Molecular Mechanisms Involved in the Dorsal Exiting of the Spinal Accessory Motor Neuron in Embryonic Mice

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Introduction

At the tip of an axon, a specialized structure known as the growth cone acts as a type of sensor that leads the trailing axon through an environment rich in guidance cues. As well, the growth cone/axon typically travels long distances along a complex trajectory, which can be subdivided into much shorter segments by intermediate targets. (Guthrie and Pini, 1995) These targets are groups of cells that express both positive/attractive and negative/repulsive cues. Accordingly, intermediate targets initially attract growing axons and later push these same axons away and onto the next leg of their journey. (Guthrie and Pini, 1995)

Dr. Marc Tessier-Lavigne discovered an important attractant protein, which he named Netrin-1 (Serafini T, 1994). Netrin-1 binds a receptor located on the surface membrane of the growth cone/axon referred to as deleted in colorectal carcinoma or Dcc (Tessier-Lavigne, Goodman 1996). Through this Netrin-1-mediated interaction the growth cone exhibits cycles of protrusion, adhesion and contraction (Kandel E., Schwartz J, 1994). On the contrary, Slit, a repellent protein, triggers axons to grow away from a site by binding a receptor called Roundabout (Robo) (Dickson, 2000). Thus, it was found that an axon would grow toward a choice point, being attracted by netrin, and then repelled away from it towards another choice point by Slit, as shown in figure 1.

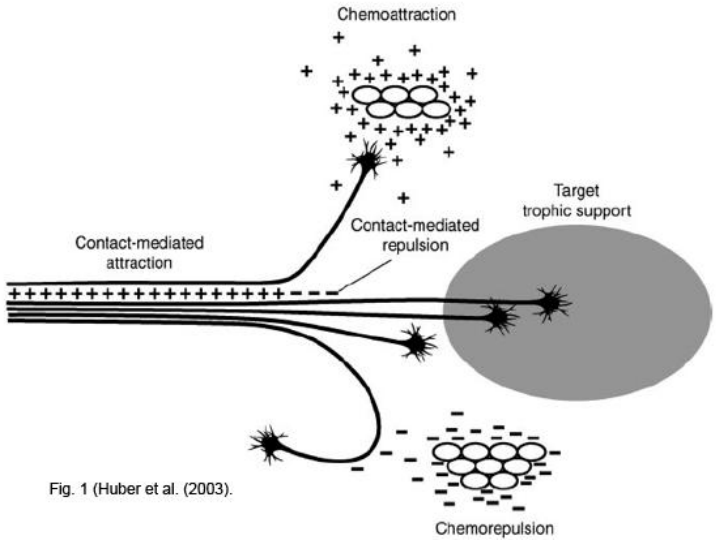


Fig. 1 (Huber et al. (2003).

In the vertebrate CNS, specific subclasses of motor neurons compose distinct neural circuits (Jessell, 2000; Landmesser, 2001; Price and Briscoe, 2004). Repressive interactions involving homeodomain and basic helix– loop– helix transcription factors give rise to several classes of postmitotic motor neurons (Shirasaki and Pfaff, 2002; Price and Briscoe, 2004) Most motor axons emerge through nearby ventral exit points. However, a subset grow for some distance toward and extend through more dorsally located exit points (Shirasaki and Pfaff, 2002).

Consistent in their initial migration away from the ventral midline, both ventrally and dorsally exiting motor axons have been shown in vitro to be repelled by floor plate tissue (Guthrie and Pini, 1995). Whereas the ventral midline-associated chemorepellent netrin-1 selectively repels dorsally exiting trochlear axons in vitro (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995), it is apparently not required for repulsion from the ventral midline in vivo (Serafini et al., 1996), ventral spinal cord- associated semaphorins are capable of repelling these axons both in vitro (Varela-Echavarria and Guthrie, 1997; Varela-Echavarria et al., 1997; Giger et al., 2000) and in vivo (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). Notably, the molecular mechanisms that facilitate the exit of either type of motor axon from the CNS are not known.

Spinal accessory motor neurons (SACMNs) are located within cervical regions of the spinal cord and selectively innervate the sternocleidomastoid and trapezius muscles in the neck and back (Greene, 1935; Wentworth and Hinds, 1978; Snider and Palavali, 1990). Unlike most spinal motor neurons, SACMNs extend dorsally projecting axons along the lateral margins of the spinal cord that extend out of the CNS through the lateral exit point (LEP) (Snider and Palavali, 1990) (Fig. 2). Subsequently, SACMN axons execute a rostral turn and assemble into the longitudinally

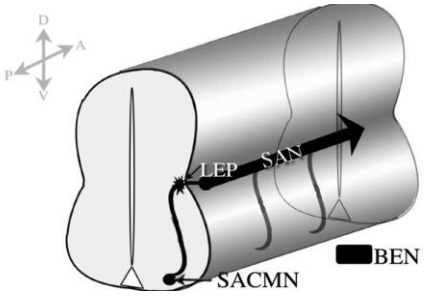


Fig 2(Dillon et al.)

oriented spinal accessory nerve (SAN) (Snider and Palavali, 1990) (Fig 2).

Although the molecular mechanisms that regulate SACMN development have not been directly investigated, all spinal motor neurons acquire SACMN-like properties in mice lacking Lhx3 and Lhx4 (Sharma et al., 1998), and Nkx2.9 null mice exhibit a truncated SAN (Pabst et al., 2003).

Here, this researcher uses anti-BEN/ALCAM/SC1/DM-GRASP/MuSC (herein referred to as BEN) as a specific marker of SACMN to utilize the steps their axons take to navigate through and out of the CNS. Moreover, this researcher uses this marker to analyze mice deficient in Robo 1. Findings include the identification of molecules that are required for the initial extension of SACMN axons, the dorsally directed migration of SACMN cell bodies, the projection of their axons toward the LEP, and the exit of SACMN axons from the CNS.

Research Question and Hypotheses

This researcher is interested in the molecular mechanisms that have a direct effect on the exiting of the spinal accessory motor neuron. The molecular mechanisms that facilitate the exit of either type of motor axon from the CNS are not known, though the transcription factor Nkx2.9 has been proven to play a vital role in this process. SACMNs axons fail to exit the spinal cord in mice lacking Nkx2.9. (Dillon et al)

Research Hypothesis: The repellent protein Robo, expressed along the cell body, plays an essential role in the dorsal exiting of the spinal accessory motor neuron in a developing mouse embryo.

Null Hypothesis: In knocked out mice (mutated mice) that lack the ability to produce the protein Robo, the spinal accessory motor neuron will “wander,” as opposed to exiting the spinal cord dorsally.

Materials and Methods

Mice

For the analyses aimed at examining BEN expression in wild-type animals, timed pregnant CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). For the analyses of the various aged Gli2 (Matise et al., 1998), netrin-1 (Serafini et al., 1996), DCC (Fazeli et al., 1997), Nkx2.9 (Pabst et al., 2003), and BEN (Weiner et al., 2004) mutant mouse embryos, pregnant mice harboring litters containing homozygous, heterozygous, and wild-type animals were generated.

Litters containing homozygous mutants were generated by intercrossing adults and were identified by PCR using yolk sac DNA (Ding et al., 1998; Matise et al., 1998). Chimeric netrin-1 mutant males were bred to CD1 females (Serafini et al., 1996), and chimeric DCC mutant males were bred to 129/Sv females (Fazeli et al., 1997). The Nkx2.9 and BEN/ALCAM-GFP lines were maintained on a C57BL/6 background (Pabst et al., 2003; Weiner et al., 2004). In all cases, pregnant dams were killed by exposure to compressed carbon dioxide or by cervical dislocation. This researcher merely observed such events and had no direct contact with any living mouse.

Embryos were removed by cesarean section and immersed in PBS (150mM Na2HPO4, 20mMNaH2PO4, and 150 mM NaCl, pH 7.4). The genotypes of the various embryos were determined as previously described for Gli2 (Matise et al., 1998), netrin-1 (Serafini et al., 1996), Dcc (Fazeli et al., 1997), Nkx2.9 (Pabst et al., 2003), and BEN (Weiner et al., 2004) mutant mice.

Antibodies

The following monoclonal antibodies were used to label cryosections and/or whole embryos: monoclonal antibody (mAb) 802C11 (Sekine- Aizawa et al., 1998), mAb 33.1 [anti-ALCAM; kind gift of Dr. Michael Bowen, Bristol-Myers Squibb (Wallingford, CT), now at Human Genome Sciences (Rockville, MD)], mAb 2H3 (Dodd et al., 1988), mAb 5272 (anti-mouse L1; rat IgG; Chemicon, Temecula, CA), mAb 347 (anti- GAP43; mouse IgG; Chemicon), and anti-DCC intracellular domain (G97–449; anti-human DCC; mouse IgG; PharMingen, San Diego, CA).

Immunohistochemistry

Cryosection preparation and immunolabeling: whole mouse embryos were fixed in 4% paraformaldehyde (PFA) for 12 h at 4°C, followed by cryoprotection in 30% sucrose for 6 h at 4°C, and embedded at -20°C in optimal cutting temperature compound (Tissue Tek; Sakura Finetek, Torrance, CA). Cryosections (16 µm) were cut using a Leica (Nussloch, Germany) cryostat (model CM3050 S) and mounted onto glass slides (Superfrost Plus; Fisher Scientific, Houston, TX). The sections were rinsed in PBS, postfixed in 4% PFA for 20 min at room temperature (RT), and blocked with 10% goat serum (HyClone, Logan, UT) in PBS for 30 min at RT.

For mAb 802C11 and mAb 5272 labeling, sections were incubated in blocking solution containing 1% Triton X-100 (Fisher Scientific). Sections were then incubated with the primary antibody diluted in blocking solution (or undiluted supernatant) for 90 min at RT and then rinsed in PBS. Primary antibodies were used as follows: mAb 802C11 (20 µg/ml), mAb 33.1 (1:10), mAb 2H3 (hybridoma supernatant), mAb5272 (10 µg/ml), mAb 347 (0.5 µg/ml), and anti-DCC intracellular domain(2.5 µg/ml). Sections were then incubated with the following concentrations of isotype-appropriate cyanine 2 (Cy2) or Cy3-conjugated secondary antibodies (Jackson

ImmunoResearch, West Grove, PA): mAb 802C11 (goat anti-Armenian hamster IgG; 1:100), mAb 33.1 (goat anti-rat IgG; 1:200) mAb 5272 (goat anti-rat IgG; 1:200), mAb 2H3 (goat anti-mouse IgG; 1:200), mAb 347 (goat anti-mouse IgG; 1:200), and anti-DCC intracellular domain (goat anti-mouse IgG; 1:200) for 60 min at RT. Slides were then mounted in a 1:1 mixture of glycerol and PBS.

Double labeling of anti-DCC and anti-BEN on the same section was performed by first performing the anti-DCC staining followed by anti-BEN labeling according to the protocols described above, because anti-BEN, but not anti-DCC, labeling requires Triton X-100 (Fisher Scientific) in the blocking solution.

Photodocumentation

Primary antibody binding to transverse cryosections was visualized under epifluorescence optics (Nikon Eclipse TE300) using Cy2 or Cy3 optical filters (Chroma Technology). Black and white images were captured with a digital camera manufactured by Optronics (Bolton, MA) and compatible Magnafire software. For color images, the brightness, contrast, and/or color balance was adjusted in Photoshop for increased visibility of nerves/axons and for color matching. For black and white images, only the brightness and contrast were adjusted for increased visibility of cell bodies and axons using Adobe Photoshop. Sections subjected to in situ hybridization were visualized using an Olympus Optical AX70 microscope. Images of these sections were captured using a digital camera and compatible Magnafire software.

Results

Expression of Spinal Accessory Motor Neuron using anti-Ben as Marker

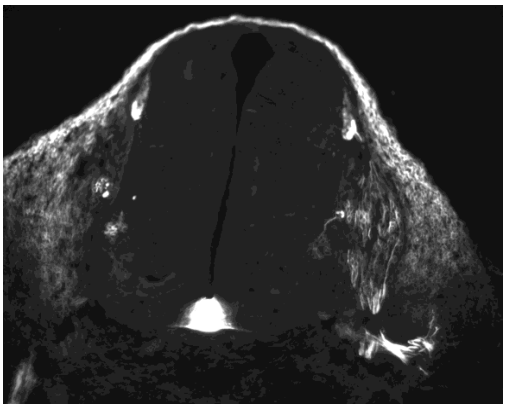


Fig. 3.1 – Ben expressed in wildtype C11.5 mouse, magnification 10x

Wildtype cyrosection using Anti-Ben to mark the SACMNs. SACMNs can be located midway between the dorsal root ganglia. Anti-Ben also marks the floor plate of the spinal cord and the dorsal root ganglia.

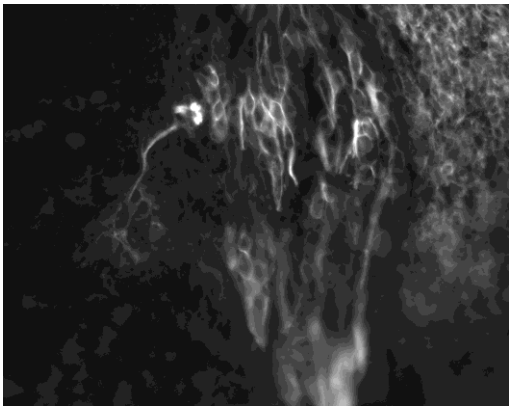


Fig. 3.2 - Ben expressed in wildtype C11.5 mouse, magnification 40x

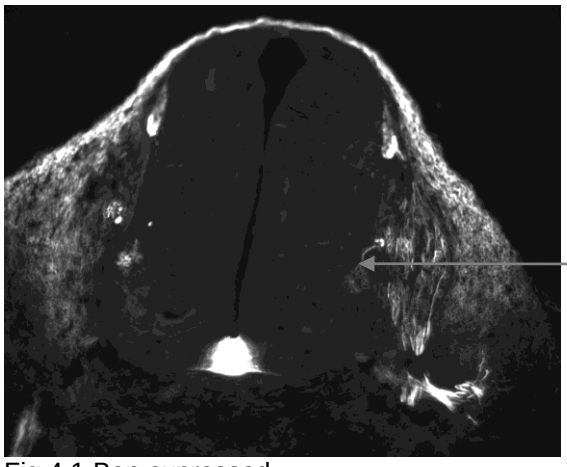
Magnification of 40x of SACMNs. This is a clear staining of the SACMNs cell bodies, as it has made its initial migration dorsally and is preparing to exit the spinal cord laterally.

Anti-BEN is a marker of SACMNs, their axons, and the SAN in the developing mouse spinal cord. At cervical levels of embryonic mouse spinal cord, SACMN cell bodies and their dorsally projecting axons selectively express the murine SC1-related protein (BEN). BEN continues to be expressed on these axons as they emerge from the spinal cord through the LEP, execute a rostral turn, and assemble into the longitudinally oriented SAN (Fig. 3.2). Anti-BEN labeling of transverse cryosections was derived from cervical spinal cord levels of various aged mouse embryos. At E9.25, anti-BEN specifically labels SACMN cell bodies that line the lateral edges of

the ventral and intermediate spinal cord and their dorsally directed axons, which have just begun to exit the spinal cord through the LEP (Dillon et al. 2006). One day later, at E10.5, anti-BEN intensely and selectively labels ventrolaterally and intermediolaterally positioned SACMN cell bodies and their dorsally projecting axons, which have extended out of the spinal cord and assembled into the longitudinally oriented SAN (Dillon et al. 2006). At E11.5, anti-BEN labeled SACMN cell bodies that have migrated to a more dorsal position just below the LEP, as well as the SAN (Fig. 3.1, 3.2). Anti-BEN immunoreactivity is also associated with dorsal root ganglia located adjacent to the LEP on either side of the spinal cord, as well as the neuroepithelium that lines the dorsal surface of the spinal cord. At each of these ages, anti-BEN also labels the floor plate and notochord (Fig. 3.1, data for notochord is not shown). At E11.5, anti-ALCAM, a different anti-BEN mAb, also labels SACMN cell bodies that have migrated to a more dorsal position just below the LEP and the SAN, as well as the floor plate (FP).

Expression of Spinal Accessory Motor Neuron using Anti-Ben as a Marker Compared to Knock Out Mouse

E11Wildtype Mouse Cyrosections

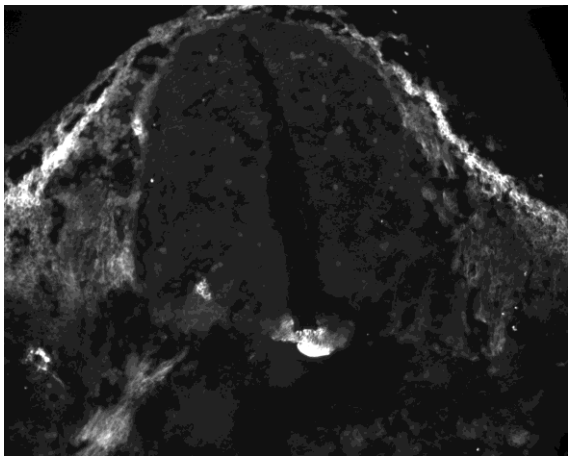


The SACMNs is preparing to exit the spinal cord laterally, after an initial dorsal migration. It has a clear direction, and is located in the correct position to be identified as the SACMNs.

Spinal accessory motor neuron

Fig 4.1 Ben expressed

E11 Knockout Roundabout Mouse Cyrosections



Though difficult to identify the SACMNs due to unsatisfactory tissue, upon further examination the SACMNs, are not affected by the knockout of Robo 1.

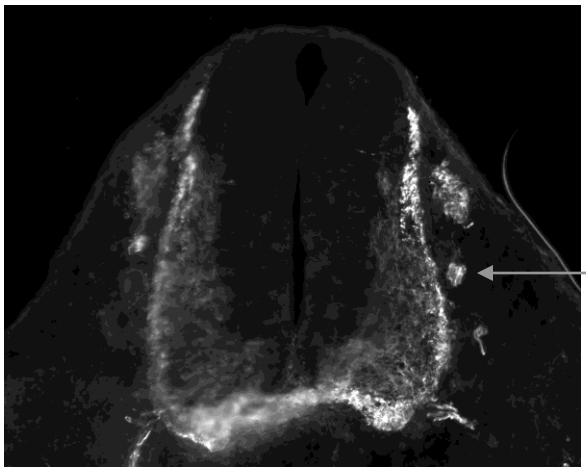
Fig 4.2 Ben expressed

It has been demonstrated that mAb SAC1 selectively labels SACMN in the embryonic rat spinal cord (Schubert and Kaprielian, 2001) and recognizes the rat ortholog of BEN/ALCAM/ SC1/DM-GRASP/MuSC, a homophilic adhesion molecule that regulates axon outgrowth/fasciculation (Leppert et al., 1999; Schubert and Kaprielian, 2001; Avci et al., 2004; Weiner et al., 2004). This researcher showed that anti-BEN (Sekine-Aizawa et al., 1998) labels mouse SACMNs, their axons, and the SAN (Fig. 3.1) and this marker was used to selectively examine SACMN development in a of mutant mice. To determine whether BEN is selectively expressed by SACMNs in mice, spinal cord-containing transverse cryosections derived from cervical regions of 11.5 days old (E11.5) mouse embryos were labeled with an anti-BEN mAb (Sekine-Aizawa et al., 1998). Anti-BEN is a marker of SACMNs, their axons, and the SAN in the developing mouse spinal cord. Schematic representation of SACMN cell bodies, the trajectory of SACMN axons, and the ascending SAN are apparent in figure 2. At cervical levels of embryonic mouse spinal cord, SACMN cell bodies and their dorsally projecting axons selectively express the murine SC1-related protein (BEN). BEN continues to be expressed on these axons as they emerge from the spinal cord through the LEP, execute a rostral turn, and assemble into

the longitudinally oriented SAN. Anti-BEN labeling of transverse cryosections derived from cervical spinal cord levels of various aged mouse embryos.

Expression of Spinal Accessory Motor Neuron using a variety of Markers Compared to Knock Out Mouse

E11Wildtype Mouse Cyrosections

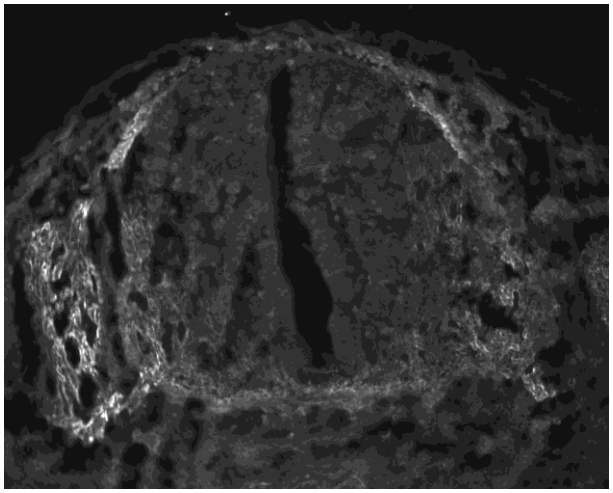


Using Neurofilimin as a marker, which labels all axons in the spinal cord, the SAN is labeled, though the SACMNs are not as apparent.

Spinal accessory nerve

Fig 4.3 Neurofilimim expressed

E11 Knockout Roundabout Mouse Cyrosections



Though difficult to identify the SACMNs due to unsatisfactory tissue, upon further examination the SACMNs, are not affected by the knockout of Robo 1.

Fig 4.4 Neurofilimim expressed

E11 Wildtype Mouse Cyrosections

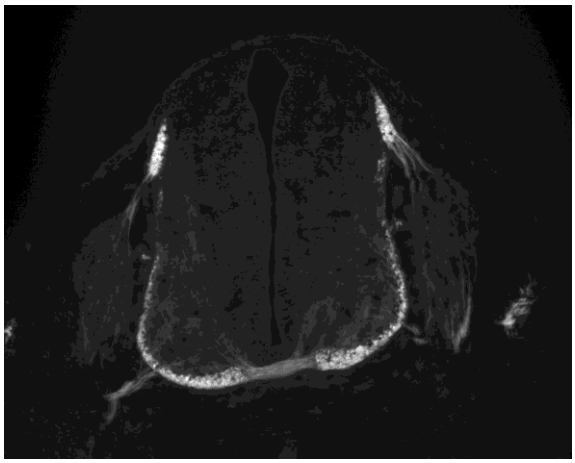


Fig. 4.7 Laminin 1

Using laminin 1 as a marker, which labels all axons in the spinal cord, the SAN is labeled, though the SACMNs are not as apparent.

This is most likely due to the specific cyrosection that was stained.

E11 Knockout Roundabout Mouse Cyrosections

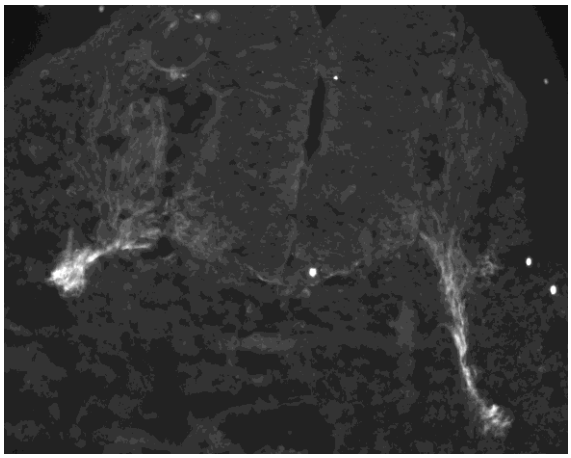


Fig. 4.8 Laminin 1

Though difficult to identify the SACMNs due to unsatisfactory tissue, upon further examination the SACMNs, are not affected by the knockout of Robo 1.

At E11.5 (fig. *3.1, 3.2*), anti BEN labels SACMN cell bodies that have migrated to a more dorsal position just below the LEP, as well as the SAN. Anti-BEN immunoreactivity is also associated with dorsal root ganglia located adjacent to the LEP on either side of the spinal cord, as well as the neuroepithelium that lines the dorsal surface of the spinal cord. At E11.5, anti-ALCAM, a different anti-BEN mAb, also labels SACMN cell bodies that have migrated to a more

dorsal position just below the LEP and the SAN, as well as the floor plate (FP). Consistent with previous findings in the rat model (Schubert and Kaprielian, 2001), BEN is transiently expressed by SACMNs in the developing mouse spinal cord.

As early as E9.25, anti-BEN labeled SACMN cell bodies and their axons, which project dorsally to the LEP, as well as the notochord and floor plate (Dillon et al., 2005) At E10.5, anti-BEN positive SACMN cell bodies lined the lateral edge of the spinal cord from a ventral position near the floor plate to a more dorsal position adjacent to the LEP (Dillon et al., 2005). Anti-BEN labeling was also observed on dorsally directed SACMN axons, including those segments that had extended out of the LEP, turned into the longitudinal plane, and assembled into the SAN. A day later, at E11.5, BEN-expressing SACMN cell bodies had migrated dorsally and were clustered in close proximity to the LEP (Fig. 4.1). To further confirm that mouse SACMNs express BEN during this period of development, transverse cryosections derived from cervical levels of an E11.5 mouse embryo were labeled with anti ALCAM, an independently generated mAb against BEN (Weiner et al., 2004). Consistent with anti-BEN labeling at E11.5, anti-ALCAM also labels SACMNs and the SAN, as well as the floor plate. The close similarity between anti-BEN and anti-ALCAM labeling supports the finding that BEN expression marks SACMNs.

Discussion

Spinal motor neurons/axons represent a well-studied model system for elucidating the molecular mechanisms that pattern neuronal connectivity. However, the full set of cues required to guide any motor axon subtype from cell body to target has yet to be defined. MAb SAC1has previously been identified as a marker of SACMNs and their axons in the developing rat spinal cord (Schubert and Kaprielian, 2001). Here it is demonstrated that a mAb specific for the mouse homolog of the SAC1 antigen, BEN (Sekine- Aizawa et al., 1998), recognizes mouse SACMNs and this marker was used to assess SACMN development in a variety of mutant mice. These

analyses show that *Robo 1* is not required for SACMN axon outgrowth thus disproving the null hypothesis. It has been previously shown that *netrin-1* and *Dcc* are necessary for the dorsal migration of SACMN cell bodies/axons toward the LEP, and *Nkx2.9* is required for the exit of SACMN axons through the LEP (Dillon et al 2005). Despite its established role as a mediator of axon outgrowth/ fasciculation (Weiner et al., 2004), it is also shown that *BEN* does not appear to be required for the assembly of SACMN axons into the SAN. Several observations support the conclusion that SACMN selectively express BEN. First, anti-BEN labels a bilaterally symmetric population of cell bodies located at cervical levels of the embryonic mouse spinal cord. Second, anti-BEN-positive cell bodies project dorsally directed axons to the LEP. These axons then emerge from the spinal cord, turn into the longitudinal plane, and fasciculate into a longitudinally projecting nerve.

In wild-type embryos, anti-BEN-positive SACMN cell bodies and their axons migrate along a dorsally directed trajectory toward the LEP. Consistent with the ability of the floor plate (Colamarino and Tessier-Lavigne, 1995b; Guthrie and Pini, 1995; Tucker et al., 1996) and netrin-1 (Varela-Echavarria et al., 1997; Alcantara et al., 2000; Hamasaki et al., 2001) to repel hindbrain or spinal motor axons/cell bodies, a subset of SACMN cell bodies/ axons fails to migrate dorsally in both *netrin-1* and *Dcc* mutants. The observation that BEN-positive SACMNs are likely to express *Dcc* mRNA supports a cell autonomous role for Dcc in mediating the presumed repulsion of SACMNs and their axons. The *in vivo* SACMN/axon migration defects that were described here are consistent with the failure of oligodendrocyte precursors to migrate dorsally in *Dcc* mutants (Jarjour et al., 2003). The abnormal migration of leutinizing hormone-releasing hormone neurons, aberrant projections of the caudal vomeronasal nerve in both *netrin-1* mutants and *Dcc* null embryos (Schwarting et al., 2001, 2004), finding that netrin-1 selectively repels dorsally projecting motor axons *in vitro* (Colamarino and Tessier- Lavigne, 1995a; Varela-Echavarria et al., 1997). Because a subset of anti-BEN-positive SACMN cell

bodies/ axons migrate appropriately toward the LEP in both *netrin-1* and *Dcc* mutants, other SACMN/axon-associated netrin-1 receptors and/or ventral spinal cord-associated chemorepellents, such as Slits and semaphorins (Varela-Echavarria et al., 1997; Brose et al., 1999; Patel et al., 2001), may also have roles in these guidance events. Previous studies in *Caenorhabditis elegans*, *Drosophila*, and *Xenopus* have shown that UNC-40/DCC binding to UNC-6/ netrin mediates ventrally directed cell body/axon migration events, whereas UNC-40/DCC operates in concert with UNC5 receptors to facilitate dorsally directed cell body/axon migration in response to netrin (Hedgecock et al., 1990; Hamelin et al., 1993; Hong et al., 1999; Keleman and Dickson, 2001; Dickson, 2002; Araujo and Tear, 2003). UNC5H2, H3, and H4 (Engelkamp, 2002) are expressed in the developing rodent CNS during neuronal migration and axogenesis (Ackerman et al., 1997; Leonardo et al., 1997; Mattar et al., 2004). Furthermore, UNC5H3 has been implicated in the migration of cranial branchiomotor neurons and Purkinje cell progenitors (Przyborski et al., 1998) and corticospinal tract development (Finger et al., 2002). Thus, SACMNs may express a mouse UNC5H receptor that cooperates with DCC to drive SACMN cell bodies and axons along a dorsally directed trajectory to the LEP.

Motor axon exit from the CNS is a poorly understood phenomenon, and the underlying molecular mechanisms remain obscure. A specialized group of neural crest derivatives, referred to as boundary cap cells, prefigure motor axon exit points (Vermeren et al., 2003). Although boundary cap cells confine motor neuron cell bodies to the spinal cord, they are not required for motor axon exit through the ventral roots (Vermeren et al., 2003). It is demonstrated that SACMN axons are incapable of exiting the spinal cord in *Nkx2.9* null embryos (Dillon et al). The finding that SACMNs are likely to arise from Nkx2.9-expressing progenitors suggests that Nkx2.9 functions cell autonomously. Mechanistically, it is proposed that Nkx2.9 regulates the expression of downstream targets that mediate the exit of SACMN axons from the spinal cord. Good candidates include receptors on SACMN axon/growth cones that respond to long- or

short-range attractive guidance cues, which may be expressed in the immediate vicinity of the LEP (Guthrie and Lumsden, 1992; Niederlander and Lumsden, 1996; Irving et al., 2002; Vermeren et al., 2003). Robo 1 falls under the category of a short-range repellent cue, though after experimentation, it is apparent that Robo 1 does not play a vital role in the exiting. In contrast to the inability of axons to emerge from the spinal cord, hindbrain-associated SACMN axons exit the CNS normally in *Nkx2.9* null embryos (data not shown), raising the possibility that another Nkx family member compensates for the loss of Nkx2.9 in the hindbrain. Notably, Nkx2.2 and Nkx2.9 are coexpressed for a longer period of time in the hindbrain compared with the spinal cord (Briscoe et al., 1999), and Nkx6 genes have evolutionarily conserved roles in motor neuron/axon development (Muller et al., 2003; Broihier et al., 2004). BEN has been shown to mediate axon outgrowth.

Conclusion

It can be concluded that roundabout, though a common chemorepellent, provides no role in the dorsal exiting of the spinal accessory motor neuron in mice. Though the tissue of the knockout mouse was not prime, there appeared to be no “wandering” of the spinal accessory motor neuron, as it was viewed with four different markers, each of which labeled either specifically the spinal accessory motor neuron, or all neurons. When compared to wildtype mice, the spinal accessory motor neuron behaved in a similar manner.

Further research could include breeding different strains of mice deficient in other attractant or repellent proteins and comparing the spinal accessory motor neuron to wildtype mice. It would be interesting to see if the affects of the removal of an attractant protein would have a positive or negative affect on the spinal accessory motor neuron.

Acknowledgements

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