Neuron, Vol. 35, 1043–1056, September 12, 2002, Copyright ©2002 by Cell Press

WNT-3, Expressed by Motoneurons, Regulates Terminal Arborization of Neurotrophin-3-Responsive Spinal Sensory Neurons

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Summary

Sensory axons from dorsal root ganglia neurons are guided to spinal targets by molecules differentially expressed along the dorso-ventral axis of the neural tube. NT-3-responsive muscle afferents project ventrally, cease extending, and branch upon contact with motoneurons (MNs), their synaptic partners. We have identified WNT-3 as a candidate molecule that regulates this process. *Wnt-3* is expressed by MNs of the lateral motor column at the time when MNs form synapses with sensory neurons. WNT-3 increases branching and growth cone size while inhibiting axonal extension in NT-3- but not NGF-responsive axons. Ventral spinal cord secretes factors with axonal remodeling activity for NT-3-responsive neurons. This activity is present at limb levels and is blocked by a WNT antagonist. We propose that WNT-3, expressed by MNs, acts as a retrograde signal that controls terminal arborization of muscle afferents.

Introduction

The formation of neuronal connections requires neurons to project to their appropriate synaptic partners and to make functional synapses. This process is initiated when neurons begin to send axons in search of their targets. Upon reaching the target field, axons branch, cease extending, and their growth cones differentiate into presynaptic terminals. A number of attractive and repulsive signals have been shown to regulate the behavior of axons in transit to their targets (Brose and Tessier-Lavigne, 2000; Cook et al., 1998; Mueller, 1999; O'Leary and Wilkinson, 1999; Perrin et al., 2001). However, little is known about the signals made by postsynaptic cells that regulate the terminal differentiation of presynaptic arbors (reviewed by Tao and Poo, 2001).

The spinal cord has provided a model system to identify the molecules and mechanisms that regulate the formation of specific neuronal circuits. Secreted molecules of the netrin, semaphorin, BMP, and Slit families guide sensory and commissural axons (Augsburger et al., 1999; Brose and Tessier-Lavigne, 2000; Kennedy et al., 1994; Messersmith et al., 1995; Serafini et al., 1994). Several classes of sensory afferents project from the dorsal root ganglia (DRG) to specific target neurons in the spinal cord (Windle and Baxter, 1936; Mirnics and Koerber, 1995; Ozaki and Snider, 1997). Each class of sensory neurons has a characteristic dorso-ventral projection. Nerve growth factor (NGF)-dependent skin thermoreceptive/nociceptive sensory neurons make central connections with cells of the dorsal horn (Figure 1B) (Crowley et al., 1994; Ruit et al., 1992; Smeyne et al., 1994). Slit2 regulates axonal extension and collateralization of NGF-dependent DRG sensory neurons (Wang et al., 1999). On the other hand, proprioceptive sensory neurons projecting to the ventral spinal cord (VSC) are of two subtypes: group Ia afferents that carry signals from muscle spindles to motoneurons (MNs; Figure 1B) and group Ib afferents that connect Golgi tendon organs to interneurons in the ventral horn (Light and Perl, 1979; Brown, 1981; Eide and Glover, 1997; Ozaki and Snider, 1997). Thermoreceptive and nociceptive axons, those that terminate in the dorsal-most layers of the spinal cord, are repelled by members of the collapsin/semaphorin family expressed in the VSC (Fan and Raper, 1995; Giger et al., 1996; Luo et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Wright et al., 1995). However, muscle afferents that are neurotrophin-3 (NT-3)-dependent (Ernfors et al., 1994; Hory-Lee et al., 1993; Klein et al., 1994; Tessarollo et al., 1994) are not inhibited (Shepherd et al., 1997) and enter the ventral territory. The stereotypic pattern of projections of muscle afferents suggests that VSC factors, such as F11 (Perrin et al., 2001), regulate their pathfinding. Muscle Ia afferents branch as they enter the ventral horn and, upon contact with MNs, cease extending and form synaptic boutons (Kudo and Yamada, 1987; Chen and Frank, 1999). Explant experiments suggest that VSC provides stop and branching signals for Ia afferents (Sharma and Frank, 1998). However, the molecular identities of ventral horn signals that control the terminal arborization of Ia afferents remain largely unknown.

Recent studies suggest that members of the WNT family of signaling proteins play a role in the formation of neuronal connections. WNT-7A, expressed by cerebellar granule cells, induces growth cone enlargement, axonal spreading, and increases the clustering of synapsin I in mossy fibers (Hall et al., 2000). More importantly, *Wnt-7A* mutant mice exhibit a delay in the morphological maturation and accumulation of synaptic proteins (Hall et al., 2000). Thus, WNT-7A acts as a retrograde signal from postsynaptic neurons to regulate axonal remodeling and expression of synaptic proteins on presynaptic terminals.

Here we have investigated the role of WNT proteins in the formation of the sensory-motor connections in the mouse spinal cord. We show that *Wnt-3* is expressed in MNs of the lateral motor column (LMC) at a time when sensory axons make contact with them. WNT-3 inhibits

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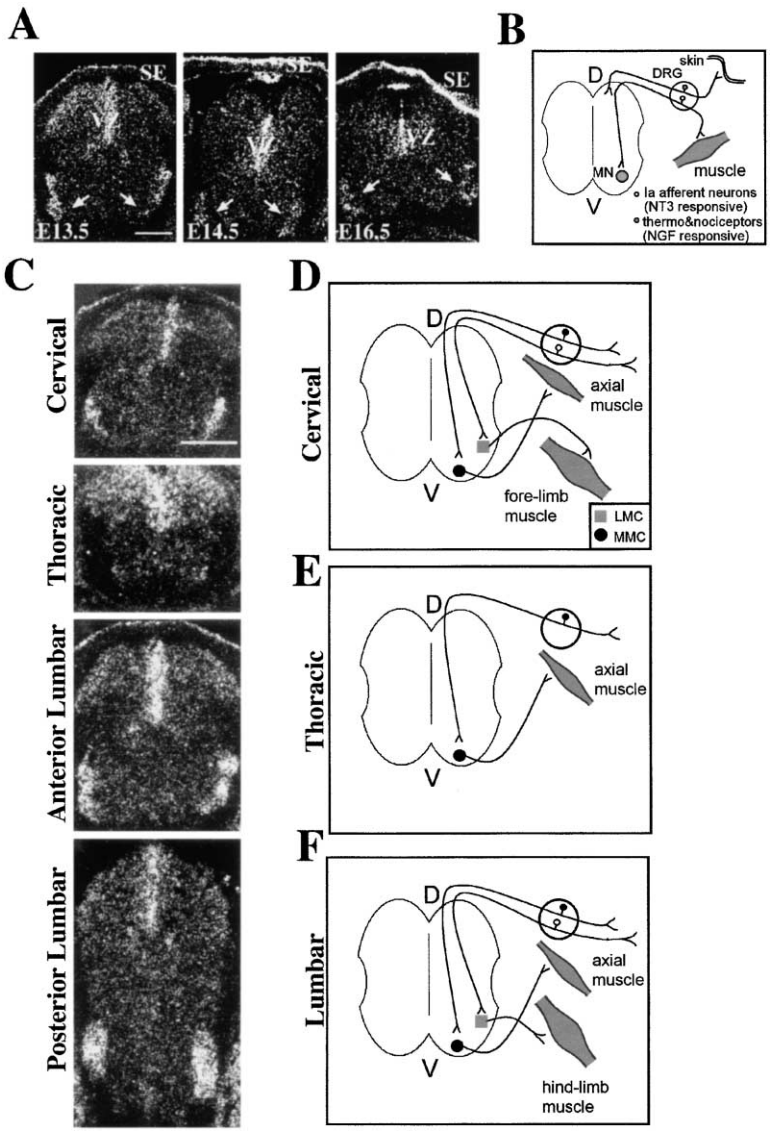

Figure 1. *Wnt-3* Is Expressed in the Lateral Motor Column of the Developing Spinal Cord

(A) In situ hybridization of transverse cervical spinal cord sections shows the expression of *Wnt-3* at E13.5, E14.5, and E16.5. *Wnt-3* expression is detected in the surface ectoderm (SE), ventricular zone (VZ), and in the ventral horn of the spinal cord, where it is restricted to MNs of the LMC. Arrows indicate MNs. Scale bar, 500 μm.

(B) Schematic representation of the sensory neuron projections in the spinal cord. The peak of *Wnt-3* expression in MNs coincides with the time when sensory axons are beginning to form sensory-motor connections. DRG, dorsal root ganglia; MN, motoneurons; D, dorsal; V, ventral.

(C) In situ hybridization of E13.5 mouse spinal cord reveals a restricted expression of *Wnt-3* along the A-P axis. *Wnt-3* expression was found in MNs at cervical and lumbar levels of the ventral spinal cord. No expression of *Wnt-3* was detected in MNs at thoracic levels. Scale bar, 500 μm.

(D–F) Schematic representations of the organization of motor columns and peripheral targets of the MNs at different A-P levels. MMC, medial motor column; LMC, lateral motor column.

axonal extension of DRG neurons. In contrast to other signals that inhibit axonal outgrowth, WNT-3 induces axonal branching and growth cone enlargement rather than growth cone collapse. The effect of WNT-3 is selective for NT-3-dependent sensory neurons, as WNT-3 does not affect NGF-responsive sensory neurons. The specificity of the response matches the expression of *Wnt-3* in MNs at limb levels, the major postsynaptic targets of NT-3-responsive sensory neurons. Interestingly, explants of cervical and lumbar, but not thoracic, VSC are significant sources of axonal remodeling activity that is blocked by the WNT antagonist, secreted Frizzled-related peptide 1 (sFRP1). Thus, our studies identify a WNT-like axonal remodeling activity in the spinal cord that is differentially expressed along the anterior-posterior (A-P) axis. Taken together, our results suggest that MN-derived WNT-3 acts as a retrograde branching and stop signal for muscle afferents during the formation of sensory-motor circuits in the spinal cord.

Results

Wnt-3 Is Expressed in Motoneurons of the Lateral Motor Column during the Formation of Sensory-Motoneuron Connections

To study a possible role for WNTs in the formation of neuronal connections in the mouse spinal cord, we examined the temporal expression pattern of several *Wnt* genes between embryonic day 12.5 (E12.5) and E17.5. In the developing mouse spinal cord, *Wnt-3*-positive cells are first detected at E12.5 in the ventral horn (Roelink and Nusse, 1991). At E13.5 and E14.5, higher levels of *Wnt-3* expression are detected in the VSC where MNs are found (Figure 1A). Expression of *Wnt-3* declines at E16.5 (Figure 1A) and E17.5 and becomes undetectable from E18.5 (data not shown).

The expression of *Wnt-3* in lateral ventral areas of the spinal cord suggested that *Wnt-3* was restricted to a subset of MNs. Spinal MNs are divided into two large subpopulations, axial and limb MNs (Pfaff and Kintner,

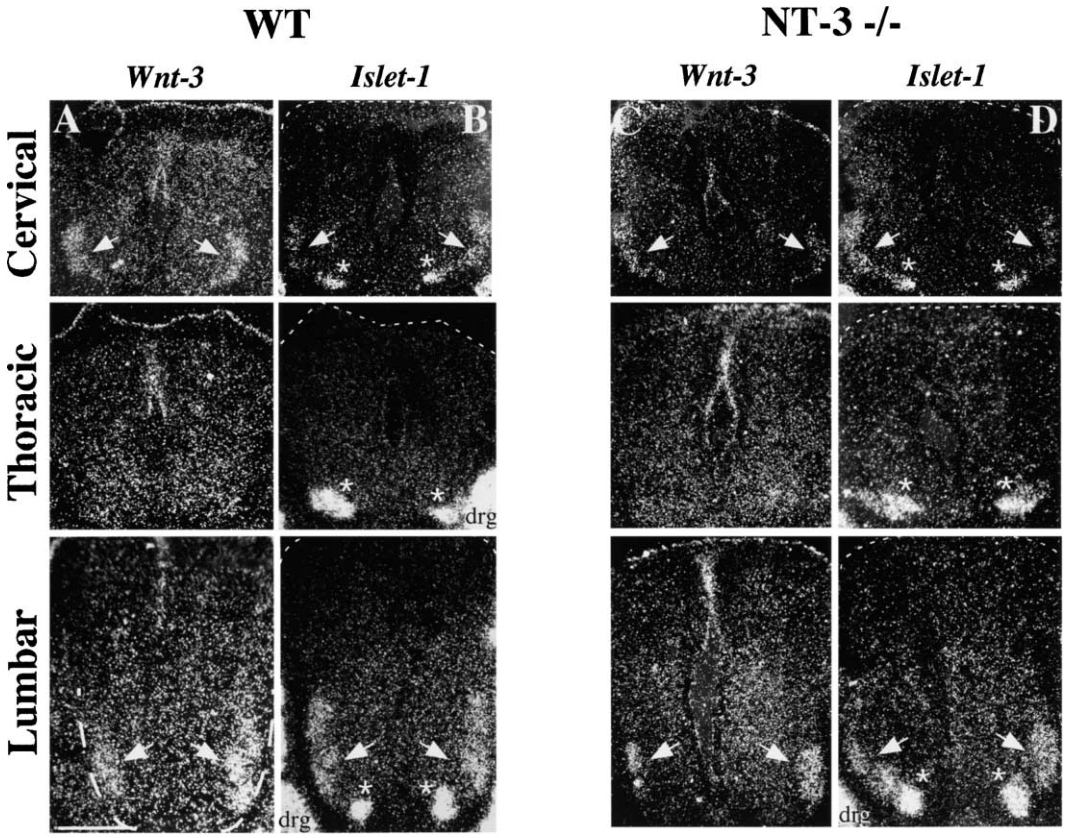

Figure 2. *Wnt-3* and *Islet-1* Colocalize in the Lateral Motor Column of Wild-Type and NT-3 Null Embryos

(A) *Wnt-3* expression along the A-P axis coincides with the expression of *Islet-1* (B) in MNs of the LMC at cervical and lumbar levels of E13.5 wild-type embryos. In contrast, no expression of *Wnt-3* is detected in MNs of the MMC that express *Islet-1* (asterisks). Note the expression of *Wnt-3* in the ectoderm and the ventricular zone and *Islet-1* expression in the DRGs. (C) The pattern and level of expression of *Wnt-3* is not altered in NT-3 null embryos. (D) Expression of *Islet-1* was also normal in these embryos. Dashed line defines the edge of the sections. Arrows, LMC; asterisks, MMC. Scale bar, 500 μm.

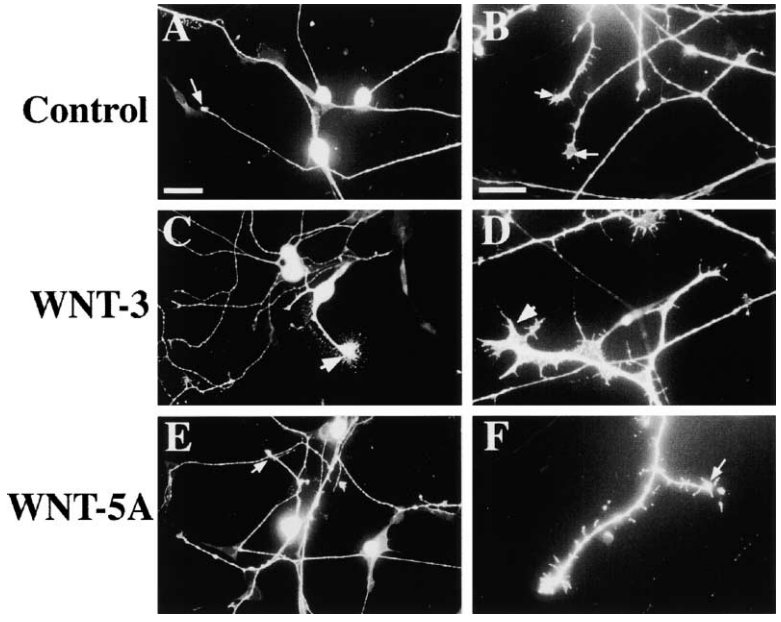

Figure 3. WNT-3 Induces Axonal Remodeling in Sensory Neurons

Embryonic sensory neurons were cultured for 24 hr in the presence of control, WNT-3, or WNT-5A conditioned medium. Cultures were immunostained for GAP-43 to visualize the neuronal morphology.

(A–B) Control sensory neurons have normal morphology with small growth cones.

(C–D) Treatment with WNT-3 induces a significant increase in growth cone size and spreading at the axon terminal.

(E–F) Treatment with WNT-5A does not affect axonal morphology. Large arrows indicate enlarged growth cones. Small arrows indicate small growth cones. Scale bars, 100 μm (A, C, and E); 50 μm (B, D, and F).

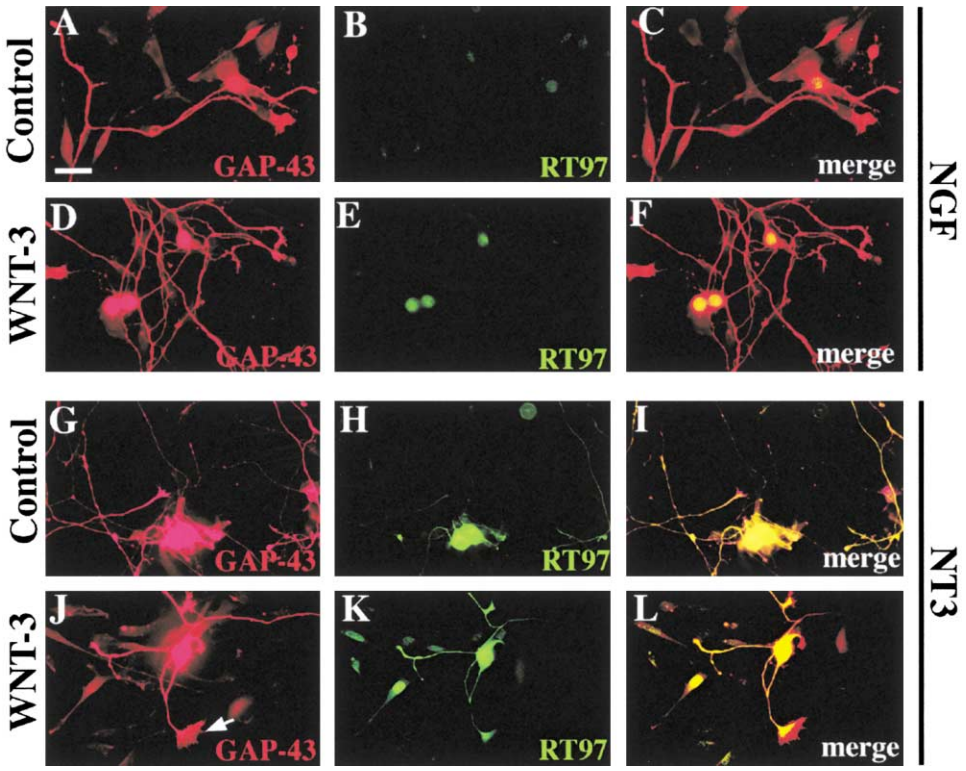

Figure 4. Neurotrophin Selection of Sensory Neurons Is Not Affected by WNT-3

Sensory neurons were grown in the presence of 25 ng/ml NGF (A–F) or NT-3 (G–L) for a total 48 hr. Control (A–C and G–I) or WNT-3-conditioned media (D–F and J–L) were added for the last 16 hr of culture. Staining for GAP-43 (A, D, G, and J) shows the cell morphology. Staining with the RT97 antibody (B, E, H, and K), a marker for NT-3-dependent sensory neurons, labels only the nucleus in NGF-selected cultures (A–F). WNT-3 treatment does not change the number of RT97-stained neurons in either NGF- (D–F) or NT-3-selected cultures (J–L). However, WNT-3 increases growth cone size in NT-3-selected sensory neurons (arrow). Scale bar, 100 μm.

1998; Hughes and Salinas, 1999; Jessell, 2000). Limb MNs form the LMC at cervical and lumbar levels (Figures 1D and 1F) (Landmesser, 1978a, 1978b; Pfaff and Kintner, 1998). To test whether *Wnt-3* expression is restricted to these two populations of MNs, we examined the expression of *Wnt-3* along the A-P axis of the spinal cord. In situ hybridization reveals that *Wnt-3* is expressed mainly in MNs at cervical and lumbar regions at E13.5 and not at thoracic levels (Figure 1). Expression of *Islet-1*, a LIM transcription factor expressed by MNs (Jessell, 2000), shows that *Wnt-3* is detected in the LMC but not in the medial motor column (MMC) (Figures 2A and 2B). This restricted pattern of *Wnt-3* expression along the A-P axis is also observed at E14.5 and E16.5 (data not shown). Furthermore, the onset of *Wnt-3* expression coincides with the arrival of sensory neuron projections to the MN territory and when MNs begin to innervate muscles in the periphery (Altman and Bayer, 1984; Snider et al., 1992; Zhang et al., 1994). Our previous studies have shown that WNT-7A is a retrograde signal capable of remodeling presynaptic axons (Hall et al., 2000). Therefore, a candidate function for WNT-3 made by MNs is to regulate the formation of neuronal connections between sensory axons and MNs.

The timing of *Wnt-3* expression suggests that innervation could regulate *Wnt-3* expression in MNs. NT-3 null mice show severe loss of NT-3-responsive sensory axons, the presynaptic partners of limb MNs, in the spinal cord (Ernfors et al., 1994; Fariñas et al., 1994). Therefore, we examined the expression of *Wnt-3* in these mice. *Islet-1* expression is normal in both MMC and LMC, suggesting that MN number is not significantly altered in this mutant (Figure 2D). More importantly, in NT-3 null mutant mice, *Wnt-3* expression in spinal MNs is indistinguishable from that in wild-type littermates (Figure 2C). Thus, *Wnt-3* expression in MNs is not dependent upon the arrival of sensory afferents to the VSC.

WNT-3 Induces Growth Cone Remodeling in Sensory Neurons

To test the possible role of WNT-3 as a retrograde signal, we examined whether WNT-3 regulates the behavior of sensory axons. Dissociated DRG neurons isolated from E13.5 mice cultured in the presence of both NGF and NT-3 were exposed to soluble WNT-3 protein. Cells grown in control medium have a normal neuronal morphology (Figures 3A and 3B). In contrast, soluble WNT-3 protein causes an increase in growth cone size in a subset of DRG neurons (Figures 3C and 3D). WNT-5A, which is not expressed in the spinal cord (A. McMahon, personal communication) has no effect on axonal morphology (Figures 3E and 3F). These results suggest that WNT-3 has an axonal remodeling activity for a subset of spinal sensory neurons.

WNT-3 Specifically Induces Axonal Remodeling in NT-3- but Not NGF-Responsive Sensory Neurons

Two findings suggest that a distinct population of sensory neurons responds to WNT-3. First, a small proportion of DRG sensory neurons responds to WNT-3 when cultured in the presence of both NGF and NT-3. Second, most of the cells responding to WNT-3 were those with large axon caliber and large cell bodies, characteristics of proprioceptive sensory neurons (Snider and Wright, 1996). Therefore, we used DRG cultures selected with either NT-3 or NGF to test the effect of WNT-3 on proprioceptive (NT-3-dependent) or thermo/nociceptive (NGF-dependent) neurons. NGF-responsive neurons have small cell bodies and fine axons and at E13.5 represent approximately 80% of the total number of DRG sensory neurons, with the remaining 20% being large-diameter NT-3-responsive neurons (reviewed by Snider and Wright, 1996).

As WNT factors have recently been implicated in cell survival (Chen et al., 2001), we first studied whether WNTs affect the number of neurons selected with either NT-3 or NGF. Expression of the neurotrophin receptors, TrkC and TrkA by proprioceptive and thermo/nociceptive sensory neurons, respectively, has been used to identify these two populations of DRG neurons in vivo (Lin et al., 1998; Perrin et al., 2001). The available antibodies against these receptors do not work in dissociated DRG cultures. Therefore, we used the RT97 antibody that recognizes neurofilament proteins and labels large-diameter sensory neurons characteristic of muscle afferents (Lawson et al., 1984; Perry et al., 1991). In NGF-selected cultures, on average, fewer than 10% of the cells were labeled with the RT97 antibody (Figures 4B and 4E). A low level of nonspecific nuclear staining was observed in small diameter cells (Figures 4B and 4E). In control NT-3-selected cultures, over 85% of neurons were RT97 positive (Figure 4H), indicating a substantial enrichment of distinct neuronal populations by neurotrophin selection. The presence of WNT-3 or WNT-5A does not affect the total number of neurons or the number of RT97-positive cells that survive with either NGF or NT-3 compared to control media (Figure 4 and data not shown). These results suggest that WNT-3 and WNT-5A do not affect the survival of a subpopulation of sensory neurons.

We then examined whether WNT-3 selectively affects the morphology of sensory axons. NGF-selected neurons have the same morphology in the presence of control or WNT-3 containing media (Figures 5A and 5C). In contrast, treatment with WNT-3 increases the size of growth cones of NT-3-selected neurons (Figure 5D). Quantification of the growth cone area shows that WNT-3 significantly increases the average size of growth cones (see also Figure 8C). Analysis of the growth cone area distribution shows that WNT-3 induces a shift that results in a decrease of small growth cones, while increasing the percentage of large growth cones ($p < 0.001$; Figure 5F).

Visual inspection of NT-3-selected cultures suggested that WNT-3 also affects axon length in NT-3-selected DRG neurons (Figure 5D). Indeed, WNT-3 induces a 25% reduction in axon length in NT-3-responsive neurons

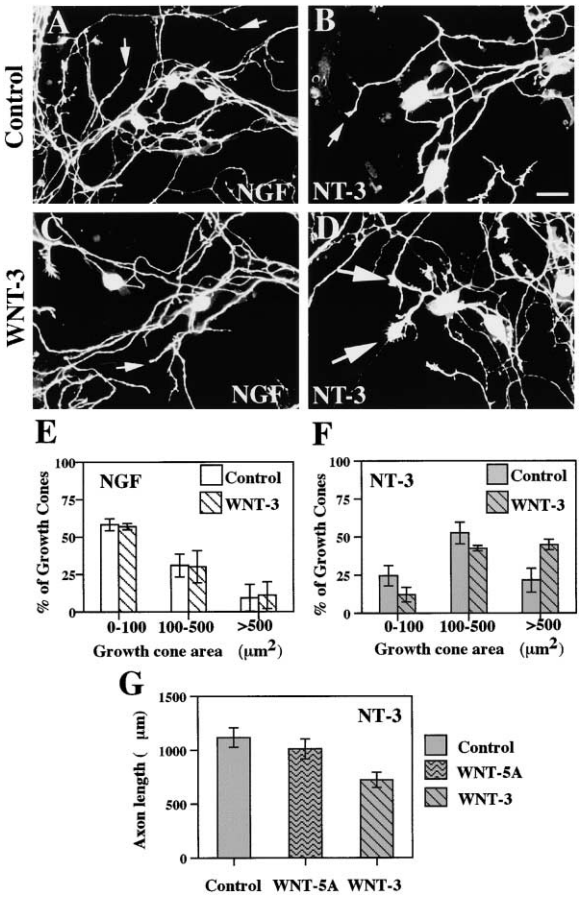

Figure 5. WNT-3 Induces Axonal Remodeling in NT-3 but Not NGF-Responsive Sensory Neurons

Sensory neurons were cultured in the presence of NGF (A, C, and E [unshaded]) or NT-3 (B, D, F, and G) for 48 hr in the presence of control or WNT-3 added for the last 16 hr. Control NGF- (A) or NT-3-selected sensory neurons (B) stained for GAP-43 have small growth cones. (C) WNT-3 does not affect axonal morphology of NGF-selected cultures. (D) WNT-3 treatment induces growth cone enlargement in NT-3-selected sensory neurons. Large and small arrows indicate enlarged and small growth cones respectively. Scale bar, 50 μm. (E) Distribution of growth cone sizes in NGF-selected cultures is unaffected by WNT-3. (F) In NT-3-selected cultures, WNT-3 increases the number of neurons with a growth cone area greater than 500 μm^2, while decreasing the number of growth cones smaller than 100 μm^2 ($p < 0.001$). (G) WNT-3 inhibits axonal extension in NT-3-selected cultures ($p < 0.001$) compared to control or WNT-5A. Values are mean ± SEM, n = 100–150.

($p < 0.001$; Figure 5G). This effect is specific for WNT-3, as WNT-5A does not affect axon length (Figure 5G). In contrast, WNT-3 has no effect on axon length on NGF-responsive neurons (data not shown). These findings demonstrate that WNT-3 induces axonal remodeling in NT-3-responsive sensory neurons.

To further analyze the specificity of WNT-3 effects in NT-3-selected sensory neurons, we examined the role of different WNT factors on axonal remodeling. In addition to WNT-5A (Figures 3E and 3F), we found that WNT-4, WNT-5B, and WNT-7B do not affect the morphology of NT-3-responsive sensory neurons (Figure 6). Although WNT-1 and WNT-6 exhibit a weak axonal re-

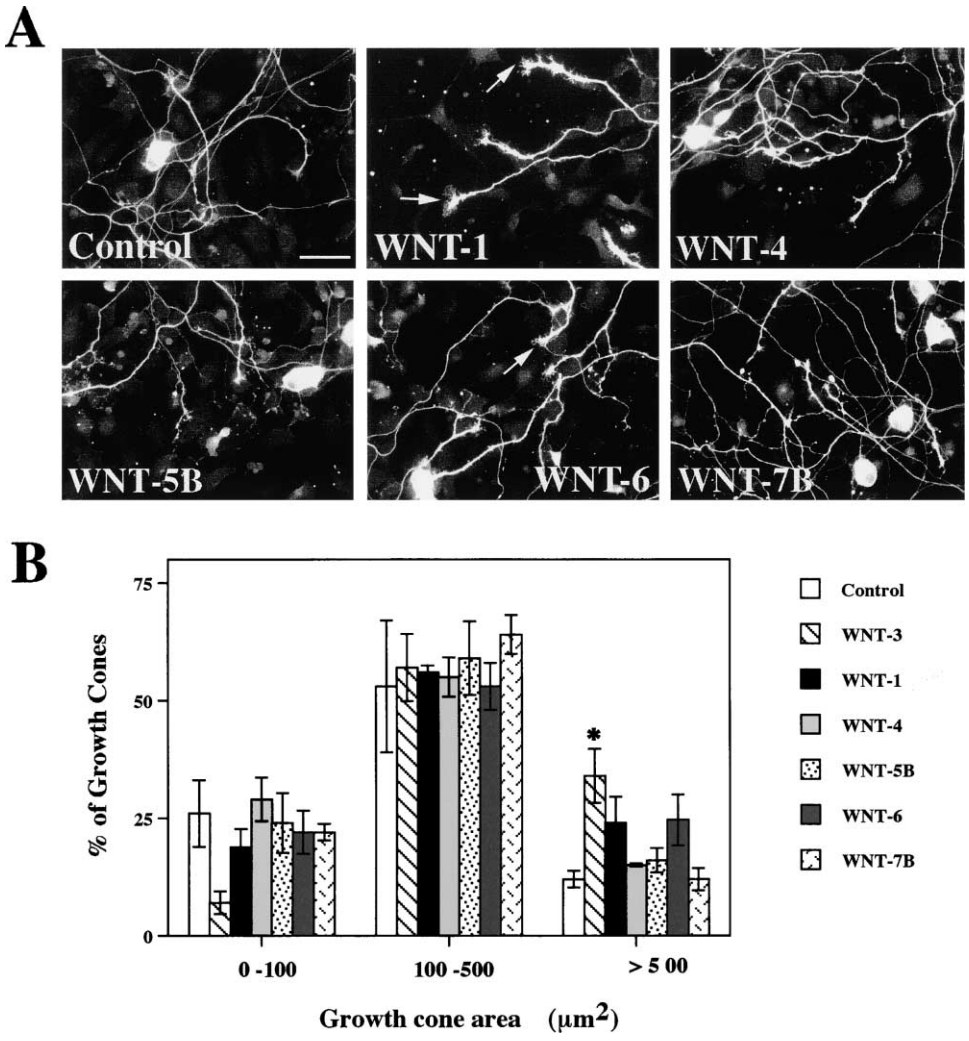

Figure 6. Several WNTs Do Not Affect Axonal Remodeling in NT-3-Responsive Sensory Neurons

Neurons were grown in 25 ng/ml NGF or NT-3 for 48 hr and WNTs added to the culture for the last 16 hr.

(A) NT-3-selected DRG neurons were cultured in the presence of CM containing different WNTs. Arrows indicate slightly enlarged growth cones. Scale bar, 100 μm.

(B) Distribution of the growth cone size in NT-3-selected DRG neurons incubated with various WNT factors. With the exception of WNT-3, none of the WNT factors tested significantly increases the size of growth cones. Asterisk denotes significance for WNT-3 ($p < 0.01$). Values are mean ± SEM, n = 100–150.

modeling activity, these effects were not statistically significant (Figures 6A and 6B). Thus, NT-3-dependent sensory neurons are unresponsive to a number of WNT proteins.

WNT-3 Induces Axonal Branching in Sensory Neurons

In the VSC, sensory axons branch as they reach the motor nuclei (Kudo and Yamada, 1987). Since *Wnt-3* is expressed during this period, we tested whether WNT-3 has a branching activity on DRG neurons. In NT-3-selected cultures, control neurons are mainly bipolar with few branches (Figure 7A), whereas WNT-3-treated neurons show an increase in the number of branches (Figure 7B). To quantify this effect, we measured the number of primary and higher order branches per neurite. In NGF-selected cultures, no significant differences were found in the number of primary, secondary, and higher order branches between control and WNT-treated cultures (Figure 7C). In NT-3-selected neurons, WNT-3 increases the number of secondary, tertiary, and higher order branches (Figure 7D), although it does not affect the number of primary branches (data not shown). In contrast, WNT-5A has no effect on axon branching (Figures 7C and 7D). The increased branching suggests that WNT-3 affects the axon shaft to generate a new area of axon growth. Taken together, our findings indicate that WNT-3 acts as a stop and axonal branching signal for a select population of sensory neurons, the Ia afferents.

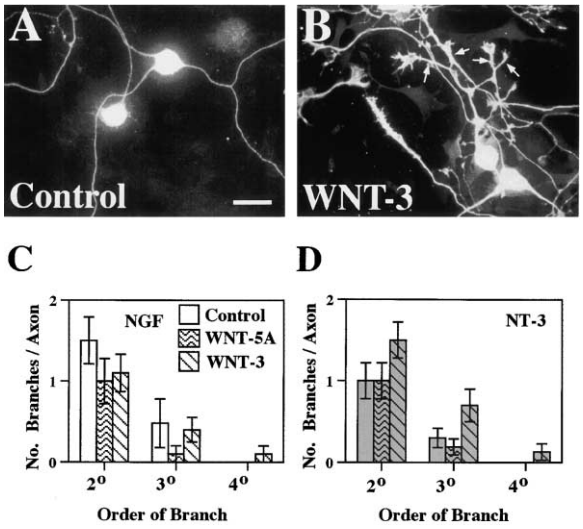

Figure 7. WNT-3 Induces Axonal Branching in NT-3-Responsive Sensory Neurons

(A) NT-3-selected neurons (50 ng/ml for a total 72 hr) treated with control medium are predominantly bipolar with some secondary branching.

(B) WNT-3 induces more secondary branching and the appearance of higher order branches (arrows). Scale bar, 50 μm.

(C and D) Quantification of the number of different order branches per axon in NGF- (C) and NT-3- (D) selected cultures. WNT-5A does not affect significantly the number of branches in NGF- or NT3-selected sensory neurons. WNT-3 does not increase the number of branches in NGF-selected neurons. However, WNT-3 increases the number of secondary order branches in NT-3-selected neurons by 50%, whereas third order branches increase by almost three-fold. Values are mean ± SEM, n = 30–50.

sFRP1 Antagonizes WNT-3 Axon Remodeling Activity

Due to the lack of purified WNT factors, all our experiments were performed with conditioned media (CM) from expressing cells. Therefore we analyzed whether the effect observed with WNT-3 CM is due to the direct action of WNT-3 or alternatively due to the presence of another factor induced by the expression of WNT-3. To demonstrate the direct action of WNT-3, we used the secreted WNT antagonist, sFRP1 (Finch et al., 1997; Rattner et al., 1997). In the presence of WNT-3, the average growth cone size was doubled when compared to control cultures (Figures 8A and 8C). However, preincubation of WNT-3 with sFRP1 reverses the effect of WNT-3 by reducing the average growth cone size to control values (Figures 8B and 8C). sFRP1 alone does not affect axonal morphology when compared to control media (data not shown). We also analyzed the effect of sFRP1 on WNT-3-induced branching activity. WNT-3, when added alone, increases axonal branching (Figures 8A and 8D). We noticed a higher branching activity in these sets of experiments, which correlates with a higher level of WNT-3 in the media (data not shown). Addition of sFRP1 to WNT-3 blocks most of the axonal branching activity of WNT-3 (Figures 8B and 8D), as sFRP1 inhibits the formation of secondary and tertiary branches to almost control levels (Figure 8D). These experiments strongly support the notion that WNT-3 directly mediates axonal remodeling in NT-3-responsive sensory neurons.

WNT-3 Increases Synapsin I Clustering In Sensory Neurons

The changes in the morphology of sensory axons induced by WNT-3 are similar to those observed when

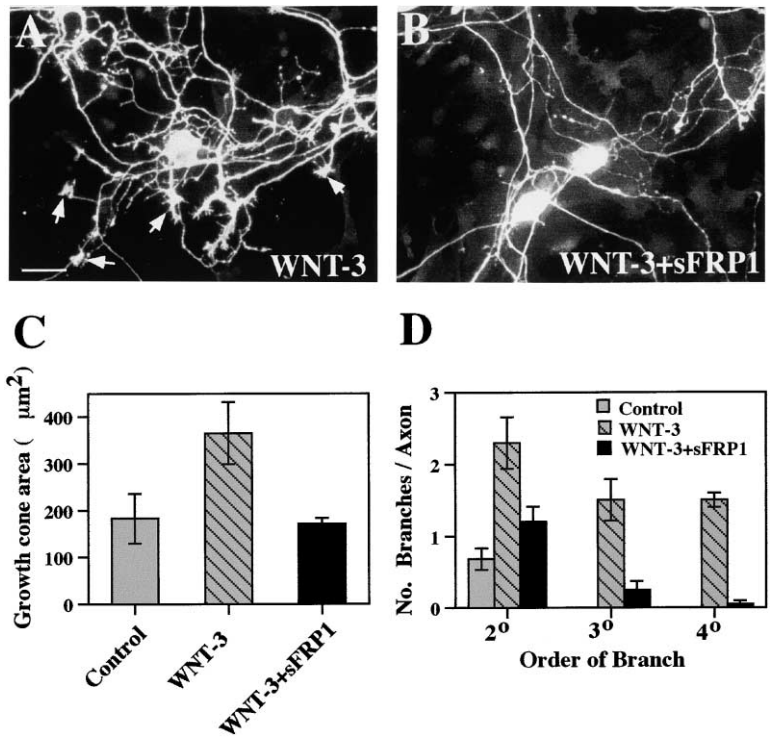

Figure 8. The WNT Antagonist sFRP1 Blocks the Effect of WNT-3 on Axonal Remodeling

WNT-3 CM diluted with control (A) or sFRP1 CM (B) were used to treat NT-3-selected sensory neurons. Arrows indicate enlarged growth cones.

(B) The preincubation of sFRP1 with WNT-3 blocks the effect of WNT-3. Scale bar, 50 μm.

(C) Quantification of the average growth cone area shows that the addition of sFRP1 to WNT-3 reverts growth cone size to that of control values.

(D) The effect of WNT-3 on branching is also blocked by sFRP1, with reduction of secondary and higher order branching to almost control levels. Values are mean ± SEM, n = 50–100.

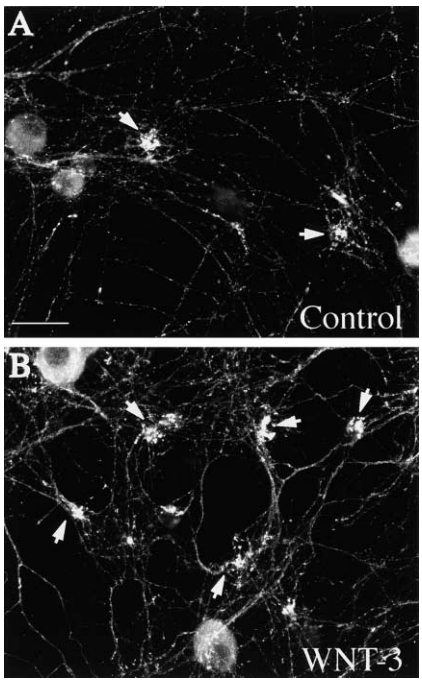

Figure 9. WNT-3 Increases Synapsin I Clustering in Embryonic Sensory Neurons

Sensory neurons were cultured for 6 days in vitro and in the last 16 hr were treated with control or WNT-3 CM.

(A) Control cultures exhibit small synapsin I clusters along the axon and few large clusters in areas where axons cross.

(B) WNT-3 increases the number of large and small synapsin I clusters (arrows) compared to controls. Scale bar, 50 μm.

axons come into contact with their target and begin to form synapses. To begin to address the possible role of WNT-3 in synapse formation, we examined the effect of WNT-3 in the clustering of synapsin I, a presynaptic protein involved in synapse formation and function (Chin et al., 1995; Rosahl et al., 1995). DRG neurons were cultured for 6 days and exposed to control or WNT-3 containing media for the last 16 hr of the culture period. In control sensory neurons, synapsin I is localized in small clusters along the axon and in very few large clusters that formed in areas where several axons cross (Figure 9A). In contrast, neurons cultured in the presence of WNT-3 formed numerous large and small synapsin I clusters (Figure 9B). WNT-3 also increases the overall level of synapsin I along the axon (Figure 9B). The effects of WNT-3 on axonal remodeling and synapsin I clustering suggest a role for WNT-3 in presynaptic differentiation.

Inhibition of GSK-3β by Lithium Mimics the Effect of WNT-3 In Sensory Neurons

The lack of response of NGF-selected sensory neurons to WNT-3 raised the question of how these cells differ from the NT-3-selected population. To address this issue we activated the canonical WNT signaling pathway downstream of the receptor using lithium, a direct inhibitor of GSK-3β (Klein and Melton, 1996). Lithium mimics WNT signaling in early development and during axonal extension (Hall et al., 2000; Klein and Melton, 1996; Lucas and Salinas, 1997). Control neurons were cultured in the presence of 10 mM sodium chloride. We observed a slight increase in growth cone size in NGF-selected DRG neurons treated with sodium when compared to control media (compare Figures 5E and 10E). However, sodium chloride treatment does not affect the distribution of growth cones in NT-3-selected cultures (Figures 5F and 10F). Lithium causes growth cone enlargement in NT-3-responsive neurons when compared to sodium ($p < 0.001$; Figures 10B and 10D). Unlike WNT-3, lithium affects NGF-selected neurons ($p < 0.001$; Figures 10A and 10C). In both populations there was a shift in the distribution of growth cone size toward growth cones greater than 500 μm^2 compared to control (Figures 10E and 10F).

Lithium treatment also reduces axon length by 53% in NGF-selected and 48% in NT-3-selected neurons (Figure 10G) and increases axonal branching in both NT-3 and NGF-responsive sensory neurons (Figure 10H). Thus, lithium mimics WNT-3 signaling in NT-3-dependent sensory axons. However, the effect of lithium is not restricted to this specific neuronal population. These results indicate that axonal remodeling is a consequence of GSK-3β inhibition and suggest that NGF-dependent neurons lack functional components upstream of GSK-3β.

Cervical and Lumbar Ventral Spinal Cord Explants Have Growth Cone Remodeling Activity Antagonized by sFRP1

The pattern of *Wnt-3* expression in the LMC and the effect of WNT-3 on NT-3-selected sensory neurons suggest that WNT-3 could act as a retrograde signal from MNs to regulate the formation of motor-sensory neuron connections. To begin to address the role of WNT-3 in vivo, we examined whether VSC at limb levels is a source of WNT-like endogenous activity. Cervical, thoracic, and lumbar VSC explants (Figure 11A) were cultured for 24 hr in serum-free media. CM from these explants were then used to treat NT-3- or NGF-selected sensory neurons. We found that secreted factors from cervical and lumbar levels induce growth cone enlargement in NT-3-selected DRG neurons ($p < 0.001$ for both conditions; Figures 11B and 11D). This effect can be observed by the reduction in the percentage of growth cones with a size less than 100 μm^2 to 15% for cervical and 13% for lumbar compared to 27.5% in control (Figure 11E). Furthermore, growth cones with an area greater than 500 μm^2 increase to 37% and 35% respectively, compared to 20% in control (Figure 11E). The shift in growth cone area distribution is similar to that observed in the presence of WNT-3. In contrast, CM from thoracic levels, where *Wnt-3* expression is undetected, does not have a significant effect on the growth cone size distribution in NT-3-responsive neurons (Figures 11C and 11E). Furthermore, CM from VSC does not affect growth cone size of NGF-responsive neurons (Figure 11F), as observed with WNT-3. These findings suggest that VSC from cervical and lumbar regions secretes factors with a WNT-like activity.

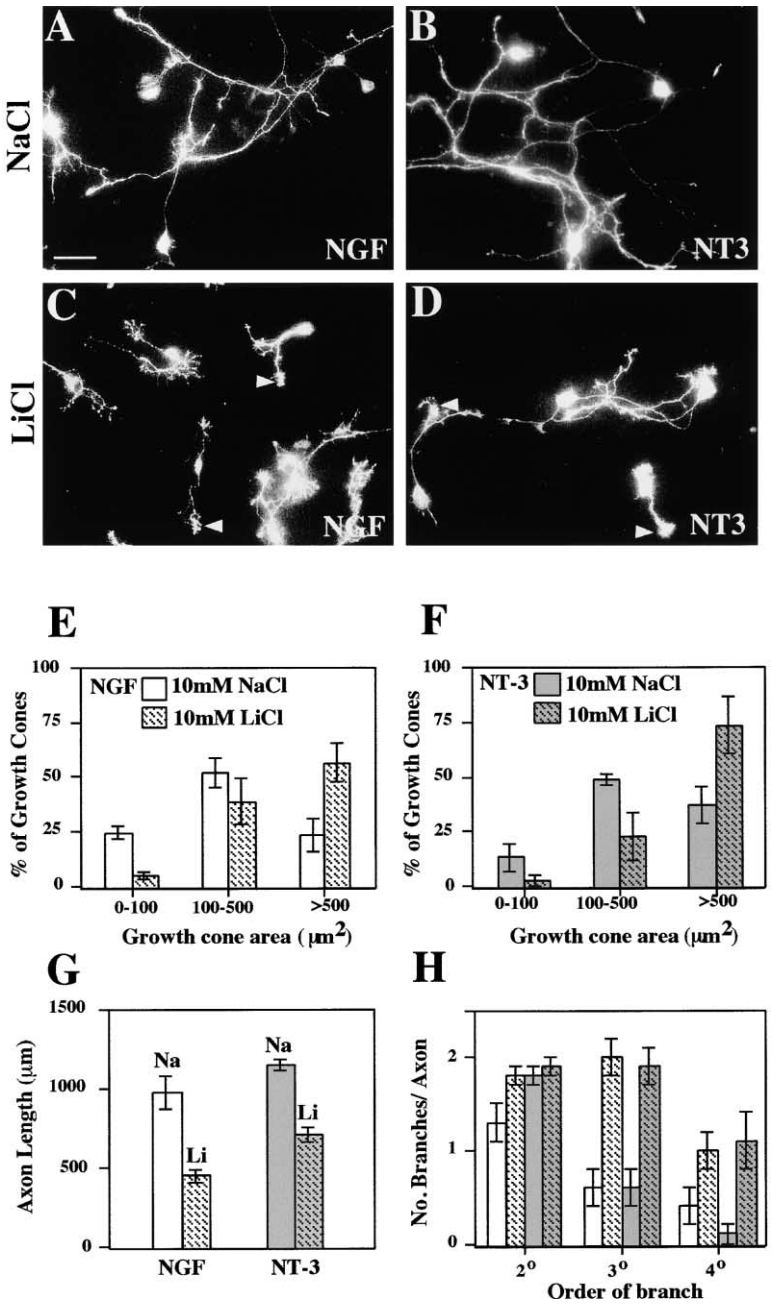

Figure 10. Inhibition of GSK-3β by Lithium Mimics the Effect of WNT-3

Sensory neurons were cultured in the presence of NGF or NT-3 for 48 hr with 10 mM NaCl or 10 mM LiCl added for the last 16 hr. NaCl has little effect on axonal remodeling in either NGF- (A) or NT-3- (B) selected cultures, whereas lithium induces axon shortening, growth cone enlargement (arrowheads), and increased branching in both NGF- (C) and NT-3- (D) selected cultures. Scale bar, 100 μm. (E and F) The distribution of growth cone sizes in NGF- (E) and NT-3- (F) selected cultures is shifted by lithium treatment, with a higher percentage of growth cones greater than 500 μm^2 ($p < 0.001$ for both conditions). (G) Lithium treatment inhibits axonal extension by 53% in NGF- (unshaded) and 48% in NT-3-selected cultures (shaded) ($p < 0.001$). (H) Quantification of axonal branching shows that lithium increases the number of secondary and higher order branches in both NGF- (unshaded) and NT-3-selected cultures (shaded) when compared to control (NaCl). Values are mean ± SEM, n = 100–200.

To investigate if this activity was due to the presence of a WNT protein, we tested whether the WNT antagonist sFRP1 could block the axonal remodeling activity of VSC. We found that sFRP1 blocks the growth cone remodeling activity of cervical and lumbar VSC-secreted factors in NT-3-selected sensory cultures. Addition of sFRP1 to CM from cervical and lumbar explants reverts growth cone size to control values ($p < 0.001$ for both conditions; Figure 11E). Thus, VSC at limb levels has an axonal remodeling activity that can be blocked by sFRP1. Since *Wnt-3* is expressed at cervical and lumbar levels in MNs of the LMC, WNT-3 is likely to mediate at least in part this endogenous growth cone remodeling activity of the VSC.

Discussion

The formation of functional motor connections in the spinal cord requires matching of specific sensory neurons with their appropriate MN targets. Here we present data that suggest a role for WNT-3 from MNs in regulating the terminal arborization of muscle sensory afferents in the spinal cord. *Wnt-3* is expressed by MNs at cervical and lumbar levels at the time when sensory neurons come into contact with MNs. WNT-3 inhibits neuronal outgrowth while increasing growth cone size and axonal branching in NT-3-responsive sensory neurons, the presynaptic targets for MNs. Thus, the behavior induced by WNT-3 resembles that observed in sensory axons as

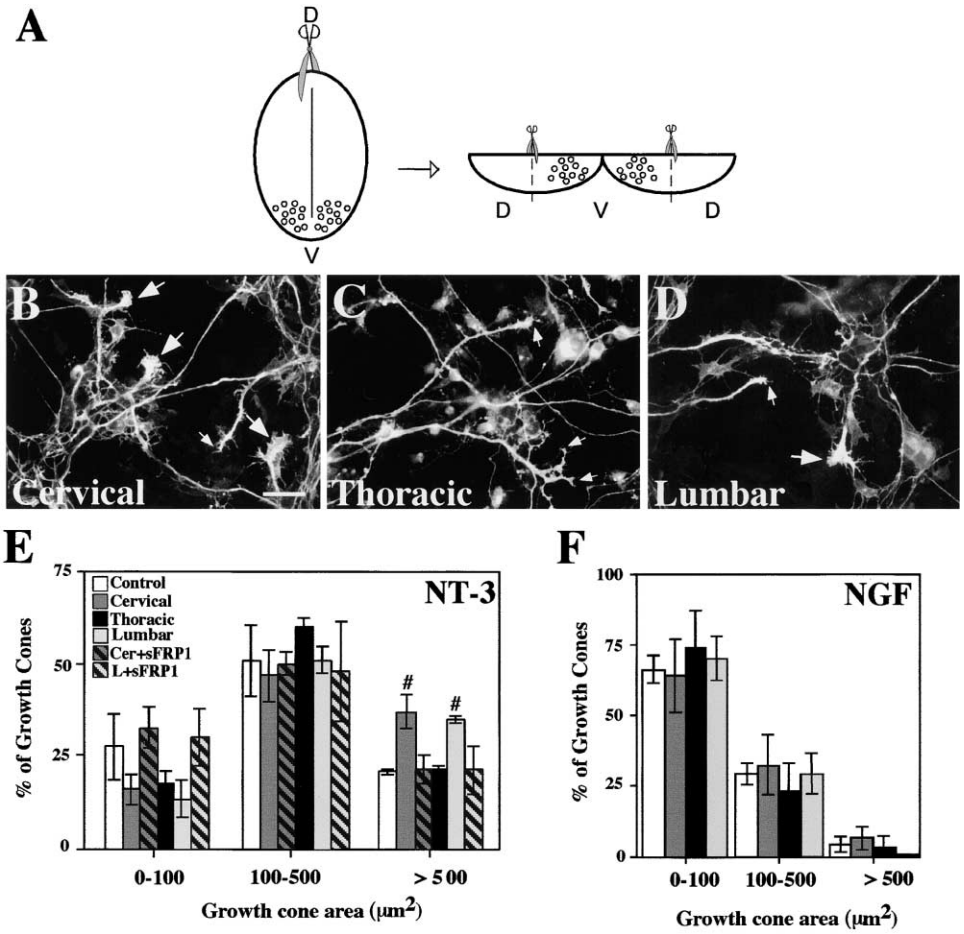

Figure 11. Ventral Spinal Cord at Limb Level Is a Source of Axon Remodeling Activity Blocked by sFRP1

(A) Schematic representation of the isolation of ventral spinal cord explants. Left, the schematic spinal cord viewed in cross-section. Spinal cord was opened from the dorsal side. Small circles denote MNs. Right, the dorsal parts of the resulting plate were dissected out leaving the ventral region containing MNs. D, dorsal; V, ventral.

(B–D) NT3-selected DRG neurons were cultured for the last 20 hr with media conditioned by VSC explants from cervical (B), thoracic (C), and lumbar (D) regions. CM from cervical and lumbar VCS induce growth cone enlargement in NT3-selected neurons (B and D). CM from thoracic VSC explants does not affect axonal morphology of NT3-selected DRG neurons (C). Small arrows indicate small growth cones. Large arrows indicate enlarged growth cones. Scale bar, 100 μm.

(E and F) Distribution of the growth cone size of NT-3- (E) and NGF-selected (F) DRG neurons incubated with CM from E13.5 VSC explants from different A-P levels. Incubation with the CM from cervical and lumbar VSC explants increases the number of growth cones with an area greater than 500 μm^2 in NT-3- selected cultures ($p < 0.001$), as indicated by #. Preincubation with recombinant sFRP1 blocks this effect. The effect of thoracic VSC explants was not significantly different from control. NGF-selected cultures were not affected. Values are mean ± SEM, n = 100–150.

they project to the motor nuclei in the VSC. Importantly, cervical and lumbar VSC explants exhibit a WNT-like axonal remodeling activity that can be blocked by the WNT antagonist, sFRP1. Thus, motoneuronal WNT-3 may act as a selective stop and branching signal for muscle afferents in the developing VSC.

WNT-3 Inhibits Axonal Extension while Increasing Growth Cone Size and Axonal Branching

WNT-3 decreases axonal length while increasing growth cone area and axonal branching. These results suggest that WNT-3 is bifunctional within the same axon: while inhibiting growth in length of the primary axon, WNT-3 promotes the growth of branches along the axon shaft. How can these two activities be elicited by a uniform concentration of WNT-3? Local differences in the response to WNT-3 may reflect intrinsic differences in cytoskeleton dynamics in different regions of the neurite. Alternatively, WNT-3 could signal through different receptors localized at the growth cone and the axon shaft. We believe that the latter is unlikely, as treatment with lithium, a direct inhibitor of GSK-3β (Klein and Melton, 1996), the kinase that acts downstream of the WNT receptor complex, mimics the effect of WNT-3 at both the growth cone and axon shaft. Lithium, like WNT-3, induces growth cone enlargement and axon branching and inhibits axon length. The implications of these findings are three-fold. First, axonal remodeling induced by WNT-3 is likely to be mediated through GSK-3β. Second, the ability of lithium in contrast to WNT-3 to induce

axonal remodeling in both NT-3- and NGF-responsive sensory neurons suggests that NGF-dependent sensory neurons do not express a functional receptor for WNT-3. Third, the differences in axon growth at the growth cone and axon shaft lie downstream of GSK-3β. Differential distribution of GSK-3β targets such as MAPs or other cytoskeleton components along the axon may contribute to the different response to WNT-3. Thus, local changes in composition of the cytoskeleton may explain the different behaviors induced by WNT-3 signaling.

Axon guidance molecules also induce distinct changes in the behavior of the axon shaft and growth cone. Slit proteins increase axonal extension and branching of sensory neurons (Wang et al., 1999). Eph molecules and Semaphorin 3A inhibit axonal extension via growth cone collapse, but promote collateral branching of retinal ganglion cells (Davenport et al., 1999). This contrasts with WNTs and FGF-2 that inhibit axonal extension, yet increase the size of growth cones (Szebenyi et al., 2001; Hall et al., 2000; Lucas and Salinas, 1997). Several mechanisms may contribute to the diversity of responses. One possibility is that signaling molecules affect the actin cytoskeleton and the microtubule organization to different degrees, leading to cessation of axon growth and growth cone collapse in some instances, but inhibiting axonal extension and increasing growth cone size in others. Detailed analyses of the cytoskeleton reorganization and the localization and activation of signaling components in response to different guidance molecules may provide an understanding of the diversity of behavior in developing axons.

WNT-3 and Sensory-Motoneuron Connectivity

Sensory neurons from DRG represent a heterogeneous population of neurons that innervate different targets and respond differentially to signaling molecules (Snider and Wright, 1996). Thermoreceptive and nociceptive sensory neurons project to and synapse with neurons of the dorsal horn and do not extend into the VSC (Mu et al., 1993; Ruit et al., 1992) due to the presence of repulsive signals such as semaphorin III (Messersmith et al., 1995). In contrast, muscle afferents are not repelled by semaphorin III and form extensive branches as they navigate to the motor nuclei (Ozaki and Snider, 1997). Recent studies demonstrate that F11/F3/contactin, a member of the immunoglobulin superfamily of cell adhesion molecules, is required for the proper navigation of proprioceptive sensory axons to their targets (Perrin et al., 2001). Upon encounter with the MNs, muscle afferents cease extending and initiate formation of synapses.

Transplant experiments have suggested that short-range signals from the VSC induce the terminal arborizations of muscle afferents (Sharma and Frank, 1998). We show that E13.5 VSC explants contain a WNT-like axonal remodeling activity for a subset of muscle afferents, those that primarily innervate limb MNs. This activity is restricted to cervical and lumbar levels coinciding with the location of limb MNs in the LMC. In contrast, thoracic VSC explants do not exhibit detectable axonal remodeling activity for NT-3-responsive neurons. Spinal cord from thoracic level contains MNs from the MMC that synapse with axial muscles, but not from the LMC. The expression of *Wnt-3* specifically in the LMC and its effect only on NT-3-responsive neurons suggest that WNT-3 contributes to the axonal remodeling activity of the VSC. Although the contribution of factors other that WNT-3 cannot be ruled out, our data strongly suggest that WNT-3 is mediating the activity of VSC. First, the expression of *Wnt-3* along the A-P axis of the spinal cord correlates with the presence of the endogenous axonal remodeling activity of the VSC. Second, both WNT-3 and VSC factors affect NT-3- but not NGF-responsive sensory neurons. Third, both activities are blocked by sFRP1, a secreted WNT antagonist. Thus, our studies support the notion that WNT-3 from MNs can act as a retrograde signal that regulates the terminal arborization of the muscle afferents that innervate the LMC.

The peak of expression of *Wnt-3* coincides with the arrival of NT-3-responsive axons to the VSC, raising the possibility that sensory input may regulate *Wnt-3* expression in MNs. Therefore, we used NT-3 null mice which exhibit a severe loss of NT-3-responsive sensory neurons (Ernfors et al., 1994; Fariñas et al., 1994). The apparent normal expression pattern of *Islet-1* in the VSC supports previous findings that lack of NT-3 does not significantly affect MN survival (Snider, 1994). The presence of *Wnt-3* mRNA in the LMC of the NT-3 mutant rules out the possibility that *Wnt-3* expression in MNs depends upon sensory innervation.

NT-3, which itself is expressed by MNs, was proposed to regulate terminal arborization of DRG neurons (Zhang et al., 1994; Lentz et al., 1999). However, NT-3 expressed in muscle is sufficient to rescue proprioceptive neurons in the NT-3 null mice, and these undergo extensive terminal arborization in the ventral horn (Wright et al., 1997). These findings argue against the role of motoneuronal NT-3 in the differentiation of Ia afferents and suggest that another signal from MNs, unaffected by the lack of NT-3, regulates sensory arborization. Therefore, the presence of *Wnt-3* in the NT-3 mutant further supports the role of WNT-3 in the formation of sensory-MN connections.

The question remains, however, what is the function of the restricted expression of *Wnt-3*? The expression of *Wnt-3* in the LMC suggests that WNT-3 regulates sensory neurons that synapse with limb MNs but not those that synapse with axial MNs. A unified model for neuronal matching is now emerging (Jessell, 2000) in which cadherins may facilitate the formation of selective synapses (Yagi and Takeichi, 2000; Price et al., 2002). Interestingly, cadherin expression is regulated by ETS, a family of transcription factors that are implicated in moto-sensory neuron connectivity (Price et al., 2002). As WNT factors regulate cadherin expression (Bradley et al., 1993), our results raise the possibility that WNT-3 is involved in synaptic matching between specific sensory neurons and their synaptic MN targets.

The inhibition of axonal extension and increased growth cone size induced by WNT-3 suggests a role for WNT-3 in presynaptic differentiation in sensory neurons. Consistent with this idea, we found that WNT-3 increases the clustering of synapsin I, a presynaptic protein involved in synapse formation and function, in sensory neurons. We have previously shown that WNT-7A regulates presynaptic differentiation of pontine mossy fibers (Hall et al., 2000). Thus, the behavior induced by

WNT-3 signaling is consistent with the role of WNT-3 in the formation of specific sensory-MN synapses. As *Wnt-3* null mice are embryonic lethal (Liu et al., 1999), establishing the in vivo function of WNT-3 in neuronal connectivity will require the generation of conditional mutants. Our studies on WNT-3 and WNT-7A suggest that WNTs play a general role in the formation of neuronal connections in the vertebrate CNS. The well-characterized WNT signaling pathway provides a unique opportunity to elucidate the mechanisms that control the terminal differentiation of an actively growing growth cone into a functional presynaptic terminal.

Experimental Procedures

RNA In Situ Hybridization

Frozen or wax-embedded embryos fixed in 4% paraformaldehyde were cut in 10–12 μm sections. Specimens were postfixed, treated with 20 μg/ml proteinase K, washed, refixed with 4% paraformaldehyde, acetylated, and dehydrated (Lucas and Salinas, 1997). [^{35}S]-UTP riboprobes were generated from noncoding sequences of *Wnt-3* (Roelink and Nusse, 1991). Sense probes were used in all experiments and showed no signal. The genotype of NT-3 null embryos was determined by DNA blot analysis (Fariñas et al., 1994).

Production of Soluble WNTs and sFRP1

Soluble WNT-3 and WNT-5A were obtained using QT6 cells transiently transfected with a pCS2+ expression vector containing hemagglutinin (HA)-tagged *Wnt-3*, *Wnt-5A*, or green fluorescent protein (control) cDNAs using the Ca^{2+} phosphate method or from stably transfected Rat1B cells. Transfected cells were cultured 16 hr in the presence of serum-free media. The CM was immediately added to sensory neurons, and these were cultured for a further 16 hr. The levels of WNT protein in the CM were determined by Western blotting using an anti-HA antibody (Roche Diagnostics). The higher levels of WNT-3 in the CM correlated with higher axonal remodeling activity.

QT6 cells were transfected with myc-tagged sFRP1 cDNA expressed in pRK5. WNT-3 CM was incubated for 30 min at room temperature with an equal volume of medium conditioned with sFRP1-expressing cells or mock-transfected cells before addition to neuronal cultures. The presence of sFRP1 in the CM was confirmed by Western blotting using the c-Myc antibody 9E10 (Sigma). The dilution of WNT-3 CM with control medium did not alter axon remodeling and branching activities of WNT-3.

Neuronal Cultures

DRGs were isolated from E13.5 mice according to Kleitman et al. (1991). Neurons were plated at 180 cells/mm^2 (10,000 cells/well) in Lab-Tek chamber slides (Nunc) precoated with poly-D-lysine (100 μg/ml) and laminin (50 μg/ml) and cultured in the presence of both NGF and NT3 (50 ng/ml). For neurotrophin selection we used two different methods: embryonic DRG neurons were cultured either in the presence of 25ng/ml NGF or NT-3 (Promega) for 48 hr or, alternatively, in the presence of 50 ng/ml NGF or NT-3 for 72 hr. Neurons were then exposed to CM from control or WNT-expressing cells for the last 16 hr of the culture period. Similar results were obtained using these two selection methods based on RT97 staining. For neurotrophin selection experiments chamber slides were coated with poly-D-lysine only and cells plated at 10,000 or 30,000 cells/well for NGF and NT-3 treatments, respectively. In lithium experiments neurotrophin selection for 48 hr was used. The culture medium was then supplemented with 10 mM LiCl or 10 mM NaCl for the last 16 hr. Treatment with 10 mM NaCl increases slightly the size of growth cones compared to normal media in NGF-selected cultures. For analyses of synapsin I clustering, DRGs, plated at 13,000 cells/well and cultured for 6 days, were treated with CM from control or WNT-3 expressing cells for the last 16 hr in culture.

Ventral Spinal Cord Explants

Spinal cords from E13.5 mice were dissected according to Figure 11A. DRGs, meninges, and dorsal spinal cord were removed before ventral portions of the spinal cord were divided into three segments corresponding to cervical, thoracic, and lumbar regions (Kandel et al., 1991). The cervical segment corresponded to the same A-P position as the 12 most rostral DRGs, thoracic level to the following 7 DRGs, and lumbar corresponding to the 11 most caudal DRGs. Each segment was then cut into pieces ~0.5 mm in length (explants). Five to seven explants from a single region were cultured for 24 hr in 150 μl of serum-free medium. Ventral spinal cord (VSC) CM was added to neurotrophin-selected (25 ng/ml NT-3 or NGF) embryonic DRG cultures between 24 and 44 hr in vitro prior to fixation. Human recombinant sFRP1 was used at 2.5 μg/ml and preincubated with the corresponding CM (30 min at room temperature) before addition to the cultures. Measurements of the growth cone area were performed blind.

Immunocytochemistry

Sensory neuron cultures were fixed in 4% paraformaldehyde and stored in PBS at 4°C. Cells were permeabilized prior to immunostaining with increasing ethanol concentrations and blocked in PBS containing 5% goat serum, 5% horse serum for 1 hr. Cultures were immunostained overnight using polyclonal anti-GAP-43 and monoclonal anti-neurofilament RT97 antibodies or synapsin I monoclonal antibodies (Serotec) followed by a 1 hr incubation with fluorescent secondary antibodies (Vector Laboratories or Molecular Probes).

Image and Statistical Analysis

Sensory neuron cultures were photographed on 1600 ASA Kodak Ektachrome film, scanned into Adobe Photoshop 4.0, and analyzed using the public domain NIH 1.62 Image program (available at http://rsb.info.nih.gov/nih-image). For the quantification of axon length, an individual axon was measured from the axon hillock to the most distant growth cone along a given route. DRG neurons in control cultures were predominantly bipolar. WNTs did not affect the number of primary axons. The number of branches was quantified by defining the first point of divergence from the main axon as secondary branches. The route of the axon was then followed along one of the secondary branches to the next point of divergence and similarly the number of these tertiary branches was counted. Quaternary and higher order branches were also identified and counted along the entire length of the axon. Statistical analysis was performed on the raw measurements data using Mann-Whitney test. Data from at least three independent experiments were used for statistical analysis.

Acknowledgments

We thank Drs. J. Rubin for recombinant sFRP1, J. Nathans for sFRP1 cDNA, T. Jessell for Islet-1 DNA, A. Münsterberg and J. Kitajewski for Wnt-expressing Rat1B cells, G. Wilkin and B. Anderton for antibodies, and our colleagues in the lab for helpful discussion. We thank Dr. I. Fariñas and M. Cano for generously providing us with NT-3 null embryos. The Wellcome Trust (P.C.S), BBSRC (P.C.S), the MRC (S.M.H., J.H.), Muscular Dystrophy Campaign (S.M.H), and an Erwin Schrödinger Fellowship from the Austrian Research Council (E.E.) supported this work. P.C.S. and S.M.H. are members of MRC Centre of Developmental Neurobiology, KCL.

Received: July 13, 2001
Revised: July 18, 2002

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