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En1 directs superior olivary complex neuron positioning, survival, and expression of FoxP1

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Abstract

Little is known about the genetic pathways and transcription factors that control development and maturation of central auditory neurons. En1, a gene expressed by a subset of developing and mature superior olivary complex (SOC) cells, encodes a homeodomain transcription factor important for neuronal development in the midbrain, cerebellum, hindbrain and spinal cord. Using genetic fate-mapping techniques, we show that all En1-lineal cells in the SOC are neurons and that these neurons are glycinergic, cholinergic and GABAergic in neurotransmitter phenotype. En1 deletion does not interfere with specification or neural fate of these cells, but does cause aberrant positioning and subsequent death of all En1-lineal SOC neurons by early postnatal ages. En1-null cells also fail to express the transcription factor FoxP1, suggesting that FoxP1 lies downstream of En1. Our data define important roles for En1 in the development and maturation of a diverse group of brainstem auditory neurons.

Keywords

hearing; brainstem; auditory; deafness; nucleogenesis

Introduction

The mammalian auditory brainstem contains a diverse set of neurons organized into several discrete nuclei. In mice, these neurons are generated on embryonic days 9.5-13.5 (E9.5-E13.5) by neuroepithelial precursors located in rhombomeres 2-5 (r2-5) of the developing

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brainstem (Bruce et al., 1997; Di Bonito et al., 2013; Farago et al., 2006; Maricich et al., 2009; Marín and Puelles, 1995; Pierce, 1973). Nascent auditory neurons migrate to the developing cochlear nuclei, superior olivary complex (SOC) and ventral nuclei of the lateral lemniscus (VNLL). Collectively, these neurons play central roles in sound processing important for all aspects of hearing.

The genetic factors that generate auditory neuron heterogeneity are incompletely understood. In the cochlear nucleus, the basic helix-loop-helix transcription factors Atoh1 and Ptf1a are instrumental for generating excitatory glutamatergic and inhibitory GABAergic/glycinergic neurons, respectively (Fujiyama et al., 2009). Glutamatergic SOC neuron specification also requires Atoh1 (Maricich et al., 2009; Rose et al., 2009), while development of cholinergic (Ach) olivocochlear neuron projections relies upon the zinc finger transcription factor GATA3 (Bruce et al., 1997; Pata et al., 1999). However, genes that regulate production of GABAergic, glycinergic and mixed neurotransmitter phenotype SOC neurons are unknown. Recently, four transcription factors (En1, Foxp1, MafB and Sox2) were found to be expressed in the SOC of developing and adult mice (Marrs et al., 2013; Simon et al., 2001). These genes are important for neuronal development in several CNS regions, but what roles they play in SOC development are unknown.

En1 encodes a homeodomain transcription factor that is conserved across multiple species (Davis et al., 1991; Joyner and Martin, 1987). In Drosophila, engrailed deletion causes homeotic transformations that lead to aberrant cell fate decisions (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975). Similar transformations also occur in developing limbs of En1-null mice (Loomis et al., 1996). In the mouse CNS, En1 is necessary for neuronal specification in the cerebellum, tectum, brainstem noradrenergic and serotonergic systems, and in the spinal cord. En1-null precursor cells in these regions do not adopt aberrant fates, but rather exhibit developmental arrest followed by death (Matise and Joyner, 1997; Simon et al., 2001; 2005; Wurst et al., 1994). En1 is also required for survival but not specification of midbrain dopaminergic neurons and for survival of serotonergic neurons during late embryonic development (Fox and Deneris, 2012; Simon et al., 2001).

We recently showed that conditional deletion of En1 in r3/5 led to the absence of medial and ventral nuclei of the trapezoid body (MNTB and VNTB) neurons in adult mice (Jalabi et al., 2013). However, it is not clear why these cells are missing or whether En1 deletion affects other SOC neurons. Here, we used transgenic mouse models to investigate whether En1 deletion affected SOC neuron specification, cell fate acquisition or survival. We show that subsets of glycinergic, cholinergic and GABAergic SOC neurons require En1 for proper nucleogenesis, survival and expression of FoxP1, establishing En1's importance for SOC neuron development.

Materials and Methods

Mice and mating paradigms

All mice were housed at the Case Western Reserve University or Children's Hospital of Pittsburgh of UPMC Animal Care Facilities in accordance with IACUC guidelines. Mice were housed under pathogen-free conditions in a temperature-and humidity-controlled

environment with 12hr light/dark cycles and given access to food and water ad libitum. Generation of $Egr2^{Cre}$, $En1^{Cre}$, $En1^{flox}$, $ROSA^{LacZ}$ and $ROSA^{tdTomato}$ mice were described previously (Maricich et al., 2009; Sgaier et al., 2007; Soriano, 1999; Voiculescu et al., 2000). All mice were maintained on a C57Bl/6J strain background except for $En1^{Cre}$ mice, which were maintained on both C57Bl/6J and mixed genetic backgrounds, and $En1^{flox}$ mice, which had a mixed genetic background.

En1 conditional knock-out ($En1^{CKO}$) mice were generated by mating $Egr2^{Cre/+}$ mice with $En1^{flox/flox}$ mice to generate $Egr2^{Cre/+}$; $En1^{+/flox}$ double-transgenic animals. These mice were mated with $En1^{flox/flox}$ mice to generate transgenic mice of four genotypes: $Egr2^{+/+}$; $En1^{+/flox}$, $Egr2^{+/+}$; $En1^{flox/flox}$, $Egr2^{Cre/+}$; $En1^{+/flox}$, and $Egr2^{Cre/+}$; $En1^{flox/flox}$. Only $Egr2^{Cre/+}$; $En1^{flox/flox}$ (Egr2; $En1^{CKO}$) mice lack En1 expression in the Egr2 distribution. Mice of the other three genotypes are collectively referred to as “control” because they displayed no abnormal phenotypes and their SOC histology and immunostaining for all markers tested was indistinguishable at all ages examined.

En1-null mice were generated by intercrossing $En1^{Cre}$ mice. Unlike their 129/Sv counterparts that die during the neonatal period, C57Bl/6J En1-null mice sometimes survive to adulthood and have no cerebellar or midbrain abnormalities (Bilovocky et al., 2003; Wurst et al., 1994).

For fate mapping experiments, $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice were intercrossed to generate $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice. Alternately, $En1^{Cre/+}$ mice were mated to $En1^{+/+}$; $ROSA^{LacZ/LacZ}$ or $En1^{flox/flox}$; $ROSA^{Lac/LacZ}$ mice to generate $En1^{Cre/+}$; $ROSA^{LacZ/+}$ or $En1^{Cre/flox}$; $ROSA^{LacZ/+}$ mice, respectively. This allowed fate mapping of En1-lineal cells in the presence or absence of En1 gene function using two different genetic reporters.

Tissue harvesting and processing

For embryonic tissue, pregnant dams were sacrificed, embryos dissected into cold 1× PBS and brains isolated and immersion-fixed overnight at 4°C in fresh 4% paraformaldehyde (PFA)/0.1M phosphate buffer. Postnatal day 0 (P0) and adult mice were transcardially perfused with 4% PFA and tissues post-fixed for 2h at 4°C in the same fixative. For glycine and GABA immunohistochemistry, adult mice were perfused with 4% PFA and 0.2% glutaraldehyde. For paraffin sections, tissues were dehydrated and embedded in TissuePrep (Thermo Fisher Scientific) then serially-sectioned at 6μm onto Superfrost/Plus slides (Thermo Fisher Scientific) using a Leica microtome. For frozen sections, brains were cryoprotected in 30% sucrose/1× PBS for 48 h then embedded in Tissue-Tek O.C.T. (Sakura Finetek), serially-sectioned at 10-25μm on a Leica CM1950 cryostat (Leica Microsystems, Wetzlar, Germany) and sections collected on Superfrost/Plus slides and stored at -80°C. Group sizes were n=2-3 mice/genotype/age.

Histology

Embryonic and adult tissues were stained for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-•-D-galactopyranoside (X-gal) for 4-24h at 37°C followed by 1× PBS washes and overnight fixation in 4% PFA at 4°C. Tissue sections were counterstained with

Cresyl violet or nuclear fast red, dehydrated and mounted with Cytoseal 60 (Richard Allan Scientific). For morphological analyses, paraffin brain sections were stained with Cresyl violet. For all histological analyses series of slides were processed to allow exact matching of SOC levels between control and mutant brains using nVII as a reference. This allowed us to designate nuclear subdivisions within the SOC in the absence of En1. SOC images from several brainstem levels are shown in the figures to illustrate all of the En1-lineal cells.

Immunohistochemistry

Frozen sections were rinsed in 1× PBS then blocked for 1h at room temperature (RT) in 1× PBS/0.3% Triton X-100/3% normal donkey or goat serum (blocking solution). Slides were incubated overnight at 4°C with primary antibodies in blocking solution at the following dilutions: rabbit anti-ALDH1L1 (Abcam) 1:500; rabbit anti-calretinin (Millipore) 1:500; goat anti-ChAT (Millipore) 1:100; rabbit anti-cleaved caspase 3 (Biocare Medicare) 1:250; rabbit anti-FoxP1 (Abcam Inc.) 1:400; guinea pig anti-GABA (Abcam) 1:100; rabbit anti-glycine (Millipore) 1:100, goat anti-MafB (Santa Cruz) 1:1000; chicken anti-MAP2 (Abcam Inc.) 1:5000; anti-Olig2 (EMD Millipore) 1:250; rabbit anti-Sox2 (Millipore) 1:200; mouse anti-TUJ1 (Abcam) 1:500. Antigen retrieval consisting of 95°C citrate buffer, pH 6 for 15 min was performed prior to immunostaining for calretinin and ChAT. When ChAT immunostaining was performed on tissue expressing tdTomato, antigen retrieval was not done because heating destroys the tdTomato signal. Sections were rinsed in 1× PBS and secondary antibodies conjugated to DyLight 488 or 549 (Jackson Immunoresearch) were used at a 1:500 dilution applied for 1h at RT. All slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or NeuroTrace fluorescent Nissl stain (Molecular Probes). Sections were rinsed, mounted with ProLong Gold and imaged using a Leica DM5500B epifluorescence microscope (Leica Microsystems, Exton, PA) or an inverted Zeiss Axio Observer on a PerkinElmer UltraVIEW VoX spinning disk confocal with a Hamamatsu C9100-13 camera and Volocity software.

In situ hybridization

Postnatal mice were perfused, brains dissected, post-fixed with 4% PFA/PBS overnight at 4°C, then equilibrated in 30% sucrose/PBS at 4°C, embedded in O.C.T. Compound (Tissue-Tek) and cryostat sectioned at 25μm. Slides were air dried at room temperature for 2 h and stored at -80°C.

A 642bp probe for GlyT2 was generated using PCR primers flanked with T7/T3 sequences (forward T3, 5′-AATTAACCCTCACTAAAGGGAATGTGTGCATCTGTGTATGCA-3′; reverse T7, 5′-GTAATACGACTCACTATAGGGCCGGTATGGTAGTGGTGGCCACG-3′). Probes were transcribed using the Ambion Maxiscript transcription kit (Invitrogen) and Digoxigenin -11-UTP (Roche). After precipitation with 4M LiCl and 100% Ethanol, probes were centrifuged at 4°C for 20 minutes, pellets rinsed with 70% ethanol, air dried, and resuspended in 30 μl DEPC water. In situ hybridization was performed as previously described (Domowicz et al., 2008). Briefly, frozen mouse brain sections were post-fixed with 4% PFA for 15 minutes, followed by riboprobe incubation overnight at 55°C in hybridization buffer (50% formamide, 5×SSC, 1% SDS, 500μg/ml tRNA and 200μg/ml heparin). Sense probe

hybridization was used as a negative control. Post-hybridization washes were performed at 65°C in solution X (50% formamide, 2×SSC, 1% SDS). Sections were then blocked with lamb serum for 1h, and incubated with anti-digoxigenin antibody (Roche Applied Science) for 2h. Color development was processed with NBT/BCIP (Roche Applied Science) incubation. After staining, sections were dehydrated with ethanol and mounted using Cytoseal.

Cell counts

ChAT+ SOC neurons were counted in 25μm-thick serial sections through the entire SOC (n =3 mice/genotype). Raw counts were corrected using the Hendry method (Hendry, 1976). X-gal+ cell counts were conducted in 6 representative 10μm-thick sections through the middle of the SOC (n =2 mice/genotype/age, 6 sections/mouse). In cell death experiments, the number of caspase 3+/tdTomato+ cells and tdTomato+ cells with pyknotic nuclei were quantified in the MNTB of $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice and in the ectopic cell group of $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice at P0 (n=2 mice/genotype, 6 sections/mouse). In all cases, data are reported as means ±SEM, and genotype means were compared using student's t-test.

Results

En1-lineal SOC cells are neurons with multiple neurotransmitter phenotypes

In the SOC, En1-lineal cells are found in the LSO, LNTB, MNTB and VNTB (Marrs et al., 2013)(Fig. 1). We confirmed these findings in adult $En1^{Cre/+}$; $ROSA^{tdTomato/+}$ and $En1^{Cre/+}$; $ROSA^{LacZ+}$ mice, where all cells that express En1 at any point during their development are irreversibly labeled by tdTomato or β-galactosidase, respectively (Figs. 2A, 3A). Immunostaining with the neuronal marker MAP2 revealed that all En1-lineal cells were neurons (Fig. 2B-B″). These data indicate that En1 expression is restricted to a subset of neurons in these brain regions.

Specific transcription factors (for example Atoh1, Pet1 and Ptf1a) are necessary for the specification/maturation of brainstem neurons with particular neurotransmitter phenotypes (Fujiyama et al., 2009; Hendricks et al., 2003; Hoshino et al., 2005; Rose et al., 2009). We reasoned that this might also be true for the En1 neuronal lineage. We previously showed that En1-lineal neurons are not glutamatergic, as the number and distribution of glutamatergic SOC neurons were unaffected following conditional deletion of En1 (Jalabi et al., 2013). Since glycinergic, cholinergic and GABAergic SOC neurons are found in the same regions as En1-lineal neurons, we immunostained brainstem sections from adult $En1^{Cre/+}$; $ROSA^{tdTomato/+}$ mice for glycine, choline acetyltransferase (ChAT) or GABA. All tdTomato+ LSO, MNTB, and VNTB neurons were glycine+ (Fig. 2C-E″), and En1-lineal tdTomato+ VNTB neurons were also ChAT+ (Fig. 2F-F″, Supplemental Fig. 1), and all En1-lineal neurons in the LNTB were GABA+ (Fig. 2G-G″). Glycinergic and GABAergic neurons of the superior paraolivary nucleus (SPN) (Fig. 2H-I″) and ChAT+ neurons that contribute to the lateral and medial olivocochlear bundles (LOCB and MOCB) (Fig. 2J-K″) were tdTomato-, showing that they were not derived from the En1-lineage. These data demonstrate that En1-lineal neurons have multiple neurotransmitter phenotypes (Fig. 2L).

Furthermore, they show that glycinergic, cholinergic and GABAergic SOC neurons derive from at least two separate lineages.

Subsets of glycinergic, cholinergic and GABAergic SOC neurons are missing following En1 deletion

We previously reported that adult Egr2$^{Cre/+}$; En1$^{flox/flox}$ (Egr2; En1^{CKO}) mice, where En1 is deleted in rhombomeres 3 and 5 at E7.5 (Voiculescu et al., 2000), have no MNTB or VNTB neurons (Jalabi et al., 2013). En1 deletion in the limb causes aberrant cell fate decisions (Loomis et al., 1996), so we reasoned that En1-null cells normally destined for these and other regions of the SOC may have adopted alternative cellular fates. To address this possibility, we compared the distribution of En1-lineal cells in adult En1$^{Cre/+}$; ROSA^{LacZ} (control, Fig. 3A) vs. En1$^{Cre/flox}$; ROSA^{LacZ} mice (Fig. 3B,K), where “self-deletion” of En1 occurs shortly (within 24 hours) after the initiation of En1 expression (Sgaier et al., 2007). No Xgal+ cells were present in the SOC of adult En1$^{Cre/flox}$; ROSA^{LacZ} mice (Fig. 3B), suggesting that these cells did not adopt alternative fates but rather that they were either never generated or that they died during development.

We next sought to determine how En1 deletion affected the distribution of glycinergic, cholinergic and GABAergic neurons in the SOC. In situ hybridization for the glycine transporter GlyT2 revealed that glycinergic neurons were completely absent from the LSO and regions that normally contain the MNTB and VNTB of Egr2; En1^{CKO} mice, while GlyT2+ neurons remained in the LNTB and SPN (Fig. 3C, D, K). ChAT immunostaining revealed a >90% decrease in ChAT+ VNTB neuron numbers (4.6±3.0 vs. 53.76±8.9; $p<0.01$) in Egr2; En1^{CKO} mice compared to control littermates, while LOCB (317±26 vs. 272±23; $p=0.26$) and MOCB (57±13 vs. 72±11; $p=0.49$) neuron numbers were similar in the two genotypes (n=3 mice/genotype) (Fig. 3E-H, K). Furthermore, GABAergic neurons were present in the LNTB of control animals but completely absent from the LNTB of Egr2; En1^{CKO} mice (Fig. 3I-K). These data 1) corroborate our fate-mapping data; 2) together with previous work demonstrating that SOC neurons derive from r4 and r5 (Farago et al., 2006; Karis et al., 2001; Maricich et al., 2009) show that all En1-lineal SOC neurons derive from r5; and 3) suggest that En1-lineal SOC neuron creation or survival cell-autonomously depends on En1 expression.

En1 is required for proper positioning and survival, but not specification, of SOC neurons

We next sought to determine whether En1 deletion caused failure of neuronal specification and/or cell death. To distinguish between these possibilities, we compared the distribution of En1-lineal cells in En1$^{Cre/+}$; ROSA^{LacZ} and En1$^{Cre/flox}$; ROSA^{LacZ} mice at E12.5, E15.5 and P0. En1-lineal cells were first seen in the developing SOC at E12.5 and were present in similar distributions in both genotypes (Fig. 4A, B). However, at E15.5, medially-located Xgal+ SOC cells in En1$^{Cre/flox}$; ROSA^{LacZ} embryos did not coalesce into a single well-defined group (presumably the nascent MNTB) as they did in En1$^{Cre/+}$; ROSA^{LacZ} embryos (Fig. 4C, D). Quantitatively, there were no differences in the number of X-gal+ cells between En1$^{Cre/+}$; ROSA^{LacZ} and En1$^{Cre/flox}$; ROSA^{LacZ} at E12.5 (432 ±6 vs. 428 ±17; $p=0.82$) or E15.5 (1069 ±3 vs. 1064 ±31; $p=0.87$)(n=2 mice/genotype/age). By P0, En1-lineal cells of En1$^{Cre/+}$; ROSA^{LacZ} embryos were found in their adult positions (Fig. 4E). In

contrast, far fewer Xgal+ cells were present in the SOC of $En1^{Cre/flox}$; $ROSA^{LacZ}$ mice (1080 ±29 vs. 459 ±3, $p<0.01$), and the remaining En1-lineal SOC cells formed an ectopic medial cell group (Fig. 4F). This medial cell group was also observed in Cresyl violet-stained sections from P0 Egr2; $En1^{CKO}$ and En1-null mice (Fig. 4G-I), but by P3 it had vanished (data not shown). The percentage of tdTomato+ SOC cells with pyknotic nuclei as revealed by DAPI staining was far greater in P0 $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (Fig. 5B-B′″) than P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice (21.4% ±0.42 vs. 0.3% ±0.36, $p<0.001$; n=2 mice/genotype) (Fig. 5A-A′″). Moreover, several ectopic tdTomato+ cells in P0 $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice were also positive for the apoptotic cell death marker activated caspase-3, while no tdTomato+/caspase-3+ cells were found in $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice (7.5% ±2.1 vs. 0, $p<0.05$; n=2 mice/genotype). These data demonstrate that En1 is required for survival, but not specification, of En1-lineal SOC neurons. Furthermore, these data suggest that En1 plays a role in En1-lineal SOC neuron positioning.

En1-null cells maintain a neuronal identity

En1 deletion in the limb causes a homeotic transformation that alters cell fate decisions (Loomis et al., 1996). To determine whether death of En1-lineal SOC neurons occurred secondary to a change in cell fate away from a neuronal identity, we immunostained tissue sections from E14.5 and P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice with neuronal and glial cell markers. Immunostaining with TUJ1, which recognizes neuron-specific β-III tubulin, demonstrated that all tdTomato+ SOC cells were TUJ1+ in E14.5 and P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (Fig. 6A-D″). All tdTomato + SOC cells in P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice were negative for astrocyte (AldH1L1) and oligodendrocyte (Olig2) markers (Fig. 6E-H″). These data demonstrate that En1 does not specify or maintain neuronal identity of En1-lineal SOC cells.

Expression of FoxP1, but not Sox2 or MafB, is altered in En1-null cells

En1-lineal SOC neurons express the transcription factors FoxP1 and Sox2 during embryonic and early postnatal development (Marrs et al., 2013). To determine whether En1 deletion affected FoxP1 and/or Sox2 expression, we immunostained SOC tissue sections of E14.5 and P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice for these proteins. The majority of FoxP1+ cells were also tdTomato+ in the SOC of E14.5 and P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice (Fig. 7A-A″, Supplemental Fig. 2A-A″). In contrast, FoxP1+ cells were limited to the developing SPN of E14.5 and P0 $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice, and all of these cells were tdTomato- (Fig. 7B-B″, Supplemental Fig. 2B-B″). Sox2+/tdTomato- and Sox2+/tdTomato+ cells were present in the SOC of E14.5 and P0 mice of both genotypes (Fig. 7C-D″, Supplemental Fig. 2C-D″). These data suggest that FoxP1, but not Sox2, requires En1 function for the initiation and maintenance of its expression.

To further verify that En1-deletion effects were cell-autonomous, we analyzed the distribution of MafB+ cells, which become glutamatergic neurons of the LSO and MSO (Rose et al., 2009). MafB+ cells were found in similar distributions in the SOC of E14.5 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (Fig. 7E-F″). These data are

consistent with our data in adult mice suggesting that En1 deletion effects are cell-autonomous in SOC neurons.

Discussion

Our data establishes En1's importance for SOC neuron survival. This function is similar to that of En proteins in dopaminergic midbrain neurons and late embryonic serotonergic neurons (Fox and Deneris, 2012; Simon et al., 2001), but differ from the gene's role in the specification of the mid/hindbrain anlage, noradrenergic neurons and serotonergic neurons (Simon et al., 2005; Wurst et al., 1994). Interestingly, En1 deletion also disrupts SOC nucleogenesis, a phenotype similar to that seen in serotonergic neurons of the dorsal raphe nucleus (Fox and Deneris, 2012). We hypothesize that this occurs secondary to inability of undifferentiated En1-lineal cells to respond to local stop signals along their migrational path. En proteins control Eph/ephrin signaling important for retinotectal mapping (Logan et al., 1996; Shigetani et al., 1997), and En1 is necessary for proper spinal cord ventral interneuron projections, possibly through regulation of netrin-1 signaling (Saueressig et al., 1999). Ephs, ephrins and netrin-1 are expressed by developing SOC neurons and are necessary for axon pathfinding in the brainstem auditory system (Cramer et al., 2000; Howell et al., 2007; Hsieh et al., 2010; Nakamura et al., 2012). Further studies are needed to determine whether disruption of one of these pathways underlies a migrational phenotype. Regardless of the mechanism, the aberrant positioning of these cells might underlie their death secondary to disrupted connectivity or to the ectopia itself (Clarke and Cowan, 1976).

We found similar phenotypes in the SOC following constitutive and conditional deletion of En1. This argues that deletion effects are cell-autonomous, fully penetrant and consistent across deletion strategies and strain backgrounds. Importantly, phenotypic rescue did not occur on the C57Bl/6J strain background as it does in the cerebellum/midbrain (Bilovocky et al., 2003; Wurst et al., 1994), something we used to our advantage to analyze adult En1-null mice. The identical phenotypes seen in these and $En1^{Cre/flox}$ mice also suggest that En1 function in survival and differentiation is consistent over the first 24 hours (Sgaier et al., 2007). It is not clear from our study whether En1 gene function changes after this early epoch, an important question considering that the gene is expressed by SOC neurons through early adulthood (Atlas, n.d.). Future experiments will directly address this possibility.

Our data also provide insight into regulatory interactions between transcription factors expressed by developing SOC neurons (Fig. 7G). First, our data suggest that FoxP1 lies downstream of En1, providing the first identification of a transcriptional hierarchy potentially important for SOC neuron development. Whether this regulation is direct or indirect is presently unclear. Interestingly, FoxP1 deletion causes aberrant neuronal development in the forebrain and midbrain, and the gene also plays a role in positioning of ventral spinal cord motor neurons (Bacon et al., 2014; Palmesino et al., 2010; Rousso et al., 2008). However, effects of FoxP1 deletion on the auditory system have not been studied, so it is possible that some or all of the phenotypes resulting from En1 deletion are directly controlled by loss of FoxP1 expression. Second, our data demonstrates that Sox2 expression in SOC neurons is unaffected by En1 deletion, suggesting that Sox2 either lies upstream of

En1 or that it sits in a parallel regulatory pathway. Sox genes in general and Sox2 in particular are involved in precursor cell development and cell fate choices in several developing organ systems (Sarkar and Hochedlinger, 2013). Whether Sox2 or other factors control SOC neuron specification and adoption of a neural fate, which our data show are not controlled by En1, requires further study.

Interestingly, the En1 lineage gives rise to SOC neurons that vary in neurotransmitter phenotype (Fig. 7G). Most of these neurons are glycinergic (LSO, MNTB, VNTB) and inhibitory; others are cholinergic (VNTB) and likely excitatory (Fujino and Oertel, 2001); and the small number found within the LNTB are GABAergic. These neurons are known to have radically different projection patterns: glycinergic LSO neurons project to the ipsilateral inferior colliculus (Benson and Cant, 2008; Glendenning et al., 1992; Saint Marie et al., 1989); LNTB neurons project ipsilaterally to the MSO, LSO and possibly the inferior colliculus (Kuwabara and Zook, 1992; Willard and Ryugo, 1983); MNTB neurons project locally to the ipsilateral LSO, MSO and SPN (Bledsoe et al., 1990); and VNTB neurons project bilaterally to the cochlear nuclei, contralateral LSO, and possibly to the ipsilateral inferior colliculus (Frisina et al., 1998; Ostapoff et al., 1997; Sherriff and Henderson, 1994; Willard and Ryugo, 1983). Given these data, En1-lineal neurons must serve different functions within the auditory system, yet the developmental and evolutionary relationship of these neurons to one another is a mystery. In addition, the existence of En1-lineal and En1-non-lineal SOC glycinergic, cholinergic and GABAergic neurons uncovers previously unrecognized ontogenetic heterogeneity within the SOC. In the ventral cholinergic system, this lineage division aligns with functional division: large, non-En1-lineal ChAT+ neurons project in the MOCB and innervate contralateral cochlear outer hair cells, while small, En1-lineal ChAT+ neurons project to the ipsilateral cochlear nucleus (Campbell and Henson, 1988; Godfrey et al., 1987a; 1987b; Sherriff and Henderson, 1994; Yao and Godfrey, 1998). Potential functional divisions within the SOC GABAergic and glycinergic systems are less clear. This situation contrasts with the finding that all SOC glutamatergic neurons derive from the Atoh1 lineage (Maricich et al., 2009; Rose et al., 2009), and that expression of Atoh1 and Ptf1a parse cochlear nucleus neurons into glutamatergic and GABAergic/glycinergic populations (Hoshino et al., 2005). Given that Atoh1 and Ptf1a are both basic helix-loop-helix (bHLH) transcription factors, it is possible that an as yet unidentified bHLH protein lies upstream of En1 and plays a similar role in inhibitory SOC neurons. Further experiments are needed to identify factors both upstream and downstream of En1 that control SOC neuron neurotransmitter phenotype, morphology and projection patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- En1 deletion causes death of MNTB, VNTB and subsets of LSO and LNTB neurons.
- En1 is necessary for establishing correct SOC neuron positioning in the brainstem.
- The transcription factor FoxP1 lies downstream of En1 in En1-lineal SOC neurons.

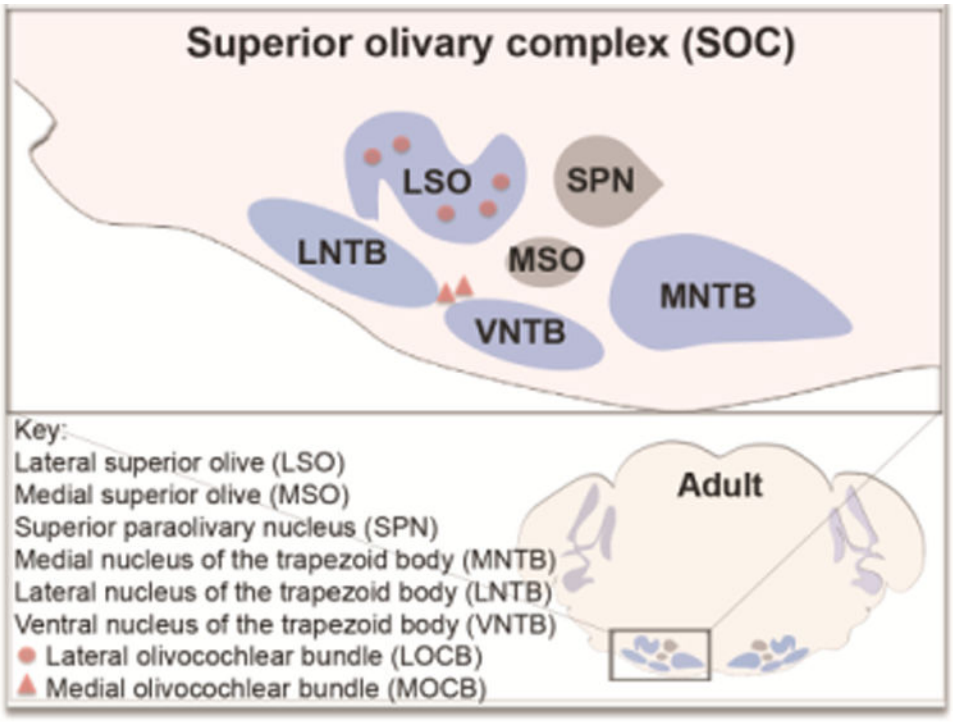

Figure 1.

SOC nuclear morphology. Cartoon of a coronal section through the brainstem shows the six nuclei that comprise the superior olivary complex (SOC). These include the lateral (LSO) and medial (MSO) superior olives, superior paraolivary nucleus (SPN) and lateral (LNTB) medial (MNTB) and ventral (VNTB) nuclei of the trapezoid body. Also indicated are the lateral (LOCB) and medial (MOCB) olivocochlear bundle neurons that reside within the SOC.

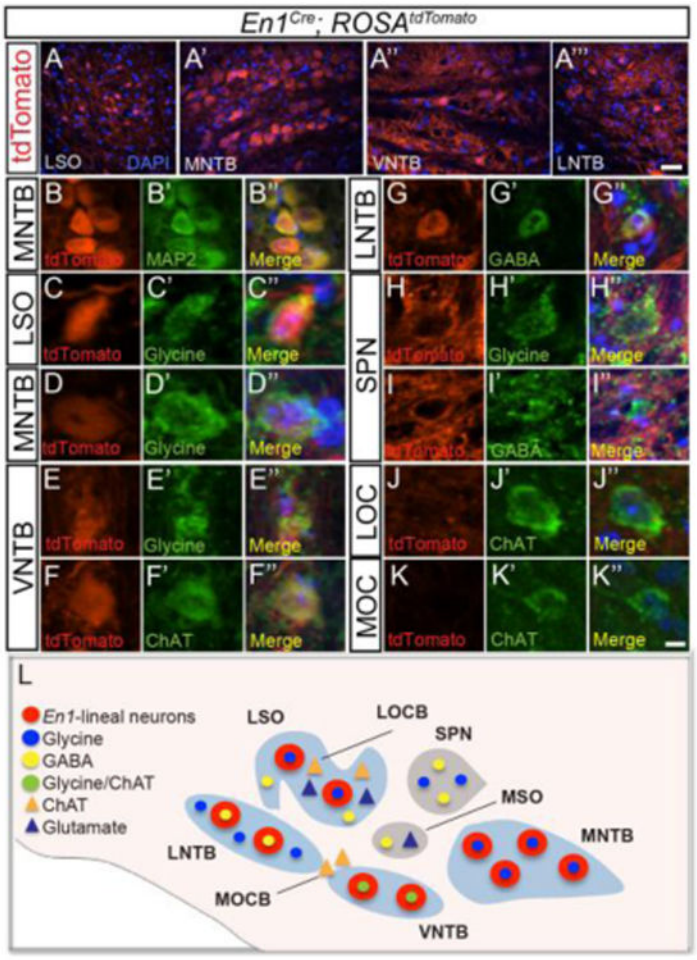

Figure 2.

En1-lineal SOC cells are glycinergic, cholinergic and GABAergic neurons. (A-A‴) Coronal brainstem section from adult En1^{Cre}; ROSA^{tdTomato} mouse showing endogenous tdTomato signal with DAPI nuclear labeling. En1-lineal neurons are found in the LSO (A), MNTB (A′), VNTB (A″) and LNTB (A‴). Endogenous tdTomato signal (B) and immunostaining for MAP2 (B′) in the MNTB reveals that all tdTomato+ cells are also MAP2+ (B″). Immunostaining of En1^{Cre}; ROSA^{tdTomato} mouse brainstem sections for glycine shows that all tdTomato+ cells in the LSO, MNTB, and VNTB are glycinergic neurons (C-E″). Immunostaining for ChAT shows that En1-lineal VNTB neurons are also cholinergic (F-F″). Within the LNTB, all tdTomato+ neurons are GABA+ (G-G″). Glycinergic and GABAergic SPN neurons receive inputs from tdTomato+ cells (red perineuronal signal in H, I), but these neurons and ChAT+ neurons that contribute to the LOCB and MOCB are tdTomato- (J-K″). In panels B″-J″, the merged images contain DAPI staining in blue. (L) Schematic summarizing results. Scale bar: 40μm (A), 13μm (A′-A‴), 3.7μm (B-K″).

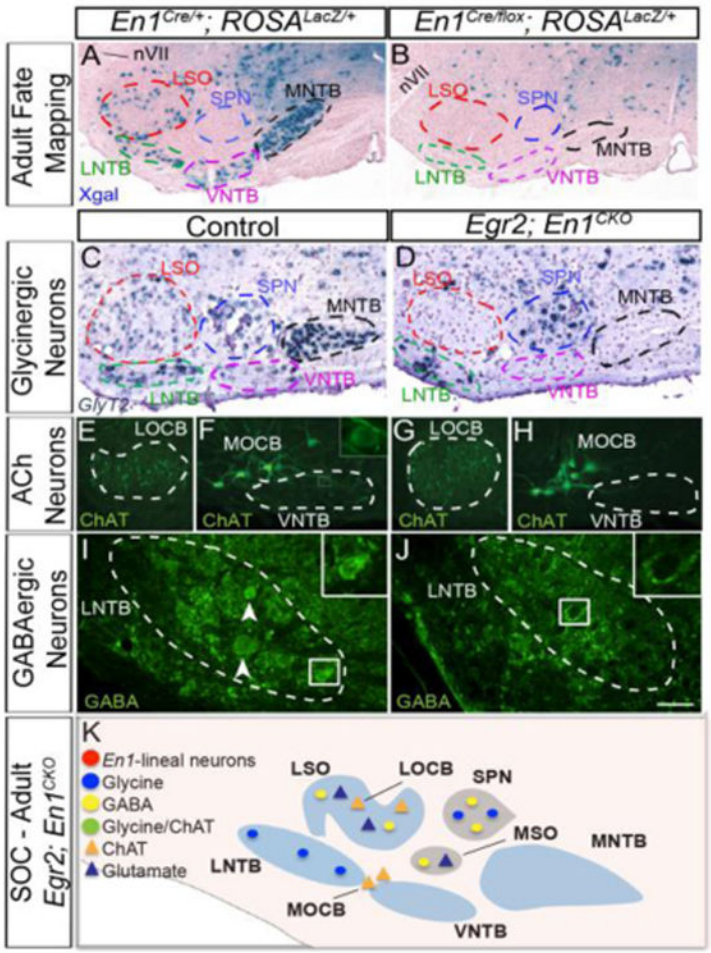

Figure 3.

En1-lineal neurons are absent from the SOC of adult mice following En1 deletion. Color-coded dotted lines delineate SOC subdivisions in this and subsequent Figures. In animals with constitutive or conditional En1 deletion subdivisions are shown based on where they should be compared to control. Xgal+ cells are present in the SOC of adult $En1^{Cre/+}$; $ROSA^{LacZ}$ (A) but not $En1^{Cre/flox}$; $ROSA^{LacZ}$ mice (B). Comparison of in situ hybridization for the glycinergic neuron marker Glyt2 counterstained with Cresyl violet in adult control (C) and Egr2; $En1^{CKO}$ mice (D) indicates a loss of glycinergic neurons in the LSO, VNTB and MNTB of Egr2; $En1^{CKO}$ mice. (E-H) ChAT immunostaining demonstrates that LOCB and MOCB neurons are present in adult Egr2; $En1^{CKO}$ mice, but that cholinergic (Ach) neurons within the confines of the VNTB are absent. Immunostaining for GABA revealed GABAergic neurons present in the LNTB of control animals (I) were missing in Egr2; $En1^{CKO}$ mice (J). GABAergic boutons are present on cell bodies of both genotypes. Boxes show areas of small insets in (I, J). (K) Schematic summarizing results. Scale bar: 375μm (A-D), 150μm (E-H), 40μm (I, J), 15μm (insets).

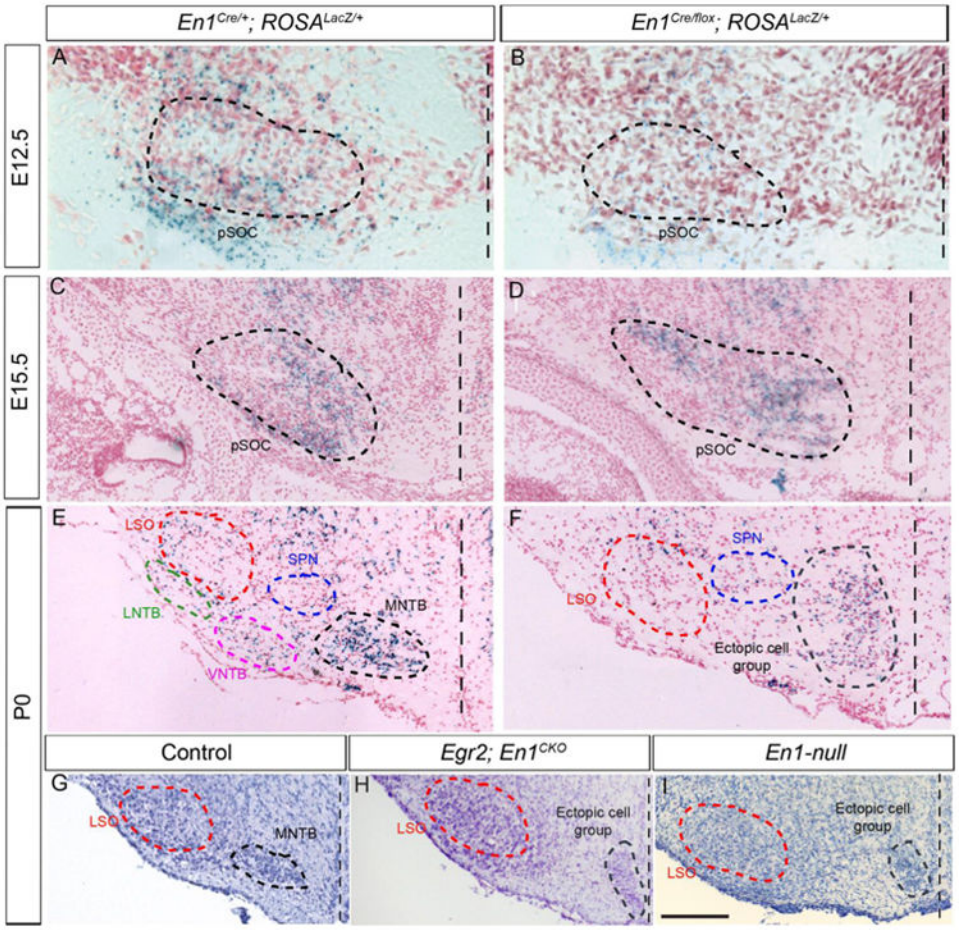

Figure 4.

En1-null SOC cells migrate aberrantly during development. Dotted vertical lines mark the brainstem midline. At E12.5, Xgal+ cells are found in similar locations in the presumptive SOC (pSOC) of the two genotypes (A, B). At E15.5, the distribution of Xgal+ cells in the medial pSOC is more diffuse in $En1^{Cre/flox}$; $ROSA^{LacZ}$ (D) compared to $En1^{Cre/+}$; $ROSA^{LacZ}$ mice (C). At P0, Xgal+ cells in the SOC are found in the adult distribution of $En1^{Cre/+}$; $ROSA^{LacZ}$ mice (E). In contrast, reduced numbers of Xgal+ cells are present in the SOC of $En1^{Cre/flox}$; $ROSA^{LacZ}$ mice (F), and these cells form an ectopic medial cell group. Compared to P0 control mice (G), Cresyl violet staining of Egr2; $En1^{CKO}$ (H) and En1-null (I) mice shows presence of an ectopic cell group close to the brainstem midline. Scale bar: 250μm (C-I), 150μm (A, B).

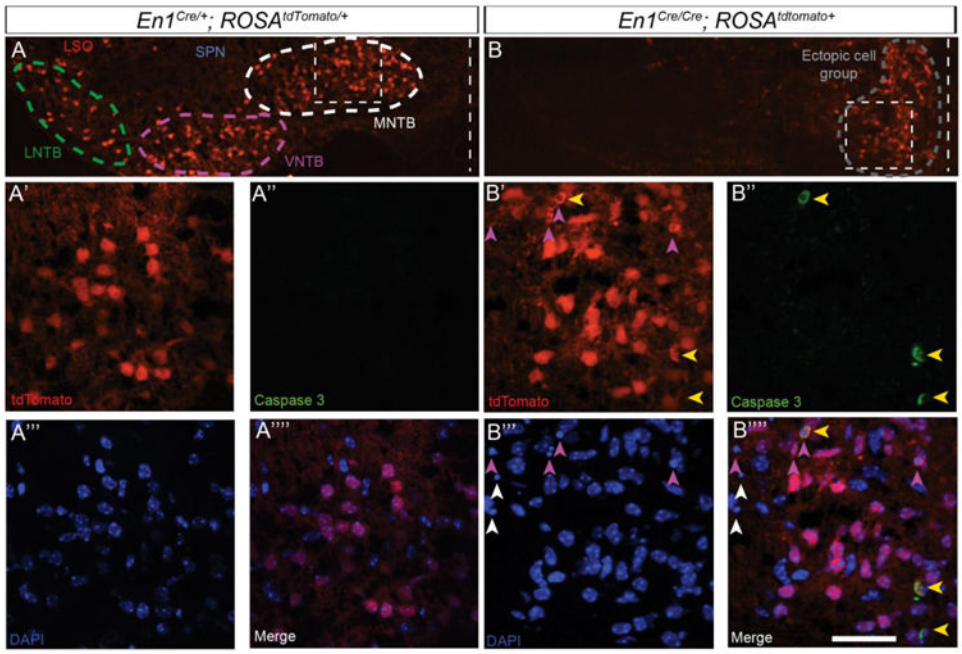

Figure 5.

Ectopic cells derive from the En1-lineage and die in En1-null mice. Coronal brain sections through the SOC of P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ (A) and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ (B) mice showing endogenous tdTomato fluorescence. Dotted vertical lines mark the brainstem midline and boxed areas the regions of (A′-A′′′′) and (B′-B′′′′). Activated caspase-3 immunostaining (A″, B″) and DAPI staining (A‴, B‴) show the presence of apoptotic cells and pyknotic nuclei in $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ but not $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice. Yellow, purple and white arrowheads (B′-B‴) denote caspase-3+/tdTomato+ cells, pyknotic tdTomato+ cells, and pyknotic tdTomato- cells, respectively. Scale bar: 120μm (A, B), 25μm (A′-B‴).

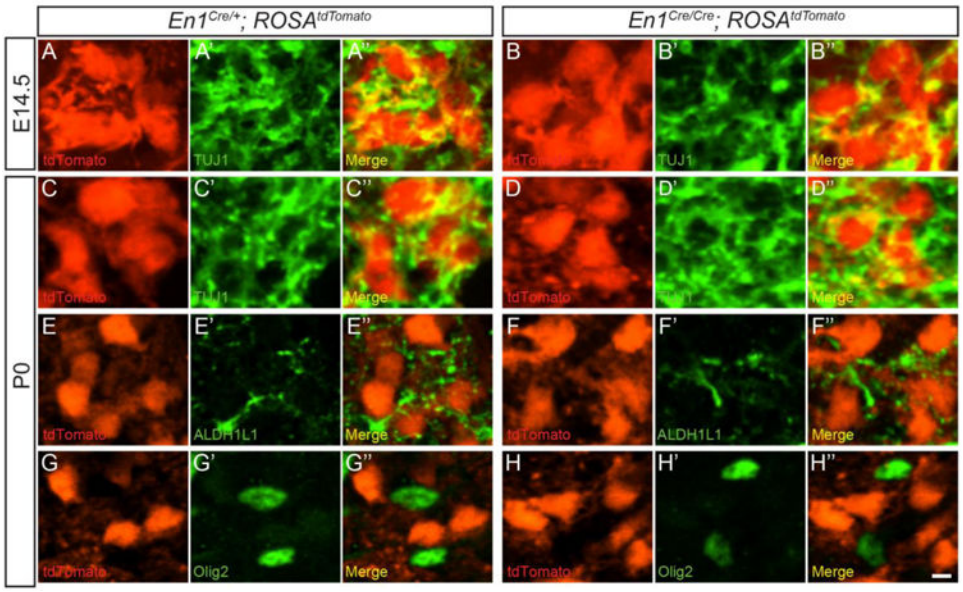

Figure 6.

En1 deletion does not affect neural cell fate in the SOC. Single-plane confocal microscope images of coronal brainstem sections through the presumptive SOC from E14.5 $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (A-B″), MNTB of P0 $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice (C-C″, E-E″, G-G″) and ectopic cell group of P0 $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (D-D″, F-F″, H-H″) showing endogenous tdTomato signal (A-H), immunostaining for the neural marker TUJ1 (A′-D′), the astrocyte marker ALDH1L1 (E′, F′), the oligodendrocyte marker Olig2 (G′, H′) and merged images (A″-H″). TUJ1 and tdTomato are colocalized in $En1^{Cre/+}$; $ROSA^{tdTomato}$ and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice, while no signal overlap is seen with the glial cell markers. Scale bar: 12μm.

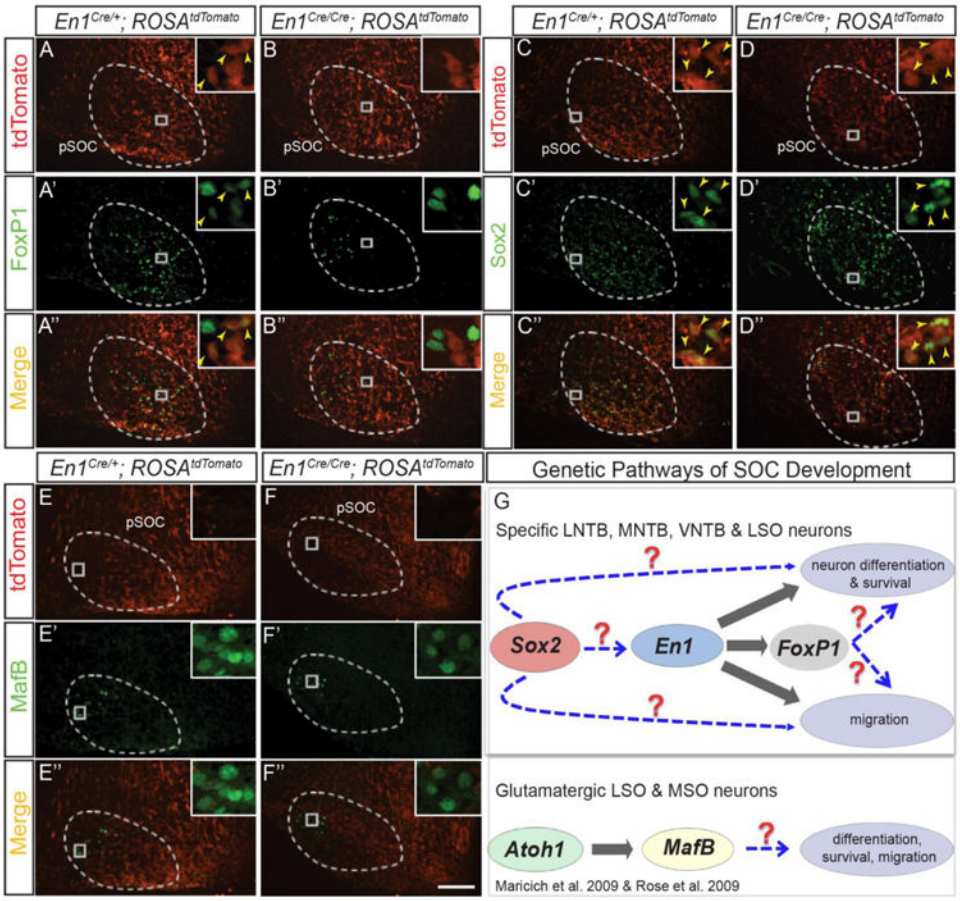

Figure 7.

FoxP1 expression, but not Sox2 or MafB expression, depends on En1 in the SOC. Boxed areas are shown in insets. Yellow arrowheads indicate double labeled neurons (A-D″). Coronal sections demonstrate endogenous tdTomato (A-D) and FoxP1 immunostaining (A′-D′) in the presumptive SOC of E14.5 $En1^{Cre/+}$; $ROSA^{tdTomato}$ (A-A″) and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ (B-B″) mice. FoxP1+ cells are found throughout the presumptive SOC of $En1^{Cre/+}$; $ROSA^{tdTomato}$ mice but are limited to the nascent SPN of $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice. tdTomato+/FoxP1+ cells are absent from the presumptive SOC of $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice. The MNTB marker Sox2 labels tdTomato+ cells in both $En1^{Cre/+}$; $ROSA^{tdTomato}$ (C-C″) and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ mice (D-D″). The LSO and MSO neuron marker MafB is present in the presumptive SOC at E14.5 in $En1^{Cre/+}$; $ROSA^{tdTomato}$ (E-E″) and $En1^{Cre/Cre}$; $ROSA^{tdTomato}$ (F-F″) but is not co-expressed in tdTomato+ neurons in either genotype. (G) Summary graphic placing En1 into genetic hierarchies that regulate SOC neuron development. Scale bar: 120μm (A-F″), 12μm (insets).