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WNT-7a Induces Axonal Remodeling and Increases

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WNT factors play a key role in early patterning of the embryo. However, expression of *Wnt* genes after cell commitment suggests additional roles in later developmental processes. We report here that *Wnt-7a* is expressed in cerebellar granule cell neurons as they begin to extend processes and form synapses. WNT-7a increases axonal spreading and branching in cultured granule cells. Moreover, WNT-7a increases the levels of synapsin I, a presynaptic protein involved in synapse formation and function. Lithium mimics WNT-7a in granule cells by inhibiting GSK-3β, a component of the WNT signaling pathway. These results suggest a direct effect of WNT-7a in the regulation of neuronal cytoskeleton and synapsin I in granule cell neurons. We propose that WNT proteins have a novel function in the formation of neuronal connections.

INTRODUCTION

Neuronal connections are formed by a gradual process that requires proper axonal extension, target recognition, and the formation of synapses. In the past few years, short- and long-range signaling molecules with attractive and repulsive properties have been identified and shown to regulate these developmental processes (Tessier Lavigne and Goodman, 1996). These molecules include members of the immunoglobulin superfamily and the laminin, semaphorin, netrin, and Eph families (Goodman, 1996; Tessier Lavigne and Goodman, 1996; Keynes and Cook, 1995). In addition, growth factors and neurotrophins have been recognized to play an important role in axon guidance, target selection, and synaptogenesis (Kennedy and Tessier Lavigne, 1995). However, little is known on how these signaling molecules regulate the cytoskeleton to generate precise and complex neuronal networks.

The cerebellum has a well-characterized pattern of neurogenesis, cell migration, and formation of synapses and therefore offers a good model system to identify and examine the role of signaling molecules in the formation of neuronal connections (Hatten and Heintz, 1995; Baptista *et al.*, 1994). In particular, the formation of synapses between granule cells and their synaptic partners has been examined *in vivo* and *in vitro* (Baptista *et al.*, 1994; Baird *et al.*, 1992; Sotelo, 1990). This process occurs after cerebellar granule cells (GCs) have migrated from the proliferative outer layer of the postnatal cerebellum, the external granular layer (EGL). GCs migrate downward while their axons are left behind in the forming molecular layer. Upon arrival in the internal granular layer (IGL), GCs begin to make contacts with their synaptic partners, the Purkinje cells (PCs) and mossy fiber (MF) axons (Altman, 1972a,b). After contact, GCs induce cytoskeletal changes in both PCs and MFs (Hamori and Somogyi, 1983). The formation of the PC dendritic tree depends on GCs (Altman, 1972b) and the relatively smooth surface of MF growth cones becomes multilobulated on contact with GCs (Hamori and Somogyi, 1983). This structure, called a glomerular rosette, represents a multisynaptic region (Hamori and Somogyi, 1983). Although the interactions between GCs and PCs or MFs have been characterized extensively at the cellular level, the molecules involved in this process remain unknown (Baptista *et al.*, 1994; Baird *et al.*, 1992).

Recently, we found that *Wnt* genes, encoding signaling proteins, are expressed in defined neuronal populations in the cerebellum during neurite extension and synaptogenesis (Salinas *et al.*, 1994). WNT growth factors have been extensively studied in different developmental systems for their role in early patterning (Moon *et al.*, 1997; Roelink, 1996; Millen *et al.*, 1995; Parr and McMahon, 1994; Nusse and Varmus, 1992). Analyses of mutations in the *Drosophila Wnt* gene, *wingless (wg)*, mouse *Wnt* genes, and in *C. eleg-*

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ans have demonstrated their role in cell fate decisions (Joyner, 1996; Parr and McMahon, 1995; Herman *et al.*, 1995; Ingham and Martinez, 1992). Therefore, expression of *Wnt* genes after cells are committed to a particular fate poses the question: what is the role of WNTs at later stages of CNS development? As the WNT signaling pathway has been well characterized (Nusse, 1997), study of the function of WNTs during neuronal maturation offers the possibility of unraveling important aspects in axonogenesis, synaptogenesis, and synaptic function. In this paper, we show that *Wnt-7a* is expressed in mouse cerebellar GCs during axonal extension and the formation of synaptic connections between GCs and their synaptic partners. Moreover, we found that in cultured GCs WNT-7a induces axonal remodeling and increases the levels of synapsin I, a protein involved in synapse formation and synaptic transmission. These results suggest a novel role for WNTs in the formation of neuronal connections.

MATERIALS AND METHODS

Neuronal Cultures

GCs were isolated from P1–P5 cerebellum according to Hatten (1985). Cultures contain a 97–99% GC population, based on staining for Pax-6 and TAG-1, GC markers (Stoykova and Gruss, 1994; Gao and Hatten, 1993). DRG neurons were isolated from mouse embryos (E15) according to Kleitman *et al.* (1991) and cultured in serum-free medium. For coculture experiments, C17.2 cells (Snyder *et al.*, 1992) were stably or transiently transfected with pJ3Ω vector carrying the *Wnt-7a* cDNA or *wg* cDNA under the SV40 promoter and pCEP4 vector carrying the hygromycin resistance gene for selection. Mock-transfected clones were generated by transfection with pJ3Ω and pCEP4. The Ca^{2+} phosphate method was used for stable transfections, while lipofection (lipofectamine) was used for transient transfections. Expressions of *Wnt-7a* and *Wnt-1* were assessed by Northern blots, while *wg* expression was determined using specific Wg antibodies (Roel Nusse). GCs were plated at low density (3×10^4 cells/cm^2) onto a monolayer of *Wnt-7a*-expressing or mock-transfected C17.2 cells and grown in serum-free medium (Hatten, 1985) for 1–4 days. DRG neurons were cultured for 2–4 days. For lithium experiments, GCs were plated on laminin-coated 16 well-Labteks at 1×10^4 cells/well. Lithium was added immediately at a 5, 10, or 20 mM final concentration. *Myo*-inositol (Sigma) was added at 20 mM concentration. Control cultures were grown in the presence of 20 mM NaCl. Coculture experiments show that WNT-7a induces a small increase in GC survival (15%) after 3 days of culture. Therefore, for analysis of synapsin I, GCs were plated at a lower density in cultures exposed to WNT-7a than control to obtain an equal number of cells under different conditions.

Antibody Staining and Image Analysis

Axonal spreading was examined by staining GCs with a rabbit anti-GAP-43 antibody followed by anti-rabbit IgG–peroxidase or with anti-rabbit IgG–FITC or Texas Red from Vector. Cell spreading was determined by scanning photographic slides into Photoshop and using NIH Image software to measure areas of individual cells. Granule cells cultured on stably transfected cells and transiently transfected cells were used. Similar results were obtained with different stable *Wnt-7a*-expressing clones and with transient transfected clones.

Expression and distribution of synapsin I were determined by immunofluorescence. Cocultures were stained with the mouse monoclonal anti-synapsin I tail region at 1:100. The number of synapsin I clusters per GC was counted using a fluorescence microscope under a 100× objective; only cells with processes were counted.

Western Blot Analysis

Analyses of β-catenin and GSK-3β levels were done from GCs grown for 2 days on laminin-coated dishes. Medium containing NaCl or LiCl at a concentration of 20 mM was added for 4 h at the end of the culture period. Cells were lysed in sample buffer and run on a 4–15% gradient SDS–PAGE. For analysis of synapsin I levels, GCs cocultured on monolayer cells were lysed and run on a 12% SDS–PAGE. Parallel cultures were stained for GAP-43 and synapsin I to make sure that equal numbers of GCs were present under different conditions. Proteins were transferred to nitrocellulose membranes. Primary antibodies were diluted in block solution containing 0.1% Tween 20 in Tris–Cl-buffered saline and incubated for 1 h. Secondary antibodies from Amersham were used. Proteins were visualized using the ECL system (Amersham). For coculture experiments, the level of proteins from neurons was normalized to acetylated tubulin. For lithium experiments, samples were normalized to total protein levels. β-Catenin-labeled membranes were stripped and probed for GSK-3β. Synapsin I-labeled membranes were stripped and probed for acetylated tubulin. The levels of synapsin I were determined by scanning radiographs on a Molecular Dynamics densitometer and normalized to acetylated tubulin levels. Antibodies were obtained from Jackie Papkoff, SUGEN (β-catenin), Scott Nicol (synapsin I, tail region), Graham Wilkin (GAP-43), Transduction Laboratories (GSK-3β), and Sigma (acetylated tubulin).

Analysis of Wnt-7a Expression by Northern Blot and in Situ Hybridization

For Northern blot analysis, RNAs from cerebella dissected at different postnatal stages were isolated by the acid–guanidinium–phenol–chloroform method (RNAzol) (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA was electrophoresed through a formaldehyde–agarose gel and blotted to nitrocellulose membranes. Membrane filters were hybridized with random primer-labeled *Wnt-7a* cDNA probes overnight and subsequently washed in 0.1× SSC, 0.1 SDS at 65°C. Filters were exposed for 4 days. For *in situ* hybridization, animals were perfused with 4% paraformaldehyde and brains were dissected and processed for wax embedding according to Salinas *et al.* (1994). In summary, brains were washed in PBS, dehydrated through a series of ethanols, treated with toluene and xylene, and then embedded in wax. Specimens were cut in 10-μm sections, xylene-treated, and rehydrated. Specimens were then treated with 20 μg/ml proteinase K, washed, fixed with 4% paraformaldehyde, acetylated, and dehydrated. [^{35}S]UTP riboprobes were generated from noncoding sequences of *Wnt-7a* (Parr and McMahon, 1995). Sense probes were used in all experiments and showed no signal. For non-radioactive *in situ* hybridization, granule cells cultured *in vitro* for 2–7 days were fixed with 4% paraformaldehyde and stored in 70% ethanol at −20°C. Specimens were pro-

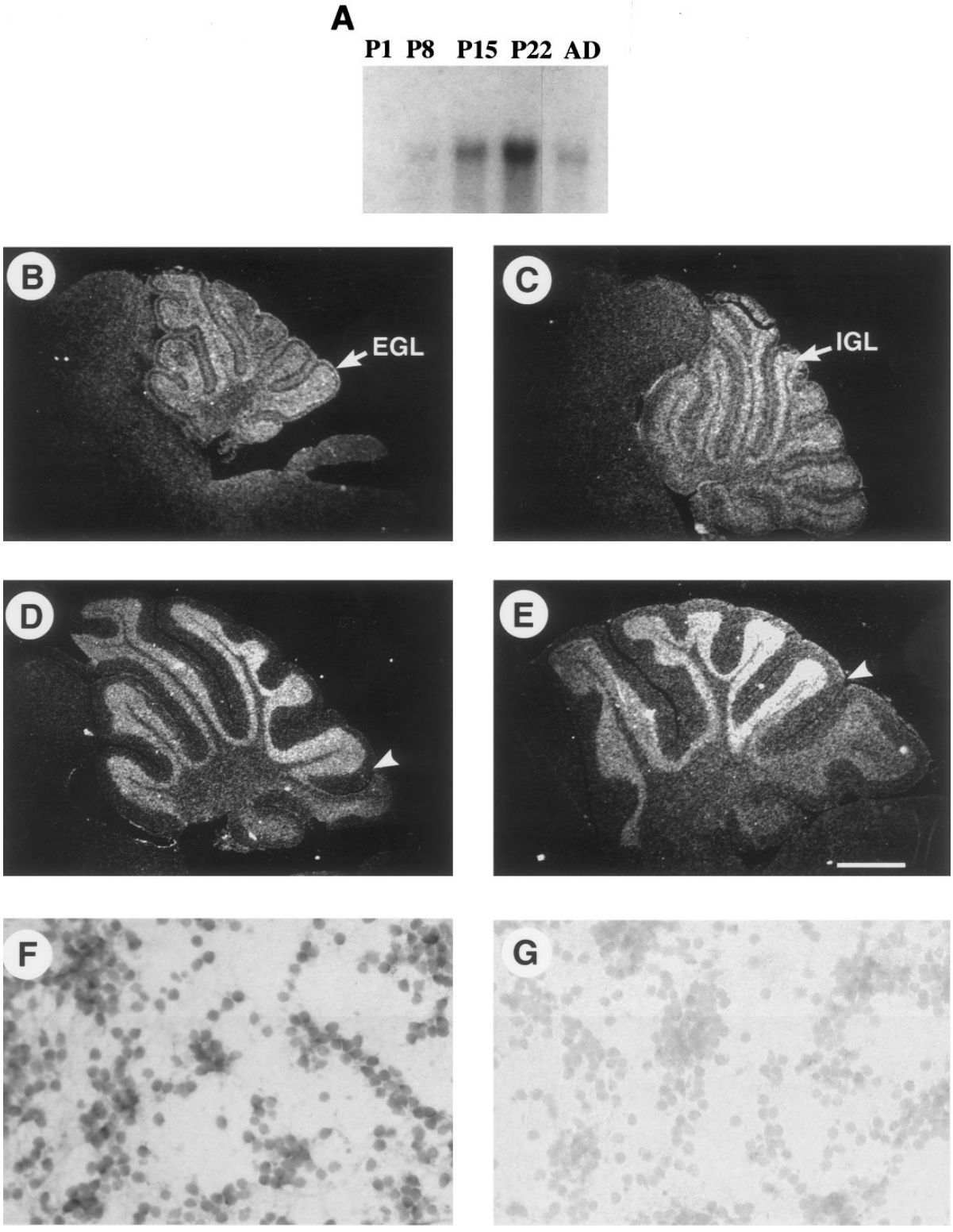


FIG. 1. Expression of *Wnt-7a* during postnatal cerebellar development. (A) Northern blot analysis of mouse cerebellar RNA isolated from postnatal day 1-, 8-, 15-, 22-, and 2-month-old mice (AD). *Wnt-7a* expression increases from P8 to a maximum at P22 and then declines to low levels in the adult (AD). (B–E) *In situ* hybridization shows that *Wnt-7a* is expressed in cerebellar GCs. (B) At P6 low levels of expression are detected in the IGL and EGL. (C) At P15 *Wnt-7a* expression increases in the IGL. (D) At P22 high levels of *Wnt-7a* expression are detected in the most mature GCs. However, there is a downregulation of *Wnt-7a* expression in the vestibulocerebellum. (E) At P28 *Wnt-7a* is highly expressed in most of the IGL but the regional differences are more marked. (F) Non-radioactive *in situ* hybridization shows that GCs grown for 3 days *in vitro* express *Wnt-7a.* (G) Negative control for non-radioactive *in situ* hybridization using sense probe. EGL, external granular layer. IGL, internal granular layer. Arrowheads, the regional boundary of *Wnt-7a* expression in the IGL. Scale bar, 650 μm.

cessed according to Baird *et al.* (1994). Digoxygenin-labeled probes were generated from noncoding sequences of *Wnt-7a.*

RESULTS

Wnt-7a is Expressed in Differentiating Cerebellar Granule Cells

During screening for *Wnt* genes expressed in the mouse cerebellum, we found that *Wnt-7a* is expressed during postnatal development. Northern blot analysis shows that *Wnt-7a* is expressed in postnatal day 8 (P8) mouse cerebellum, increases to a maximum level around P22, and then declines in the adult (Fig. 1A). *In situ* hybridization reveals that before P6, *Wnt-7a* is not expressed in the cerebellum (data not shown). At P6, *Wnt-7a* is expressed in the IGL and at low levels in the EGL (Fig. 1B). Therefore, *Wnt-7a* expression appears when GCs become bipolar, start to send processes, and migrate to their final position in the IGL (Altman, 1972a). A further increase in *Wnt-7a* expression was ob-

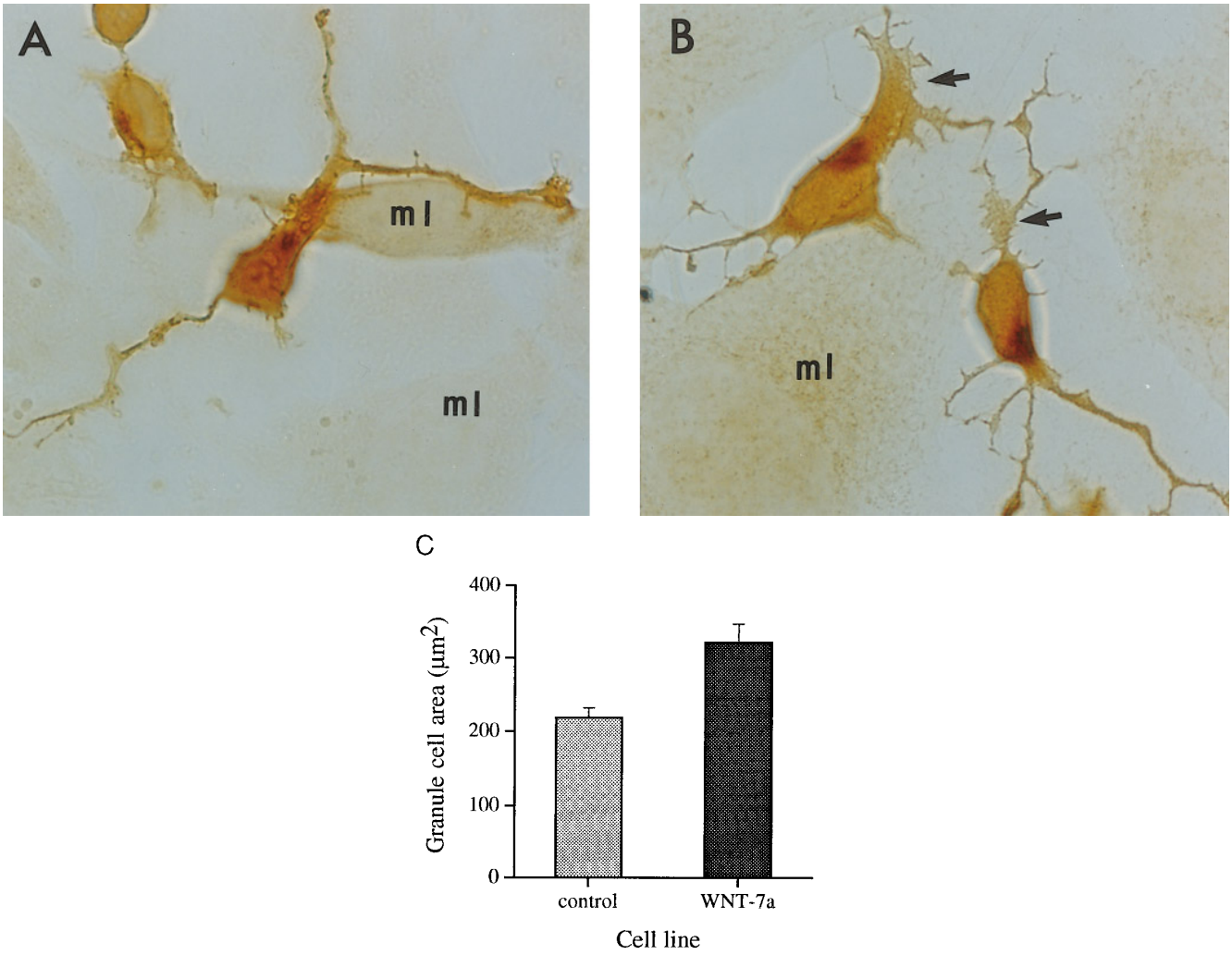
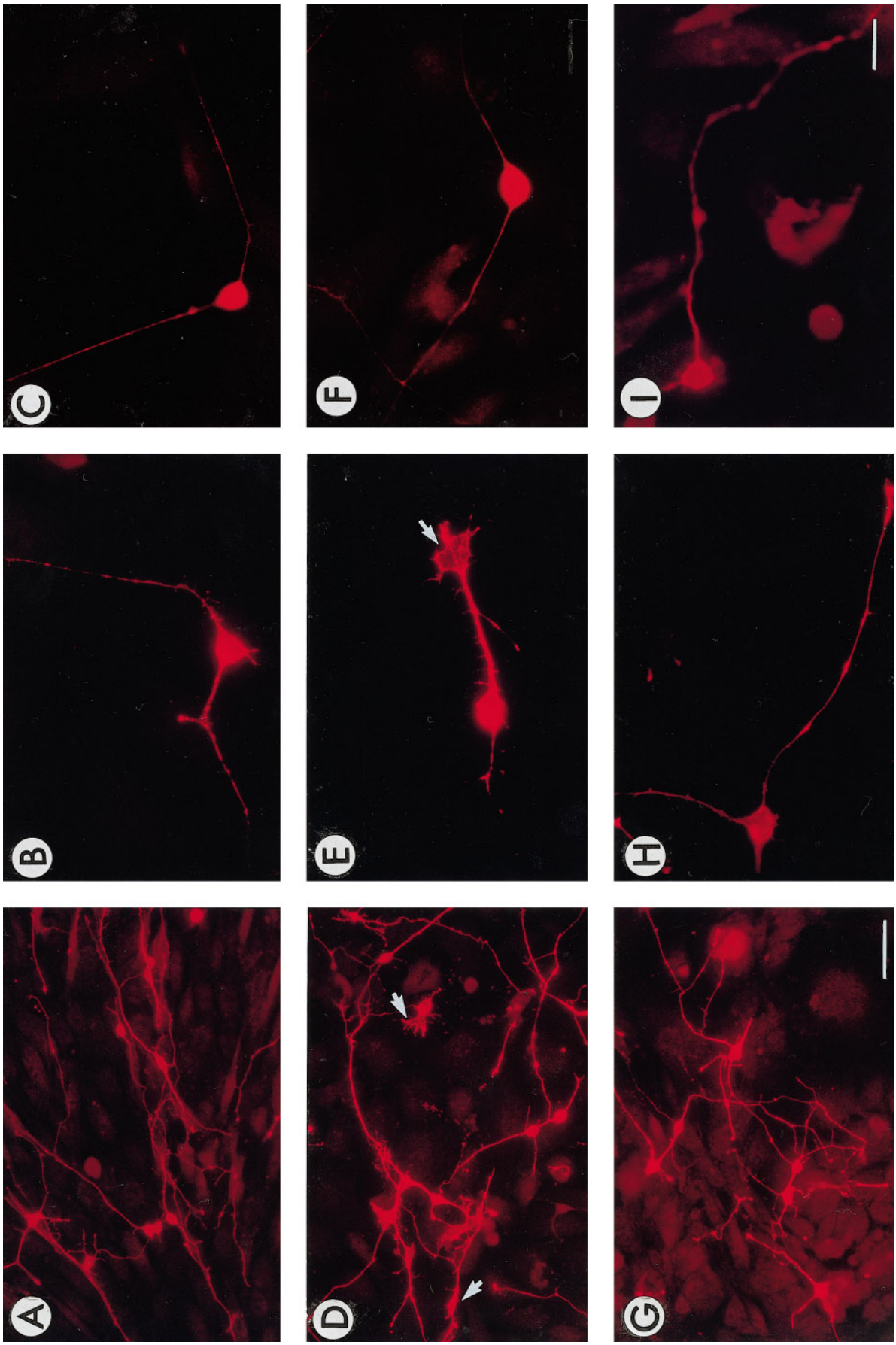


FIG. 2. WNT-7a increases neurite spreading and branching in GC neurons. GCs were grown for 20 h on a monolayer of cells and stained for GAP-43. (A) GCs on a monolayer of mock-transfected cells. (B) GCs grown on *Wnt-7a*-expressing cells exhibit increased neurite spreading and branching and increased number of filopodia. Arrows, spread areas. ml, monolayer cell. (C) GCs exposed to WNT-7a show a 47% increase in surface area ($P = 0.0022$, Student's t test).

FIG. 3. WNT-7a induces specific responses in cerebellar GCs. GCs and DRG neurons were grown on monolayers of transiently transfected cells and stained for GAP-43. (A and B) GCs grown on mock-transfected cells. (C) DRG neuron grown on mock-transfected cells. (D and E) GCs grown on *Wnt-7a*-expressing cells show increased spreading and increased number of filopodia (arrows). (F) DRG neurons grown on Wnt-7a-expressing cells show no changes in neurite spreading and branching. (G and H) GCs grown on *wg*-expressing cells have the same morphology as GCs cultured on a mock-transfected monolayer. (I) GC neuron grown on *Wnt-1*-expressing cells shows the same morphology as GCs grown on mock-transfected cells. Arrows, spread processes. Scale bars, 50 μm (A, D, G) and 20 μm (B, C, E, F, H, I).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served in the IGL from P15 onward (Figs. 1C–1E), coinciding with the period of synapse formation between GCs and MFs (Hamori and Somogyi, 1983; Altman, 1972a). From P22, *Wnt-7a* expression increases in the IGL with the exception of the most posterior areas of the cerebellum, called the vestibulocerebellum (Ito, 1984) (Figs. 1D and 1E). This region, which includes the flocculus, ventral paraflocculus, nodulus, and uvula, receives distinct innervation from primary vestibular nerve fibers (Ito, 1984). The anterior increase in *Wnt-7a* expression does not become evident before P22 (compare Figs. 1C and 1D), coinciding with the initial period of synaptic remodeling between GCs and MFs (Hamori and Somogyi, 1983). At later stages, *Wnt-7a* expression decreases to very low levels in the whole cerebellum. In summary, the timing of expression of *Wnt-7a* correlates with the period of neurite extension and/or the formation of synapses between GCs and their synaptic partners.

To confirm that *Wnt-7a* is expressed in cerebellar granule cells, we performed non-radioactive *in situ* hybridization on pure granule cells. Using antisense probes, we found that *Wnt-7a* is expressed in granule cells (Fig. 1F), while no signal was detected with sense probes (Fig. 1G). We have also examined the expression of *Wnt-7a* in the granule cell-deficient mice, *weaver* (Gao *et al.*, 1992). We found that *Wnt-7a* is not expressed in the deeper layer of the cerebellum, where normally granule cells migrate to (data not shown). These results show that expression of *Wnt-7a* in the cerebellar granule cell layer reflects expression in the granule cell population.

WNT-7a Induces Axonal Spreading and Branching in Cerebellar Granule Cells

Experiments on cultured GCs have demonstrated that their proliferation and neurite outgrowth are mediated by membrane-bound signals or factors with a short range of action produced by GCs themselves (Alder *et al.*, 1996; Gao *et al.*, 1992). As WNT factors have been shown to act in an autocrine fashion (Hooper, 1994) and *Wnt-7a* is expressed during axonal extension in GCs, we decided to test whether WNT-7a could affect this process. GCs were grown on monolayers of either *Wnt-7a*-expressing or mock-transfected cells. After a 20-h culture period, neuronal morphology was examined by staining for GAP-43, a protein present in actively growing processes (Goslin *et al.*, 1990). WNT-7a does not increase axon extension but instead increases axonal spreading and axonal branching (Figs. 2A and 2B). We quantified axonal spreading and found that WNT-7a induces a 47% increase in GC surface area (Fig. 2C). The neurons with this unusual morphology were clearly GCs because (a) these cells are labeled with antibodies to neuronal marker NF200 and the GC markers TAG-1 and Pax-6 (data not shown) and (b) the proportion of spread neurons is over 90% in this 95–97% pure GC population. The possibility that WNT-7a is toxic to GCs and prevents them from sending out processes was ruled out because the survival of these neurons was comparable to control cultures (data not shown). Moreover, the neurons develop complex processes after 3–4 days *in vitro* in the presence of WNT-7a (Fig. 5). In conclusion, we have shown that WNT-7a induces cytoskeletal changes in cerebellar GCs that lead to axonal spreading and branching. To determine whether other WNT proteins could induce neurite spreading, we tested Wg and WNT-1 on cerebellar GCs. Wg, a *Drosophila* WNT protein, has the ability to signal in mammalian cells (Cook *et al.*, 1996; Stambolic *et al.*, 1996; Ramakrishna and Brown, 1993). WNT-1 is the most studied member of the family (Joyner, 1996; Parr and McMahon, 1994; Nusse and Varmus, 1992). GCs were cultured on a monolayer of transiently transfected *Wnt-7a*-, *wg*-, and *Wnt-1*-expressing cells. Although WNT-7a induces axonal spreading and branching (Figs. 3A, 3B, 3D, and 3E), Wg and WNT-1 do not affect axonal morphology in GCs (Figs. 3G–3I). Thus, cerebellar GCs can respond to *Wnt-7a*-expressing but not *wg*- or *Wnt-1*-expressing cells, suggesting that GCs express receptors that recognize WNT-7a but not Wg or WNT-1.

The changes in morphology induced by the *Wnt-7a*-expressing monolayer could be due to the direct action of WNT-7a or to an indirect effect through changes in the adhesion properties of the *Wnt-7a*-expressing monolayers themselves. To begin to assess this issue, we examined whether WNT-7a could induce axonal spreading and branching in other neurons. We exposed cultured dorsal root ganglion neurons to WNT-7a and found no changes in their morphology (Figs. 3C and 3F). Thus, WNT-7a appears to induce cytoskeletal changes that lead to axonal spreading and branching specifically in cerebellar GCs.

Lithium Mimics the Effect of WNT-7a in Granule Cells

To determine whether WNT-7a has a direct effect on GCs, we examined the role of GSK-3β, a serine/threonine kinase and a component of the WNT signaling pathway (He *et al.*, 1995), in axonal morphology. In several developmental systems, WNT signaling results in the inhibition of GSK-3β that is manifested by the increased stability of β-catenin, a possible substrate for GSK-3β and a component of the WNT pathway (Nusse, 1997; Papkoff, 1997; Peifer *et al.*, 1994). Lithium has recently been shown to inhibit GSK-3β directly (Klein and Melton, 1996) and to mimic WNT signaling (Stambolic *et al.*, 1996). Therefore, we tested whether lithium could mimic WNT signaling by inducing cytoskeletal changes in cerebellar GCs. Lithium treatment results in an increased axonal spreading and branching similar to that observed with WNT-7a (Figs. 4A–4D). Thus, lithium can mimic WNT-7a action on cerebellar GCs.

The action of lithium on GCs is likely to be through inhibition of GSK-3β as this is a component of the WNT signaling pathway. However, lithium could act by an alternative pathway. Lithium inhibits inositol monophosphatase and inositol polyphosphate phosphatase, resulting in depletion of the inositol pool (Berridge *et al.*, 1989). Yet, inositol depletion does not mimic the effect of GSK-3β inhi-

bition by lithium in *Xenopus* (Klein and Melton, 1996). However, the role of inositol in the mechanism of action of lithium in *Xenopus* remains unclear (Hedgepeth *et al.*, 1997). Exogenous *myo*-inositol has been shown to rescue the effect of lithium on the inositol pathway by restoring the inositol pool (Atack *et al.*, 1995). To determine whether the increased axonal spreading and branching observed in lithium-treated GCs was due to the effect of lithium on the inositol pathway, *myo*-inositol was added during lithium treatment. GCs were exposed to 20 mM *myo*-inositol alone or in combination with lithium. Control *myo*-inositol alone does not affect GC morphology (Fig. 4E) and, in addition, *myo*-inositol does not prevent the effect of lithium (Fig. 4F). Therefore, lithium action on cerebellar GCs is not due to depletion of the inositol pool.

We confirmed that lithium affects GSK-3β activity in GCs by examining the levels of β-catenin protein, a known target of GSK-3β in the WNT pathway (Papkoff, 1997; Miller and Moon, 1996; Peifer *et al.*, 1994). WNT signaling results in the inhibition of GSK-3β which in turns leads to increased stability of β-catenin (Papkoff, 1997; Peifer *et al.*, 1994). We found that lithium increases the levels of β-catenin in cerebellar GCs (Fig. 4G), supporting the view that lithium inhibits GSK-3β in GCs. Taken together, our results strongly suggest the direct action of WNT-7a in axonal remodeling and implicate GSK-3β in the regulation of the axonal cytoskeleton in developing granule cells.

WNT-7a Increases the Number of Clusters and the Levels of Synapsin I

The peak of *Wnt-7a* expression coincides with both changes in axonal morphology and the formation of synapses between GCs and their synaptic partners (Hamori and Somogyi, 1983; Altman, 1972a,b). To test a possible function of WNT-7a in synaptogenesis, we examined the effect of WNT-7a on the expression of synapsin I, a presynaptic protein involved in synapse formation and synaptic transmission (Chin *et al.*, 1995; Rosahl *et al.*, 1995). Synapsin I is first detected in GCs at low levels after 2 days *in vitro*. After 3 days, GCs exposed to WNT-7a have more synapsin I clusters than control cells (Figs. 5A–5D). Often, synapsin I clusters were concentrated at spread areas and growth cones (Figs. 5E–5H). To quantify the effect of WNT-7a in the whole neuronal population, we counted the number of synapsin I clusters per neuron. Analysis of the cluster distribution from three different experiments shows that WNT-7a increases the frequency of GCs with numerous synapsin I clusters (Fig. 6A). In the presence of WNT-7a, GCs frequently have larger synapsin I clusters than control cells. The increased number of clusters could be due to a redistribution, or an increased level, of synapsin I. To distinguish between these two possibilities, Western blot analyses were performed. WNT-7a increases the levels of synapsin I fourfold in cells cultured for 3 days and sixfold after 4 days in culture (Fig. 6B). Neither GCs grown on *wg*-expressing or *Wnt-1*-expressing cells nor mouse dorsal root ganglia (DRG) neurons exposed to WNT-7a showed changes in the level of synapsin I (data not shown). Thus, WNT-7a increases the number of clusters and the levels of synapsin I in GCs during their period of synaptogenesis in culture.

To determine whether lithium mimics the WNT-7a effect on the expression of synapsin I, cerebellar GCs were cultured in the presence of 5 mM lithium for 3 to 4 days. At this concentration, lithium has a weak but noticeable effect on axonal spreading and branching after 20 h (data not shown), but is more apparent in 3- and 4-day cultures (Figs. 7B and 7D). We found that lithium increases the number of synapsin I clusters in GCs (Figs. 7A–7D). As we found in GCs exposed to WNT-7a, synapsin I clusters were frequently found in the spread areas of lithium-treated cells (Fig. 7D). These results suggest a novel function of GSK-3β in modulating synapsin I levels in maturing GC neurons. Moreover, these findings strongly suggest the direct action of WNT-7a in regulating synapsin I during the formation of synapses.

DISCUSSION

In this paper we demonstrate a novel function for WNT-7a in the regulation of neuronal morphology and the levels of synapsin I. WNT-7a has two effects that suggest its role in the formation of synapses in the postnatal cerebellum. First, WNT-7a induces axonal spreading and branching. Second, it increases the levels of synapsin I, a presynaptic protein involved in synaptic function and synapse formation.

WNT-7a Regulates Axonal Remodeling

WNT-7a increases axonal spreading and branching in cultured cerebellar GCs using a monolayer of *Wnt-7a*-expressing cells. These effects can be detected as early as 20 h after exposure to WNT-7a. However, the *Wnt-7a*-expressing monolayer does not induce axonal spreading and branching in dorsal root ganglion neurons. Moreover, Wg and WNT-1 do not affect axonal morphology in cerebellar GCs. These findings suggest that the increased axonal spreading and branching induced by *Wnt-7a*-expressing cells in cerebellar GCs is unlikely to be due to changes in the adhesion properties of the monolayer. To determine whether this effect was due to the direct action of WNT-7a on cerebellar GCs, we examined downstream components of the WNT signaling pathway. We reason that if we were able to mimic WNT-7a by modulating a component of the WNT pathway, it would provide evidence for a direct effect of WNT-7a. Unfortunately, we were unable to detect the effect of *Wnt-7a* on β-catenin levels in granule cells due to the high levels of β-catenin in the underlying C17.2 monolayer. We decided to concentrate our analysis on GSK-3β, a serine/threonine kinase required for WNT function. In the presence of WNT signaling, GSK-3β is inhibited resulting in the accumulation of β-catenin, also a component in the pathway. In the absence of WNT signal, active GSK-3β leads to the phos-

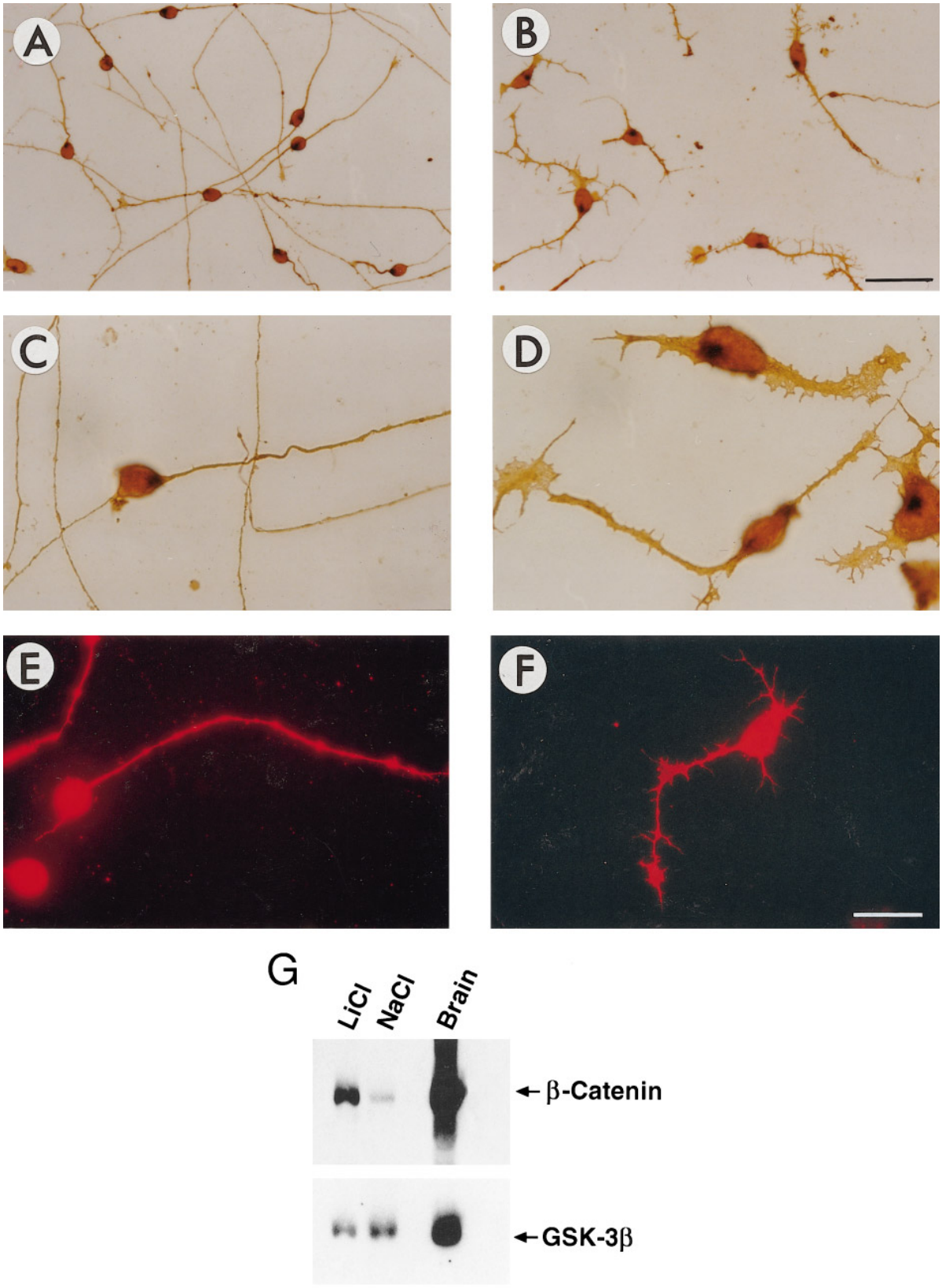
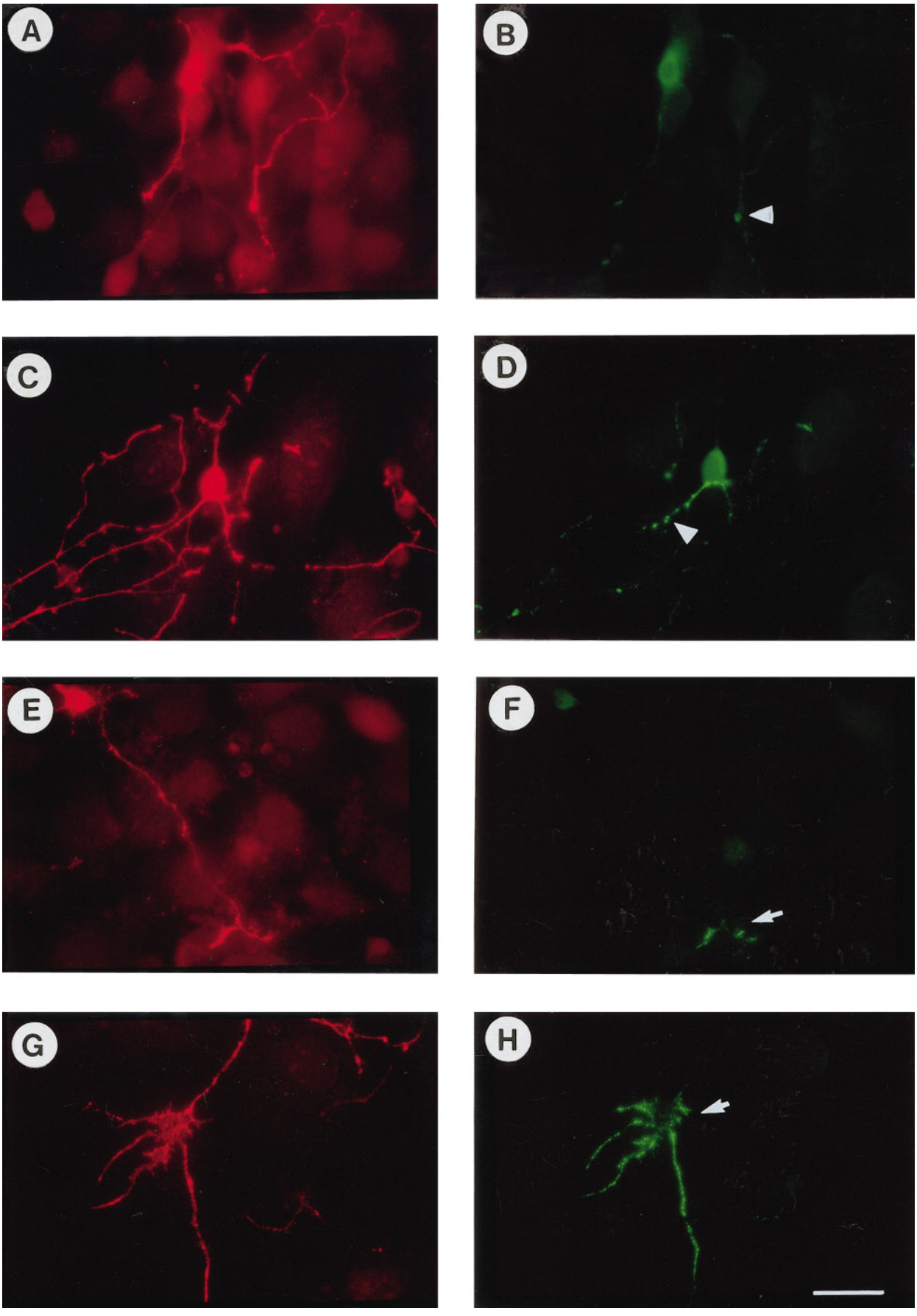


FIG. 4. Lithium mimics the effect of WNT-7a in cultured granule cells by inhibiting GSK-3β. GCs cultured for 2 days and stained for GAP-43. (A and C) GCs grown in 20 mM NaCl show a typical morphology with long axonal processes. (B and D) The presence of 20 mM lithium induces neurite spreading and branching, as observed in the presence of WNT-7a. (E) *myo*-inositol does not affect GC morphology. (F) Addition of *myo*-inositol with lithium does not rescue the effect of lithium on neurite spreading and branching. (G) Western blots show that lithium increases the levels of β-catenin in GCs. Lane LiCl, protein extracts from GCs grown in 20 mM LiCl show high levels of β-catenin. Lane NaCl, protein extracts from GCs treated with 20 mM NaCl show low levels of β-catenin. Lane brain, protein extract from P5 cerebellum run as control. GSK-3β levels show that LiCl does not affect the levels of GSK-3β in GCs. Scale bars, 50μm (A, B) and 20 μm (C–F).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phorylation of β-catenin and this modification results in β-catenin degradation (Papkoff, 1997; Yost *et al.*, 1996; Peifer *et al.*, 1994). Lithium has been shown to mimic WNT signaling in *Xenopus* and mammalian cells by inhibiting the activity of GSK-3β (Klein and Melton, 1996) and thereby increasing the levels of β-catenin (Stambolic *et al.*, 1996). To test the role of GSK-3β, lithium was added to cultured GCs. We found that lithium, like WNT-7a, induces axonal spreading and branching in cultured GCs. Several results support the hypothesis that lithium is mimicking WNT-7a in cerebellar neurons. First, lithium induces identical morphological changes to those induced by WNT-7a. Second, *myo*-inositol does not rescue the effect of lithium in cerebellar GCs, indicating that depletion of endogenous inositol pools, a known effect of lithium (Berridge *et al.*, 1989), is not the cause of cell shape changes in GCs. Third, lithium increases the levels of β-catenin in cerebellar GCs, a response observed when GSK-3β is inhibited in other systems. In summary, these results strongly suggest the direct action of WNT-7a via GSK-3β in the control of cell shape in developing cerebellar GCs. Moreover, these findings suggest a role for GSK-3β in axonal remodeling.

WNT could induce cell shape changes in neurons by affecting the cytoskeleton in a number of ways. Increased levels of β-catenin as a result of WNT signaling could induce changes in the actin cytoskeleton as β-catenin has been shown to bind to actin filaments through fascin (Tao *et al.*, 1996). β-Catenin could also cause cytoskeletal changes by binding to APC (Su *et al.*, 1993), a protein that associates with microtubules (Munemitsu *et al.*, 1994; Smith *et al.*, 1994). Alternatively, GSK-3β could cause cytoskeletal changes by phosphorylating other cytoskeletal proteins. Several groups have demonstrated that GSK-3β phosphorylates both tau and neurofilament proteins (Guidato *et al.*, 1996; Wagner *et al.*, 1996; Mandelkow *et al.*, 1995). Phosphorylation of tau by GSK-3β affects the binding capacity of tau to microtubules and possibly regulates microtubule dynamics (Wagner *et al.*, 1996). Increased microtubule assembly would lead to axonal extension, whereas increased disassembly would result in axonal spreading. These two processes may act concurrently in different areas of the axon resulting in the formation of complex axons. Our data are consistent with the hypothesis that WNT increases microtubule disassembly leading to enhanced axonal spreading. Although GCs exhibit axonal spreading and branching along the entire axon, the release of WNT-7a *in vivo* could be locally restricted such that cytoskeletal changes occur in defined areas of the neuron. This restricted action of WNT-7a, resulting in local microtubule instability, may cause axonal branching and sprouting. Whatever the case, our results show that modulation of GSK-3β activity by WNT is an important mechanism for regulating neuronal morphology during development.

WNT-7a and Synapse Formation

WNT-7a increases the levels of synapsin I, a protein involved in synapse formation, maturation, and synaptic vesicle trafficking (Chin *et al.*, 1995; Rosahl *et al.*, 1995). Although GCs respond to a WNT-7a signal by increasing axonal spreading as early as 20 h after exposure, effects on synapsin I levels were first detected after 3 days in culture. This delay probably reflects a period of neuronal maturation as a similar timing of synapsin I expression was observed in maturing cultured hippocampal neurons (Fletcher *et al.*, 1994). WNT-7a does not change the timing of synapsin I expression in GCs but rather increases the levels of synapsin I when GCs become competent to express it. As WNT-7a first induces changes in cell shape, the effect on synapsin I levels could be linked to cytoskeletal reorganization. GSK-3β activity is important in this process as lithium also increases synapsin I clusters. Thus, WNT-7a could induce cytoskeletal changes through GSK-3β that lead to the localization and/or increased stability of synaptic proteins at synaptic sites. Alternatively, GSK-3β could regulate synaptic protein levels directly, but this aspect of GSK-3β function remains to be explored. The increase in synapsin I levels suggests that WNT-7a plays a role in the formation of synapses in the developing cerebellum.

Expression of *Wnt-7a in vivo* shows regional differences in the cerebellar cortex. *Wnt-7a* is expressed in the EGL of the cerebellum from P6 onward, when GCs become postmitotic and begin to put out axonal processes (Altman, 1972a). The expression of *Wnt-7a* increases further after GCs reach their final position in the IGL and when they begin to make contact with Purkinje cells and MF axons (Altman, 1972a,b). Subsequently, *Wnt-7a* expression decreases in the most posterior lobules of the cerebellum from P22. This region maps precisely with the vestibulocerebellum, innervated by MFs of vestibular origin (Ito, 1984). In contrast *Wnt-7a* remains expressed in anterior lobules innervated by MFs of spinal and cortical cerebellar tracts (Ito, 1984).

FIG. 5. WNT-7a induces clustering of synapsin I in GCs. GCs were grown for 3 days on a monolayer of nonexpressing or *Wnt-7a*-expressing cells and double-stained for GAP-43 and synapsin I. (A) GCs grown on a monolayer of mock-transfected cells and stained for GAP-43. (B) The same GCs as in A stained for synapsin I show the presence of few synapsin I clusters (arrowhead). (C) GC grown on a monolayer of *Wnt-7a*-expressing cells and stained for GAP-43 shows relatively complex processes. (D) The same GC as in C stained for synapsin I shows that WNT-7a increases the number of synapsin I clusters. (E) Growth cone of a GC grown on mock-transfected cells and stained for GAP-43. (F) Shows the presence of few synapsin I clusters at growth cones of the same GC shown in E. (G) Staining for GAP-43 reveals increased spreading at the growth cone of a GC cultured on a *Wnt-7a*-expressing monolayer. (H) Staining for synapsin I of the same GC depicted in G shows that WNT-7a increases the number of synapsin I clusters at the growth cone and at processes extending from it. Arrowheads, synapsin I clusters. Arrows, growth cones with synapsin I clusters. Scale bar, 20 μm.

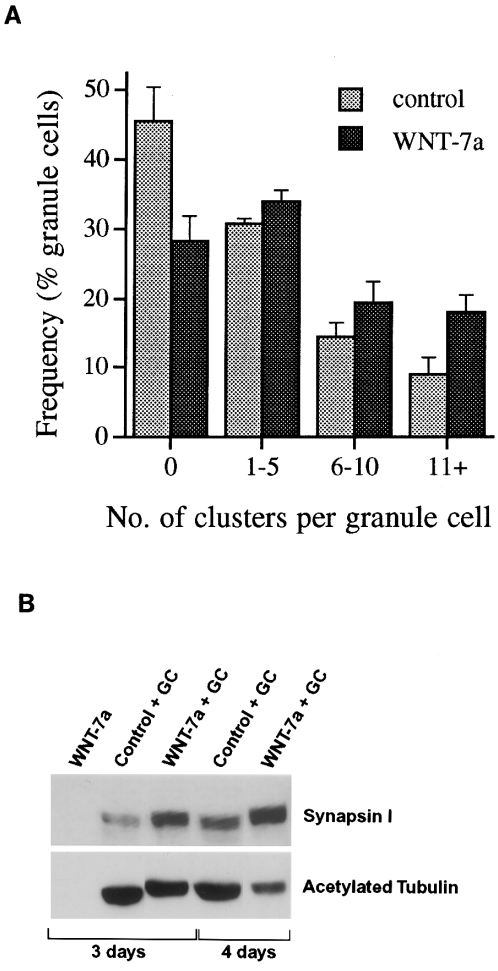

FIG. 6. WNT-7a increases the number of cells with numerous synapsin I clusters and levels of synapsin I in GCs. (A) Analysis of the distribution of synapsin I clusters in the GC population (n = 180 in each neuronal population) shows that WNT-7a increases the frequency of GCs with numerous synapsin I clusters (more than 11 clusters/cell), while there is a decrease in the number of cells with no clusters. (B) Western blots show that WNT-7a increases the levels of synapsin I. The ratio of synapsin I to acetylated tubulin shows that WNT-7a induces a fourfold increase after 3 days and a sixfold increase after 4 days.

Regional differences in *Wnt-7a* expression may be due to intrinsic differences in the GC population. This hypothesis is supported by the finding that genes such as *Otx-1* and *Otx-2* are expressed in restricted areas of the EGL, before MFs enter the cerebellar cortex (Frantz *et al.*, 1994). Alternatively, regional differences in the properties of MFs may regulate *Wnt-7a* expression in GCs. Indeed, vestibular MFs express higher levels of acetylcholine transferase than MFs that innervate the spino- and corticocerebellum (Jaarsma *et al.*, 1996). In addition, GCs of the vestibulocerebellum express higher levels of acetylcholine receptors than other GCs demonstrating the presence of cholinergic synapses in this region (Ito, 1984). Thus, the regional expression of *Wnt-7a* reveals two distinct GC populations within the maturing GC layer and suggests that *Wnt-7a* expression is differentially modulated by the synaptic properties of the MFs.

The effect of WNT-7a on axonal morphology and the levels of synapsin I suggest a role for WNT-7a in the formation of neuronal connections in the developing cerebellum. As *Wnt-7a* is expressed *in vivo* during axonal extension and the formation of synapses between GCs and their presynaptic partners, we suggest that WNT-7a made by GCs could induce axonal remodeling on neighboring GCs and other neurons by modifying the activity of GSK-3β during the formation of cerebellar synapses. Interestingly, GSK-3β is highly expressed in the postnatal CNS, including cerebellar GC axons (Takahashi *et al.*, 1994). In addition, WNT-7a could act on GC synaptic partners such as Purkinje cells and MFs. We have evidence that WNT-7a induces a similar increase in axonal spreading and the levels of synapsin I in MFs (Lucas and Salinas, unpublished results). MF growth cones adopt a complex morphology similar to those observed during the formation of the glomerular rosette, a multisynaptic region between a MF axon and several GCs (Hamori and Somogyi, 1983). These findings suggest that WNT-7a may contribute to the formation of GC–MF connections. Thus, WNT-7a through GSK-3β may be involved in the formation of neuronal networks in the developing cerebellum.

Although a function for WNTs in the regulation of neuronal cytoskeleton and synaptic proteins has not been demonstrated *in vivo*, experiments in *Drosophila* suggested that WNT proteins affect axon behavior. Overexpression of the *Drosophila* DWnt-3, a protein localized to axon tracts, leads to increased axon fasciculation (Fradkin *et al.*, 1995). Analysis of this new function for WNTs *in vivo* awaits careful characterization of WNT mutant animals in postnatal life. For example, analysis of the *Wnt-7a*-mutant mouse (Parr and McMahon, 1995), which exhibits a defect in limb patterning, could reveal important aspects of WNT function in neuronal maturation. Other *Wnt* genes are also expressed during maturation of many neuronal populations. *Wnt-3* is expressed in Purkinje cells and in pontine neurons during axonal extension and synapse formation (Salinas *et al.*, 1994). These findings are consistent with a novel role for WNTs in cytoskeletal changes associated with synapse formation in diverse areas of the developing brain. In summary, our findings that WNT proteins affect the neuronal cytoskeleton and the level of synapsin I support a link between extracellular signaling, cytoskeletal reorganization, and synapse formation.

WNTs, Lithium, and Bipolar Syndrome

Lithium has been used for some time in the treatment of uncomplicated mania and mild bipolar depression (Atack *et al.*, 1995). Although the mechanism of action of lithium in these processes is unknown, inositol depletion leading to altered IP3 metabolism has been proposed (Atack *et al.*,

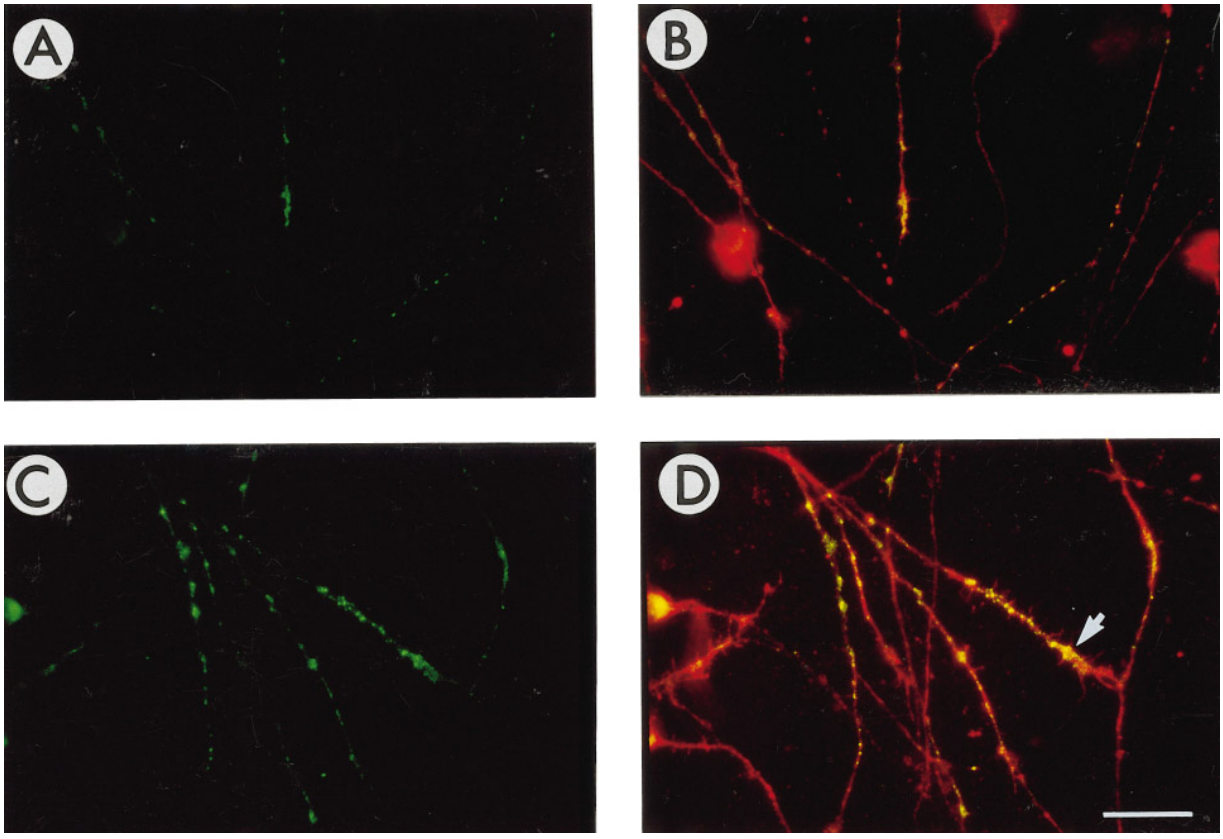

FIG. 7. Lithium increases the number of synapsin I clusters in GCs. GCs were cultured for 4 days in the presence of 5 mM NaCl or 5 mM LiCl. (A) In NaCl, GCs have a few synapsin I clusters. (B) Double staining for GAP-43 and synapsin I shows that synapsin is located along the axon process. (C) In the presence of 5 mM LiCl, there is an increase in the number of synapsin I clusters. (D) Double staining for GAP-43 and synapsin I shows that lithium induces neurite spreading and localization of synapsin I clusters in spread areas. Arrow, spread areas. Scale bar, 20 μm.

1995). Our findings that lithium, like WNT-7a, affects axonal morphology and synapsin I suggest that lithium could modulate synaptic function by regulating GSK-3β activity *in vivo*. Changes in GSK-3β activity could result in reorganization of the axonal cytoskeleton and in increased clustering of synaptic proteins with possible consequences in synaptic transmission. Therefore, lithium could act through GSK-3β to modulate synaptic activity during the treatment of bipolar depression. WNTs, by controlling synaptic protein levels, may regulate a wide variety of brain functions, including those altered in manic depression and mood disorders. Modulation of GSK-3β activity is therefore a potential therapeutic route that should be explored.

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