C-type natriuretic peptide (CNP) is a bifurcation factor for sensory neurons

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Neuronal circuits are shaped during development by the coordinated action of guidance factors and signals that regulate axonal branching. Unlike guidance cues, the molecules and signaling cascades that underlie axonal branching remain to be resolved. Here we show that the secreted molecule C-type natriuretic peptide (CNP) induces a cGMP signaling cascade via its receptor particulate guanylyl cyclase Npr2 which is essential for sensory axon bifurcation at the dorsal root entry zone (DREZ) of the spinal cord. In contrast, another form of sensory axon branching—collateral formation—is not affected by this pathway. We also demonstrate that cGMP signaling via the nitric oxide-stimulated soluble guanylyl cyclase system (NO-GC) is dispensable for sensory axon branching. Functionally, the bifurcation error in CNP mutant mice is maintained at mature stages and results in a reduced input on secondary neurons as detected by patch-clamp recordings.

axon branching and pathfinding | cGKI | cGMP signaling | Npr2

The processes of growth cone guidance and axonal branching are key mechanisms in the formation of axonal pathways during the period of axonal outgrowth resulting in the complex wiring pattern of the mature nervous system (1). Branching enables an individual neuron to innervate several distinct targets providing the physical basis for the distribution and integration of information. Different modes of axon branch formation may be distinguished including: 1) ramifications that are a result of the activity of the growth cone (growth cone splitting and delayed branching) and that could lead to a bifurcation as well as complex terminal arbors and 2) generation of collateral branches at the axon shaft by budding (also termed interstitial branching) (2). The latter appears to be the dominating branching mode of projecting cortical and thalamocortical axons (3, 4).

Axonal guidance factors such as Semaphorin 3A and Slit2 but also neurotrophins, growth factors, morphogens, focal adhesion kinase (FAK), and adhesion proteins have been shown to be implicated in branching in vitro (5–11). Furthermore, neuronal activity via calcium influx (12–15) as well as cytoskeletal proteins that affect axon stability influence branch formation (16–19).

Despite this progress, the intracellular signaling cascades that underlie both modes of axonal branching in vivo have remained poorly understood. We have therefore studied arborisation of sensory axons projecting into the spinal cord where both modes of branching can be visualized. As sensory axons enter the developing spinal cord at the dorsal root entry zone (DREZ), they split into a rostral and caudal arm. They grow over several segments but remain confined to lateral regions of the cord (20). Collaterals that grow into the gray matter are then generated after a waiting period by budding from these stem axons. Nociceptive collaterals are confined to the dorsal horn while proprioceptive collaterals terminate in the ventral cord.

In our previous investigations we demonstrated that in the absence of the receptor guanylyl cyclase Npr2 (also known as GC-B or NPR-B) or of the cGMP-dependent kinase I (cGKI, also termed PKGI) sensory axons are unable to bifurcate at the DREZ, indicating that cGMP signaling is important for branching (21, 22). Here, we identify C-type natriuretic peptide (CNP) that is expressed in the dorsal horn of the embryonic spinal cord as a ligand that induces bifurcation of ingrowing sensory axons via its receptor Npr2.

Results

Expression of CNP in the Dorsal Spinal Cord During the Ingrowth of Sensory Neurons. Preferential activation of Npr2 occurs via the natriuretic peptide ligands (CNP≫ANP≫BNP) (23) or weakly via the small GTPase Rac and the p21-activated kinase pathway (24). To identify factor(s) responsible for sensory axon bifurcation at the DREZ via activation of guanylyl cyclase Npr2 we concentrated on natriuretic peptides. In vitro stimulation of wild-type DRG with CNP-22 increased the level of cGMP after 12.5 min of incubation in an Npr2-dependent manner (Fig. 1 *A* and *B*). In line with published observations (25), CNP but not ANP or BNP was found in the dorsal spinal cord as revealed by RT-PCR, in situ hybridization, or β-galactosidase staining of a targeted CNP mutant mouse in which exon I of the CNP gene was replaced by a lacZ expression cassette (Fig. 1). Expression starts weakly at E9 in the hindbrain and the spinal cord forming a rostrocaudal gradient as development proceeds (Fig. 1 *K–M*). In transverse sections, CNP expression was initially observed in the entire dorsal quarter of the spinal cord, but at more advanced stages, it was concentrated within the dorso-medial parts of the cord. Roof plate cells were negative for CNP at this stage of development. At E14.5, CNP expression becomes restricted to cells located adjacent to the dorsal part of the central canal. Additionally at E17.5, β-galactosidase-positive cells are found interspersed within the dorsal and ventral horn of the spinal cord (Fig. 1 *N–S*). Taken together, the timing and pattern of localization as well as the biochemical data suggest that CNP might be the relevant factor for activation of Npr2 on sensory axons to affect their branching at the DREZ.

Lack of Bifurcation of Sensory Axons in the Absence of CNP. In transverse sections of E13.5 spinal cords stained with an antiserum to trkA, we detected a reduced size of the dorsal funiculus in CNP-deficient mice as compared to the wild-type (Fig. 2 *A–C*). Additionally, a small percentage of CNP mutant sensory axons were found to extend directly toward the central canal. To analyze whether the smaller dorsal funiculus might be caused by a disturbed bifurcation we traced single axons at the dorsal root entry zone by the application of the lipophilic dye DiI to E12–13 DRG in whole mount dissections of spinal cords of wild-type and mutant mice (CNPlacZ−/−). Sensory axons of CNP-deficient

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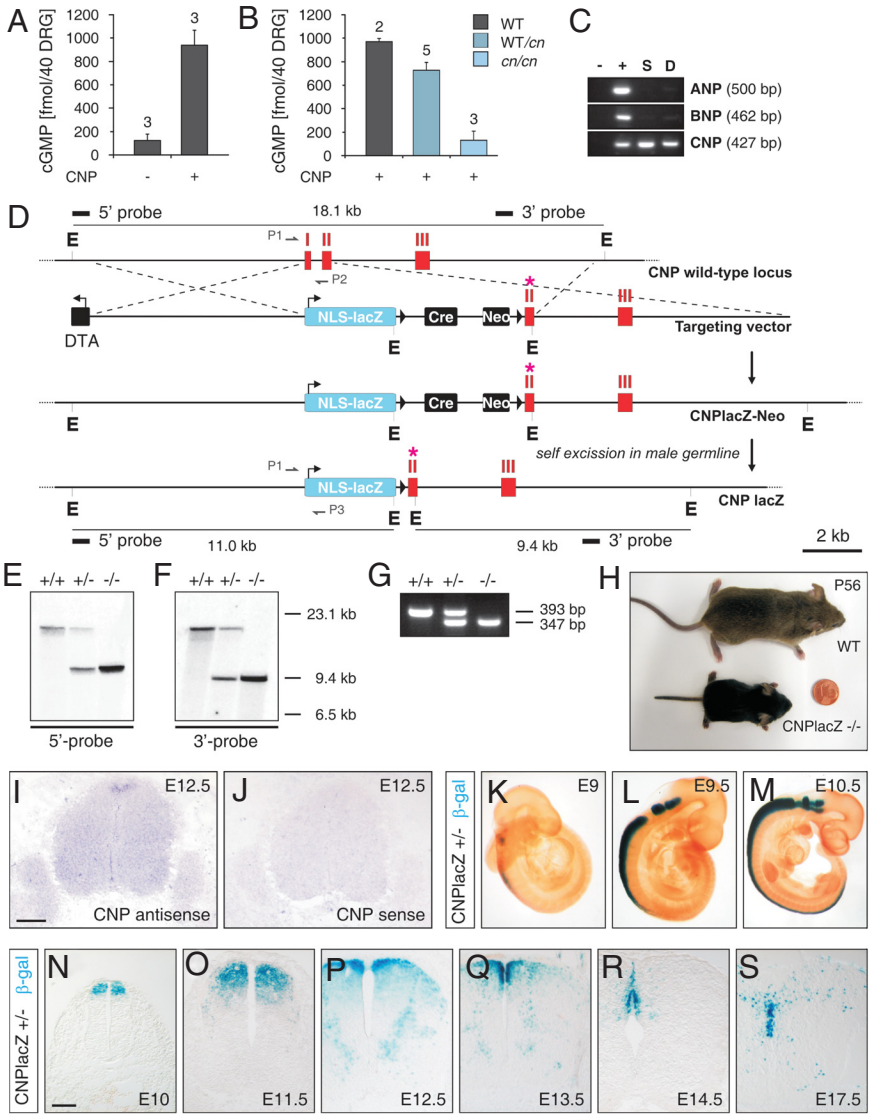

Fig. 1. CNP is localized in the dorsal region of the developing spinal cord and hindbrain. (*A* and *B*) CNP induces the formation of cGMP in wild-type (WT) but not in Npr2 (*cn*/*cn*) mutant mice. (*C*) Transcripts of CNP but not ANP or BNP are detectable in mouse E12.5 DRG (D) and spinal cord (S) by RT-PCR, − no template, + E17 mouse embryo cDNA library (Clontech). (*D*) Targeting strategy for the generation of the CNPlacZ−/− (*Nppc*$^{lacZ/lacZ}$) mouse strain. The MC1-diphtheria toxin A (*DTA*), NLS-lacZ (blue), the self-excision neomycin cassette (Cre, Neo), loxP sites (arrowheads), additional stop codons introduced in all three reading frames in exon II (star), and *Eco*RI (E) restriction sites are depicted. Black lines indicate the expected fragment sizes after *Eco*RI digestion of genomic DNA. Black bars represent the sequences used as probes for Southern blot analysis shown in *E* and *F*. Arrows P1, P2, and P3 show the positions of the primers used for genotyping as depicted in *D*. (*E* and *F*) Southern blot analysis of genomic DNA digested with *Eco*RI from F1 wild-type, CNPlacZ+/−, or CNPlacZ−/− mice. (*G*) PCR analysis of genomic DNA with oligonucleotides P1, P2, or P3 as indicated in *D*. A 393-bp product is generated from wild-type allele and a 347-bp product from the targeted allele. (*H*) A wild-type and a CNP-deficient mouse at P56 are shown revealing that CNP-deficient mice are short-statured due to abnormal bone formation as described previously (43). (*I* and *J*) Expression of CNP transcripts in transverse sections of mouse E12.5 spinal cord by in situ hybridization. (Scale bar, 200 μm.) (*K*–*M*) Expression pattern of β-galactosidase in whole mounts of CNPlacZ+/− embryos representing CNP localization. (*N*–*S*) β-galactosidase staining of transverse sections of CNPlacZ+/− spinal cord. (Scale bar, 100 μm.)

mice completely lacked T- or Y-shaped branches at all trunk levels at the DREZ (Fig. 2 *D*, *E*, and *G*). Axons entering the cord turned either in rostral or caudal direction without leaving the funiculus. Identical errors were observed in the *lbab* (long bone abnormality) mouse (Fig. 2 *F* and *H*), which contains a single point mutation in the CNP gene, resulting in an exchange of an arginine that is critical for the binding of CNP to an acidic pocket in the Npr2 receptor (26–28). Consistent with the results of the DiI tracings at embryonic stages, a bifurcation failure is also found at mature stages for all sensory axons as revealed by analyzing crosses of Thy1-YFP-H (29) reporter mice with CNP-deficient mice (Fig. 2 *I*–*K*). These findings indicate that compensatory mechanisms for the correction of the branching error do not exist at later developmental stages.

Collateral Formation Is Not Affected by the Absence of CNP. After bifurcation of sensory axons at the DREZ, the two resulting stem axons extend in opposite directions at the dorsolateral margin of the cord. Collaterals are then generated from these stem axons after a waiting period by the second type of branch formation—interstitial branching (20). Staining of transverse sections by anti-trkA or anti-parvalbumin indicated that in CNP mutant mice, nociceptive as well as proprioceptive cells extend collaterals at appropriate medio-lateral positions that grow to the

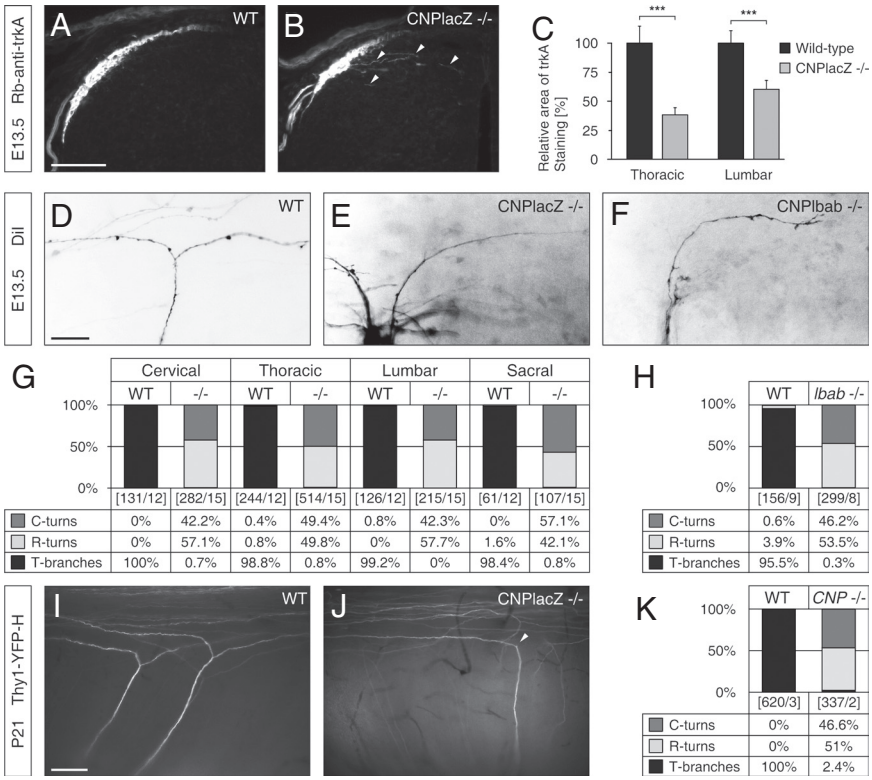

Fig. 2. CNP is required for sensory axon bifurcation at the DREZ. (*A* and *B*) Anti-trkA staining of spinal cord cross sections showing a small percentage of nociceptive axons growing directly to the central canal (arrow heads). (Scale bar, 100 μm.) (*C*) Reduction of the dorsal funiculus in E13.5 CNPlacZ$-/-$ mice as deduced from anti-trkA staining. Quantification of the relative trkA-positive area of the developing dorsal funiculus in transverse sections ($n = 28$ dorsal funiculi analyzed for each spinal level from two embryos of each genotype) of wild-type and CNP knock-out embryos at thoracic or lumbar trunk levels. $P < 0.001$, Mann-Whitney *U* test. (*D–F*) DiI tracing of CNPlacZ$-/-$ and *lbab* mice at E13.5 and quantification of bifurcation errors (*G* and *H*). (Scale bar, 25 μm.) (*I–K*) Lack of bifurcation in adult Thy1-YFP-H reporter mice in the absence of CNP indicating that additional compensation does not occur at mature stages. (Scale bar, 50 μm.) In *G*, *H*, and *K*, the total number of individual sensory axons counted followed by the number of embryos analyzed is given in brackets.

dorsal or ventral horn of the spinal cord, respectively (Fig. 3 *A–D* and Fig. S1). Thus, no gross changes in collateral formation are observed in the CNP mutant indicating that CNP signaling is not required for branch formation at the axon shaft.

CNP Deficiency Causes a Reduced Input on Dorsal Neurons. To examine the functional consequences of the bifurcation error, we recorded glutamatergic mEPSCs from neurons in the superficial laminae of the dorsal horn in slices from P10–P13. The overall layering within the dorsal spinal cord was not affected as deduced from CGRP, vGLUT1, Isolectin B4, anti-TAG-1, or anti-L1 staining (Fig. 3 *E–H* and Fig. S2 *A–D*). Application of capsaicin showed a decrease in the fraction of capsaicin-responsive neurons as well as a reduced mEPSC frequency (Fig. 3 *I* and *K*), while the basic postsynaptic parameters such as amplitude were not affected (Fig. 3*J*). This reduction of functional connectivity on neurons in the superficial dorsal horn in the absence of CNP is in line with our anatomical data on the bifurcation error.

Sensory Axon Bifurcation Does Not Require cGMP Signaling via NO-GC. Recent in vitro studies indicated a possible role for soluble guanylyl cyclases in the branching of DRG neurons (30). However, deletion of the β_1-subunit as the common dimerizing subunit of soluble guanylyl cyclases, which eliminates completely NO/cGMP signaling (31, 32), does not compromise sensory axon branching (Fig. 4 *A–F*) nor the formation of other axon tracts in the embryonic spinal cord (Fig. S2 *E–H*). In the absence of NO-GC activity sensory axons bifurcate and generate collaterals indistinguishable from wild-type mice. cGMP signaling induced by CNP via Npr2 is therefore sufficient to induce the splitting of the growth cone at the DREZ.

Discussion

Axonal guidance and branching are critical steps for the establishment of neuronal circuits. Both processes might be intimately interlinked in cases when branching occurs at the growth cone (growth cone splitting or delayed branching) (2, 12, 33). In contrast to axonal guidance cues the extracellular signals inducing the various types of branch formations remain to be determined. Our studies establish CNP—previously reported to be important for long bone formation (23)—as a factor essential for circuit formation by regulating sensory axon bifurcation at the DREZ. CNP has been shown to be expressed in the embryonic central nervous system, but its function in the nervous system remained to be resolved so far (23). Here, we reveal that in embryonic DRG neurons CNP increases cGMP levels via Npr2. Furthermore, comparison of the errors found at the DREZ in CNP-deficient mice with those lacking Npr2-activity or cGKI revealed quantitatively and qualitatively a complete overlap in all aspects analyzed (21, 22), suggesting that these three components act together in a cascade that governs bifurcation as one form of axonal branching (Fig. 4 *G* and *H*). In addition, in the absence of this cGMP signaling cascade, the majority of axons remains correctly confined to the lateral region of the cord and extends in rostral or caudal direction. Only a very small population of trkA-positive axons is misguided by growing directly to the central canal. In contrast to bifurcation, collateral formation (interstitial branching) from the stem axons running in the dorsal funiculus is not affected in all three mutant mice indicating that

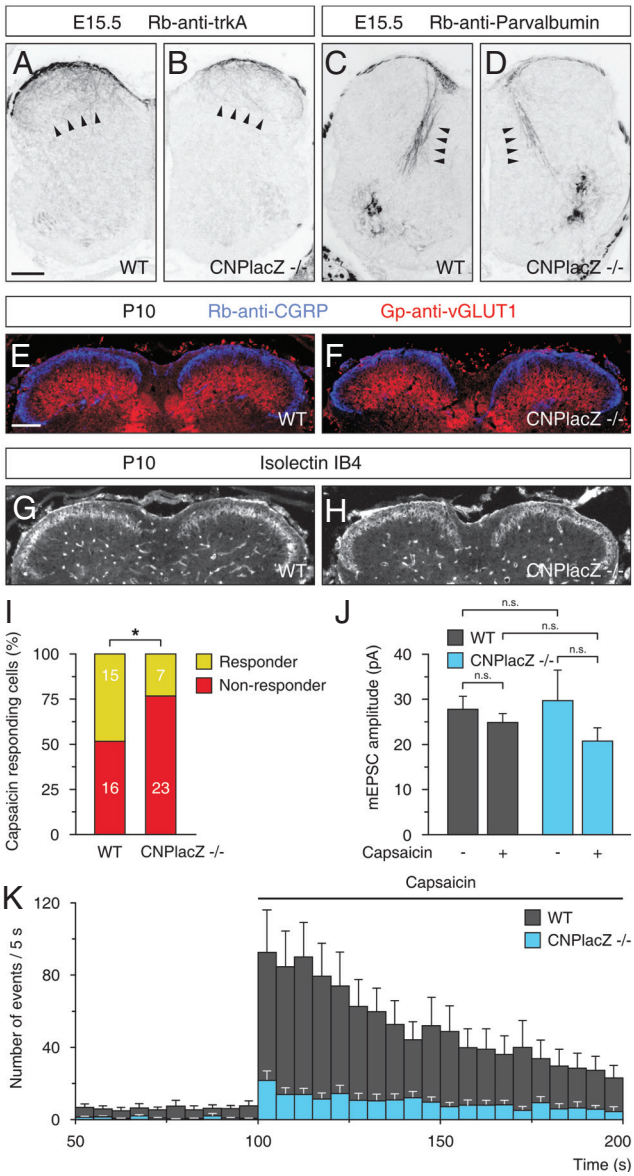

Fig. 3. The absence of CNP results in a reduced input of dorsal horn neurons. (*A–D*) Nociceptive (anti-trkA) and proprioceptive (anti-parvalbumin) collaterals are generated in CNP-deficient mice. (*E–H*) The overall laminar organization of the spinal cord is not affected in the absence of CNP as revealed by staining of P10 cross sections with anti-CGRP, anti-vGLUT1, or IB4–488. *A–D* and *E–H* depict representative stainings of each two wild-type and two CNP-deficient embryos analyzed. (Scale bar, 100 μm.) (*I* and *K*) Reduced number of responding neurons and of mEPSC frequency after capsaicin treatment as detected by patch-clamp recordings in slice preparations at P10–13 from six wild-type and four CNP-deficient mice, respectively. The number of cells measured is given in the columns. (*J*) Amplitudes of mEPSCs in the absence of CNP are indistinguishable from those of WT P10–13 slice preparations.

distinct signaling cascades exist for these different branching modes already in one neuron type (21, 22)—an observation that appears not to be unexpected since most likely intrinsic programs as well as multiple extrinsic factors might regulate branching. Similarly, the molecular analysis of axonal guidance has revealed an enormous complexity of components implicated in the steering of growth cones.

CNP is initially generated by a large population of neuronal precursor cells in the dorsal horn at an early developmental period (E10–E11.5) when the majority of sensory axons is approaching and entering the spinal cord. At more advanced stages, it becomes restricted to dorso-medial parts (E12.5–E13.5) of the cord and is later found in cells adjacent to the central canal and scattered throughout the cord where it might subserve other functions. CNP is generated as a prepropeptide of 126 amino acid residues in length. After cleavage of the signal peptide, further conversion results in the biologically active peptide CNP-53 (53 C-terminal residues of CNP). This active form is secreted by cells and then subsequently further processed to CNP-22 by an extracellular protease. The activation process is crucial for CNP; however, the enzyme(s) responsible for these conversions are not known. In HEK293 cells, intracellular processing of pro-CNP occurs by the endoprotease furin, which is predominantly localized in the transGolgi network (34). Similar to other diffusible factors CNP might interact with extracellular matrix glycoproteins and therefore might be able to influence growth cone activity in conjunctions with other components located in the dorsal horn such as the Slit proteins (5, 30). It might regulate axonal pathway formation in two ways: (1) directly as a bifurcation signal or (2) indirectly by triggering an altered interpretation of guidance signals caused by a change in cGMP levels in the growth cone (35). Interestingly, in different in vitro assays, CNP was found not to induce bifurcation of sensory axons (22). Although it is conceivable that the in vitro systems do not reflect the in vivo situation, making it difficult to reproduce the in vivo conditions for bifurcation, these observations suggest that CNP-induced cGMP signaling might indeed act in concert with other signaling cascades to generate T-shaped branches. However, the robust phenotype observed to affect all sensory axons in the absence of cGMP signaling has not been reported in other knockout mice lacking Slit, Netrin, or Semaphorin signaling. In contrast to recent in vitro studies (30), our data also show that soluble guanylyl cyclase signaling is dispensable for bifurcation and collateral formation of sensory axons in vivo.

The identification of downstream targets of cGMP signaling within sensory growth cones is of great interest since it might allow mechanistic insights into the formation of the two axonal branches and the characterization of cytoskeletal elements that provide the machinery for bifurcation. It is also conceivable that this knowledge might also establish a link to other branching modes of axons although our data argue against a common branching pathway. While several targets of cGKI have been identified by biochemical methods including GSK3 (30), genetic information for their implication in bifurcation are still lacking. In this context, it will be interesting to see whether the CNP signaling axis also influences the branching of other neuronal populations via Npr2 and cGKI or whether this pathway is restricted to sensory axons.

After bifurcation, further branching of sensory axons at the DREZ appears to be suppressed suggesting an immediate termination of CNP signaling. It is known that cGMP can regulate its own degradation into inactive 5′-guanosine monophosphate by binding to GAF domains present in several phosphodiesterases. In vertebrates, 11 gene families of phosphodiesterases with at least 21 members are transcribed. They degrade either cGMP or cAMP or both, and some of these might therefore establish a link between cGMP and cAMP signaling (36, 37) that modulates growth cone responses in vitro (38).

As a functional consequence of the absence of one longitudinal stem axon we observed a reduced input on second-order neurons of the spinal cord as detected by patch-clamp recordings. Thus, understanding the mechanism of CNP-induced axon bifurcation might be of relevance for nerve regeneration after spinal cord injury.

Materials and Methods

Generation of Mutant Mice. The CNPlacZ−/− ($Nppc^{lacZ/lacZ}$) mouse strain was generated by standard procedures replacing exon 1 of the CNP gene by a lacZ

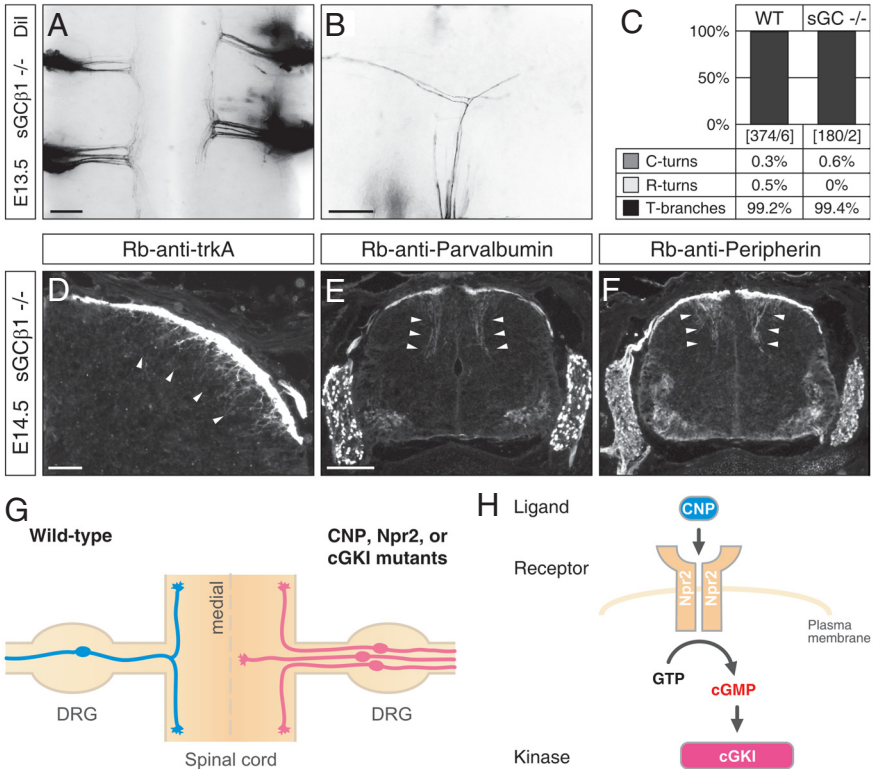

Fig. 4. NO-GC signaling is not required for bifurcation. (*A–C*) No bifurcation errors are found at the DREZ in the absence of the β1-subunit of sGC. In *C* the total number of individual sensory axons counted followed by the number of embryos analyzed is given in brackets. Collaterals are generated in the absence of the β1 subunit as revealed by anti-trkA (*D*), anti-parvalbumin (*E*), or anti-peripherin (*F*) staining ($n = 2$ embryos analyzed). [Scale bars, 50 (*D*) and 200 μm (*E* and *F*).] (*G*) Summarizing scheme of branching errors found at the DREZ in CNP, Npr2, or cGKI mutant mice: instead of bifurcation sensory axons turn in rostral or caudal direction only. A small percentage of axons prematurely enters the gray matter of the spinal cord. Collateral formation is not impaired. (*H*) Signaling components involved in CNP-induced sensory axon bifurcation.

expression cassette. The 129/Sv mouse PAC clone RPCIP711H05333Q2 (Resource Center/Primary Database, http://www.rzpd.de) containing *Nppc* was isolated from the RPCI-21 library (39). A genomic 14-kb fragment containing the *Nppc* gene was isolated by gap repair (40). Homologous recombination in bacteria (40) was used to fuse an *NLS-lacZ* cassette to the ATG of *Nppc*, to introduce the self-excision neo cassette (41), to delete the coding sequence of *Nppc* in exon 1, and to introduce additional stop codons in all three forward reading frames in exon 2. In addition, the MC1-diphtheria toxin A (*DTA*) cassette was placed at the 5′ end of the vector and was used for negative selection. E14.1 ES cells (129/Ola) were electroporated, and colonies that had incorporated the targeting vector into their genome were selected by G418 and analyzed for homologous recombination after digestion with *Eco*RI by Southern blot analysis using 5′ and 3′ sequences that lie outside of the targeting vector (see Fig. 1). Blastocysts were injected and chimeras that transmitted the mutant $Nppc^{lacZ}$ gene were identified by mating to C57BL/6J (B6) females. The mutant strain was subsequently expanded by mating $Nppc^{lacZ}$ males to B6 females. Routine genotyping was performed by PCR using oligonucleotides P1 (5′-AAGATGACATCAGCGGCAG-3′), P2 (5′-GCTTTGAGGGAGCAAGTCC-3′), and P3 (5′-CCTCTTCGCTATTACGCCAG-3′) and, occasionally, genotypes were verified by Southern blot hybridization. Lines deriving from two independently targeted ES cells were established. One line was used for the characterization of *Nppc1* function, and the other used for verification of the phenotype.

DNA fragments of natriuretic peptides were amplified using oligonucleotides *Nppa* 5′ (5′-CAAACATCAGATCGTGCCC-3′), *Nppa* 3′ (5′-CTTGGCTGTTATCTTCGGTACC-3′), *Nppb* 5′ (5′-CCACCAGTGCACAAGCTG-3′), *Nppb* 3′ (5′-TGCAGCCAGGAGGTCTTC-3′), *Nppc* 5′ (5′-ACCATGCACCTCTCCCAG-3′), and *Nppc* 3′ (5′-AGTGCACAGAGCAGTTCCC-3′).

The β1-NO-GC (*Gucy1b3*$-/-$) knock-out mouse, the Npr2 loss of function mutant (*cn/cn*), the *lbab* strain, and the Thy1-YFP-H reporter line have been described (28, 29, 31, 42). Routine genotyping of CNP*lbab* mice was carried out by PCR using oligonucleotides P1 (5′-AGCTGGTGGCAATCAGAAAA-3′) and P2 (5′-CTCTTGGGTGCAGAGCTAGG-3′) followed by a *Bsa*WI (New England BioLabs, Inc.) restriction enzyme digest that results in fragment sizes of 106 and 212 base pairs (wild-type), 106, 212, and 318 base pairs (heterozygous), and 318 base pairs (CNP*lbab* homozygous), respectively.

Immunohistochemistry, Axon Tracing, and Determination of cGMP Levels. DiI tracing, immunohistochemistry of cryostat sections using antibodies or isolectin IB4, RT-PCR, in situ hybridization, statistical calculations, and patch-clamp recordings were done essentially as described previously (22).

cGMP-levels of E13 DRG after 12.5 min of incubation with or without 0.5 μM CNP-22 (Calbiochem) were determined using the cGMP Biotrak assay from GE Healthcare and was performed according to protocol 4 of the manufacturer product booklet.

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