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BMP6 is axonally transported by motoneurons and supports their survival in vitro

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The regulation of motoneuron survival is only partially elucidated. We have sought new survival factors for motoneuron by analyzing which receptors they produce. We report here that the type II bone morphogenetic receptor (BMPRII) mRNA is one of the most abundant receptor mRNAs in laser microdissected motoneurons. Motoneurons were intensely stained by an anti-BMPRII antibody, indicating the presence of BMPRII protein. One of its ligands (BMP6) supported the survival of motoneurons in vitro. BMP6 was produced by myotubes and mature Schwann cells and was retrogradely transported in mature motor axons. BMP6 thus joins a list of known Schwann-cell-derived regulators of motoneurons, which includes GDNF, CNTF, LIF and TGF-β2. The control of the production of these factors by Schwann cells and the direction of their movement in motor axons is diverse. This suggests that the multiplicity of motoneuron factors is because cells use different factors to regulate different aspects of motoneuron function.

Keywords: BMPRII; ALK2; ALK3; Axonal transport; Glia; Motoneuron

Introduction

The survival of motoneurons is controlled by multiple factors. Skeletal muscle fibers and Schwann cells are key sources of these factors as the absence of either of these cell types causes a near total loss of developing motoneurons (Grieshammer et al., 1998; Riethmacher et al., 1997). This dual dependency also indicates that the survival factors produced by developing Schwann cells and immature muscle fibers cannot fully substitute for each other.

The requirements of mature motoneurons are less clear. Mature motoneurons survive axotomy, which deprives them of muscle-derived signals (Jiang et al., 2000a; Pollin et al., 1991). This may indicate that motoneurons cease to be dependent on the factors produced by muscle fibers as they mature. However, Schwann cells in injured nerves are able to up-regulate their production of factors such as transforming growth factor-beta2 (TGF-β2) (Jiang et al., 2000a), which are normally produced by the synaptic portions of muscle fibers (McLennan and Koishi, 1994; Murakami et al., 1999). Irrespective of the mechanism operating here, it is clear that mature motoneurons are dependent on peripheral-deprived factors as the complete removal of a motor nerve by avulsion causes a slow but progressive loss of motoneurons (Jiang et al., 2000b; Li et al., 1995).

Many neuronal cell types are controlled by a small number of survival factors, with the genetic deletion of a single factor leading to a profound loss of neurons (Lindsay, 1996). Motoneurons, in contrast, appear to be controlled by numerous survival factors. The null mutation of a single, or even multiple, factors only leads to partial loss of motoneurons (Liu and Jaenisch, 2000; Oppenheim et al., 2000, 2001). This may indicate that there are distinct subpopulations of motoneurons (Oppenheim et al., 2000). Equally, it implies that both Schwann cells and muscle fibers produce more than one regulator of motoneurons as the consequence of the genetic deletion of either cell type is more profound than the deletion of any growth factor.

Several members of the TGF-β superfamily, including Müllerian Inhibiting Substance (MIS) and TGF-β2, are involved in the regulation of motoneuron survival (McLennan and Koishi, 2002; Wang et al., 2005). Other members of the family, such as the bone morphogenetic proteins (BMPs) and the activins, affect various neurons (Ebendal et al., 1998; Hall and Miller, 2004), but their actions on motoneurons have not been extensively studied. We have therefore undertaken a systematic study of the expression of the TGF-β superfamily receptors in motoneurons as a step towards defining all of the factors that motoneurons can respond to.

The TGF-β superfamily signals through a complex involving a type I and a type II receptor. In general, the type II receptors control the ligand binding specificity, while the type I receptors determine which downstream signaling pathway is activated. There are five type II receptors and seven type I receptors (Shi and Massague, 2003). The type II receptors associate with the subfamilies of the superfamily and are referred to as the TGF-β (TβRII), BMP (BMPRII), MIS (MISRII) and activin (ActRIIA, ActRIIB) type II receptors. The type I receptors are shared by the

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various subfamilies and are termed the activin receptor-like kinases (ALKs) 1 through 7.

We report here that motoneurons produce high levels of BMPRII, with BMP6 from mature Schwann cells and myotubes being one of the ligands that may activate it.

Result

The type II BMP and MIS receptors are abundant in motoneurons

Motoneurons are known to produce various members of the TGF-β superfamily (Charytoniuk et al., 2000; Jiang et al., 2000a; Wang et al., 2005), but the relative abundance of these receptors is unknown. We therefore quantified the mRNA copy number of TGF-β superfamily receptors in motoneurons that had been isolated by laser capture microdissection. The abundance of glial fibrillary acidic protein mRNA in the motoneuron samples was not significantly different to zero, indicating that glial contamination was negligible (not illustrated).

The motoneurons contained mRNA for all of the TGF-β receptors, although the abundances of the receptors varied significantly. In all cases, the type II receptors were more abundant than the type I receptors which associate with them (Fig. 1). MISRII and BMPRII were the most abundant of the type II receptors, with mRNA copy numbers that were 25 to 40 times higher than for TβRII and ActRIIA/B (Fig. 1A). Similarly, ALK3, which associates with both MISRII and BMPRII (Jamin et al., 2003; Shi and Massague, 2003), was the most abundant of the type 1 receptors (Fig. 1B). The actions of the TGF-β subfamily are mediated by either ALK1 or ALK5 (Shi and Massague, 2003). ALK5 was the more abundant of these two receptors (Fig. 1B). The ALK3 copy number was slightly higher in the motoneurons isolated from female mice, but no sex dimorphism was observed with the other receptors (unpublished data and Wang et al., 2005).

The presence of BMPRII protein in motoneurons was verified using immunohistochemistry (Fig. 2). BMPRII immunoreactivity in the motoneurons was perinuclear (arrows) and in scattered vesicle-like structures (double-headed arrows, Figs. 2B, C). Intense perinuclear staining is commonly observed in motoneurons (Jiang et al., 2000a; Wang et al., 2005) and reflects the large size of the Golgi apparatus in these neurons. Lower levels of BMPRII immunoreactivity were associated with the circumferences of the neurons (arrowheads, Fig. 2C), which is consistent with staining of the plasma membrane. Similar staining was observed in embryonic motoneurons (Fig. 3C). ALK3 protein has previously been shown to be present in motoneurons (Wang et al., 2005).

BMP6 is expressed in the neuromuscular system

The comparatively high abundance of BMPRII and ALK3 mRNA in motoneurons suggested that motoneurons are regulated by at least one BMP. A search of the literature pointed to BMP6 being a candidate for this role. It is produced by Schwann cells (Schluesener et al., 1995) and skeletal muscle (Hall et al., 2002), but has no ascribed function at these locations. We therefore verified that BMP6 is produced within the neuromuscular system. Low levels of BMP6 transcripts were detected throughout the neuromuscular system, including the spinal cord, skeletal muscle and nerve (Fig. 4). BMP6 mRNA could not, however, be detected in isolated motoneurons, indicating that the BMP6 mRNA in the spinal cord is produced by cells other than motoneurons (Fig. 4).

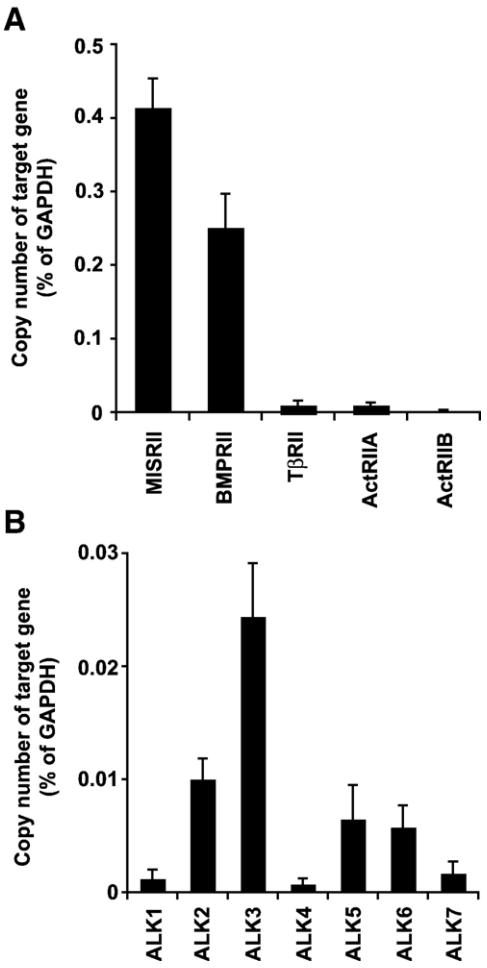

Fig. 1. Quantitative analysis of the mRNAs encoding the type I and II receptors of the TGF-β superfamily in spinal motoneurons. Motoneurons were dissected from the lumbar spinal cord by laser microdissection, and the mRNAs in them were analyzed by real-time PCR. The mRNAs from 11 mice were separately analyzed and normalized relative to the copy number of GAPDH. The values are shown as the mean±SEM. The data for MISRII and the ALKs are from Wang et al., 2005.

BMP6 protein is present in mature Schwann cells but not muscle fibers

The location of the BMP6 protein in the neuromuscular system was examined by immunohistochemistry to determine if it was associated with motoneurons in cells. The anti-BMP6 antibody intensely stained the Schwann cells of both the sciatic (arrows, Figs. 5A, B) and the hypoglossal nerves (not illustrated). No BMP6 immunoreactivity was associated with muscle fibers, in either their synaptic or extrasynaptic regions (Figs. 5C, D). BMP6 was, however, detected in the interstitial cells of muscles (arrowhead, Figs. 5C, D) and some of the cells associated with the muscle spindles (Fig. 5D), thus accounting for the presence of BMP6 mRNA in the muscle samples (Fig. 4). Low levels of BMP6 immunoreactivity were present in the vicinity of motoneurons (small arrows, Fig. 5E) with higher levels of stain associated with the circumference of the motoneurons (arrowheads, Figs. 5E, F). BMP6 immunoreactivity was not detectable within the cell bodies of motoneurons.

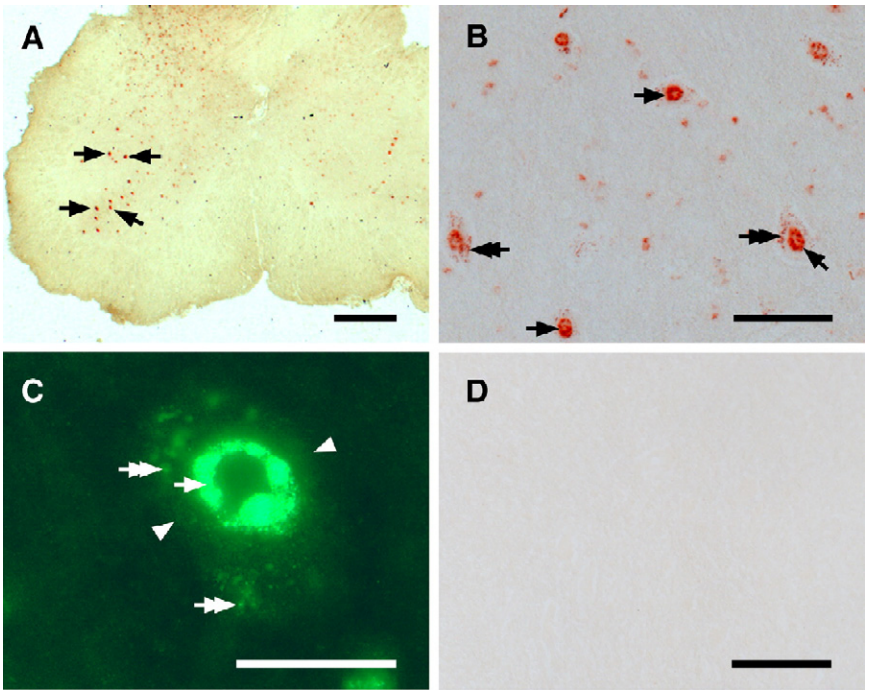

Fig. 2. Immunohistochemical localization of BMPRII in the lumbar spinal cord. (A) Low magnification view of a spinal cord, in which immunoreactivity was developed with AEC, which gives a red stain. (B) Higher magnification view of a part of panel A. The arrows point to perinuclear immunoreactivity, whereas the double-headed arrows point to scatter vesicle-like structures. (C) High magnification view of a single motoneuron using a fluorescent-linked secondary antibody. The arrows and arrowheads are as for panel B. The arrowheads point to a low level of stain on the circumference of the motoneuron. (D) Control section in which the anti-BMPRII antibody has been replaced with non-immune IgG. Scale bars = 300 μm (A), 50 μm (B, D) and 25 μm (C).

BMP6 protein is transported in the motoneurons

If Schwann cells use BMP6 to regulate motoneurons then BMP6 should be axonally transported, like other Schwann-cell-derived neurotrophic factors (Jiang et al., 2000a; Russell et al., 2000). The sciatic and hypoglossal nerves were therefore double ligated to determine whether BMP6 was transported in the axon. BMP6 immunoreactivity was associated with the distal ligation in both nerves (Figs. 6A, C, D), indicating that BMP6 was being retrogradely transported. Much lower levels of BMP6 immunoreactivity were also detected in the proximal portion of the axons (Figs. 6A, B). This is consistent with a minor anterograde transport of BMP6. The immunoreactivity at both ligation sites appeared to be in axons. This was further verified by cutting transverse sections of the nerve, which allows for clear delineation between axonal and Schwann cell proteins (Jiang et al., 2000a; Russell et al., 2000). In the transverse sections, the motor axons can be unambiguously identified (Fig. 6F). The motor axons contained intense BMP6 immunoreactivity (arrows, Fig. 6E), with lower levels of immunoreactivity in the Schwann cells that surround them.

BMP6 protein is present in myotubes

The cellular locations of BMP6 protein during the period of naturally occurring motor neuron cell death were markedly different to that in the adult. The major nerves in embryonic limbs did not contain detectable levels of BMP6 immunoreactivity (Figs. 3A, B), indicating that immature Schwann cell produces little or no BMP6. Myotube when they first formed (around E12 (McLennan, 1994)) also failed to bind the anti-BMP6. However, as they matured the myotubes became strongly immunoreactive (Figs. 3A, B).

BMP6 support motoneuron survival in vitro

MIS and BMP6 both activate ALK3 (Jamin et al., 2003; Shi and Massague, 2003), suggesting that they may produce similar effects on motoneurons. MIS supports the survival of embryonic motoneurons in vitro (Wang et al., 2005). We therefore examined the response of embryonic motoneurons to BMP6 in vitro. Addition of rBMP6 to the medium caused a dose-dependent increase in neuronal survival (Fig. 6D). The maximum extent of survival produced by BMP6 was similar to that produced by MIS and GDNF (Fig. 6D). The BMP6-treated motoneurons were islet-1 positive and usually extended long branched neurites (not illustrated).

Discussion

This study implicates members of the BMP family as regulators of embryonic and adult motoneurons. The survival of embryonic motoneurons in vitro was promoted by BMP6, to an extent that was similar to the classical motoneuron survival factor GDNF. This indicates that motoneurons express all of the components required for BMP signaling, including appropriate type I and type II receptors.

The BMP family can utilize BMPRII, ActRIIA or ActRIIB as their type II receptors and ALK2, ALK3 or ALK6 as their type I receptors (Miyazono et al., 2005; Shi and Massague, 2003). Motoneurons expressed detectable levels of all of these receptors.

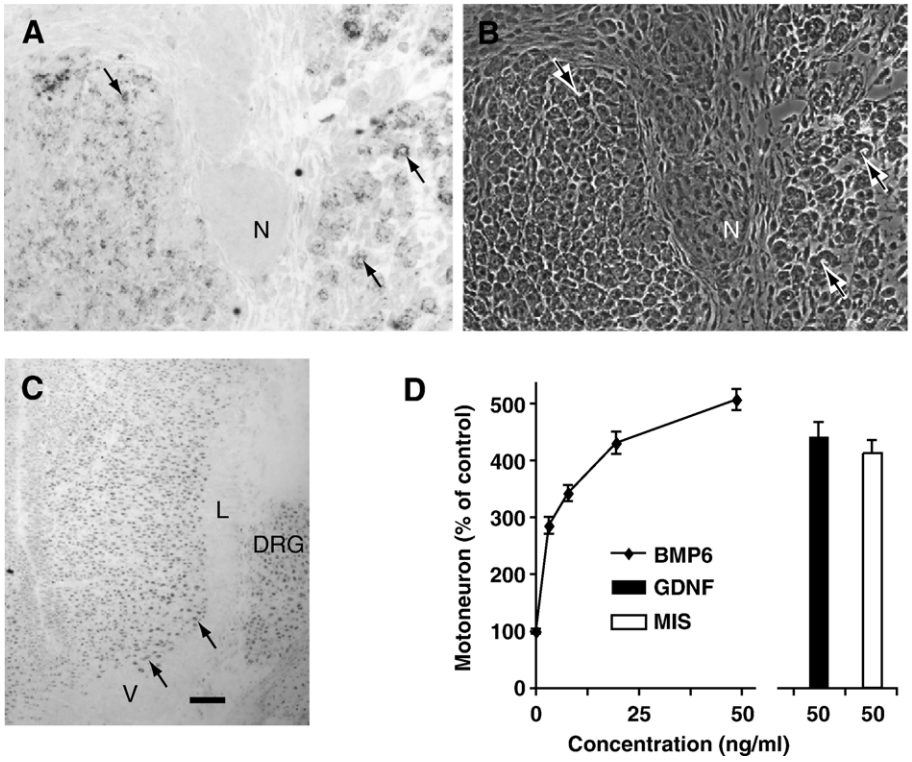

Fig. 3. BMP6 in the developing neuromuscular system. (A and B) Transverse section of the leg of an E17 embryos, stained with anti-BMP6. B is a phase-contrast image of A. The major nerves (N) were not stained. The arrows point to mature myotubes that were BMP6-immunoreactive. (C) Transverse section of a spinal cord stained with anti-BMPRII. All neurons were BMPRII-immunoreactive. The arrows point to motor neurons, whereas "DRG" labels the first lumbar DRG. The lateral and ventral white matter is labeled "L" and "V", respectively. (D) A graph showing the effect of BMP6 on the survival of embryonic motoneurons. Motoneurons were isolated from E13–E15 mouse embryos and cultured for 4 days in defined medium with BMP6, GDNF, MIS or vehicle. The surviving cells were stained with an antibody to the motoneuron marker, Islet-1, and then counted as described in the Materials and methods. The values are the mean ± SEM of triplicate wells. Similar data were obtained in 3 other replicate experiments.

BMPRII was the most abundant of the type II receptors, with a copy number that was 26 times higher than that of ActRIIA and 130 times higher than that of ActRIIB (Fig. 1). However, the mRNA levels of ActRIIA and ActRIIB were comparable with that of TβRII, which mediates the potent neurotrophic effects of TGF-β2 on motoneurons (Jiang et al., 2000b; McLennan and Koishi, 2002). It is thus possible that all of the receptors are involved, although the combination of BMPRII and ALK3 is likely to be the dominant pathway.

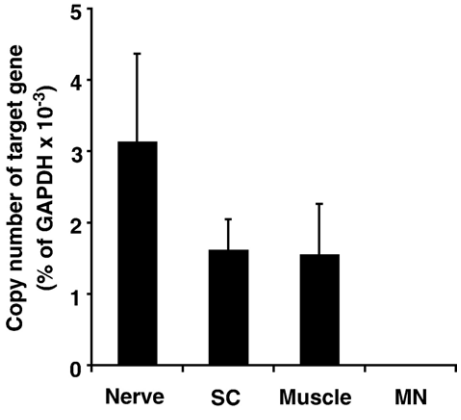

Fig. 4. Quantitative analysis of the mRNA encoding BMP6 in the neuromuscular system. mRNA was isolated from the sciatic nerve (Nerve), spinal cord (SC) and the extensor digitorum longus (Muscle). The isolated motoneurons (MN) were the samples described in Fig. 1. The copy numbers of BMP6 from either 4 (tissues) or 11 (MN) mice were separately analyzed by real-time PCR and normalized relative to the copy number of GAPDH. The values are shown as the mean ± SEM.

BMP6 may be a physiological regulator of mature motoneurons

Our studies implicate BMP6 as a Schwann-cell-derived regulator of mature motoneurons. Its mRNA is present in nerves (Fig. 4) and BMP6 protein was present in Schwann cells (Fig. 4 and Schluesener et al., 1995). Most importantly, ligation of the sciatic and hypoglossal nerves led to the accumulation of BMP6 protein at the distal tie (Fig. 6). The hypoglossal nerve is a pure motor nerve. This implies that there is a continuous flow of BMP6 from the periphery to the cell body of motoneurons. The Schwann cells appear to be the only possible source of this BMP6. BMP6 is present in skeletal muscle (Hall et al., 2002). However, the BMP6 in skeletal muscles is located in interstitial cells and not in muscle fibers. In particular, BMP6 immunoreactivity was not present in the post-synaptic domain of the neuromuscular junction (Fig. 5). Muscle fibers are thus an unlikely source for the BMP6 in motor axons. Motoneurons themselves are also unlikely sources as BMP6 mRNA was not detected in isolated motoneurons and as the direction of transport of BMP6 was predominantly towards rather than away from the cell body.

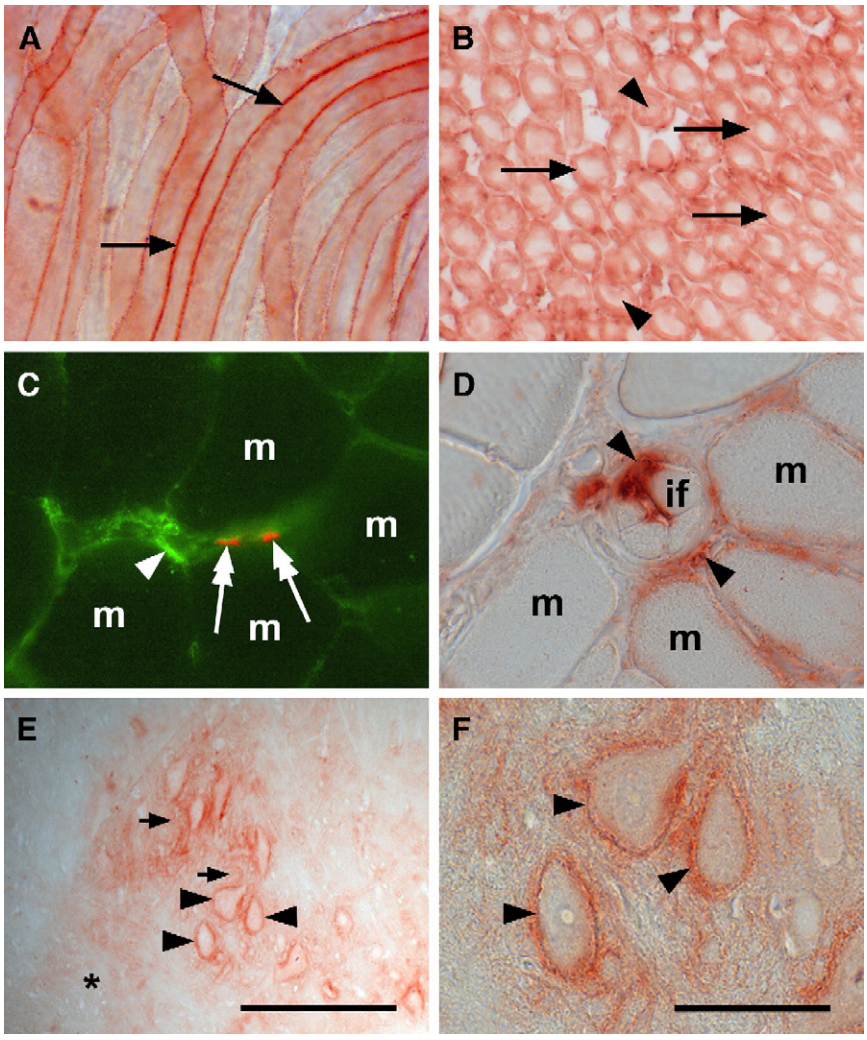

Fig. 5. Immunohistochemical localization of BMP6 in the neuromuscular system. A whole mount sciatic nerve (A), cross-section of ventral nerve root (B), extensor digitorum longus muscle (C, D) and lumbar spinal cord (E, F) were stained with an anti-BMP6 antibody. The sections were visualized using a color reaction product (AEC), except for C where a green fluorescent-linked secondary antibody was used. The section in C was also incubated with rhodamine-conjugated α-bungarotoxin (red) to mark neuromuscular junctions. (A, B) The arrows point to Schwann cells. The motor axons (arrowheads) had only trace levels of stain. (C, D) The arrowheads point to BMP^{+ve} interstitial cells. The extrafusal muscle fibers are marked by "m" and the intrafusal fibers of a muscle spindle are marked by "if". The double-headed arrows point to α-bungarotoxin-stained neuromuscular junctions. (E, F) The arrowheads point to the circumference of motoneurons, whereas the small arrows point to stain between the cell bodies of motoneurons. The "*" marks the white matter ventral–lateral to the motor nuclei. The magnifications of A, B and D–F are the same. Scale bars = 150 μm (C) and 50 μm (F).

BMP6 mRNA was also detected the spinal cord and BMP6 immunoreactivity was observed in close apposition to motoneurons (Figs. 4,5). This suggests that all of the glia associated with motoneurons, and not just Schwann cells, use BMP6 to communicate with motoneurons.

Schwann cell control of mature motoneurons

Multiple growth factors have been implicated in the communication between Schwann cells and motoneurons. This begs the question of why so many factors are needed for this communication. The answer to this question is unclear, although the available evidence points to Schwann cells using different factors in different contexts and/or to regulate different aspects of motoneuron function. The characteristics of the axonal transportation of Schwann-cell-derived growth factors are consistent with this concept (Fig. 7).

As noted above, BMP6 is largely transported towards the cell body, which fits with classical neurotrophic theory where peripheral-derived growth factors control motoneuron gene expression (Reynolds et al., 2000). GDNF from Schwann cells, in marked contrast, is mainly transported towards the nerve terminal, leading to an abundance of GDNF-rich vesicles within the intramuscular branches of motor axons (Russell et al., 2000).

The movement of BMP6 and GDNF in motor axons can be detected by ligation of the nerve, indicating that motor axons receive a chronic supply of these factors from Schwann cells. This is consistent with these factors being involved in the day-to-day regulation of motoneurons, but with each of the factors differentially affecting different compartments within the neuron. In contrast, significant levels of LIF and TGF-β2 are only present in Schwann cells after nerve damage, pointing to them being specialized injury factors (Curtis et al., 1994; Jiang et al., 2000b).

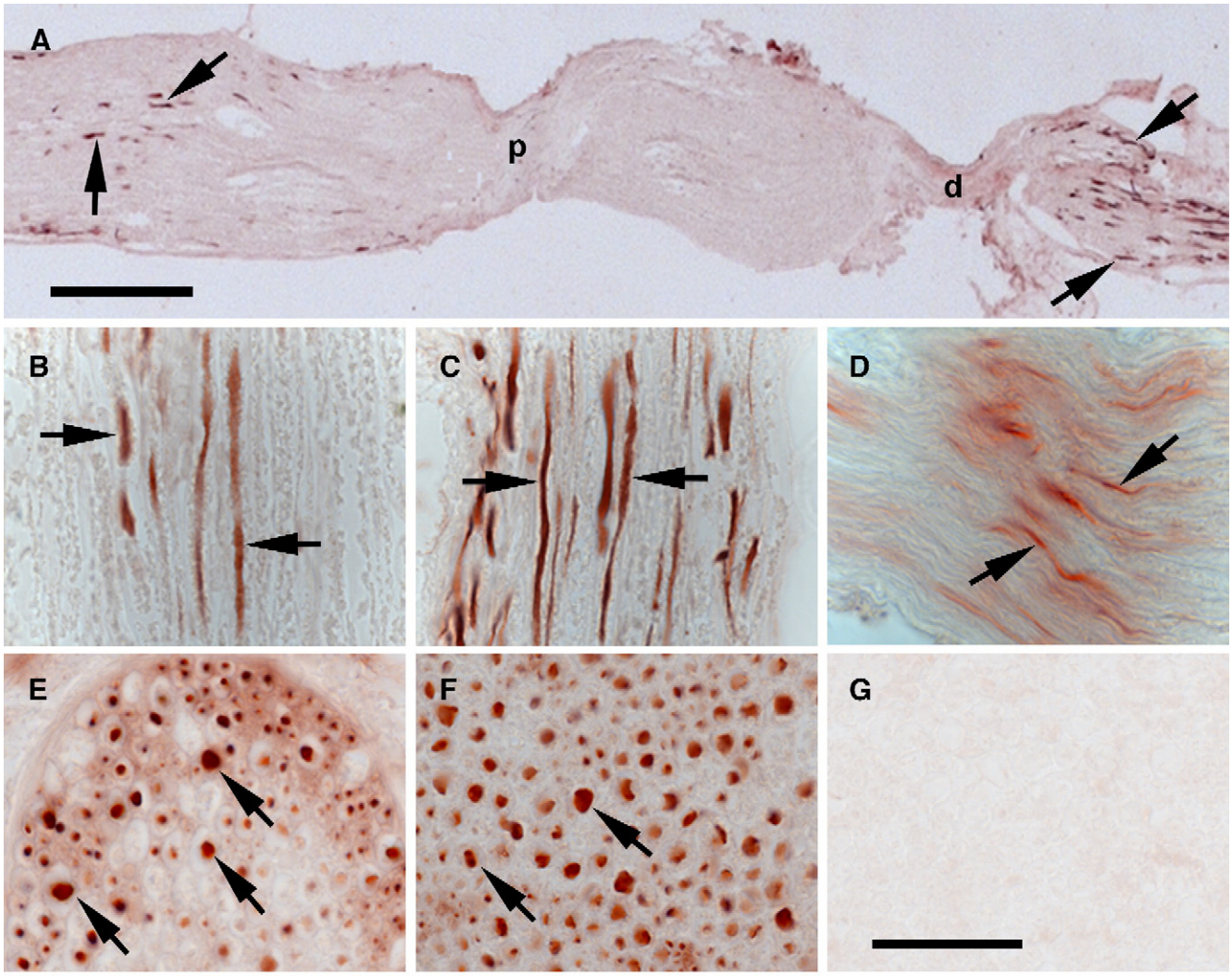

Fig. 6. Immunohistochemical localization of BMP6 in ligated nerves. Longitudinal sections (A–D) and cross-sections (E–G) of ligated sciatic (A–C, E–G) and hypoglossal (D) nerves were stained with an anti-BMP6 (A–E), anti-neurofilament to mark axons (F) or non-immune IgG (G). The proximal and distal ligations are marked in (A) by a "p" and a "d", respectively. B is a higher magnification of the proximal region illustrated in A, whereas C–E illustrate distal regions. The arrows point to axons in A–F. The magnifications of B–G are the same. Scale bars = 300 μm (A) and 50 μm (G).

LIF and TGF-β2 differ from each other in that TGF-β2 is a muscle-derived factor (McLennan and Koishi, 1994; Murakami et al., 1999) and its presence in damaged nerve may serve to substitute for muscle-derived TGF-β2 until axon regeneration is complete.

Although BMP6, GDNF, LIF and TGF-β2 have some distinct differences, they have in common an ability to support the survival of motoneurons in various assays. In this study for instance, BMP6 was as effective as GDNF in supporting the survival of isolated motoneurons. Thus, although each of the Schwann-cell-derived factors may mediate distinct aspects of motoneuron physiology, they may also have common functions, including promoting the survival of the motoneuron.

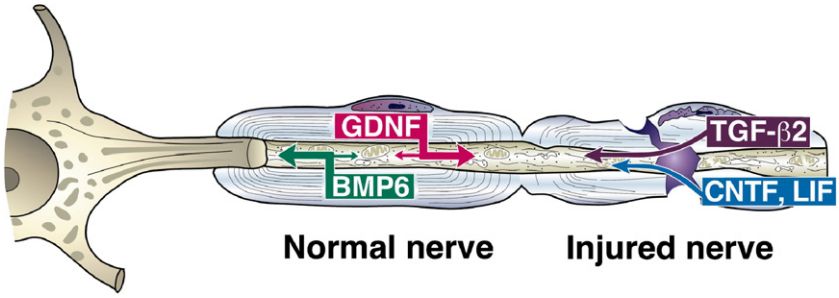

Fig. 7. Illustration showing the transport of neuronal survival factors in motor axons.

Myotubal control of developing motor neurons

The survival of motor neurons during development has an absolute dependency on muscle-derived factors. BMP6 may be one of the factors that muscle uses to support the survival and differentiation of motor neurons. It is present in myotubes (immature muscle fibers) (Figs. 3A, B), motor neurons express receptors for it (Fig. 3C) and it promotes the survival of embryonic motor neurons in vitro (Fig. 3D).

The cells that produced BMP6 were different in the embryo and adult. This is also consistent with the known cellular biology of motor neurons. As outlined in the Introduction section, immature Schwann cells do not appear to synthesize the factors produced by immature muscle, which is the pattern observed with BMP6. In the adult, however, Schwann cells are able to support motor neurons in the absence of muscle. This has been attributed to Schwann cells acquiring the capacity to produce muscle-derived signals: BMP6 was produced by myotubes and mature Schwann cells.

Other BMPs

Mice with null mutations of BMP6 are viable and do not exhibit an overt motor deficit (Solloway et al., 1998). This indicates that the function of BMP6 is not indispensable. This may be due to functional redundancy with other growth factors. There are at least 14 BMPs, and null mutations of them often have a mild phenotype. In contrast, the loss of BMPRII is embryonic lethal (Beppu et al., 2000). Additionally, BMP6 activates the same type I receptors as the male-specific hormone MIS (Teixeira et al., 2001), which is a motoneuron survival factor in vitro (Wang et al., 2005) and in vivo (Wang et al., 2006). This very large range of growth factors creates the opportunity for subtle context-dependent regulation of motoneurons. For instance, during development motoneurons in female embryos will be exposed to BMPs but only trace levels of MIS, whereas male embryos will be exposed to BMPs plus high levels of MIS in the serum. If MIS acted alone, then motoneurons would be expected to be very dimorphic as only MIS would be activated in the type I BMP receptors. In contrast, the combined concentrations of BMPs plus MIS will only have a slight male bias, which would be expected to give rise to a subtle sexual dimorphism, which is what occurs in lumbar spinal motoneurons (Wang et al., 2006).

Summary

Motoneurons are responsive to various members of the CNTF, FGF, neurotrophin, and TGF-β families of growth factors. This study indicates that the BMP family of growth factors is also important. The BMP family is large. BMP6 from myotubes, mature Schwann cells and satellite glia appears to be one of the BMPs that are physiological regulators of motoneurons. Other members of the family may also be involved. This large variety of motoneuron regulators creates the possibility for subtle context-dependent regulation of motoneurons.

Experimental method

Animals

The University of Otago's Animal Ethics Committee approved all experiments. C57/B6 mice were bred and maintained in M.I.C.E.™ cages (Animal Care Systems, Littleton, CO) and their food sterilized by gamma irradiation. The room had a 14 h white light/10 h dark–sodium light phase (McLennan and Taylor-Jeffs, 2004), with the dark phase beginning at 1 pm. Murine fetuses were from time-mated dams.

Isolation of motoneuron RNA

Motoneuron mRNA was isolated using laser capture microdissection as previously described (Wang et al., 2005). Briefly, lumbar spinal cords were sectioned in a cryostat, stained with cresyl violet and their motoneurons harvested using the PixCell 2 LCM System and CapSure HS LCM Caps (Arcturus Engineering, Mountain View, CA). Typically, 4–500 motoneurons were collected from each mouse. The purity of the mRNA was confirmed by measuring the abundance of a glial marker, glial fibrillary acidic protein.

RNA preparation, cDNA synthesis and real-time PCR

Total RNA fractions were isolated using TRIzol reagent (Invitrogen, Groningen, Netherlands) for whole tissues or PicoPure RNA isolation kit (Arcturus Engineering) for laser-captured motoneurons. The isolated RNA fractions were initially treated with RQ-DNase I (Promega, Madison, WI) to remove genomic DNA contamination. The cDNA was synthesized with SuperScript II RNase H^- (Invitrogen) and oligo-$d(T)_{15}$ as the primer. The real-time PCR reactions were performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), SYBR Green Master Mix (Applied Biosystems) and gene specific primers (Supplementary Table 1). A two-step PCR reaction was carried out with denaturation at 95 °C for 15 s, annealing and extension combined at 60 °C for 1 min with a total of 40–50 cycles. The uniqueness of the amplicons was analyzed using dissociation curves and by nucleotide sequencing. A standard curve was generated for each gene, and the copy number of the mRNA transcripts calculated.

Nerve ligation

Adult mice were anesthetized by subcutaneous injection of Domitor (1 mg/kg, Novartis Animal Health Australasia, Pendle Hill, Australia) and ketamine (75 mg/kg, Parnell Laboratories, Auckland, New Zealand). The left hypoglossal or sciatic nerve was double ligated as previously described (Jiang et al., 2000a). A midline incision was made in the ventral neck and the tendon of the digastric muscle sectioned to expose the hypoglossal nerve. The nerve was then ligated in two places, 1–2 mm apart, using fine surgical thread. The sciatic nerve was exposed by separating the anterior border of the biceps femoris from other structures and ligated as described for the hypoglossal nerve. Eighteen to twenty hours after ligation, the animals were killed in a CO_2 chamber and their nerves dissected.

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde, post-fixed in 4% paraformaldehyde in 0.6 M sucrose at 4 °C and then incubated in 30% sucrose at 4 °C overnight. The perfused tissues and the tissues from non-perfused mice were embedded in OCT medium (VWR, San Diego, CA) and snap frozen. Sections of lumbar spinal cords, skeletal muscles (extensor digitorum longus or soleus), sciatic nerves and hypoglossal nerves from adult mice and lumbar spinal cords and legs from E12, E14 and E17 fetuses were cut in a cryostat at a thickness of 10 μm. The sections were stained by immunohistochemistry, as previously described (Russell et al., 2000). Briefly, fixed tissues were washed in 0.1M glycine, incubated in 5% donkey serum and then sequentially incubated with either goat anti-BMPRII (R&D System, Minneapolis, MN) or goat anti-BMP6 (sc-7406, Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated donkey anti-goat IgG antibody (Sigma, St. Louis, MO), methanol/H_2O_2 and streptavidin biotinylated–horseradish peroxidase complex (Amersham Pharmacia Biotech, Uppsala, Sweden). The immunoreactivity was visualized using 3-amino-9-ethylcarbamide (AEC) (Sigma) as the chromogen. In a few

experiments, the immunoreactivity was visualized using a fluorescent BODIPY-fl conjugated secondary antibody (Molecular Probes, Eugene, OR). Neuromuscular junctions were labeled with rhodamine-conjugated β-bungarotoxin (Molecular Probes). Non-specific binding was controlled for by replacing the primary antibody with non-immune IgG (Sigma). The antibodies to BMP6 bind uniquely to BMP6 in Western blots of tissues (Peretto et al., 2002), indicating its suitability for use in immunohistochemistry.

Motoneuron cultures

The embryonic motoneurons were purified and cultured as previously described (Wang et al., 2005). Briefly, the spinal cords of 13 to 15-day-old (E13–15) fetuses were disassociated in Dulbecco's PBS (pH 7.2, Sigma) containing 10 μM β-mercaptoethanol, 0.05% trypsin (Sigma) and 0.04% EDTA. Motoneurons were purified using 10.4% Optiprep (Sigma) in DPBS and cultured in serum-free medium containing neurobasal medium (Invitrogen), B27 supplement (Invitrogen) and 500 μM glutamine at 37 °C with 5% CO_2. Growth factors including rhBMP6 (R&D System), rhMIS (Ragin et al., 1992) and recombinant human glial-cell-line-derived neurotrophic factor (GDNF) (Alomone Laboratories, Jerusalem, Israel) were added immediately after seeding. Half the volume of medium was changed after 2 days. Four days after plating, the cultures were stained with an antibody to the motoneuron marker, anti-islet-1 (39.4D5, Developmental Studies Hybridoma Bank) (Ericson et al., 1992). The numbers of surviving motoneurons were determined by counting islet-1^{+ve} neurons with a neurite in three randomly selected fields in each well. Three wells were used for each concentration of factor and the experiment was replicated four times.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.01.008.

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