

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

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**S**ensory 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).

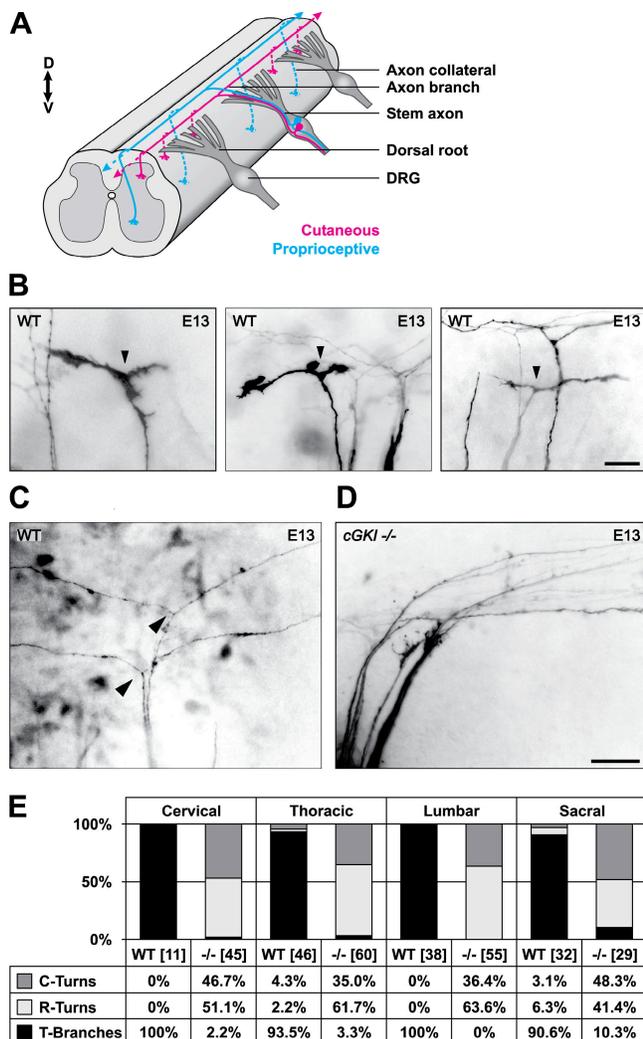
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Abbreviations used in this paper: cGKI, cGMP-dependent protein kinase I; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene-related peptide; CNP, C-type natriuretic peptide; Dil, 1,1'-diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate; DREZ, dorsal root entry zone; DRG, dorsal root ganglion; E, embryonic day; GC, guanylyl cyclase; mEPSC, miniature excitatory postsynaptic current; NOS, NO synthases; P, postnatal day; PDE, phosphodiesterase.

The online version of this article contains supplemental material.



**Figure 1. Bifurcation of sensory axons at the DREZ is impaired in the constitutive cGKI knockout.** (A) Schematic drawing of the trajectories of sensory axon projections within the spinal cord (D, dorsal; V, ventral). (B) Sensory growth cones in the process of bifurcation at the DREZ in wild-type mice. Bar, 20  $\mu$ m. (C and D) Dorsal views of DiI-labeled axons of wild-type or cGKI-deficient mice are shown. Fluorescence images are inverted, lateral is at the bottom and caudal is at the right. Arrowheads in B and C indicate bifurcations in the wild-type. Bar, 50  $\mu$ m. (E) Quantification of T-shaped branches, single rostral, or caudal turns in wild-type or cGKI-deficient mice. The numbers of counted single axons are given in brackets for different trunk levels for each genotype. In total, 25 wild-type and 17 cGKI-deficient embryos were analyzed blind with regard to the genotype. To test whether the preference for rostral turns is statistically significant, the binomial test was used and showed that the distribution is other than 50:50 ( $P = 0.05$ ).

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* promoter, 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  $\alpha$  and  $\beta$  subunits that are activated by nitric oxide (NO) and found to be colocalized with NO synthases (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 *cGKI $\alpha$*  (Fig. 2, B–E), whereas *Gucy2e* mRNA was found to be beyond the detection limits using two distinct probes for hybridization (unpublished data). *cGKI $\beta$*  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*,

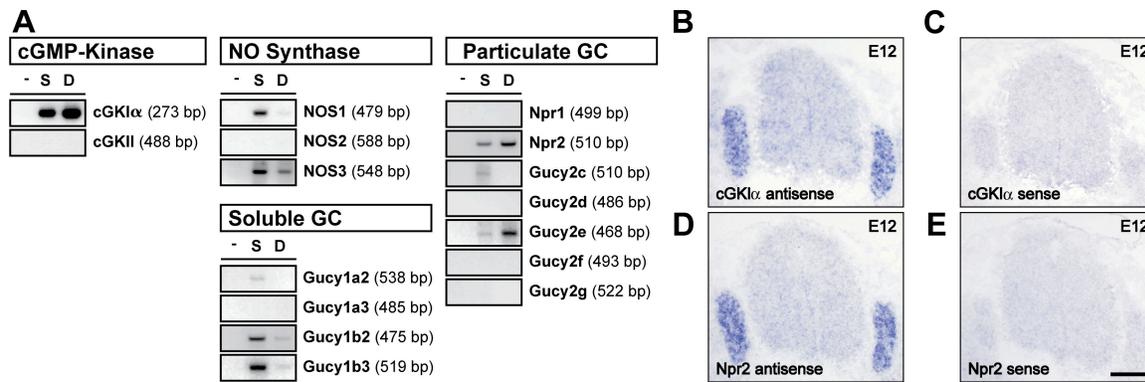


Figure 2. **The receptor GC Npr2 is selectively expressed in developing DRG neurons.** (A) RT-PCR analysis of cGMP-dependent protein kinases, NOSs, sGCs, or pGCs in mouse E12 spinal cord or DRG. S, spinal cord; D, DRG; -, no template in the reaction mixture. The sizes of the PCR amplification products are given in parenthesis. (B–E) Expression pattern of Npr2 and cGKIα transcripts in transverse sections of mouse E12 spinal cord by in situ hybridizations. Sense probe served as control. Bar, 200 μm.

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 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 (Tsuji and Kunieda, 2005). DiI 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.

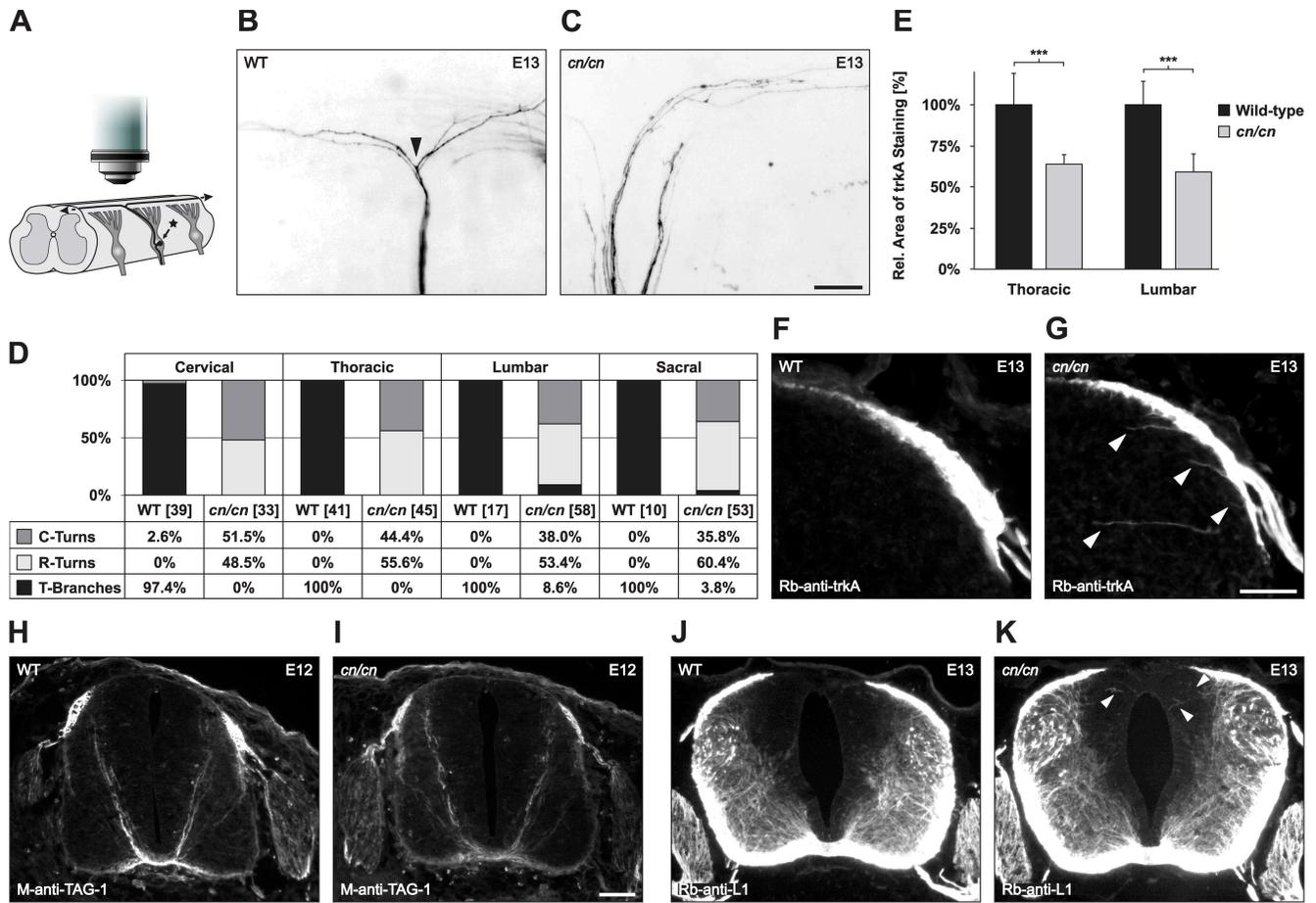
A lack of bifurcation at the DREZ would result in a significant reduction of axon numbers in the developing dorsal funiculus. As a measure of axon number, anti-trkA immunoreactivity was quantified in transverse sections of E13 spinal cords (Fig. 3 E). The trkA-labeled area in the dorsal funiculus of *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 path-finding 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 peripherin 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, DiI tracing experiments revealed that neither blocked cGMP formation in mice with inactive Npr2,



**Figure 3. Bifurcation of sensory axons at the DREZ in *Npr2* loss-of-function mutants is impaired.** (A) Schematic representation of microscopic analysis of bifurcation errors at the DREZ. The star indicates a single highlighted sensory neuron within a DRG. (B and C) To visualize the longitudinal branching of single primary afferent axons, whole mounts of E12–14 spinal cords were labeled with the lipophilic tracer Dil (Honig and Hume, 1989). Lateral is to the bottom and caudal to the right. Some bifurcations are indicated by an arrowhead in the wild type. Fluorescence images were inverted. (D) Quantification of T-shaped branches, single rostral (R-Turns) or caudal turns (C-Turns) in wild-type or *cn/cn* mice. The numbers of counted single axons for each genotype are given in brackets for different trunk levels. In total, 13 wild-type and 8 mutant embryos were analyzed blind with regard to the genotype. To test whether the preference for rostral turns is statistically significant, the binomial test was used and indicated a 50:50 distribution. (E) Quantification of the relative area of the developing dorsal funiculus in transverse sections stained by anti-trkA antibodies. The values are means of 16 transverse sections for wild-type and *cn/cn* embryos, each at thoracic (64.1%; SD 5.3%; \*\*\*,  $P < 0.001$ , Mann-Whitney U test) or lumbar (59%; SD 11.1%; \*\*\*,  $P < 0.001$ , Mann-Whitney U test) levels. (F and G) Dorsolateral regions of transverse sections of the spinal cord stained by antibodies to trkA revealed premature ingrowth of axon fascicles (arrowheads) into the dorsal horn in the direction of the central canal. (H–K) Transverse sections of embryonic spinal cord stained by antibodies to TAG-1 or to L1 revealed that the pattern of TAG-1-positive axons or of L1-positive axon tracts is not affected in *Npr2*-inactive mutants. Arrowheads in K indicate misguided axon fascicles in the dorsal horn also observed by anti-trkA staining (G). Bars: (B, C, F, and G) 50  $\mu$ m; (H–K) 100  $\mu$ m.

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 $\alpha$  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 Dil 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

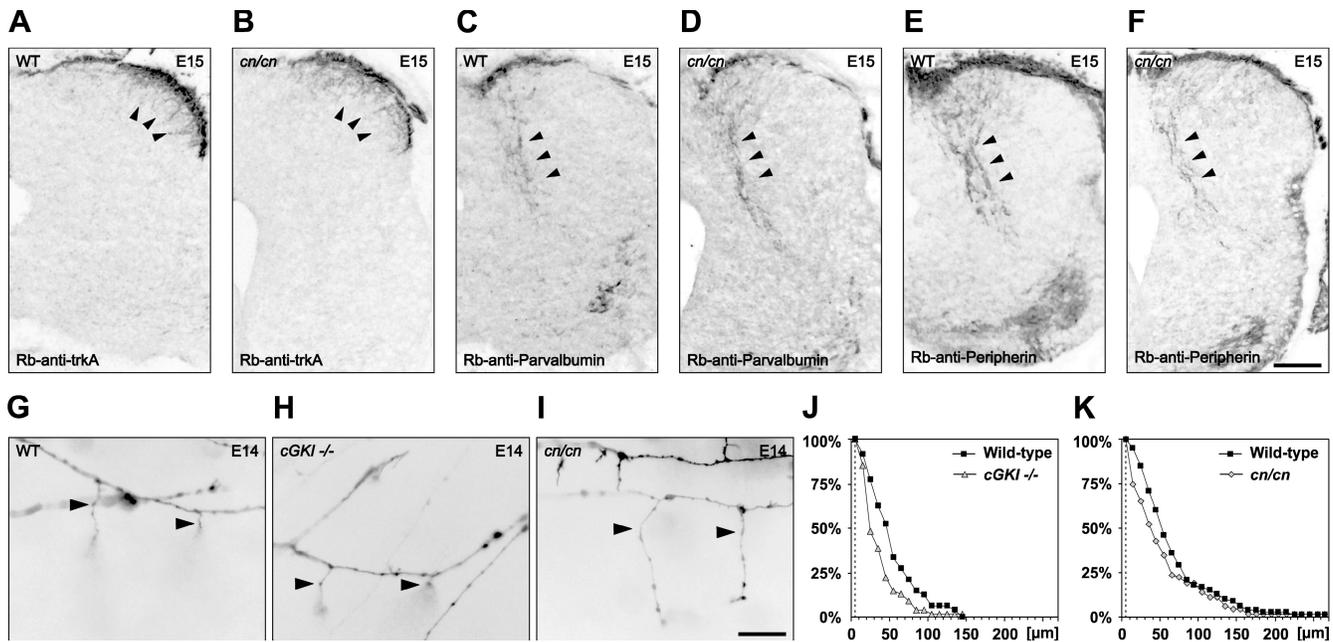


Figure 4. **Collaterals of sensory axons are generated in the absence of cGKI or in *Npr2* loss-of-function mutants.** (A–F) Staining of cross sections by antibodies to trkA, parvalbumin, or peripherin to visualize collaterals of sensory axons. Arrowheads indicate nociceptive or proprioceptive collaterals. Bar, 50  $\mu\text{m}$ . (G–I) Single axons labeled by Dil and their developing collaterals are shown from E14 embryos (arrowheads). A dorsolateral view is presented. Bar, 20  $\mu\text{m}$ . (J and K) Quantification of the distances between two collaterals in microscopic view fields of wild-type and mutant mice at E14. Frequency cumulation plots are shown for distances  $>5 \mu\text{m}$ . Distributions of distance lengths are significantly different for mutant mice compared with the corresponding wild-type data ( $P = 0.019$  for *Npr2*;  $P = 0.001$  for cGKI; Mann-Whitney U test). The numbers of measured distances were 54 for cGKI-deficient mice (five embryos) and 48 for the corresponding wild type (five embryos), and 63 for *cn/cn* mice (four embryos) and 78 for the wild type (11 embryos). Note that *cn/cn* and cGKI mutant mice were from a different genetic background.

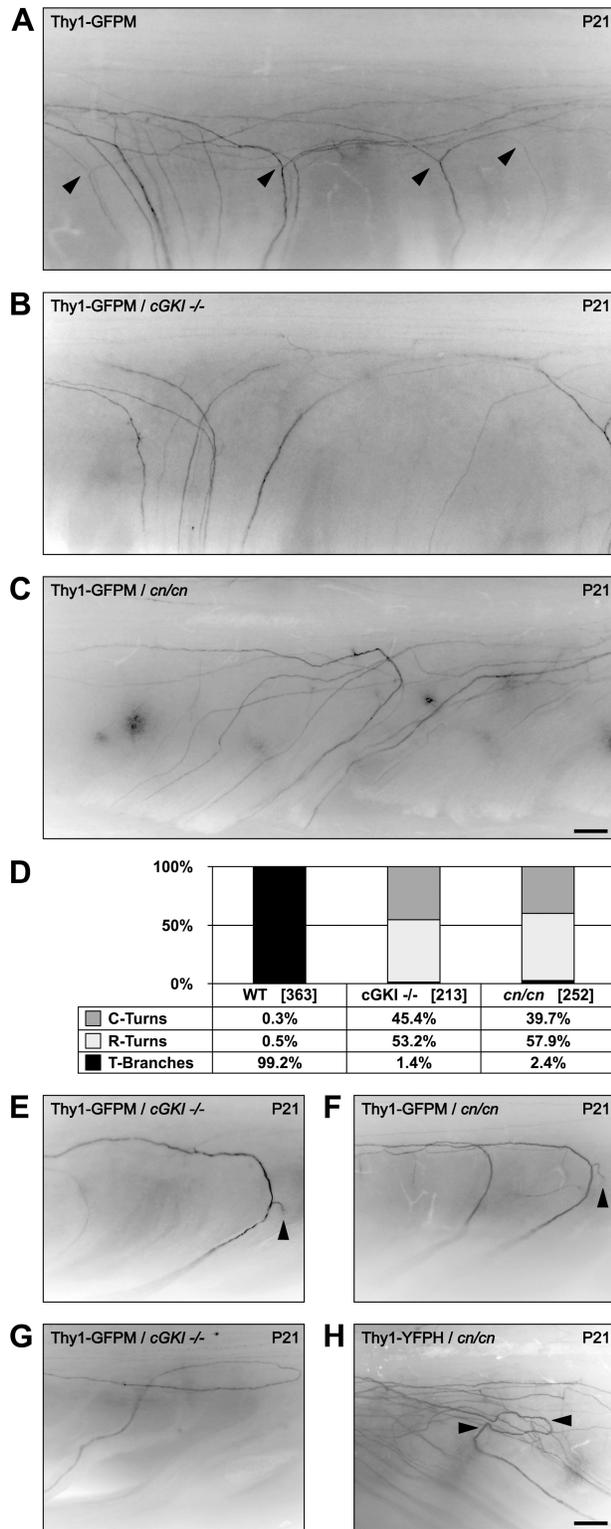
at a stage when axons enter the cord persists to mature stages, and no compensation seems to exist at the DREZ, at least until P56 (Fig. 5 and not depicted), when the connectivity of the spinal cord is quasimature in mice. However, in addition to the bifurcation error, we sometimes observed aberrant 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  $\mu\text{m}$  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];  $P < 0.01$ ,  $\chi^2$  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,  $21.36 \pm 1.39 \text{ pA}$ ,  $n = 24$ ; *cn/cn*,  $24.19 \pm 2.17 \text{ pA}$ ,  $n = 24$ ; decay time constant: wild-type,  $2.09 \pm 0.15 \text{ ms}$ ;  $n = 24$ ; *cn/cn*,  $1.90 \pm 0.12 \text{ ms}$ ;  $n = 24$ ).

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.



**Figure 5. The failure of sensory axons to bifurcate persists in mature cGMP-signaling mutant mice.** (A–C) A *Thy1-EGFP* allele (Feng et al., 2000) was crossed into *cn/cn* or *cGKI*-deficient mice. Mice were analyzed at P21 and dorsal views are shown. Arrowheads indicate bifurcations in the wild type. (D) Quantification of T-shaped branches, single rostral, or caudal turns in the wild type, *cGKI*-deficient mice, or *Npr2* loss-of-function mutants. The numbers of counted single axons are given in brackets for each genotype. 12 animals were analyzed in total. (E–H) A very small percentage of sensory axons showed an unusual growth behavior at the DREZ in mutant mice of the cGMP signaling cascade. Arrowheads indicate short

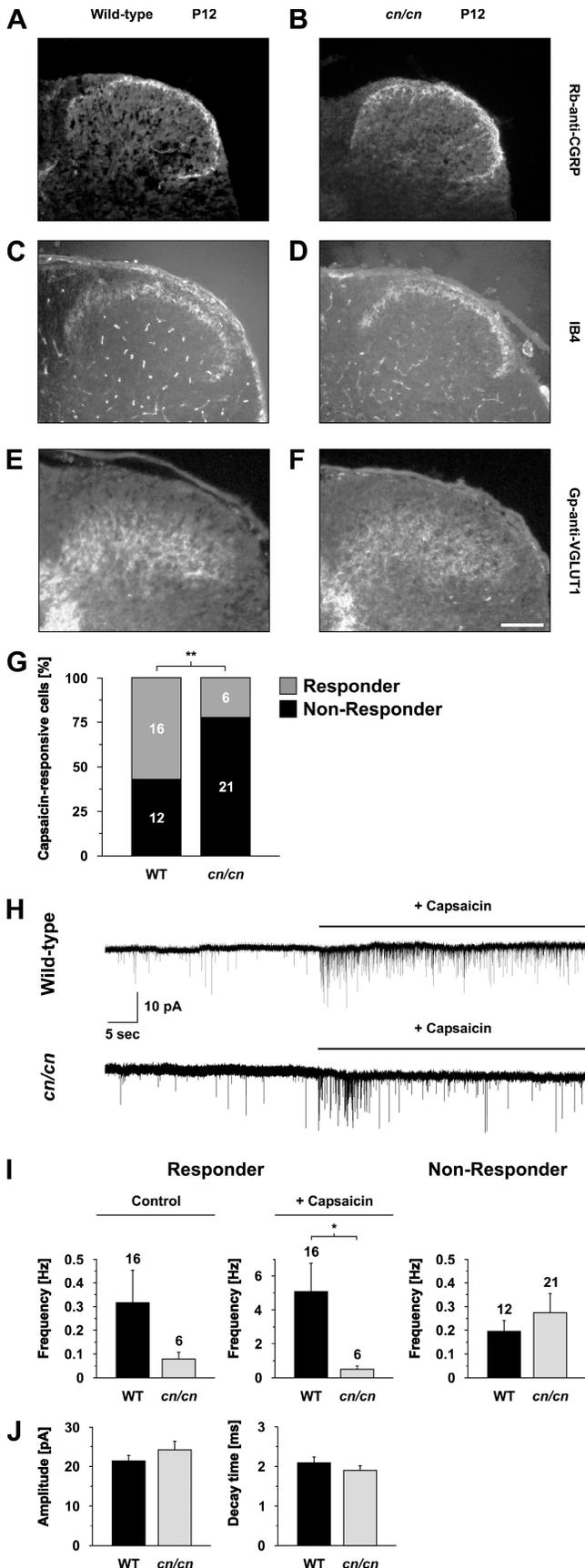
## Discussion

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 *cGKI* $\alpha$ . 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).

processes (E and F) and an axon that grew in one direction then looped backwards at the DREZ (H). Bars, 100  $\mu$ m.



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 cGKI $\alpha$  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

**Figure 6. Connectivity with second order neurons in the superficial dorsal horn of the spinal cord is reduced in *cn/cn* mice.** (A–F) The overall laminar organization in the dorsal horn is not affected in *cn/cn* mice as depicted by isolectin B4 staining or anti-CGRP and anti-VGLUT1 antibodies. Bar, 100  $\mu$ m. (G) Relative number of capsaicin-responding and nonresponding cells is reduced in *cn/cn* in comparison with wild-type mice. (\*\*,  $P < 0.01$ ,  $\chi^2$  test). The numbers of cells measured are indicated within the columns. mEPSCs were recorded from neurons in the superficial dorsal horn in the presence of 1  $\mu$ M strychnine, 100  $\mu$ M picrotoxin, and 100  $\mu$ M APV to block glycinergic and GABAergic input as well as NMDA receptor-mediated currents. 1  $\mu$ M tetrodotoxin was used to block action potential-dependent neurotransmitter release. (H) Example current traces of mEPSCs at resting conditions and after application of 10  $\mu$ M capsaicin in wild-type and *cn/cn* mouse. (I) Application of capsaicin leads to a significant increase in the frequency of mEPSC in wild-type as compared with *cn/cn* neurons (\*,  $P = 0.032$ , Mann-Whitney U test). Under resting conditions, a reduced but not significantly different frequency was observed in capsaicin-responding neurons of *cn/cn* mice ( $P = 0.055$ , Mann-Whitney U test). The frequency of capsaicin nonresponding cells is not affected by the absence of Npr2 activity. (J) The amplitude and decay time constant of mEPSCs in current remains unchanged in Npr2 mutant mice. Numerical data are reported as mean  $\pm$  SEM.

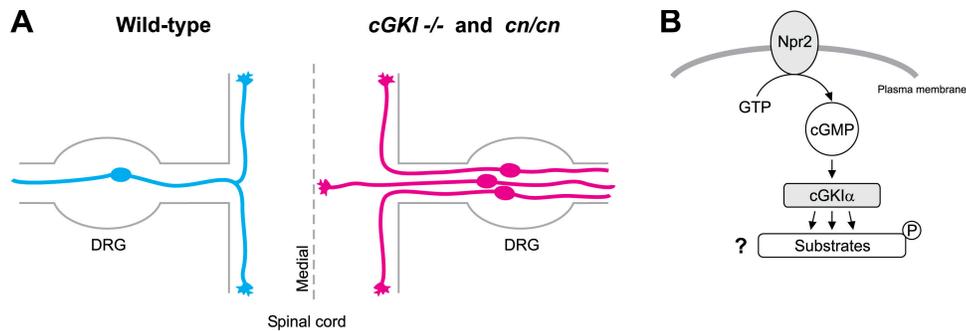


Figure 7. **Scheme summarizing the observed bifurcation errors in the absence of cGMP signaling of cGKI or Npr2 mutant mice.** (A) In wild-type mice sensory axons bifurcate as soon as they enter the spinal cord and stay in the oval bundle of His, whereas in mutant mice only turns into rostral or caudal directions were observed. A small proportion of axons were found to grow directly to the central canal in both genotypes. (B) For the activation mechanism of the receptor GC Npr2 and downstream phosphorylation targets of cGKI $\alpha$ , see Discussion.

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 cGKI $\alpha$  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- $\mu$ M 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 offspring 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)<sub>25</sub> (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 *cGKI $\alpha$  5'* (5'-AAAAATG-AGCGAACTGGAG-3'), *cGKI $\alpha$  3'* (5'-GACCTCTCGGATTAGTGAAC-3'), *cGKI $\beta$  5'* (5'-GTTCCGGAAGAGTGGAGCTTG-3'), *cGKI $\beta$  3'* (5'-CTTTGTGA-AGAATGACCTCGGG-3'), *NOS1 5'* (5'-CGTCAATGATCGGCCCTGTGTA-3'), *NOS1 3'* (5'-CTTTGGCTGGTCCCCCTCTGTG-3'), *NOS2 5'* (5'-GGGACCTGGCCACTTGTTC-3'), *NOS2 3'* (5'-TGCGGCTGGAC-TTTCACTCTGC-3'), *NOS3 5'* (5'-CAAAGGGGGCAGGCATCACCAG-3'), *NOS3 3'* (5'-CACCGCTCGAGCAAAGGCACAGA-3'), *Gucy1a2 5'* (5'-AAGGGTCAACCTGGACTCAC-3'), *Gucy1a2 3'* (5'-ATAGATTCTCT-TCTGTCAGCC-3'), *Gucy1a3 5'* (5'-AGAAAGACAAGCCGCAACAG-AGT-3'), *Gucy1a3 3'* (5'-GCAGCCGCTTAATGATACCAG-3'), *Gucy1b2 5'* (5'-ACTGGACCAGTCTAGCAGG-3'), *Gucy1b2 3'* (5'-TCTCATCTG-TGTGATGACTGG-3'), *Gucy1b3 5'* (5'-GAGAAGGGGCCATGAAGAT-TGTC-3'), *Gucy1b3 3'* (5'-CCTCCGTGCCTGATTTTCTCTG-3'), *Npr1 5'* (5'-CTTCCACTGGATGTGTTG-3'), *Npr1 3'* (5'-CACGCCATCAG-CTCCTG-3'), *Npr2 5'* (5'-TGGCGCCTTCCCCTCTGACT-3'), *Npr2 3'* (5'-CTGGGCTGTTCCTGGGTTCCG-3'), *Gucy2c 5'* (5'-TGCTGATTGCC-CTCCTTGTCT-3'), *Gucy2c 3'* (5'-ATTGAGACGCCCGTGGACTCT-3'), *Gucy2d 5'* (5'-CTGTGTTTGGAGTGC-3'), *Gucy2d 3'* (5'-TGCTCT-GGTGAGAGGACAC-3'), *Gucy2e 5'* (5'-GATCACTGCACCCCAAGAC-3'), and *Gucy2e 3'* (5'-TGCTCAGGTGAGGTCAAG-3'). The second set of primers for *Gucy2e* were *Gucy2e 5'* (5'-GGACTGGATGTTCAAGTCTCC-3'), *Gucy2e 3'* (5'-CAGGTCTACCACCTCAATAGGC-3'), *Gucy2f 5'* (5'-GGC-CTCAGGATTTGTTGG-3'), *Gucy2f 3'* (5'-TACATCATAGGGGACAAAG-ACG-3'), *Gucy2g 5'* (5'-TCGTTTTATCCTCTTGCAAG-3'), and *Gucy2g 3'* (5'-ATGAGGTGTTTCGAGTCAATCC-3'). For control purposes all primer pairs were tested with a cDNA template from a whole mouse E17 embryo. The amplification products were subsequently cloned into a pBluescriptKS vector (Stratagene) and their identity was verified by sequencing.

In situ hybridization studies on 25- $\mu$ m transversal sections of mouse E12 spinal cord using DIG-labeled riboprobes to *cGKI $\alpha$*  and *Npr2* were performed as described previously (Ausubel et al., 2004).

#### Immunohistochemistry

For immunohistochemical detection, cryostat sections of formaldehyde-fixed embryos were stained by indirect immunofluorescence using rabbit antibodies to *trkA* (Upstate Biotechnology), parvalbumin (Swant), CGRP (Peninsula Laboratories, Inc.), L1 (Rathjen and Schachner, 1984), peripherin (Chemicon), guinea pig anti-VGlu1 (Chemicon), mouse monoclonal anti-TAG-1 (4D7; Developmental Studies Hybridoma Bank; Dodd et al., 1988), or isolectin GS-IB<sub>4</sub> Alexa Fluor conjugate (Invitrogen). The mAb 4D7 was purified from hybridoma supernatant by affinity chromatography and applied at a concentration of 4  $\mu$ g/ml. Secondary Cy3-conjugated antibodies were obtained from Dianova. All images were obtained at room temperature using a microscope (Axiovert 135 or 200) equipped with Neofluar/Acroplan objectives (5, 10, 20, or 40 $\times$  magnification with numerical apertures 0.15, 0.25, 0.5, or 0.75, respectively), a charged-coupled device camera (AxioCam HRC), and acquisition software (Axiovision 3.1; all from Carl Zeiss MicroImaging, Inc.). The area labeled by anti-*trkA* was measured using Image 1.37v software (National Institutes of Health). Contrast and brightness were adjusted in some images using Photoshop (Adobe) but no further processing was performed. Figures were assembled using CorelDraw (Corel).

#### Electrophysiology

The spinal column was removed from P10–14 mice and placed in an ice-cold dissection solution with a reduced calcium concentration consisting of 125 mM NaCl, 4 mM KCl, 10 mM glucose, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 0.1 mM CaCl<sub>2</sub>, and 3.0 mM MgCl<sub>2</sub> (Baccei et al., 2003; Jüttner et al., 2005). After laminectomy, the spinal cords were removed and embedded in 2.5% agarose (SeaPlaque; Cambrex Bio Science), and 180- $\mu$ m transversal slices were prepared by vibratome cutting. Slices were maintained at room temperature for at least 1 h before recording. Whole-cell patch-clamp recordings of mEPSCs of neurons located in the superficial dorsal horn were performed as described previously (Baccei et al., 2003). AMPA receptor-mediated mEPSCs were isolated pharmacologically by blocking glycinergic, GABAergic input, and NMDA receptor-mediated currents (1.0  $\mu$ M strychnine; 100  $\mu$ M picrotoxin; 100  $\mu$ M APV). Action potential-dependent neurotransmitter release was blocked by 1  $\mu$ M tetrodotoxin. All experiments were performed at room temperature. During recordings, slices were perfused at a flow rate of 2 ml/min with a bath solution of 125 mM NaCl, 4 mM KCl, 10 mM glucose, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.0 mM CaCl<sub>2</sub>, and 1.0 mM MgCl<sub>2</sub>. The patch pipette solution contained 120 mM CsCl, 4 mM NaCl, 5 mM glucose, 5 mM ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.5 mM CaCl<sub>2</sub>, and

4 mM MgCl<sub>2</sub>, pH 7.3. Capsaicin (Sigma-Aldrich) was applied by a superfusion pipette for 100 s at a concentration of 10  $\mu$ M. During recordings at a holding potential of  $-70$  mV the effective access resistance was in the range of 10–40 M $\Omega$  and was controlled throughout the experiment by using a short depolarizing pulse. Recordings were only accepted if the access resistance was  $<40$  M $\Omega$  and did not change more than 20% during the experiment. All electrophysiological experiments were performed blind with regard to the genotype of the animals. Recordings were made using a patch clamp amplifier (EPC-9; HEKA Elektronik). Signals were sampled at a rate of 10 kHz and analyzed off-line using WinTida 5.0 (HEKA Elektronik). Postsynaptic currents were filtered at 3 kHz and analyzed by MiniAnalysis (Synaptosoft, Inc.).

#### Online supplemental material

Fig. S1 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.200707176/DC1>.

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