Developmental Biology 399 (2015) 2–14

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Loss of Npn1 from motor neurons causes postnatal deficits independent from Sema3A signaling

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form
24 November 2014
Accepted 25 November 2014
Available online 13 December 2014

Keywords:
Npn1
Sema3A
Motor neuron
Postnatal development

ABSTRACT

The correct wiring of neuronal circuits is of crucial importance for the function of the vertebrate nervous system. Guidance cues like the neuropilin receptors (Npn) and their ligands, the semaphorins (Sema) provide a tight spatiotemporal control of sensory and motor axon growth and guidance. Among this family of guidance partners the Sema3A-Npn1 interaction has been shown to be of great importance, since defective signaling leads to wiring deficits and defasciculation. For the embryonic stage these defects have been well described, however, also after birth the organism can adapt to new challenges by compensational mechanisms. Therefore, we used the mouse lines *Olig2-Cre;Npn1*^{cond} and *Npn1*$^{Sema-}$ to investigate how postnatal organisms cope with the loss of Npn1 selectively from motor neurons or a systemic dysfunctional Sema3A-Npn1 signaling in the entire organism, respectively. While in *Olig2-Cre*$^{+}$*;Npn1*$^{cond-/-}$ mice clear anatomical deficits in paw posturing, bone structure, as well as muscle and nerve composition became evident, *Npn1*$^{Sema-}$ mutants appeared anatomically normal. Furthermore, *Olig2-Cre*$^{+}$*;Npn1*^{cond} mutants revealed a dysfunctional extensor muscle innervation after single-train stimulation of the *N.radial*. Interestingly, these mice did not show obvious deficits in voluntary locomotion, however, skilled motor function was affected. In contrast, *Npn1*$^{Sema-}$ mutants were less affected in all behavioral tests and able to improve their performance over time. Our data suggest that loss of Sema3A-Npn1 signaling is not the only cause for the observed deficits in *Olig2-Cre*$^{+}$*;Npn1*$^{cond-/-}$ mice and that additional, yet unknown binding partners for Npn1 may be involved that allow *Npn1*$^{Sema-}$ mutants to compensate for their developmental deficits.

Introduction

The establishment of a precisely working nervous system is of crucial importance for all vertebrates in order to interact with their environment. Thus, during development billions of neurons have to extend axons towards their target regions and establish the proper connections, which they do in a highly specific manner and with stunningly few errors. To enable this high accuracy of axon growth and guidance those processes underlie tight spatiotemporal regulation by specific cues that allow for axon–axon interaction and communication of the axons with their environment (Huber et al., 2003). During the late embryonic and early postnatal phase the carefully established circuits then undergo several changes in the

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http://dx.doi.org/10.1016/j.ydbio.2014.11.024

process of circuit maturation, since axons compete for target derived trophic support. It is during this phase that misprojecting axons are identified and eliminated and synapses undergo activity-dependent modulation by potentiation or repression of their signals (Hensch, 2005; Lichtman and Colman, 2000; Vanderhaeghen and Cheng, 2010).

Since the development of well-functioning neuronal circuits is such a complex process it is of great interest how the organism as a whole can cope with axonal wiring defects after the initial phase of circuit establishment. In this respect we investigated the defects resulting from deficient signaling of the axon guidance cue Semaphorin 3A (Sema3A) and its receptor Neuropilin 1 (Npn1). This ligand–receptor interaction has been shown to play important roles in several distinct aspects of axon guidance, including timing of growth, selective fasciculation, and mediation of the interaction between sensory and motor axons (Huber et al., 2005; Huettl et al., 2011; Kolodkin and Tessier-Lavigne, 2011). Therefore, we used the mouse lines *Olig2-Cre; Npn1^{cond}*, in which the Npn1 receptor is specifically ablated from motor neurons, and *Npn1^{Sema-}*, where point mutations in the ligand-binding region of the receptor selectively abolish the interaction with Sema3A in the entire organism, however, without interfering with the interaction with VEGF receptor 2 (Gu et al., 2003).

During embryonic development, this interaction has been investigated intensively, especially in respect of its consequences on limb innervating motor axons of the lateral motor column (LMC). Thus, it has been shown that at E10.5 the interaction of this ligand–receptor pair prevents a precocious ingrowth of axons, since the repulsive ligand Sema3A is expressed in the entire limb mesenchyme. Later, the expression pattern of Sema3A changes and consequently a distinct path is cleared for the axons to guide their way through the developing limb in a surround repulsion manner (Huber et al., 2005; Kolodkin and Tessier-Lavigne, 2011). Accordingly, this signaling is responsible for the accurate timing of sensory and motor axon ingrowth into the limb. Subsequently, axon trajectories are determined by other interaction partners, like the ephrins and their Eph receptors (Egea and Klein, 2007; Kao et al., 2012), GDNF and its receptor c-Ret (Kramer et al., 2006), or Sema3F-Npn2 signaling (Huber et al., 2005), that work together in order to guide the developing axons through the limb to their appropriate targets in a stepwise manner. Given the important role of this interaction it is not surprising that its elimination leads to severe pathfinding deficits. Recent studies by Huettl et al., show that the conditional removal of Npn1 from sensory neurons affects the fasciculation of motor and sensory fibers while motor axon trajectories are still established correctly. In contrast, depletion of Npn1 specifically from motor neurons disrupts the fasciculation of motor axons. In these mutants also the dorsal–ventral pathfinding of motor axons is affected and the most advanced defasciculated motor projections hardly reach the distal forelimb (Huettl et al., 2011). In a former study by Huber et al., *Npn1^{Sema-}* mice were investigated and also here motor and sensory axons were defasciculated within the plexus even though individual nerve branches were still found in a roughly correct position distal to the plexus. Nevertheless, retrograde tracing from the ventral forelimb musculature also revealed dorsal–ventral pathfinding deficits of Npn1 expressing motor neurons. Additionally, these mice displayed deficits in sensory axon fasciculation and a precocious ingrowth of both, motor and sensory axons, into the limb (Huber et al., 2005). At later embryonic and early postnatal stages, defasciculation is still evident in intercostal and sciatic nerves in this mouse line (Haupt et al., 2010). Thus, while the embryonic defects resulting from the deficient Sema3A-Npn1 signaling are already well described, it is still unclear how the postnatal organism deals with these impairments and if the loss of connections can be balanced by compensational mechanisms.

In order to approach this question, we analyzed general health, bone structure, and muscle composition in *Olig2-Cre;Npn1^{cond}* mice and assessed the functionality of their brachial nerves by electrophysiological stimulation. Furthermore, we compared their nerve composition and their performance in several sensory and motor behavioral tests to that of mice of the *Npn1^{Sema-}* line. Our data suggest that loss of Sema3A-Npn1 signaling in motor neurons is not the only cause for the observed anatomical and functional deficits in *Olig2-Cre^{+};Npn1$^{cond-/-}$* mice and that additional, yet unknown binding partners for Npn1 may be involved in the wiring of the sensory-motor circuitry and compensate the developmental deficits in *Npn1^{Sema-}* mutants.

Material and methods

Ethic statement

Mice were handled according to the federal guidelines for the use and care of laboratory animals, approved by the Helmholtz Zentrum München Institutional Animal Care and Use Committee. All experimental procedures were approved by and conducted in adherence to the guidelines of the Regierung von Oberbayern.

Mouse lines (mus musculus)

The following mouse lines on a C57BL/6 background were used: *Hb9::eGFP* (Wichterle et al., 2002), *Npn1^{cond}* (Gu et al., 2003), *Npn1^{Sema-}* (Gu et al., 2003), *Olig2-Cre* (Dessaud et al., 2007). Genotyping was performed as described previously (Huettl et al., 2011). Since *Olig2-Cre^{wt};Npn1$^{cond-/-}$* and *Olig2-Cre$^{+/-}$;Npn1$^{cond\ wt}$* animals did not reveal any differences in their phenotype or motor neurons innervating the forelimb extensors (Fig. S1), only *Olig2-Cre^{wt};Npn1$^{cond-/-}$* animals were used as controls.

Electrophysiological analysis

Electrophysiological experiments were carried out in adult animals under Ketamine (0.1 mg/g, i.p., Bela-Pharm GmbH & Co. KG, Vechta, Germany) and Xylazine (0.01 mg/g, i.p., cp-pharma mbH, Burgdorf, Germany) anesthesia. An additional dose of anesthesia was given if required during the experiment. After the experiment, mice were euthanized without regaining consciousness.

The nerve stimulation procedure was modified from a previous protocol (Udina et al., 2008). The nerves of interest (i.e. *N. musculocutaneus, N. radial, N. median, N. ulnar*) were identified according to the literature (Greene, 1963) and stimulated with single bipolar electric pulses (100 μs duration) using custom made stimulation electrodes with 2 hooks (stainless steel, 0.2 mm in diameter with 1 mm in between). For the generation of the pulses the following instruments were used: Master-8 pulse generator, A.M.P.I, Jerusalem, Israel; Amplifier P511 DC; Astromed GmbH, Rodgau, Germany; Digidata 1440A Digitizer, Molecular devices, Sunnyvale, USA. Stimulation intensity was increased from 0 to 0.1 mA in 0.05 intervals (Isoflex-Flexible stimulus isolator, A.M.P.I). The elicited movement in the limb was described in terms of direction and body parts involved and afterwards compared between control and mutant animals.

Immunohistochemistry

For fluorescent immunohistochemistry tissue was fixed in 4% paraformaldehyde (PFA) overnight and cryoprotected in 30% sucrose. Spinal columns and arms of P0 animals were cryosectioned in 20 μm slices as series of 4. Spinal cords were sectioned at 40 μm sections using a sliding microtome (Leica) and every second section was analyzed. Immunohistochemistry was performed as described previously (Huber et al., 2005). For staining the following antibodies were used: rabbit anti-GFP (1:4000, Invitrogen), rat anti-myelin basic