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The transforming growth factor-betas: multifaceted regulators of the development and maintenance of skeletal muscles, motoneurons and Schwann cells

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ABSTRACT This review discusses the roles of the transforming growth factor-betas (TGF-βs) as part of a complex network that regulates the development and maintenance of the neuromuscular system. The actions of the TGF-βs often vary depending on which other growth factors are present, making it difficult to extrapolate results from *in vitro* experiments to the *in vivo* situation. A new approach has therefore been needed to understand the physiological functions of the TGF-βs. The behaviours (proliferation, fusion, apoptosis) of many of the cells in the neuromuscular system have a complex pattern which varies in space and time. The actions of growth factors in this system can thus be deduced based on how well their pattern of expression correlates with known cellular behaviours. Hypotheses based on this molecular anatomical evidence can then be further tested with genetically modified mice. From this type of evidence, we suggest that: (1) TGF-β1 is an autocrine regulator of Schwann cells; (2) maternally-derived TGF-β1 helps to suppress self and maternal immune attack; (3) TGF-β2 regulates when and where myoblasts fuse to myotubes; (4) motoneuron survival is regulated by multiple sources of TGF-βs, with TGF-β2 being the more important isoform. The concept of TGF-β1 as a regulator of secondary myotube formation is not supported by either the location of the TGF-β1 in developing muscles or by the phenotype of TGF-β1$^{-/-}$ mice. The review concludes with a discussion of whether all of these of postulated functions can occur independently of each other, within the confines of the neuromuscular system.

KEY WORDS: *myotube, connective tissue, apoptosis, myelination, null mutant*

Introduction

The neuromuscular system is the product of a complex interaction between multiple cell types. The three central cell types, the motoneuron, the Schwann cell and the muscle fibre, are interdependent. The survival of motoneurons and the pattern of their gene expression are regulated by muscle fibres and by Schwann cells. In turn, motoneurons have a dominant influence on Schwann cells and muscle fibres. These interactions between the central cell types tend to be foremost in our minds, but they are only part of a more elaborate set of regulatory influences. Muscle fibres interact with connective tissues, blood vessels, tendons and bones. Motoneurons interact with glia and other neurons, and the vasculature that surrounds them.

The interactions between the multiple cell types of the neuromuscular system are mainly mediated by diffusible factors, many of which are protein growth factors. This review focuses on a small family of growth factors, the transforming growth factor-betas (TGF-βs). It begins with a brief description of their biochemistries, before proceeding to discuss their putative roles within the neuromuscular system. These roles are very wide ranging and include the regulation of myotube formation, connective tissue patterning and differentiation, motoneuron survival, maintenance of myelin sheaths and immune rejection. Each of these putative actions of the TGF-βs have been studied in isolation of each other, and to a large extent without consideration of other regulator influences. The final parts of the review will therefore consider the TGF-βs as part of a complex network. This will involve discussion of whether the TGF-βs are able to mediate apparently diverse functions within a single anatomical system.

Abbreviations used in this paper: α-BTX, alpha-bungarotoxin; mRNA, messenger ribonucleic acid; MHC, major histocompatibility antigens; Rh, rhesus; TGF-β, transforming growth factor-beta; TβR, transforming growth factor-beta receptor; +/-, heterozygous null mutant; -/-, null mutant.

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The Transforming Growth Factor-betas

Isoforms

The TGF-βs are a small family of multi-functional growth factors, consisting of three mammalian isoforms: TGF-β1, TGF-β2 and TGF-β3 (Kingsley, 1994). Each isoform is synthesised as a latent dimeric complex that is secreted and subsequently cleaved to yield a smaller active dimer. The pro-region of the TGF-β can bind to various extracellular proteins (Munger *et al.,* 1997) enabling the TGF-βs to be stored in the extra-cellular matrix, until activated by a physiological process such as wound healing or tissue remodelling. This delayed mode of action appears to be more common with TGF-β1 than TGF-β2 or TGF-β3, although all three isoforms also mediate acute biological responses where they act immediately after release.

Most cells *in vitro* respond equivalently to the three isoforms. However, exceptions to this generalisation occur. In particular, some myogenic cell lines respond to TGF-β1 and TGF-β3 but not to TGF-β2. The molecular basis of this specificity has been described by Massague and others and is briefly outlined below. Despite the similarity of their actions *in vitro*, each of the TGF-βs appears to mediate a distinct set of actions *in vivo*. Each isoform has a different distribution *in vivo*, with only limited overlap (Schmid *et al.,* 1991). Consequently, the opportunities for redundant actions, involving two or three of the isoforms are limited, except in the neuromuscular system. Consistent with this, null mutants of each of the three isoforms produce distinct phenotypes (Letterio and Bottinger, 1998).

Receptors

The TGF-βs produce their biological actions through a heteromeric receptor complex consisting of the type I (TβR-I) and type II (TβR-II) TGF-β receptors (Wrana *et al.,* 1992). Both receptors are transmembrane proteins with an extracellular TGF-β binding site and an intracellular serine/threonine kinase domain. However, TβRI and TβRII must co-operate to transduce TGF-β signals (Wrana *et al.,* 1992), with a binding-protein named betaglycan (TβRIII) being required when the ligand is TGF-β2 (Lopez-Casillas *et al.,* 1993) (Fig. 1).

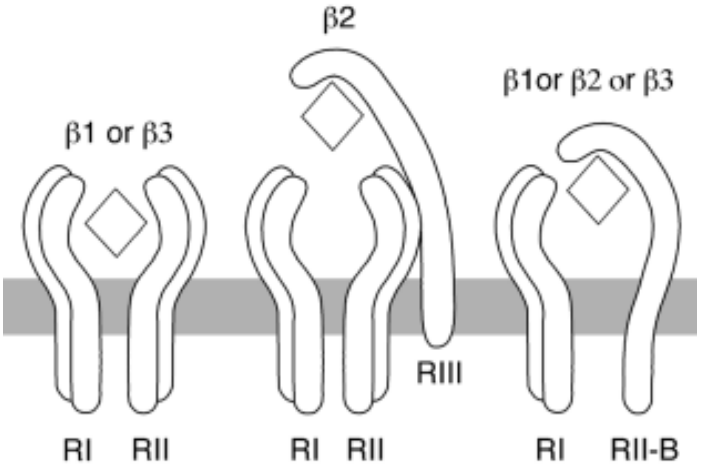

Fig. 1. An illustration of the specificity of TGF-β signalling. (A) *The combination of TβRI and TβRII is responsive to TGF-β1 and TGF-β3.* **(B)** *The addition of betaglycan (TβRIII) enables TGF-β2 to become associated with TβRI/TβRII.* **(C)** *The combination of TβRI and TβRII-B binds all three TGF-β isoforms.*

A splice variant of TβRII (TβRII-B) containing a 26 amino acid insert in the extracellular domain of the receptor has been known for some years, but has only recently been characterised (Rotzer *et al.,* 2001) (Fig. 1). Unlike TβRII, TβRII-B is able to bind TGF-β2 in the absence of betaglycan, although it also binds TGF-β1 and TGF-β3 (Rotzer *et al.,* 2001). TβRII-B is associated with tissues where the predominant isoform is TGF-β2 (Rotzer *et al.,* 2001) but there is currently insufficient evidence to conclude that it is principally a receptor for TGF-β2.

The Formation of Muscle Fibres

Cell Biology

Muscle precursor cells become determined to one of several myogenic lineages while in the somite. They subsequently migrate to the muscle primordium where they differentiate into either early (embryonic), late (fetal) or adult (satellite) myoblasts. Myoblasts are the immediate precursors of muscle fibres and are able to either divide or fuse, depending on signals in their local environment. The fates of myoblasts vary in space and time, enabling the formation and growth of muscle fibres to be divided into a number of steps, which are illustrated in Fig. 2 (McLennan 1990; 1994). Several aspects of this are commented on below.

First, the fusion of two myoblasts to form a binucleated myotube is rare, being restricted to two short temporal windows (steps 1 and 3) and to the synaptic portion of developing muscles (step 3) (Duxson and Sheard, 1995; Zhang and McLennan, 1995). Myotubes rarely fuse with one another *in vivo*. Consequently, the fusion of two myoblasts is a significant phenomenon as it determines the number of fibres in a muscle. *In vitro*, all myoblasts are able to fuse with other myoblasts, indicating that this type of fusion is actively suppressed *in vivo*, except at the particular times and the particular locations where myotubes form.

Second, myotubes/fibres continually absorb myoblasts until the mature state of the muscle is reached (Wigmore *et al.,* 1992; Zhang and McLennan, 1995). However, the rate at which new nuclei are added to myotubes/fibres varies with the stage of development, as does the site of addition of the new nuclei. During some stages, myoblasts preferentially fuse with the ends of myotube/fibres whereas at other stages they mainly fuse in the middle of the myotube (Zhang and McLennan, 1995).

Third, myoblast-to-myoblast fusion only occurs at sites where no myotubes exist (stage 1) or where the fusion of myoblasts to myotube formation is low (stage 3). This may indicate that existing myotubes/fibres suppress the formation of new myotubes by absorbing myoblasts as soon as they become competent to fuse.

Fourth, the later stages of muscle formation are profoundly dependent on the innervation of the muscle. Acetylcholine-stimulated contraction of primary myotubes is an important component of the neural regulation of muscle formation (McLennan, 1983). However, this does not preclude a role for the various peptides factors produced by motoneurons (McLennan, 1994), including TGF-β2 (see below).

TGF-βs regulate Myoblast In Vitro

The TGF-βs are best known as potent inhibitors of the proliferation and fusion of myoblasts and myogenic cell lines (Olson *et al.,* 1986). However, in apparent contradiction of this notion, C2C12 myoblasts that express a truncated TβRII are unable to fuse *in vivo*.

This suggests that the TGF-βs are essential promoters of myoblast fusion *in vivo* (Filvaroff *et al.,* 1994). The underlying cause of this conflicting data is that the response of myogenic cells to the TGF-βs is profoundly influenced by other regulator influences. For instance, TGF-β either inhibits, stimulates or has no effect on the proliferation of adult myoblasts depending on whether platelet-derived growth factor, fibroblast growth factor or insulin-like growth factor is included in the culture medium (Cook *et al.,* 1993).

The complex interaction between the TGF-βs and other growth factors makes extrapolation from *in vitro* to *in vivo* myogenesis difficult. Despite this, the concepts derived from *in vitro* studies have only rarely been challenged by *in vivo* experiments. We are attempting to do this using a combination of approaches. In our view, the location of growth factors *in vivo* are an under valued tool. As noted above, one of the salient features of muscle formation is that the behaviours of myoblasts (differentiation, proliferation and fusion) vary in space and in time. This suggests that the quantities and distributions of regulatory factors also vary in space and time. If so, the functions of growth factors *in vivo* may be tested (or predicted) by determining whether their pattern of expression correlates with the behaviour of myoblasts *in vivo*. Second, the putative functions of a growth factor can then be further tested by analysing muscle formation in mice with a null mutation of the growth factor.

Does TGF-β1 control the Onset of Secondary Myotube Formation?

Early myoblasts are relatively insensitive to the inhibitory effects of TGF-β1 *in vitro* (Cusella-De Angelis *et al.,* 1994). This has lead to the hypothesis that TGF-β1 regulates the pattern of myotube formation by suppressing the proliferation and fusion of late myoblasts until primary myogenesis (steps 1 and 2) is complete. Consistent with this, addition of TGF-β1 to explant cultures of limb buds suppresses secondary myotube formation (step 3) whereas neutralising antibodies to TGF-β have the opposite effect (Cusella-De Angelis *et al.,* 1994).

Although the *in vitro* evidence for this hypothesis is very attractive, our *in vivo* studies do not support it. First, the hypothesis requires that TGF-β1 levels in the developing limb drop immediately prior to the formation of secondary myotubes (step 3) (Cusella-De Angelis *et al.,* 1994). In contradiction of this, the concentration of TGF-β1 mRNA in developing limbs is constant throughout muscle development (Fig. 3). Similarly, the amount TGF-β1-immunoreactivity in developing muscles does not decrease immediately prior to secondary myotube formation (McLennan, 1993). Second, secondary myotube formation is normal in the absence of fetal TGF-β1, as evidenced by quantitative electron-microscopic studies of the muscles of TGF-β1$^{-/-}$ mice (McLennan *et al.,* 2000).

TGF-β1 protein crosses the placenta and binds to connective tissues, including those in developing skeletal muscles (Letterio *et al.,* 1994; McLennan *et al.,* 1999; McLennan *et al.,* 2000). However, the time of onset of myotube formation varies considerably between proximal and distal muscles, indicating that it is controlled by local factors rather than by a systemic factor, such as maternal TGF-β1. Consistent with this, mice born to TGF-β1$^{-/-}$ mothers show no gross abnormalities in their skeletal muscles, despite the absence of maternal TGF-β1 protein (McLennan *et al.,* 2000).

Does TGF-β2 regulate When and Where Myoblasts fuse In Vivo?

The location and concentration of TGF-β2 protein in developing muscles varies in space and time. This is in marked contrast to the other TGF-β isoforms, which are more uniformly distributed. As discussed above, this variability is consistent with TGF-β2 regulating some aspect of myogenesis.

The TGF-β2 protein in developing and regenerating muscles is principally associated with myoblasts and myotubes (McLennan and Koishi, 1997; unpublished observations). As development proceeds, the distribution of TGF-β2 immunoreactivity within myotubes varies along their long axis. For instance, during secondary myotube formation, the levels of TGF-β2 immunoreactivity are higher at the ends than in the middle of myotubes. This, in conjunction with the proliferation of non-myogenic cells which lack TGF-β2, leads to a sharp decrease in the concentration of TGF-β2 mRNA in the limb bud (Fig. 3). Immediately after the cessation of myotube formation, this distribution changes with TGF-β2-immunoreactivity becoming uniformly distributed along the length of the immature muscle fibres (McLennan and Koishi 1994). This pattern of TGF-β2 distribution parallels that of myoblast-to-myotube fusion. On this basis, we suggest that myotubes release TGF-β2 to stimulate adjacent myoblasts to fuse with them. If so, this would explain why myoblasts with disrupted TGF-β receptors are unable to fuse *in vivo* (Filvaroff *et al.,* 1994).

There are, however, several unresolved issues with respect to the potential role of TGF-β2 in myogenesis. First, the regions where

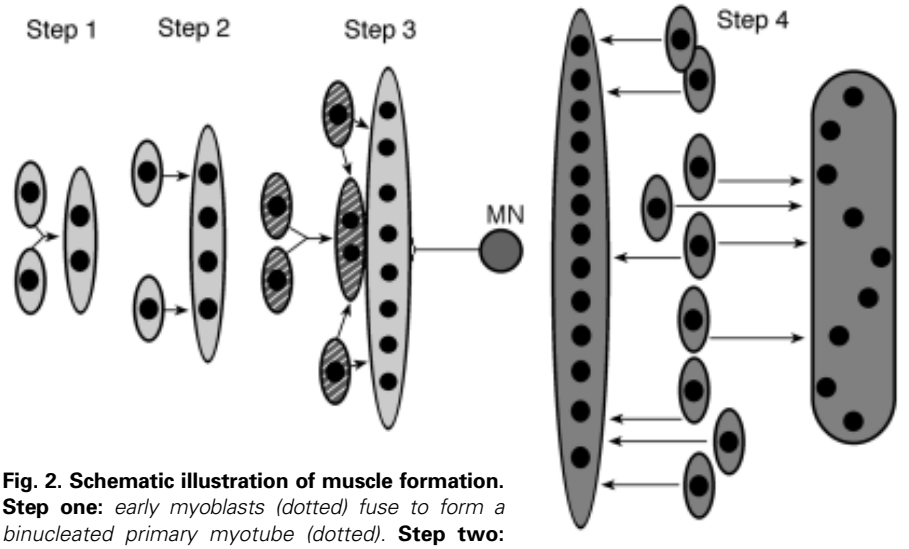

Fig. 2. Schematic illustration of muscle formation. Step one: *early myoblasts (dotted) fuse to form a binucleated primary myotube (dotted).* **Step two:** *myotube formation ceases, with the remaining early myoblasts fusing with existing primary myotubes.* **Step three:** *late myoblasts (striped) fuse with each in synaptic region to form secondary myotubes (striped). In the extra-synaptic region, the late myoblasts fuse with primary and secondary myotubes.* **Step four:** *primary and secondary myotubes have matured to myofibres (hatched) which are absorbing adult myoblasts (hatched). The adult myoblasts preferentially fuse with the ends of the fibres as it elongates, whereas they preferentially fuse with the middle of the fibre as it hypertrophies.*

myotubes have the lowest levels of TGF-β2 during secondary myogenesis (step 3) surround their synaptic regions, where myoblast-to-myoblast fusions occur. The synaptic region is, however, not necessarily devoid of TGF-β2. In the adult, motoneurons produce TGF-β2 and transport it down their axons, toward the synapse (Jiang *et al.,* 2000a). It is unknown whether immature motoneurons also transport TGF-β2 down their axons. If so, TGF-β2 released from nerve terminals could be one of the factors that trigger secondary myotube formation. We are currently testing this hypothesis, by examining the limbs of TGF-β2-/- fetuses.

Another unresolved issue is whether all myoblasts are able to respond to TGF-β2. This issue arises as one of the rodent satellite cell lines (L6) expresses TβRI and TβRII but does not express TβRII-B or betaglycan (Lopez-Casillas *et al.,* 1993; Rotzer *et al.,* 2001). L6 myoblasts are therefore responsive to TGF-β1 and TGF-β3, but not to TGF-β2. This lack of responsiveness to TGF-β2 may be a consequence of the L6 cells having been transformed. Nevertheless, it points to the need for studies to establish whether the responsiveness of myoblasts to TGF-β2 varies with their state of differentiation and/or type (early/late/adult).

Does TGF-β1 regulate the Formation of Connective Tissues?

Skeletal muscles are highly ordered arrays, with distinct myogenic and connective tissue zones. Initially the muscle primordia contain a mixture of myoblasts, fibroblasts and other cell types. The formation of the major connective tissue zones (epi- and perimysium) involves regions of the muscle primordia becoming zones of connective tissue, where the fibroblasts become concentrated and differentiate. Myogenic development must be inhibited in these regions

The creation of connective tissue zones within developing muscles has been postulated to be under the control of TGF-β1

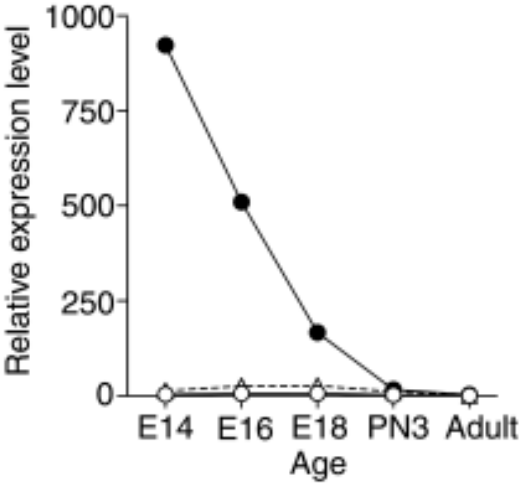

Fig. 3. A graphic illustration of the levels of *TGF-β* mRNA during the development of the hindlimb. *The levels of the messages were measured using semi-quantitative RT-PCR as described in Koishi* et al. *(2000). The copy numbers of the messages were estimated from the signal intensities and were normalised relative to the quantities of* GAPDH *mRNA. The relative levels of* TGF-β1, TGF-β2 *and* TGF-β3 *mRNAs are indicated by open circles, filled circles and triangles, respectively.*

(McLennan, 1993). The accumulation of TGF-β1 is one of the earliest events in skeletal muscle formation, occurring around the time that fibroblasts begin to accumulate in the presumptive connective tissues. At all stages, TGF-β1 is restricted to the developing connective tissues (McLennan, 1993). *In vitro*, fibroblasts migrate towards sources of TGF-β1 (Lucas and Caplan, 1988). If this occurs *in vivo*, then fibroblasts would be attracted to the TGF-β1-rich regions, creating distinct myogenic and connective tissue zones. *In vitro*, TGF-β1 promotes fibroblasts to differentiate (Ignotz and Massague, 1986) and may inhibit myogenic development (see above). Thus the location of TGF-β1 *in vivo* and its actions *in vitro* appear to be consistent with each other.

The putative importance of TGF-β1 for connective tissue formation is however not supported by the phenotype of TGF-β1$^{-/-}$ mice. The connective tissues of these mice develop normally (Poussart *et al.,* 1998), as do the connective tissues of mice that lack maternal TGF-β1 (McLennan *et al.,* 2000). The lack of phenotype in null mutant mice can be due to redundancy in signalling pathways. This is clearly possible here due to the existence of twin (fetal and maternal) sources of TGF-β1 (Letterio *et al.,* 1994; McLennan *et al.,* 1999). However, it is equally possible that the physiological functions of TGF-β1 *in vivo* are much more limited than its actions *in vitro* appear to be. In this context, it is important to note that the accumulation of TGF-β1 in developing connective tissues could be related to TGF-β1's role as an immune regulator (see below).

TGF-β1 and Immune Rejection

The TGF-βs are very potent regulators of the immune system, affecting both cell and antibody-mediated responses (Letterio and Roberts, 1998). One function of TGF-β1 is to regulate the recognition of self and non-self, by controlling the expression of major histocompatibility (MHC) antigens. In TGF-β1$^{-/-}$ mice, the expression of MHC antigens is suppressed by maternal TGF-β1 that is transferred to the fetus via the placenta and to the neonate via milk (Letterio *et al.,* 1994; McLennan *et al.,* 1999). When weaned, TGF-β1$^{-/-}$ mice lose maternal TGF-β1, leading to up-regulation of MHC and death within days due to a generalised auto-immune attack (Boivin *et al.,* 1995).

In addition to controlling autoimmunity, TGF-β1 may also be involved in preventing maternal rejection of a pregnancy. Concepti are as foreign to the mother as tissue transplanted from her children. Consequently, the mother's immune system is able to destroy a conceptus and will do so unless actively prevented. The placenta is the primary defence against the mother's immune system. However, the TGF-βs in amniotic fluid/fetal blood act as potent immunosuppressants (Lang and Searle, 1994). The TGF-β1 in these sources is of dual maternal and fetal origin, with the maternal source being the larger (McLennan *et al.,* 1999).

Some TGF-β1$^{-/-}$ mice die in utero around the time of yolk sac formation. The extent of this early embryonic loss is related to genetic background (Kallapur *et al.,* 1999). However, in one of our colonies (TGF-β1$^{-/-}$, nude $^{+/-}$) the embryonic loss is strongly related to parity. That is, the TGF-β1$^{-/-}$ mutants survive to birth during the mother's first pregnancy but not during subsequent pregnancies, ala anti-Rh (rhesus factor) disease in humans. This suggests that the TGF-β1$^{+/-}$ murine mothers become immunised to their concepti during the first pregnancy, leading to immune rejection of their concepti during subsequent pregnancies.

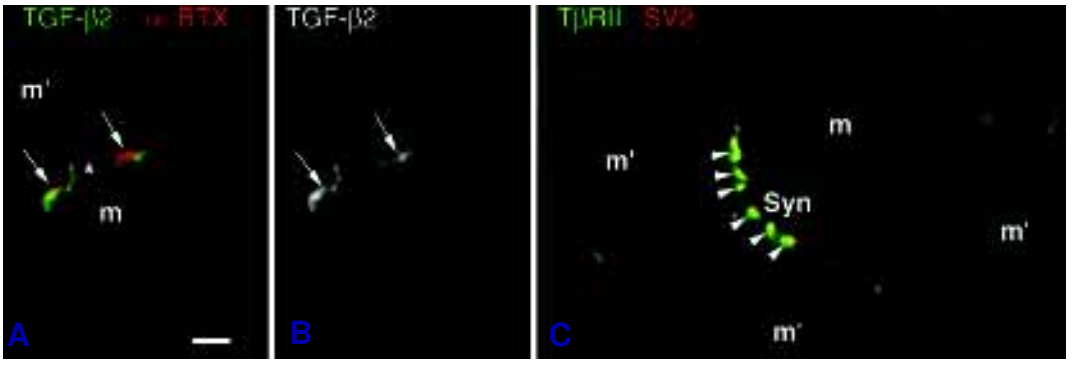

Fig 4. Immunohistochemical localisation of TGF-β2 and TβRII in mature skeletal muscle. (A) *TGF-β2 is concentrated in the post-synaptic domain of a muscle fibre (m). Transverse section stained with anti-TGF-β2 (green channel, arrows) and rhodamine-conjugated alpha-bungarotoxin (α–BTX, post-synaptic membrane, red channel). The TGF-β2-immunoreactivity surrounds a sub-synaptic nucleus, which is marked with an asterisk. The adjacent muscle fibres (m') are unstained.* **(B)** *Green channel of (A) to show the full extent of TGF-β2 immunoreactivity.* **(C)** *TβRII is concentrated in nerve terminal associated the synaptic (syn) portion of a muscle. Transverse section stained with anti-TGF-β2 (green channel) and a marker of synaptic vesicles (anti-SV2, red channel). The nerve terminals are marked by arrowheads. For experimental details, see McLennan and Koishi, 1994 (A/B) and Jiang et al., 2000a (C). The magnification is the same for each panel. Bar, 2 μm. Panels A/B are reprinted from Neuroscience Letters, 177, McLennan IS and Koishi K, "Transforming growth factor-beta-2 (TGF-β2) is associated with mature rat neuromuscular junctions", pp.151-154 (1994), with permission from Elsevier Science.*

TGF-β Regulation of Motoneuron Survival

The Survival and Death of Motoneurons

During development, extensive cell death occurs in motor pools, with over 50% of the motoneurons dying shortly after their axons reach their designated muscle (McLennan 1988; Oppenheim, 1991). The developmental death of neurons appears to involve a competition for a limiting amount of survival factor (Oppenheim, 1991). In the case of motoneurons, the survival factor is provided from the periphery as amputation of the limb bud leads to massively increased death of motoneurons (McLennan and Hendry, 1981; Comans *et al.,* 1988). Until recently, the muscle was generally presumed to be the source of the survival factor, with primary myotubes being of particular importance (McLennan, 1982; 1988). However, recent studies of transgenic mice point to Schwann cells having a much greater role than previously thought.

As motoneurons mature their biological imperatives change. Whereas death is an essential component of the development of the motor nervous system its prevention is the hallmark of the mature system. Adult motoneurons have extremes of size and metabolism that place them at risk of accumulated damage, leading to death. However, they are irreplaceable, with their death leading to a loss of function. To ensure their survival, mature motoneurons have extensive repair mechanisms and receive trophic support from all the cells that they interact with, including central glia and other neurons. Consequently mature motoneurons are resilient to damage even though they are primed for apoptosis, like immature motoneurons (Oorschot and McLennan, 1998).

The search for the growth factors that control motoneuron survival has been long and extensive because of their potential use as therapeutic agents for diseases such as amyotrophic lateral sclerosis. At the start of the search, it was common to talk about "the motoneuron survival factor". The challenge was to find the factor. Today, we are faced with a bewildering abundance of putative motoneuron survival factors. The challenge now is to determine which of these factors are physiologically important and to understand how these multiple factors combine to regulate motoneurons during the different stages of their life.

Most of the putative motoneuron survival factors have been discovered on the basis of sequence similarity to other neuronal survival factors and/or by the use of *in vitro* bioassays of immature motoneurons. TGF-β2 is an exception to this, with its function as a putative motoneuron survival factor being initially postulated on the basis of its anatomical location in mature muscle (McLennan and

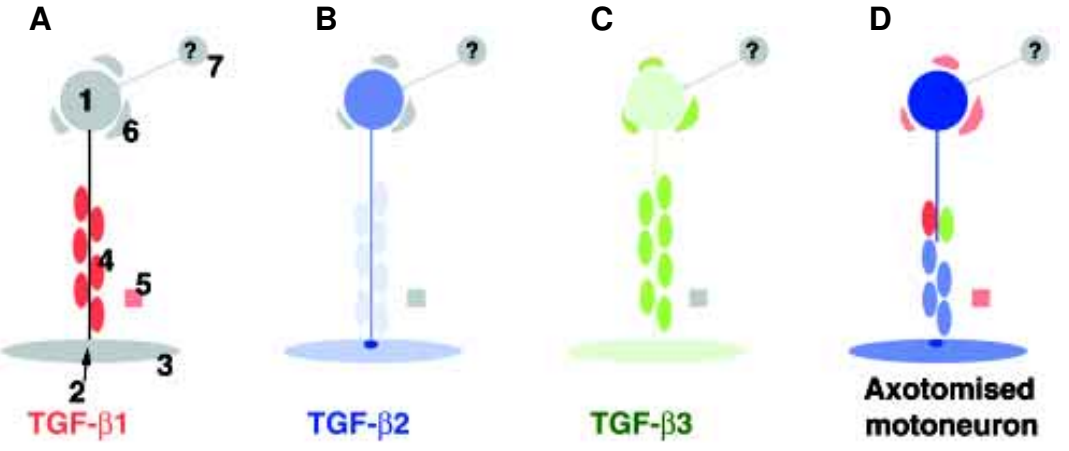

Fig 5. Schematic illustration of the localisation of the TGF-βs in the mature neuromuscular system. (A) *TGF-β1 (red).* **(B)** *TGF-β2 (blue).* **(C)** *TGF-β3 (green).* **(D)** *TGF-β1, TGF-β2 and TGF-β3 after axotomy. The intensity of the colour indicates the relative abundance of the growth factor. Cells with no-expression are shown in light grey whereas a "?" indicates that the levels are unknown. The cells are labelled as follows: 1, the motoneuron; 2, the synaptic region of a skeletal muscle fibre; 3, muscle fibre; 4, Schwann cell; 5, plasma; 6, glia associated with the motor nucleus; 7, neurons which innervate motoneurons.*

Koishi, 1994). We will thus review this anatomical evidence, before discussing the actions of the TGF-βs in the traditional bioassays of motoneuron survival.

TGF-β2 is concentrated in the Post-Synaptic Domain of Adult Fibres

In the adult, the proteins involved in post-synaptic function are concentrated beneath the synapse, with minimal expression in the extra-synaptic portions of muscle fibres. A priori, it is reasonable to assume that any muscle-derived motoneuron survival factor will be similarly located. Most of the putative motoneuron survival factors that are present in skeletal muscles fail this simple criterion, being either located in non-myogenic cells, such as resident macrophages, or being uniformly distributed along the length of muscle fibres (Koishi *et al.,* 1999; Russell *et al.,* 2000; McGeachie *et al.,* 2001). In this respect, our discovery that TGF-β2 is concentrated in the post-synaptic domain of mature rodent and human muscle fibres (McLennan and Koishi, 1994; McLennan *et al.,* 1998; Murakami *et al.,* 1999) sets TGF-β2 apart from most other putative motoneuron survival factors.

Motoneurons express TGF-β Receptors

If the TGF-β2 present in skeletal muscle fibres acts on motoneurons, then the motor nerve terminal must contain all the necessary TGF-β receptor proteins. This is clearly the case for TβRII as antibodies to it intensely stain motor nerve terminals, but not the muscle fibres themselves (Jiang *et al.,* 2000a). We are currently investigating which splice variant of TβRII is present in nerve terminals. However, TβRI and betaglycan are produced by motoneurons and transported anterogradely down their axons, making it likely that they are also present in the nerve terminal (Jiang *et al.,* 2000a). Consequently, nerve terminals are expected to be responsive to TGF-β2 irrespective of which variant of TβRII is present.

The insertion of transmembrane proteins into the axonal and nerve terminal membranes is controlled via sorting signals. This system is analogous to that which regulates the entry of proteins to the nucleus or mitochondria. Consequently, the presence of TβRII in the nerve terminal is very suggestive that it is exposed to a TGF-β. However, TβRII-immunoreactivity is strong on both the muscle and Schwann cell side of the synapse and in the axons (Jiang *et al.,* 2000a), suggesting that motoneurons may be exposed to TGF-βs along their full length and not just from muscle fibres. Consistent with this, motoneurons are surrounded by cells that produce TGF-βs (see below).

Motoneurons may be Exposed to Multiple Sources of TGF-βs

The distribution of TGF-βs in the neuromuscular system is illustrated in Fig. 5. There are several unusual features of this distribution. First, motoneurons are potentially exposed to all three isoforms: TGF-β1 from the blood and Schwann cells; TGF-β2 from muscle fibres; and low levels of TGF-β3 in Schwann cells (Bottner *et al.,* 2000; Jiang *et al.,* 2000a). Second, this supply appears to have mechanisms that compensate for the loss of any of these sources. For instance, axonal damage isolates the motoneuron from skeletal muscle-derived TGF-β2 and from Schwann cell-derived TGF-β1. But, it also leads to new sources of TGF-β by inducing the glia associated with motor nuclei to produce TGF-β1 (Kiefer *et al.,* 1993) and the Schwann cells in the damaged nerve to produce TGF-β2 (Jiang *et al.,* 2000a).

TGF-β2 may be an Autocrine Regulator of Motoneurons

Adult neurons, unlike other cell types, have exceptionally strong autocrine survival mechanisms (Acheson *et al.,* 1995), possibly because they are irreplaceable. Motoneurons produce both TGF-β2 and TGF-β receptors, which raises the issue of whether motoneurons are producing TGF-β2 to promote their own survival and/or to affect the cells adjacent to them (Jiang *et al.,* 2000a).

The TGF-β2 produced by motoneurons is transported anterogradely down axon (towards the muscle fibres), making it potentially available to the TGF-β receptors on axons (see above) (Jiang *et al.,* 2000a). The movement of a putative survival factor from the nucleus towards the periphery of a neuron is in apparent contradiction to the traditional neurotrophic hypothesis, where neuronal survival factors are collected in the periphery and retrogradely towards the cell body. However, there is a growing body of evidence that the initiation of apoptosis can occur in axons, dendrites or nerve terminals (Mattson *et al.,* 1998). Consequently, there is a need for autocrine survival mechanisms to suppress apoptosis throughout the entirety of a neuron, and not just its cell body. In this context, the movement of an autocrine survival factor would be expected to be from the perikaryon to all other parts of the neuron, as observed for TGF-β2.

TGF-β2 promotes the Survival of Injured Motoneurons

The molecular anatomical evidence suggests that motoneurons receive TGF-β stimulation *in vivo*, but what effects do the TGF-βs produce on motoneurons? To date, little work has been done on this. When mature motoneurons are damaged, their metabolism switches from a functional mode to a growth mode. This switching appears to be due to the loss of a signal from muscle fibres. TGF-β2 is present in muscle fibres but it is unlikely to control whether motoneurons are in a functional or a growth mode. This is because distal axon damage is unlikely to reduce the supply of TGF-β2 to motoneurons, due to the ability of Schwann cells to produce TGF-β2 after axon damage (see above, Fig. 5). Consistent with this, TGF-β2 does not prevent axotomised motoneurons from down-regulating their levels of the neurotransmitter synthetic enzyme, choline acetyltransferase (Jiang *et al.,* 2000b).

The multiple locations of the TGF-βs are suggestive that they are anti-apoptotic. Adult motoneurons are able to survive damage, but will slowly die if the injury is severe enough. Motoneurons can be fatally damaged by avulsion, which completely removes their axon, thus depriving motoneurons of both Schwann-cell-derived and muscle-fibre-derived factors. Under these extreme circumstances, motoneurons lose much of their endogenous supply of TGF-β. Replacement of this loss by infusion of TGF-β2 adjacent to the perikaryon of avulsed motoneurons attenuate their death (Jiang *et al.,* 2000b). In this bioassay, TGF-β2 is more potent than most other motoneuron survival factors, suggesting that it is a key regulator of motoneuron survival. Further testing of this idea is needed however, which we are currently undertaking using a range of transgenic mice.

The trophic requirements of some neurons change as they develop. Consequently, the apparent involvement of the TGF-βs in the survival of adult motoneurons does not prove that they are similarly involved during development. The evidence for this is considered below.

Developing Motoneurons may be exposed to Multiple Sources of TGF-βs

Immature motoneurons appear to have access to abundant sources of TGF-β. At the onset of motoneuron cell death, the levels of TGF-β2 mRNA and protein in developing muscles are high. As discussed previously in connection with muscle fibre formation, the levels of TGF-β2 in the synaptic region become low during secondary myogenesis (step 3, Fig 2). However, motoneuron cell death is largely complete before this time (McLennan, 1982; 1988). Furthermore, there are other sources of TGF-βs in the developing neuromuscular system which motoneurons may be exposed to. These are TGF-β2 from the immature motoneurons and TGF-β1 from fetal plasma (McLennan *et al.,* 1999) and immature Schwann cells (McLennan, unpublished observations).

During neuronal cell death, the supply of survival factor is postulated to be limiting. It is unclear how this occurs for any survival factor working on any neuron, including the archetype survival factor, nerve growth factor. Nevertheless, the apparent abundance of TGF-βs raises questions about whether they act as traditional neuronal survival factors. These concerns are amplified by recent studies using various *in vitro* and *in vivo* assays of neuronal survival.

Are the TGF-βs Apoptotic or Anti-Apoptotic for Developing Motoneurons?

The survival of immature motoneurons appears to be regulated by the TGF-βs, but it is currently unclear what the essential nature of this regulation is. Some of the actions of the TGF-βs are non-classical whereas others are paradoxical.

First, most neuronal survival factors only affect the survival of a comparatively small number of neuronal cell types. In marked contrast to this, the TGF-βs influence the survival of many types of neurons. This was initially overlooked as TGF-β1 is a hormone, and is therefore present in the various sera included in culture medium.

Second, in the absence of TGF-β some neurons lose their ability to respond to other neuronal survival factors. Conversely, in the absence other survival factors, the TGF-βs fail to stimulate the survival of neurons *in vitro*, with motoneurons being the only known exception to this (Bottner *et al.,* 2000).

Third, in contrast to the above, Krieglstein *et al.* have recently shown that administration of either a TGF-β antagonist or anti-TGF-β antibodies to chicken embryo reduces rather than increases the extent of motoneuron cell death (Krieglstein *et al.,* 2000). This has been interpreted to suggest that the TGF-βs have pro-apoptotic functions on neurons, as is known to occur with some non-neuronal cell types (Krieglstein *et al.,* 2000). However, other explanations are possible. The TGF-βs regulate the synthesis of various growth factors. Consequently, neutralisation of TGF-β *in vivo* could increase neuronal survival by increasing the production/release of other neuronal survival factors.

One of the more intriguing questions raised by Krieglstein's study is whether the reduced death arise through antagonism of a particular isoform of TGF-β, or whether this phenomenon only arises when the action of all three isoforms are simultaneously inhibited. In particular, the reagents used in Krieglstein's study are more potent antagonists of TGF-β1 and TGF-β3 than TGFβ2. It could thus be argued that the observation by Krieglstein *et al.* arises due to the creation of an imbalance between TGF-β2 (via TβRII-B) and TGF-β1/β3 (TβR-II) stimulation of neurons. However, in apparent disproof of this possibility, TGF-$\beta1^{-/-}$ neonates have normal numbers of motoneurons (Jiang, 2000). Similarly, the extent of axotomy-induced loss of mature motoneurons is normal in the absence of TGF-β1 (McLennan, unpublished observations). The examination of TGF-$\beta2^{-/-}$ and other genetically modified mice is likely to resolve the issues raised above.

TGF-β1 as an Autocrine Regulator of the Myelin-Sheath

In the section above, the possibility that Schwann cells use TGF-βs to communicate with motoneurons was discussed. However, Schwann cells express TβRI and TβRII and are therefore responsive to TGF-β1 and TGF-β3 (Jiang *et al.,* 2000a). This raises the possibility that the TGF-β1 produced by Schwann cells has an autocrine function. Consistent with this, the myelin sheaths in mature TGF-$\beta1^{-/-}$, $nude^{-/-}$ mice exhibit distinct perinodal pathology (Day *et al.,* 1999). These mice were exposed to TGF-β1 until weaned, suggesting that the abnormal myelination is due to aberrant maintenance, rather than abnormal formation, of the myelin sheath. This not withstanding, TGF-β1 may also be important for the development of Schwann cells as it is expressed by immature Schwann cells and produces a variety of affects on Schwann cells *in vitro*.

Can the TGF-βs mediate Diverse Tasks in a Single Anatomical Location?

There is a large and growing body of evidence implicating the TGF-βs as regulators of motoneurons, myoblasts, myotubes, fibroblasts, Schwann cells and immune rejection. When considered in isolation, each of the putative functions of the TGF-βs seems reasonable. But can they all co-exist within the confines of a single system? For instance, can myotubes control the absorption of myoblasts by releasing TGF-β2 without simultaneously affecting the development of the connective tissues surrounding them and the motoneurons that innervate them? Similarly, can Schwann cells use TGF-β1 to regulate some aspects of their own function without affecting the survival of the motor axons they surround or without inducing fibrosis in the perineurum? When considering this type of issue, the most problematic is endocrine TGF-β1. All cells appear to need to be continually exposed to endocrine TGF-β1 in order to prevent immune rejection. However, if cells are continually exposed to endocrine TGF-β1, how can they use autocrine or paracrine TGF-βs to produce biological responses that are quite distinct from that elicited by endocrine TGF-β1?

Multiple theoretical answers exist to these questions, but there is little experimental evidence by which to judge them. It is possible that some of the actions of the TGF-βs are intracrine, rather than autocrine. It is possible that cells respond differently to different concentrations of TGF-βs and/or can differentiate between continual versus episodic exposure to TGF-βs. However, perhaps the largest issue here is whether the reductionist view of considering the TGF-βs as isolated regulators is correct. The actions of growth factors are contextual. As noted above, myoblasts and motoneurons *in vitro* respond very differently to TGF-βs depending on which other growth factors are present. If this is occurring *in vivo*, then the TGF-βs may be able to produce a vast array of biological actions within a single biological system because they work in concert with factors that are more discretely localised. If so, we need to study the

actions of the TGF-βs within their physiological context. This is now possible by using mice with cell-type- and temporal-specific-defects. Such mice may enable us to begin to understand the biology of the TGF-βs and the neuromuscular system in a more holistic manner.

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