.2	3.8
Prokr1	Prokineticin receptor 1	nt	680	60	675	73	**10.2**	1.7	3.2	2.3
Scn7a	Na^+ channel, voltage-gated, type VII, alpha	ch	3050	366	3089	351	**8.6**	2.7	10.1	2.1
Hmx1	H6 homeo box 1	tx	1766	209	1661	206	**8.3**	1.8	1.9	1.3

Gene Symbol	Gene Title	Class	Expression value				E13 WT/ KO	E11 WT/ KO	E13 WT/ E13 WT/	E13 DRG WT/ KO
			TG replicate 1		TG replicate 2					
			E13 WT	E13 KO	E13 WT	E13 KO				
Runx3	Runt related txn factor 3	tx	219	(28)	329	(40)	**8.1**	15.3	0.6	5.9
Gal	Galanin	nt	8851	1288	9107	1112	**7.5**	2.6	36.5	4.1
Gpr64	G protein-coupled receptor 64	nt	1944	238	1969	314	**7.1**	3.5	5.5	3.0
Kcnab2	K^+ voltage-gated channel, shaker-related subfamily, beta member 2	ch	2509	374	2868	422	**6.8**	1.8	4.7	3.4
Trpv1	Transient receptor potential channel, V1	ch	771	155	746	86	**6.3**	0.9	26.0	1.8
Runx1	Runt related transcription factor 1	tx	1905	310	1689	263	**6.3**	2.2	12.7	1.6
Hoxd1	Homeo box D1	tx	2797	522	3177	485	**5.9**	3.0	10.8	2.0
Ptpn3	Protein tyrosine phospha-tase, non-receptor type 3	st	7062	1112	7802	1539	**5.6**	4.3	3.4	2.2
Kcna1	K^+ channel, shaker-related subfamily, member 1	ch	3232	636	3260	645	**5.1**	2.5	3.1	2.0
Accn2	Acid sensing Na^+ channel ASIC1a	ch	2541	430	2676	609	**5.0**	0.9	4.8	1.3
Pou4f3	POU domain, class 4, transcription factor 3	tx	305	72	212	(47)	**4.3**	1.8	1.1	2.3
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3	ax	1064	263	1131	258	**4.2**	5.3	1.4	0.9
Scn10a	Na^+ channel, voltage-gated, type X, alpha	ch	312	112	289	131	**2.5**	1.7	3.4	1.5
Lxn	Latexin	nt	5857	2688	6703	2491	**2.4**	1.3	3.6	2.0
Ntrk1	neurotrophic tyrosine kinase, receptor, type 1; TrkA	ax	5214	2321	4429	2471	**2.0**	↑0.5	6.7	1.1
Prph	peripherin	ax	17231	9651	15825	10290	**1.7**	5.1	3.0	1.1

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Table 3

Selected transcripts increased in E13.5 TG and DRG of Brn3a knockout embryos

Microarray analysis was performed on duplicate samples for E13.5 TG, E11.5 TG, and E13.5 DRG from Brn3a WT and KO embryos. A subset of genes implicated in cardiac/cranial muscle development are marked in bold face. Expression values associated with absent calls appear in parentheses. Transcripts are ranked by the fold increase in E13.5 KO TG compared to WT controls. For the E13.5 TG WT to KO comparison, all transcripts shown exhibited a significant I call (p value <0.002) in both replicates. For all other comparisons, transcripts with replicated I or D change calls are indicated with an underscore. For all comparisons, fold change values for assays with an absent call in all conditions are not meaningful and are indicated by “abs”. Changes for the E11.5 TG WT to KO comparison are generally concordant with the E13.5 results. However, the failure in developmental repression of neurogenic genes such as Neurod1, Neurod4, and Neurog1 was not yet evident at E11.5. Changes for the E11.5 WT to E13.5 WT comparison are expressed such that a positive number represents the fold decrease in expression from E11.5 to E13.5. Decreasing developmental expression is highly correlated with increased expression in E13.5 KO ganglia (Figure 2). Underscored values indicate an D call in both replicates, with the exception of 5HT3a and Neurod6, for which the paradoxical developmental increase is indicated by an up arrow.

			Expression value							
			TG replicate 1		TG replicate 2					
Gene Symbol	Gene Title	Class	E13 WT	E13 KO	E13 WT	E13 KO	E13 KO/ WT	E11 KO/ WT	E11 WT/ E13 WT	E13 DRG KO/ WT
Calb2	Calbindin 2; calretinin	st	(11)	1888	(25)	2361	**116.7**	abs	abs	abs
Ankrd1	Cardiac ankyrin repeat domain 1 , CARP	cd	(9)	622	(1)	498	**115.5**	2.8	abs	1.2
Gata3	GATA binding protein 3	tx	(58)	3303	(31)	3502	**75.8**	288	abs	abs
Lmcd1	LIM and cys-rich domains 1; Dyxin	cd	(3)	382	(8)	370	**66.0**	abs	abs	abs
Tcfap2d	Transcription factor AP-2, delta	tx, cd	(12)	1051	(22)	968	**60.4**	5.3	abs	abs
Slc6a1	Gabt1, Gat1 GABA transporter	nt	(19)	512	(14)	487	**30.1**	2.6	2.6	5.1
Tcfap2b	Transcription factor AP-2 beta	tx	152	4797	178	4916	**29.4**	1.4	14.2	abs
Irx2	Iroquois related homeobox 2	tx, cd	(9)	672	47	612	**23.0**	3.9	7.3	abs
Trpa1	Transient receptor potential channel, A1	ch	(20)	301	(6)	257	**21.8**	abs	abs	abs
Sst	Somatostatin	nt	(132)	2360	(102)	2348	**20.1**	1.5	115.3	3.3
Prox2	Prospero homeobox 2	tx	167	2558	167	2980	**16.6**	9.1	0.8	3.7
Figf	C-fos induced growth factor; VEGF-D	cd	(59)	850	(61)	1021	**15.6**	12.4	1.4	2.1
Msc	Musculin; MyoR	tx, cd	308	3707	259	3696	**13.1**	5.3	2.3	4.4
Eya1	Eyes absent 1	tx	71	841	73	917	**12.1**	3.6	5.8	1.5
Neurod4	Neurogenic differentiation 4	tx	171	1614	113	1623	**11.4**	1.7	17.2	2.2

Gene Symbol	Gene Title	Class	Expression value: TG replicate 1 E13 WT	TG replicate 1 E13 KO	TG replicate 2 E13 WT	TG replicate 2 E13 KO	E13 KO/ WT	E11 KO/ WT	E11 WT/ E13 WT	E13 DRG KO/ WT
Nkx2.5	NK2 transcription factor related, locus 5	tx, cd	(45)	535	(59)	583	**10.8**	abs	abs	abs
Eya4	Eyes absent 4	tx, cd	30	383	48	459	**10.7**	1.4	2.6	abs
Prlr	Prolactin receptor	nt	(55)	522	(50)	492	**9.6**	abs	abs	abs
Pcdh8	Protocadherin 8	ax	209	1691	175	1619	**8.6**	2.0	8.3	2.2
Eya2	Eyes absent 2	tx	120	1044	196	1269	**7.3**	2.0	7.5	1.5
Angpt1	Angiopoietin 1	other	136	865	100	846	**7.3**	1.9	1.5	0.8
Olfr78	Olfactory receptor 78	nt	141	801	102	887	**6.9**	2.8	0.3	1.5
Irx1	Iroquois related homeobox 1	tx, cd	114	794	146	826	**6.2**	2.3	3.3	2.3
Htr3a	5-HT receptor 3A	nt	1078	5064	1076	6365	**5.3**	4.4	↑0.1	2.2
Zfpm2	Zinc finger protein, multitype 2; FOG-2	tx, cd	259	1340	300	1385	**4.9**	4.1	0.5	2.2
Pdlim4	PDZ and LIM domain 4; Ril	other	373	1541	346	1789	**4.6**	1.2	8.0	abs
Dach2	Dachshund 2	tx	184	635	149	668	**3.9**	0.6	4.3	abs
Dcc	Deleted in colorectal carcinoma	ax	185	736	198	734	**3.8**	1.6	1.1	2.0
Etv1	Ets variant gene 1	tx	403	1311	284	1256	**3.7**	2.2	0.8	0.7
Insm1	Insulinoma-associated 1	tx	777	2739	787	2988	**3.7**	1.3	4.8	3.1
Ccnd1	Cyclin D1	cy	482	1593	514	1623	**3.2**	1.4	4.1	1.2
Neurod1	Neurogenic differentiation 1	tx	3725	11231	3833	12811	**3.2**	1.2	3.6	1.6
Neurog1	Neurogenin 1	tx	137	537	339	901	**3.0**	1.2	17.3	abs
Neurod6	Neurogenic differentiation 6	tx	413	1218	409	1129	**2.9**	2.7	↑0.02	3.1
Six1	Sine oculis-related homeobox 1 homolog	tx	1204	2317	935	2173	**2.1**	1.5	2.2	1.7

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