The receptor guanylyl cyclase Npr2 is essential for sensory axon bifurcation within the spinal cord

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Sensory axonal projections into the spinal cord display a highly stereotyped pattern of T- or Y-shaped axon bifurcation at the dorsal root entry zone (DREZ). Here, we provide evidence that embryonic mice with an inactive receptor guanylyl cyclase Npr2 or deficient for cyclic guanosine monophosphate–dependent protein kinase I (cGKI) lack the bifurcation of sensory axons at the DREZ, i.e., the ingrowing axon either turns rostrally or caudally. This bifurcation error is maintained to mature stages. In contrast, interstitial branching of collaterals from primary stem axons remains unaffected, indicating that bifurcation and interstitial branching are processes regulated by a distinct molecular mechanism. At a functional level, the distorted axonal branching at the DREZ is accompanied by reduced synaptic input, as revealed by patch clamp recordings of neurons in the superficial layers of the spinal cord. Hence, our data demonstrate that Npr2 and cGKI are essential constituents of the signaling pathway underlying axonal bifurcation at the DREZ and neuronal connectivity in the dorsal spinal cord.

Introduction

Appropriate functioning of the mature nervous system relies on the correct development of neuronal circuitry. In the spinal cord, second order neurons integrate sensory input from a large number of primary afferents. A prerequisite to form such a high degree of connectivity is the multiple ramification of primary axon projections to allow the innervation of several distinct targets. Dorsal root ganglion (DRG) axons enter the spinal cord at the dorsal root entry zone (DREZ), where they bifurcate into a rostral and a caudal arm. These arms extend longitudinally over several segments but remain confined to the oval bundle of His. Collaterals are then generated from these stem axons to penetrate the gray matter (Mirnics and Koerber, 1995; Ozaki and Snider, 1997). Cutaneous sensory collaterals are confined to the dorsal horn, whereas collaterals of muscle spindle Ia afferents grow to the ventral cord (Fig. 1 A). Thus, from a structural point of view, sensory axons display at least two types of ramifications within the cord: bifurcation at the DREZ and interstitial branching from stem axons to generate collaterals.

So far, the signaling cascades that underlie axonal branching in vivo have remained poorly understood, although neurotrophins, semaphorin 3A, and Slit proteins were implicated in the branching of axons or dendrites in vitro (Cohen-Cory and Fraser, 1995; Gallo and Letourneau, 1998; Wang et al., 1999; Polleux et al., 2000; Whitford et al., 2002). Our earlier studies suggested that the bifurcation of sensory axons at the DREZ depends on cyclic guanosine monophosphate (cGMP) signaling via the serine/threonine kinase cGMP-dependent protein kinase I (cGKI, also termed PKGI). cGKI is strongly expressed in embryonic sensory axons at the DREZ, and its absence was shown to cause axonal misprojections at the DREZ, reduced axon numbers in the developing dorsal funiculus, premature growth of some sensory axons toward the central canal, and consequently a reduction of ventral root potentials (Schmidt et al., 2002). cGMP, a common second messenger that is produced by soluble or particulate guanylyl cyclases (sGCs or pGCs, respectively), controls a broad spectrum of physiological responses such as smooth muscle relaxation, phototransduction, olfactory transduction, bone growth, sperm motility, platelet spreading, electrolyte and water balance, and axonal pathfinding (Hofmann et al., 2000; Lucas et al., 2000; Ayoob et al., 2004; Hofmann et al., 2006).
Here, we identify the cGMP-producing receptor GC Npr2 as a molecule essential for sensory axon bifurcation. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) labeling analysis of a loss-of-function mutant of Npr2, as well as a constitutive cGKI knockout and crossbreeding experiments of these mutant mice with a mouse line expressing EGFP in sensory neurons under control of the Thy-1 promotor, demonstrate at the single axon level that the interruption of cGMP signaling in sensory neurons results in a selective bifurcation error of DRG neurons. As a likely consequence a reduction in the degree of coupling with second order neurons in the dorsal horn was observed. Thus, these findings demonstrate that cGMP signaling involving Npr2 and cGKI is crucially important for proper sensory axon bifurcation at the DREZ during nervous system development.

**Results**

In the absence of cGKI, sensory axons lack bifurcation at the DREZ of the spinal cord

Because our previous studies (Schmidt et al., 2002) gave evidence for an erroneous projection of DRG axons at the DREZ in the absence of cGKI, we analyzed the trajectories of single axons in cGKI knockout mice at different spinal levels. The lipophilic tracer DiI was applied to embryonic day (E) 12–14 DRG in a manner that allowed us to follow the trajectories of single sensory axons in whole mount dissections of the spinal cord. Interestingly, visualization of single growth cones at the DREZ in wild types indicated that the formation of the rostral and caudal arms occurs directly by splitting of the tip of the axon, the growth cone (Fig. 1 B), whereas interstitial branches, the major branch type within the brain, sprout from the axon shaft (O’Leary and Terashima, 1988). All sensory axons in wild-type and heterozygous mice bifurcated in a T- or Y-shaped manner in caudal and rostral directions; in contrast, sensory axons of cGKI-deficient mice at all spinal levels almost always lacked a bifurcation. The ingrowing axon ran either caudally, or, with slight preference, rostrally (Fig. 1, C–E). These results indicate that sensory axon bifurcation at the DREZ depends on cGKI.

**Expression of the receptor GC Npr2 and cGKI in embryonic DRG**

To identify components responsible for cGMP synthesis in embryonic sensory neurons we surveyed the expression of GCs in embryonic DRG neurons. cGMP is generated from Mg-GTP by s- or pGCs. sGCs are heterodimeric enzymes composed of α and β subunits that are activated by nitric oxide (NO) and found to be colocalized with NOS in several cases (Ding et al., 2004, 2005; Koesling et al., 2004). In contrast, the pGCs are transmembrane proteins that contain extracellular ligand-binding domains and exist as homodimers on the plasma membrane. In mammals, seven distinct pGCs are known that share a common overall domain structure (Lucas et al., 2000; Potter et al., 2006).

In a PCR screen using cDNA from E12 mouse DRG as an amplification template, we could detect transcripts for the receptor-type GCs Npr2 (also called GC-B or NPR-B) and Gucy2e (GC-E) but not for the other five receptor GCs (Fig. 2 A). The expression of Npr2 could be confirmed by in situ hybridization where Npr2 mRNA was found to be expressed in embryonic DRG in a pattern overlapping with that of cGKIIa (Fig. 2, B–E), whereas Gucy2e mRNA was found to be beyond the detection limits using two distinct probes for hybridization (unpublished data). cGKII transcripts were also not detectable using cDNA from DRG or spinal cord tissue as an amplification template. Spinal cord neurons were negative or faintly positive for Npr2,
whereas cGKIα was found in DRG cells, in preganglionic spinal neurons, and faintly in motoneurons at the mRNA as well as at the protein level (Schmidt et al., 2002). It has been reported that NOS1 (nNOS) colocalized with cGKI in rat DRG, suggesting that sGCs might be relevant to the generation of cGMP in embryonic DRG (Qian et al., 1996). Our PCR screen, as well as in situ analysis of the different NOSs and sGCs, revealed no or only weak expression of the NOS and different forms of sGCs in embryonic DRG, making it unlikely that, at this age, the NOS/sGC system is functional in DRG (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200707176/DC1).

Inactivation of the receptor GC Npr2 in cn/cn mice results in a phenocopy of the bifurcation error of cGKI-deficient mice

Based on these results on the expression pattern and other published findings (DiCicco-Bloom et al., 2004), we focused on Npr2 as a candidate protein for the generation of cGMP and thereby activation of cGKIα in embryonic DRG neurons. To test whether Npr2 might be involved in axonal bifurcation at the DREZ, we made use of cn/cn mice. These express an inactive form of Npr2 because of an amino acid substitution in the GC domain and lack an increase of the intracellular cGMP level upon stimulation (Tsujii and Kunieda, 2005). Dil tracing experiments revealed a complete phenocopy of the lack of bifurcation of sensory axon as observed in the constitutive cGKI knockout mice. Again, sensory axons turned without bifurcation in the rostral or caudal direction with a slight preference to the rostral direction (Fig. 3, A–D). The majority of the axons stayed within the oval bundle of His.

A lack of bifurcation at the DREZ would result in a significant reduction of axon numbers in the developing dorsal funiculus. As a measure of axon number, anti-trkA immunoreactivity was quantified in transverse sections of E13 spinal cords (Fig. 3 E). The trkA-labeled area in the dorsal funiculus of cn/cn E13 embryos amounted to 64.1 and 59% of the wild-type controls, determined at thoracic and lumbar trunk levels, respectively. Although this method only gives an indirect estimate of axon number, the results strongly suggest that mice lacking a bifurcation in the DREZ contain substantially fewer axons within the dorsal funiculus. In addition to these branching errors we also observed a small group of trkA-positive axons penetrating prematurely into the dorsal horn growing further in the direction of the central canal (Fig. 3 G). This erroneous trajectory of trkA-positive axons resembles the previously described misguidance in the absence of cGKI (Schmidt et al., 2002). However, in both cases the majority of axons turns and grows in the developing funiculus. Thus, the identical phenotypes in axon bifurcation at the DREZ of cn/cn and the constitutive cGKI knockout mice suggest that in embryonic DRG neurons activation of cGKIα by cGMP generated by Npr2 is crucial for triggering the bifurcation process at the DREZ. Analysis of bifurcation of double heterozygotes did not reveal significant bifurcation errors (93% T-shaped branches in double heterozygotes and 97% in wild type), indicating that there was no detectable concentration dependency in this system.

It is important to note, however, that the inactivation of cGMP signaling in Npr2 loss-of-function mutants disrupts neither the overall organization of the spinal cord nor the pathfinding of TAG-1-positive commissural axons to the ventral midline or the formation of L1-positive lateral and ventral axon tracts within the developing spinal cord (Fig. 3, H–K).

Nociceptive and proprioceptive collaterals are formed in cn/cn and cGKI-deficient mice

After a waiting period, collaterals extending into the gray matter of the spinal cord are generated by interstitial branching along the longitudinal stem axons (Ozaki and Snider, 1997). Although the collaterals of nociceptive afferents are confined to the dorsal cord, collaterals of muscle afferents grow into the ventral horn where further branching occurs. Staining of transverse sections by antibodies to trkA, parvalbumin, or peripheralin indicated that nociceptive as well as proprioceptive collaterals extend into the dorsal and ventral horn of mutant mice, respectively. The pattern of target innervation of both collateral systems was indistinguishable from that of wild-type mice (Fig. 4, A–F). Furthermore, Dil tracing experiments revealed that neither blocked cGMP formation in mice with inactive Npr2,
nor did the constitutive cGKI knockout prevent the formation of collaterals, although the distances between the points of origin from the stem axon were slightly reduced in mutant mice (Fig. 4, G–K). Thus, cGMP signaling induced by Npr2 and mediated by cGKI is not required for collateral formation, and its loss does not influence the overall trajectories of sensory collaterals (Schmidt et al., 2002). It should be noted that the overall growth pattern of sensory axons within the periphery was unchanged as well (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707176/DC1).

Lack of bifurcation of sensory axons persists until spinal cord maturity in cn/cn and cGKI-deficient mice

To seek further confirmation of the bifurcation error observed at the DREZ and clarify whether compensatory mechanisms exist to correct the bifurcation error at later developmental stages, we examined the bifurcation behavior of DRG neurons by a Thy-1–EGFP or Thy-1–YFP allele crossed into the cn/cn mutants or the constitutive cGKI knockout mice. Only small fractions of all sensory modalities of DRG neurons are labeled in the Thy-1–EGFP mouse line, designated M, which allows one to follow the trajectories of single sensory axons at the DREZ (Feng et al., 2000). Because of the late activation of the Thy-1 promoter, dissected spinal cords were analyzed at postnatal day (P) 21. Consistent with the results of the DiI tracing, we found in whole mounts from cn/cn or cGKI-deficient mice a selective bifurcation error at the DREZ. In contrast to the T- or Y-shaped axons observed in wild-type mice, the main axons of mutant mice turned either caudally or rostrally, with some preference for the rostral direction (Fig. 5, A–D). We conclude that the bifurcation error caused by the lack of cGMP signaling...
at a stage when axons enter the cord persists to mature stages, and no compensation seems to exist at the DREZ, at least until P56 (Fig. 5 and not depicted), when the connectivity of the spinal cord is quasimature in mice. However, in addition to the bifurcation error, we sometimes observed aberrant sensory axon projections at the DREZ as illustrated in Fig. 5. Axons initially turned in one direction but then looped backward to extend in the opposite direction (Fig. 5, G and H) or formed short processes of 20–50 μm in length at the DREZ (Fig. 5, E and F). Furthermore, these data were confirmed by using the Thy-1–YFP-H mouse line crossed into mutant mice (Fig. 5 H and not depicted).

Electrophysiological recordings revealed deficits in connectivity in cn/cn mice

Primary nociceptive afferents establish synaptic connections with neurons in the superficial laminae of the dorsal horn. The absence of bifurcation in cn/cn mice prompted us to examine the functional consequences of this alteration. The overall layering within the dorsal spinal cord of these mice appears not to be severed by the inactive Npr2, as indicated by staining using the isolectin B4 antibodies to the calcitonin gene-related peptide (CGRP) or the vesicular transport protein of glutamatergic synapses VGlut1 (Fig. 6, A–F). As an indicator for neuronal connectivity we recorded glutamatergic miniature excitatory postsynaptic currents (mEPSCs) from neurons in the superficial laminae of the dorsal horn in slice preparations from P10–14. To measure activity originating from nociceptive afferents we applied capsaicin, a compound preferentially selective for polymodal nociceptor cells in the superficial dorsal horn that activates presynaptic TRPV1 receptors on primary afferents (Baccei et al., 2003). It was found that the fraction of neurons responding to capsaicin was significantly higher in wild-type slices (wild type, 57.14% [16/28]; cn/cn, 22.22% [6/27]; P < 0.01, χ² test), which is consistent with the observation that the number of DRG axons was lower in the oval bundle of His in cn/cn mice (Fig. 6 G). Furthermore, dorsal spinal cord neurons of cn/cn mice displayed a significantly weaker response to capsaicin than wild-type neurons (Fig. 6, H and I; P = 0.032, Mann-Whitney U test). Even under resting conditions, i.e., in the absence of capsaicin, mEPSC frequency tended to be lower in cn/cn neurons (P = 0.055, Mann-Whitney U test). Interestingly, capsaicin-insensitive neurons did not differ with respect to their frequency. The basic postsynaptic parameters of the glutamatergic mEPSCs in the dorsal spinal cord, i.e., amplitude and time constant of decay, were not affected in cn/cn mice (Fig. 6 J; amplitude: wild type, 21.36 ± 1.39 pA, n = 24; cn/cn, 24.19 ± 2.17 pA, n = 24; decay time constant: wild-type, 2.09 ± 0.15 ms; n = 24; cn/cn, 1.90 ± 0.12 ms; n = 24).

Hence, the loss of cGMP-mediated regulation of axon bifurcation at the DREZ in embryonic development leads to a loss of functional connectivity with second order neurons within the superficial dorsal horn. Therefore, the results of electrophysiological recordings correlate with our anatomical findings on sensory axon bifurcation errors.
Discussion

Branch formation is one principle process that defines the pattern of axonal trajectories. Despite intensive research efforts, the molecular signaling pathways underlying neuronal branching have remained poorly understood. The data presented in this paper illuminate the role of cGMP signaling comprising the receptor GC Npr2 and the serine/threonine protein kinase cGKI for bifurcation of primary afferents at the DREZ. In this paper, we observed that Npr2 is expressed in embryonic DRG, largely overlapping the expression of cGKIα. We showed that interruption of cGMP signaling caused by a loss of function of Npr2 or the absence of cGKI prevents sensory axon bifurcation at the DREZ. Interestingly, interstitial branching, i.e., the sprouting of collaterals, is not affected by the interruption of cGMP-mediated signal transduction, suggesting that different sets of molecules are responsible for sensory axon bifurcation and interstitial branching. Consistently, in retinotectal axons, interstitial branching appears to depend on ephrin-EphA signaling (Yates et al., 2001; Hindges et al., 2002). The absence of bifurcation at the DREZ in turn results in a substantial reduction in the number of axons running in the dorsal funiculus and the synaptic input received by second order neurons within the superficial dorsal horn, which is the first relay station of nociceptive sensory axons (Fitzgerald, 2005).

Several genetic instructions are likely to act in concert to induce bifurcation and longitudinal growth: (a) a mechanism causing the sensory axons to avoid the gray matter when reaching and entering the cord; (b) a signal at the point of bifurcation that allows the two main branches to become distinct and enables further segregation; (c) the growth cones of the two main branches should be able to navigate independently to detect specific signals for rostral or caudal growth and therefore may express distinct sets of guidance receptors; (d) main branches growing in the same longitudinal direction must recognize each other to fasciculate in the oval bundle of His; and (e) it might be essential that after branching at the DREZ, further bifurcation is suppressed. Additional guidance cues are then required to regulate the mediolateral position of the main axon branches in the dorsal funiculus and to induce interstitial branching to generate collaterals.

Most of these processes are not fully understood, although recent investigations on the trajectories of proprioceptive and cutaneous axons and their collaterals suggest that repulsive factors, such as semaphorins and netrins, or branching/repulsive factors, such as Slit2, may shape axon growth. For instance, in the absence of plexinA1, main axons of proprioceptive neurons invade the superficial dorsal horn, whereas the expression of netrin-1 within the dorsal spinal cord prevents ingrowth and intraspinal projections of both proprioceptive and cutaneous afferents (Watanabe et al., 2006; Yoshida et al., 2006). Slit proteins that bind to robo receptors are reported to influence branching of sensory axons in a collagen culture (Wang et al., 1999).
Notably, in the absence of Slit1 and 2, mice display a partial overshooting in axonal growth toward the midline of the spinal cord, but all of the sensory axons in Slit1;Slit2 double mutants still bifurcated at the DREZ, although at a slightly different angle (Ma and Tessier-Lavigne, 2007). Furthermore, the transcription factors Runx3 and Er81 may regulate the expression of some of the surface receptors that direct proprioceptive sensory collaterals to the intermediate and ventral spinal cord (Arber et al., 2000; Inoue et al., 2002; Chen et al., 2006). Finally, antibody perturbation experiments in the chick indicated that members of the immunoglobulin superfamily influence the projection patterns of collaterals (Perrin et al., 2001).

The deficits observed in this paper suggest a signaling mechanism where, after the activation of the receptor GC Npr2, cGMP is generated from GTP, which triggers cGKIα to phosphorylate yet-unresolved targets converging in cytoskeletal rearrangements (Suga et al., 1992) in the growth cones of sensory afferents (Fig. 7). From other cellular systems it is established that the binding of C-type natriuretic peptide (CNP), the extracellular ligand of Npr2, leads to the generation of the second messenger cGMP that, in turn, activates cGMP-dependent kinases as one of its major cellular targets (Lucas et al., 2000). Because transcripts of CNP are abundant in the dorsal spinal cord of E12.5 mice (DiCicco-Bloom et al., 2004), CNP might be an attractive candidate serving as a “bifurcation signal.” However, using a variety of in vitro conditions we did not see any action of CNP (either provided as a point source or homogeneously) on the growth cone behavior of DRG axons (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200707176/DC1). There are several explanations for this finding, including the following: (a) in DRG neurons CNP is inappropriate for activation of Npr2; (b) CNP only acts in concert with other factors, e.g., repulsive components such as the Slit or netrin proteins to avoid ingrowth into the gray matter (Watanabe et al., 2006; Ma and Tessier-Lavigne, 2007); and (c) the in vitro systems do not reflect the in vivo situation, which makes it impossible to reproduce the conditions required for sensory axonal bifurcation. In this context it is noteworthy that activation of receptor GCs can also occur via the small GTPase Rac and the p21-activated
kinase pathway (Guo et al., 2007), which opens the possibility that cGMP formation by Npr2 can be induced by extracellular factors other than CNP. cGMP formation by transmembrane GCs can also be regulated via protein kinase C through a so-called heterologous desensitization mechanism leading to dephosphorylation of conserved stretches of intracellular segments of pGCs (Potter et al., 2006). The situation becomes more complex in that on the one hand, cGMP signaling can regulate its own degradation by binding to GAF domains present in several cyclic nucleotide phosphodiesterases (PDEs), where they cause allosteric activation of their catalytic domain (Bender and Beavo, 2006). Alternatively, this association can also affect the level of intracellular cAMP depending on the type of PDE present in sensory growth cones. In vitro studies showed that the relation between cAMP and cGMP modulates the responses of growth cones to external signals (Song and Poo, 1999).

Outside the nervous system, a well-characterized physiological action of CNP/Npr2 via cGKII but not GKI is the induction of long bone growth, where CNP acting on chondrocytes induces endochondral ossification. Inactivating mutations of the genes encoding for CNP (Chusho et al., 2001) or Npr2 (Npr-B) in mice (Tamura et al., 2004; Tsuji and Kunieda, 2005) or humans (Bartels et al., 2004) causes dwarfism, whereas overexpression of CNP as a transgene (Suda et al., 1998; Yasoda et al., 2004) or reduced clearance of CNP (Jaubert et al., 1999; Matsukawa et al., 1999) was shown to cause skeletal overgrowth. Interestingly, Npr2-deficient mice displayed self-clasping, priapism, and seizures, suggesting neuronal disorders (Tamura et al., 2004). Abnormalities of bone growth caused by enhanced or decreased CNP action only evolve after birth because of changes in the proliferative zones in the growth plates. Accordingly, cGKII deficiency or spontaneous inactivating mutations of this gene in rats produce dwarfism (Pfeifer et al., 1996; Miyazawa et al., 2002; Chikuda et al., 2004). Although it is unclear whether dwarfism as seen in Npr2 mutants also influences neuronal connectivity, it is unlikely to explain the present bifurcation deficits in DRG sensory axons by a reduced bone growth because cGKI-deficient mice lack the respective symptoms (Wegener et al., 2002). cGMP signaling has also been implicated in cell proliferation, but cell counts in the DRG have revealed no change in the constitutive cGKI knockout mice. The reduction of axon numbers as reflected by the stained area in the dorsal funiculus is therefore not a result of neuronal cell death (Schmidt et al., 2002).

A deeper understanding of the cGMP signaling mechanisms at the DREZ requires further knowledge on the activation and desensitization processes of the receptor GC Npr2, the regulation of cGMP concentration by PDEs, and the identification of phosphorylation targets of cGKIα in growth cones. The latter might include components regulating the actin cytoskeleton such as proteins of the Ena/VASP family (Krause et al., 2003) or the myosin phosphatase (Surks et al., 1999). It will be interesting to explore whether cGMP-mediated bifurcation is unique to sensory afferents of the spinal cord and how the complete absence of one longitudinal main branch affects the sensory-motor coordination of spinal reflex activity.

Materials and methods

Mice

cn/cn mice were obtained from Jackson Immunoresearch Laboratories and their genotyping as well as that of cGKI-deficient mice has been described previously (Wegener et al., 2002; Tsuji and Kunieda, 2005). These mouse lines were crossed with transgenic GFP-M or YFP-H mice under the control of the Thy-1 promoter (Feng et al., 2000) to get GFP-expressing cn/cn and cGKI-deficient mice, respectively.

Tracing studies

For Dil (Sigma-Aldrich) tracing, E12–14 spinal cords with attached DRG were dissected and fixed in 4% paraformaldehyde in PBS overnight followed by DRG labeling with a dye-filled glass electrode (100- or 200-μM Dil solution in ethanol). The preparations were then incubated in PBS at room temperature for 1–3 d.

For fluorescence analysis of sensory axon morphology in the off-spring of crossbreedings between GFP-M and cGKI-deficient mice or cn/cn mice, spinal cords were removed from P21 and fixed in PBS containing 4% paraformaldehyde. After mounting, spinal cords were examined using an inverted fluorescence microscope (for details see Immunohistochemistry). The evaluation was performed blind with regard to the genotype. Only labeled axons that were unambiguously identified as single axons were counted. Because of the limitations of the Dil tracing only distances between developing collaterals were counted from E14 embryos. Axons that had no or only one collateral were ignored for the quantification.

RT-PCR analysis and in situ hybridization

mRNA isolated from mouse E12 spinal cord or DRG, respectively, using Dynabeads Oligo(dT)25 (Invitrogen) according to the manufacturer’s instructions was subsequently reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). DNA fragments of elements of the cGMP signaling
pathway were amplified using oligonucleotides cGKλ3 5′-AGGATCTCGGAGTTCGAGG-3′, cGKλ3 5′-GGCACTGGGGACGAG-3′, cGKλ3 5′-CTCGGGGCTGCTAGTGCTGAC-3′, NOS1 5′-CGGAGTTCGAGG-3′, NOS2 5′-GCAGGCTGCTGCTAGTGCTGAC-3′, NOS3 5′-GGAGTTCGAGG-3′, Gucy1a3 5′-GGCACTGGGGACGAG-3′, Gucy1b3 5′-CTCGGGGCTGCTAGTGCTGAC-3′, Gucy1c 5′-CTCGGGGCTGCTAGTGCTGAC-3′, NOS1 5′-GAGGATCTCGGAGTTCGAGG-3′, Gucy1b 5′-GGCACTGGGGACGAG-3′, Gucy2a 5′-GGCACTGGGGACGAG-3′, NOS3 5′-CTCGGGGCTGCTAGTGCTGAC-3′.

The amplification products were subsequently cloned into a pBluescriptKS' vector. Primer sequences are provided in Table 1 (Table S1).

For control purposes all primer pairs were tested with a cDNA template from a mouse E17 embryo. The amplification products were subsequently cloned into a pBluescriptKS' vector (Strategene) and their identity was verified by sequencing.

In situ hybridization studies on 25-μm transversal sections of mouse E12 spinal cord using DIG-labeled riboprobes to cGKI were performed as described previously (Ausabel et al., 2004).

Immunohistochemistry

For immunohistochemical detection, cryostat sections of formaldehyde-fixed embryonic CNS tissue or P10–14 mice were incubated with primary antibodies and processed as described above. Figure 1 shows that NOS1, NOS3, and NOS1/NOS3-deficient mice do not reveal a reduced trkA-positive dorsal funiculus that is indicative of a branching error at the DREZ (A–C). The localization of NOS1 at the mRNA and protein level is revealed in D–H. Fig. S2 shows whole mounts of embryonic mice stained by an anti-neurofilament antibody that indicate no pathfinding errors of sensory axons in the periphery in the absence cGKI. Fig. S3 analyzes the growth cone behavior of sensory axons in response to the presence of CNP in in vitro cultures. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200701176/DC1.

We are grateful to Madlen Driesner and Carola Bach for their excellent technical assistance. We thank Dr. Jonathan Granthy and Gary Lewin for valuable comments on the manuscript and Drs. Rudolf Martin and Karl Zilles for discussions. We thank Drs. Tobias Bonhoeffer, Rudolf Martini, and Michael Bader for the help with the GFP, YFP, and cn/cn mice, respectively. We acknowledge the advice of Dr. Christine Eichhorn on the statistical evaluations.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB665) and PhD stipends of the Max Delbrück Centrum für Molekulare Medizin to A. Storkine and to S. Schaffler.

Submitted: 25 July 2007
Accepted: 22 September 2007

References

Auber, S., D.R. Ladle, J.H. Lin, E. Frank, and T.M. Jessell. 2000. ETS gene Ebr1 controls the formation of functional connections between group I sensory afferents and motor neurons. Cell. 101:485–498.

Ayoob, J.C., H.H. Yu, J.R. Terman, and A.L. Kolodkin. 2004. The Drosophila receptor guanylyl cyclase Gyc76C is required for semaphorin-1a-plexin A-mediated axonal repulsion. J. Neurosci. 24:6369–6366.

Baccei, M.L., R. Bardon, and M. Fitzgerald. 2003. Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. J. Physiol. 549:231–242.

Bartels, C.F., H. Bukulmez, P. Padayatti, D.K. Rhee, C. van Ravenswaaij-Ants, R.M. Pauli, S. Mundlos, D. Chitayat, L.Y. Shih, L.I. Al-Gazali, et al. 2004. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am. J. Hum. Genet. 75:27–34.

Bender, A.T., and J.A. Beavo. 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol. Rev. 58:488–520.

Chen, A.I., J. C.de Nooij, and T.M. Jessell. 2006. Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron. 49:395–408.

Chikuda, H., F. Kugimiya, K. Hoshi, T. Ikeda, T. Ogasawara, T. Shimoaka, H. Kawano, S. Kametka, A. Tsuchida, N. Yokoi, et al. 2004. Cyclic GMP-dependent protein kinase II is a molecular switch from proliferation to hypertrophic differentiation of chondrocytes. Genes Dev. 18:2418–2429.

Chusho, H., N. Tamura, Y. Ogawa, A. Yasoda, M. Suda, T. Miyazawa, K. Nakamura, K. Nakao, T. Kurhara, Y. Komatsu, et al. 2001. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc. Natl. Acad. Sci. USA. 98:4016–4021.

Cohen-Cory, S., and S.E. Fraser. 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodeling in vivo. Nature. 378:192–196.

DeCicco-Bloom, E., V. Leleivre, X. Zhou, W. Rodriguez, T. Jam, and J.A. Wasichek. 2004. Embryonic expression and multifunctional actions of the natriuretic peptides and receptors in the developing nervous system. Dev. Biol. 271:161–175.

Ding, J.D., A. Burette, P.I. Nedvetsky, H.H. Schmidt, and R.J. Weinberg. 2004. Distribution of soluble guanylyl cyclase in the rat brain. J. Comp. Neuro. 472:437–448.
Inoue, K., S. Ozaki, T. Shiga, K. Ito, T. Masuda, N. Okado, T. Iseda, S. Matsukawa, N., W.J. Grzesik, N. Takahashi, K.N. Pandey, S. Pang, M. Yamauchi, K. Krause, M., E.W. Dent, J.E. Bear, J.J. Loureiro, and F.B. Gertler. 2003. Ena/In Situ Hybridization and Immunohistochemistry. 2004. Current Protocols in

Ozaki, S., and W.D. Snider. 1997. Initial trajectories of sensory axons toward

Honig, M.G., and R.I. Hume. 1989. Dil and diO: versatile fluorescent dyes for

Hofmann, F., R. Feil, T. Kleppisch, and J. Schlossmann. 2006. Function of

Guo, D., Y.C. Tan, D. Wang, K.S. Madhusoodanan, Y. Zheng, T. Maack, J.J. Fitzgerald, M. 2005. The development of nociceptive circuits.

Feng, G., R.H. Mellor, M. Bernstein, C. Keller-Peck, Q.T. Nguyen, M. Wallace, J.M. Nerbonne, J.W. Lichtman, and J.R. Sanes. 2000. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron. 28:41–51.

Fitzgerald, M. 2005. The development of nociceptive circuits. Nat. Rev. Neurosci. 6:507–520.

Gallo, G., and P.C. Letourneau. 1998. Localized sources of neurotrophins initiate axon collateral sprouting. J. Neurosci. 18:5403–5414.

Guo, D., Y.C. Tan, D. Wang, K.S. Madhusoodanan, Y. Zheng, T. Maack, J.J. Zhang, and X.Y. Huang. 2007. A Rac-cGMP signaling pathway. Cell. 128:341–355.

Hindges, R., T. McLaughlin, N. Genoud, M. Henkemeyer, and D.D. O’Leary. 2002. EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron. 35:475–487.

Hofmann, F., A. Ammendola, and J. Schlossmann. 2000. Rising beyond NO: cGMP-dependent protein kinases. J. Cell Sci. 113:1671–1676.

Hofmann, F., R. Feil, T. Kleppisch, and J. Schlossmann. 2006. Function of cGMP-dependent protein kinases as revealed by gene deletion. Physiol. Rev. 86:1–23.

Honig, M.G., and R.I. Hume. 1989. Dil and diO: versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci. 12:333–335, 340–341.

Inoue, K., S. Ozaki, T. Shiga, K. Ito, T. Masuda, N. Okado, T. Iseda, S. Kawaguchi, M. Ogawa, S.C. Bae, et al. 2002. Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. 5:946–954.

In Situ Hybridization and Immunohistochemistry. 2004. Current Protocols in Molecular Biology. F.M. Ausuble, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, editors. John Wiley and Sons, New York. Chapter 14.

Jaubert, J., F. Jaubert, N. Martin, L.L. Washburn, B.K. Lee, E.M. Eicher, and J.L. Guenet. 1999. Three new allelic mouse mutations that cause skeletal overgrowth involve the natriuretic peptide receptor C gene (Npr3). Proc. Natl. Acad. Sci. USA. 96:10278–10283.

Jüttner, R., M. I. More, D. Das, A. Babich, J. Meier, M. Henning, B. Erdmann, E.C. Mu Ller, A. Otto, R. Grantyn, and F.G. Rathjen. 2005. Impaired synaptic function during postnatal development in the absence of CALEB, an EGF-like protein processed by neuronal activity. Neuron. 46:233–245.

Koelling, D., M. Russwurm, E. Mergia, F. Mullershausen, and A. Friebe. 2004. Nitric oxide-sensitive guanylyl cyclase: structure and regulation. Neurochem. Int. 45:613–619.

Krause, M., E.W. Dent, J.E. Bear, J.J. Loureiro, and F.B. Gertler. 2003. EGF/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu. Rev. Cell Dev. Biol. 19:541–564.

Lucas, K.A., G.M. Pitari, S. Kazeronian, I. Ruiz-Stewart, J. Park, S. Schulz, K.P. Chepenik, and S.A. Waldman. 2000. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol. Rev. 52:375–414.

Ma, L., and M. Tessler-Lavigne. 2007. Dual branch-promoting and branch-repelling actions of Slit/Robo signaling on peripheral and central branches of developing sensory axons. J. Neurosci. 27:6843–6851.

Matsukawa, N., W.J. Grzesik, N. Takahashi, K.N. Pandey, S. Pang, M. Yamauchi, and O. Smithies. 1999. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc. Natl. Acad. Sci. USA. 96:7403–7408.

Murnick, K., and H.R. Koerber. 1995. Prenatal development of rat primary afferent fibers: II. Central projections. J. Comp. Neurol. 355:601–614.

Miyazawa, T., Y. Ogawa, H. Chusho, A. Yasoda, N. Tamura, Y. Komatsu, A. Pfeifer, F. Hofmann, and K. Nakao. 2002. Cyclic GMP-dependent protein kinase II plays a critical role in C-type natriuretic peptide-mediated endochondral ossification. Endocrinology. 143:3604–3610.

O’Leary, D.D., and T. Terashima. 1988. Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and “waiting periods”. Neuron. 1:901–910.

Ozaki, S., and W.D. Snider. 1997. Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J. Comp. Neurol. 380:215–229.

Perrin, F.E., F.G. Rathjen, and E.T. Stoeckli. 2001. Distinct subpopulations of sensory afferents require F11 or axonin-1 for growth to their target layers within the spinal cord of the chick. Neuron. 30:707–723.