Elucidating the molecular mechanisms that underlie the target control of motoneuron death

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ABSTRACT Approximately half of the motoneurons generated during normal embryonic development undergo programmed cell death. Most of this death occurs during the time when synaptic connections are being formed between motoneurons and their target, skeletal muscle. Subsequent muscle activity stemming from this connection helps determine the final number of surviving motoneurons. These observations have given rise to the idea that motoneuron survival is dependent upon access to muscle derived trophic factors, presumably through intact neuromuscular synapses. However, it is not yet understood how the muscle regulates the supply of such trophic factors, or if there are additional mechanisms operating to control the fate of the innervating motoneuron. Recent observations have highlighted target independent mechanisms that also operate to support the survival of motoneurons, such as early trophic-independent periods of motoneuron death, trophic factors derived from Schwann cells and selection of motoneurons during pathfinding. Here we review recent investigations into motoneuron cell death when the molecular signalling between motoneurons and muscle has been genetically disrupted. From these studies, we suggest that in addition to trophic factors from muscle and/or Schwann cells, specific adhesive interactions between motoneurons and muscle are needed to regulate motoneuron survival. Such interactions, along with intact synaptic basal lamina, may help to regulate the supply and presentation of trophic factors to motoneurons.

KEY WORDS: Motor neuron survival, basal lamina, agrin, anoikis, trophic factor, branching, programmed cell death

Introduction

A large percentage of motoneurons undergo apoptosis or programmed cell death (PCD) during embryonic development in vertebrates. Historically, most work elucidating the molecular mechanisms that control motoneuron death stem from target ablation and pharmacological experiments in the chick. These experiments show that both trophic factors and activity of motor units (α-motoneurons and the skeletal muscle fibres they innervate) are important in regulating the number of surviving motoneurons during embryonic development. More recently, molecular genetic studies show specific cellular interactions are required for the survival of motor units. This review provides a brief background on how the motor unit controls motoneuron survival during embryonic development. We then propose a novel mechanism by which motoneurons are selected to live or die through cell-matrix interactions (anoikis) at the neuromuscular synapse. We then describe ways in which our laboratory is testing this theory with specific reference to agrin, rapsyn and MuSK-deficient mice.

The Spatio-Temporal Sequence of Motoneuron Death

The spatio-temporal succession of motoneuron death in the spinal cord is an ordered morphological event (Yamamoto and Henderson, 1999). Apoptotic nuclei are evident in the cervical region of the spinal cord followed by thoracic, lumbar and sacral motoneurons in mice and rats (Yamamoto and Henderson, 1999). The chick has a somewhat different sequence of motoneuron death with cervical motoneurons dying first, followed by lumbar, thoracic, brachial and sacral (Yamamoto and Henderson, 1999). This stereotyped sequence indicates that motoneuron death during normal embryonic development is a highly controlled process.
The first signs of cell death are in proliferating neural precursor cells and young postmitotic neuroblasts (de la Rosa and de Pablo, 2000). This period of cell death begins in neurons that are projecting toward their target and is named projecting-neuron cell death. This form of cell death is unresponsive to trophic factors and is also independent of interactions with the target, skeletal muscle (Yaginuma et al., 1996). The authors suggest that this period of cell death is cell autonomous or is dependent on other interactions within the central nervous system (CNS) (Yaginuma et al., 1996).

Projecting motoneuron death may also be explained by motoneuron-motoneuron, or motoneuron-target interactions during the “waiting period” of axonal outgrowth (Tosney and Landmesser, 1985; Wang and Scott, 2000; Eisen and Melancon, 2001). In the chick, motoneurons extend axons to their primordial limb bud and wait for approximately 24 hours until their target muscle differentiates and proliferates before innervating the muscle (Tosney and Landmesser, 1985; Wang and Scott, 2000). This waiting period also exists in the zebrafish and is known to be a selection point between two identified motoneurons where only one motoneuron may grow towards its final innervation site and the other dies (Eisen and Melancon, 2001). The selection process is thought to depend on interactions between the growing axons and its target muscle (Eisen and Melancon, 2001). Selection of motoneurons to live or die at the waiting period in mammals is yet to be studied. The study of early PCD is in its infancy making its significance in moulding the CNS unclear (reviewed in de la Rosa and de Pablo, 2000).

Although motoneurons may undergo projecting cell death early in embryonic development, most motoneurons that undergo PCD die during the period when skeletal muscle becomes functionally innervated (Hamburger, 1975; Chu-Wang and Oppenheim, 1978; Dahm and Landmesser, 1991; Hamburger and Hamilton, 1992) as described in the subsequent sections.

**Motoneurons Require the Presence of the Target Skeletal Muscle and the Schwann Cell to Survive PCD**

Shorey (1909) first documented decreases in motoneuron number after amputating the target limb, however she thought that the decrease was due to changes in early motoneuron differentiation (Shorey, 1909). It was only later that Victor Hamburger and Levi Montalcini recognised that the decrease in motoneuron number after limb ablation was an exacerbation of the normal PCD of motoneurons and concluded that the target limb may regulate motoneuron survival during embryonic development (Hamburger, 1934; reviewed in Oppenheim, 1991). As the limb is a heterogeneous mixture of many cell types it was not known what target cells promoted the survival of motoneurons. Surgical removal of somites, the developmental precursors of muscle cells, increased motoneuron death like that in limb bud ablation providing evidence that motoneuron survival is dependent on their target skeletal muscle fibres (Phelan and Hollyday, 1991). Further evidence that motoneurons depend on their target skeletal muscle to survive stems from transgenic mice that lack skeletal muscle which retain only 10% of the normal number of surviving motoneurons (Grieshammer et al., 1998). Thus, motoneurons depend on the presence of the skeletal muscle fibre to survive.

Motoneurons may also require Schwann cells to survive the period of naturally occurring PCD. Deletion of Erb-b3 gene leads to the absence of Schwann cell precursors and the formation of Schwann cells surrounding both motor and sensory neurons (Riethmacher et al., 1997). This resulted in the death of 75% of the peak number of motoneurons during the period of PCD (Riethmacher et al., 1997). The large increase in motoneuron death occurred between E15.5 and E18.5 indicating that the Schwann cells are not required for neurite growth to their target, but are required during the period when muscles become innervated and functional (Riethmacher et al., 1997). Thus, other contributing factors to motoneuron death may occur in these mice including changed electrical activity of the motoneuron or an involvement of Erb-b3 in regulating the expression of trophic factors (Riethmacher et al., 1997). To test these possibilities, the generation of Cre-conditional transgenic mice that lack specifically Schwann cells is required (Grieshammer et al., 1998). Taken together, it appears that Schwann cells are insufficient to rescue motoneurons in the absence of skeletal muscle and the muscle fibre is insufficient to rescue motoneurons without Schwann cells.

Results from the target ablation studies led to the proposal that skeletal muscle contains trophic factors that promote motoneuron survival during embryonic development (Hamburger and Levi-Montalcini 1949). Indeed trophic factors from the muscle and Schwann cell are retrogradely transported to the motoneuron cell body (Bartlett et al., 1998; Reynolds et al., 2000). Comprehensive reviews of trophic factors that promote motoneuron survival are presented elsewhere (Henderson et al., 1998; Sendtner et al., 2000). Although muscle derived trophic factors promote motoneuron survival after limb ablation, only a small proportion of motoneurons that normally die after limb ablation survive with the addition of muscle extract, which presumably contains all muscle derived trophic factors (Caldero et al., 1998). Therefore, it is likely that structural and functional aspects of muscle differentiation are also required to support motoneurons during embryonic development.

**Muscle Activity, Motoneuron Survival and Intramuscular Branching are intricately related during Embryonic Development**

Blocking neuromuscular activity with acetylcholine receptor (AChR) antagonists such as d-tubocurare, α-cobratoxin and α-bungarotoxin during embryonic development promotes motoneuron survival in the chick (Pittman and Oppenheim, 1978; Pittman and Oppenheim, 1979). In fact nearly all motoneurons survived the period of motoneuron death when muscle activity is absent (Pittman and Oppenheim, 1978; Pittman and Oppenheim, 1979). Similar responses are found in the mouse (Houenou et al., 1990) and rat (Harris and McCaig, 1984). Conversely, activating muscle and nerve during the period of PCD increased the number of dying motoneurons (Oppenheim and Nunez, 1982). These studies indicate that motoneuron survival is not only dependent on the presence of skeletal muscle but also dependent on its activity.

Blocking neuromuscular activity not only stops motoneuron death, but also increases motor neurite branching within the muscle and the number of neuromuscular synapses (Pittman and Oppenheim, 1979; Harris and McCaig 1984; Dahm and Landmesser, 1988, 1991; Houenou et al., 1990; reviewed in Landmesser, 1992). Both paralytic and sub-paralytic doses of d-tubocurare promote the survival of motoneurons, increase branching and the number of neuromuscular synapses during embryonic development (Oppenheim et al., 2000). The promotion of moto-
neuron survival, branching and neuromuscular synapse formation is significantly greater with paralytic versus sub-paralytic doses of \( \alpha \)-tubocurare indicating that changes in motoneuron survival results from the precise regulation of muscle activity (Oppenheim et al., 2000).

Furthermore, a close relationship between motoneuron survival and innervation exists when the extent of branching is decreased with endoneuraminidase (endo N; Tang and Landmesser, 1993; Usiak and Landmesser, 1999). Administration of \( \alpha \)-tubocurare increases the level of polysialic acid and neuron cell adhesion molecule (NCAM) which can subsequently be removed by endo N (Tang and Landmesser, 1993; Usiak and Landmesser, 1999). Endo N decreases branching and innervation and also decreases the number of surviving motoneurons which would otherwise be increased by \( \alpha \)-tubocurare treatment (Tang and Landmesser, 1993; Usiak and Landmesser, 1999). Interestingly, this reduction in survival and branching may be due to a change in transmitter release from the motor neurite terminals (Rafuse et al., 2000), however this needs to be directly tested. Together these studies indicate a precise relationship between motoneuron survival, intramuscular axonal branching and neuromuscular synapse formation that are regulated by muscle activity.

**Breaking the Relationship between Muscle Activity, Motoneuron Survival and Branching when comparing Agrin, Rapsyn and MuSK-Deficient Mice**

The neuromuscular synapse is a highly specialised connection between the motor nerve terminal and the skeletal muscle fibre (Sanes and Lichtman, 1999). The motor nerve terminal occupies only 0.1% of the muscle membrane and is able to transduce signals from the motor nerve terminal to the skeletal muscle fibre to induce muscle contraction. The specialisations include active zones in the pre-synaptic terminal that align adjacent to AChR clusters on muscle fibre junctional folds (Fig. 1). Acetylcholine receptors concentrate to 10,000/\( \mu \text{m}^2 \) in the synapse compared to 10/\( \mu \text{m}^2 \) in non-synaptic areas (Colledge and Froehner, 1998). During development of the neuromuscular synapse, agrin is thought to be secreted by the nerve terminal to activate its muscle specific kinase receptor (MuSK) to bring about AChR clustering through a receptor associated protein of the synapse, rapsyn (Apel and Merlie, 1995; Apel et al., 1997; Fuhrer et al., 1999; Gautam et al., 1999). Recently, the genes that encode these proteins were knocked out in mice. These mice have few or no AChR clusters underneath motor nerve terminals and show varying degrees of disruption of innervation and transcription of AChRs (Gautam et al., 1995; DeChiara et al., 1996; Gautam et al., 1996).

Rapsyn-deficient mice live for approximately six hours after birth and are thought to have the least synaptic disruption of the three mice generated (Gautam et al., 1995). AChR clusters are not found in rapsyn-deficient mice, however membrane bound AChRs are found in the centre of the muscle (Gautam et al., 1995). The restricted AChR expression is brought about by centrally located muscle nuclei (synaptic nuclei) that retain high levels of gene transcription for the AChR subunits in rapsyn-deficient mice similar to that in wild-type mice (Gautam et al., 1995). Agrin-deficient mice die at birth and are thought to have a disrupted synapse that is more severe than rapsyn-deficient, but not as disrupted as MuSK-deficient mice (Gautam et al., 1996). Agrin is not required for the initial formation of AChR clusters (Lin et al., 2001), however it is required for their maintenance and maturation (Gautam et al., 1996). Thus, agrin-deficient mice have AChR clusters at neurite terminals at the initial stages of neuromuscular synaptogenesis but these receptor clusters soon dissipate (Gautam et al., 1996). Moreover, transcriptional specialisation of AChR in the centre of muscle is lost in agrin-deficient mice so that membrane bound AChRs are found throughout the muscle (Gautam et al., 1996).

MuSK-deficient mice also die at birth and are thought to have the
most disrupted synapse of the three lines of mutant mice (DeChiara et al., 1996). MuSK-deficient mice lack AChR clusters and transcriptional specialisation of AChRs by sub-synaptic nuclei in the centre of the muscle (DeChiara et al., 1996). Thus, synapse disruption (and presumably disruption of muscle contraction) is greatest in MuSK followed by agrin followed by rapsyn (Gautam et al., 1999).

As the level of muscle activity regulates motoneuron survival, branching and synapse formation we tested whether the same applies when comparing rapsyn and agrin-deficient mice (Banks et al., 2001; Noakes et al., 2001). We found that the force of muscle contraction reflected the biochemical disruption with rapsyn-deficient mice having a significantly greater muscle contraction compared to agrin-deficient mice (Noakes et al., 2001). The decreased muscle contraction induced greater branching in agrin-deficient mice (Banks et al., 2001; Noakes et al., 2001), but not motoneuron survival compared to rapsyn-deficient mice at the end of the motoneuron death period in accordance with previous results (Banks et al., 2001; Noakes et al., 2001; Terrado et al., 2001). By quantifying motoneuron numbers throughout the period of naturally occurring PCD we found that the number of motoneurons significantly decreased at the same time as the skeletal muscle basal lamina became disorganised in agrin-deficient mice at E16.5 (Noakes et al., 2001). This does not occur in rapsyn-deficient mice (Gautam et al., 1995; Banks et al., 2001; Noakes et al., 2001). Thus, it appears that the basal lamina is important for motoneuron survival during embryonic development. Basal lamina components are known to enhance the motoneuron survival properties of trophic factors (especially CNTF) in vitro (reviewed in Sendtner et al., 2000). Recently CNTF receptor α has been found to associate with the extracellular matrix (Gould et al., 2001) and is important in regulating motoneuron survival in vitro (DeChiara et al., 1995; Bartlett et al., 2001; however see Terrado et al., 2001). The disruption of the basal lamina and subsequent inappropriate contact between the growing motor neurite terminal with muscle is likely to allow motoneuron death in agrin-deficient mice.

Motoneurons Attach to the Muscle Fibre through the Synaptic Basal Lamina

As motoneurons require the presence of the skeletal muscle to survive, it is interesting to note that motoneurons do not directly attach to the muscle fibre. Sandw iched between the motor nerve terminal and the postsynaptic membrane of the muscle is basal lamina, known as synaptic basal lamina (Fig. 1). Experiments where the muscle has first been denervated, followed by removal of both muscle and Schwann cells but leaving an intact synaptic basal lamina is enough to direct re-innervating motor neurons to stop at synaptic basal lamina and differentiate into a functional nerve terminal (Glicksman and Sanes, 1983). Likewise, if the muscle is allowed to regenerate within basal lamina tubes but the motor nerves or Schwann cells are not allowed to enter, the portion of the muscle membrane under the original synaptic basal lamina will form postsynaptic specialisations (Sanes et al., 1978). Thus, the synaptic basal lamina can mediate both pre and postsynaptic differentiation. This idea lead investigators to identify and clone molecules such as β2-laminin and neural agrin that are unique to the synaptic basal lamina and which induce pre- and postsynaptic differentiation respectively (Hall and Sanes, 1993; Noakes et al., 1995; Gautam et al., 1996; Burgess et al., 1999; Burgess et al., 2000). In this section we give a brief overview of the organisation of the muscle basal lamina before we propose how the basal lamina may function to regulate motoneuron survival.

Muscle fibres are surrounded by the sarcolemma, a highly organised structure consisting of the muscles plasma membrane, overlying basement membrane and glyocalyx (Fig 1). The basement membrane is further divided into reticular lamina and basal lamina. The basal lamina comprises an electron dense lamina densa and an electron lucent lamina rara (Sanes, 1994). At synaptic sites, only the basal lamina is present between the motor nerve terminal and underlying muscle fiber (see Fig 1). The molecular composition of the sarcolemma consists of some of the following molecules: integrins and proteins of the dystrophin associated complex in the plasma membrane; fibronectin, laminins, collagens (I, III, IV and V), entactin, and agrin in the basement membrane and glyocalyx (Sanes, 1994).

The basal lamina functions in providing a strong adhesive connection between the motor nerve terminal and the muscle fibre in an environment where fibres are continually changing length in response to motor nerve stimulation. It is also vital for the differentiation and formation of skeletal muscle as shown in many muscular dystrophy diseases. Agrin is a vital contingent of the muscle basal lamina (Bezakova and Lomo, 2001; Moll et al., 2001; reviewed in; Hagiwara and Fallon, 2001). Muscular dystrophy models that lack α2-laminin show a disrupted basal lamina that is somewhat rescued by agrin (Moll et al., 2001). This process may involve the reorganising of castomeres in the muscle fibre membrane by agrin (Bezakova and Lomo, 2001). Thus, agrin is important for basal lamina formation in non-synaptic regions of the basal lamina.

So far the role of agrin at the neuromuscular junction is best known by its ability to cluster postsynaptic AChRs, thereby promoting maturation of the synapse which provides efficient transmission from the nerve terminal to the muscle (Gautam et al., 1996; Sanes and Lichtman, 1999). We propose here that the synaptic basal lamina induced by neural agrin may also function in governing motoneuron survival during embryonic development. Agrin deposited into the synaptic basal lamina binds to laminin trimers through an interaction with the γ1 chain, a subunit present in all synaptic laminin trimers (Kammerer et al., 1999). Stabilisation and concentration of laminins, in particular laminins 9 and 11 (α4,β2,γ1 and α5,β2,γ1 respectively), by agrin could be critical for increased synaptic efficacy during development, and in part, determine motoneuron fate. In our proposed model (see Fig. 2), agrin is needed to present laminin 9 and 11 to its pre-synaptic receptors that associate with the mechanics of transmitter release from the motor nerve terminal. The pre-synaptic receptor for the α5 subunit of laminin 11 appears to be the transmembrane proteoglycan SV2 (Son et al., 2000), which is present on synaptic vesicles and becomes exposed on the pre-synaptic membrane upon fusion of these vesicles. It is proposed that the SV2-α5 interaction promotes pre-synaptic adhesion to the synaptic basal lamina (Son et al., 2000). The pre-synaptic receptor for the α4 subunit of laminin 9 is not known, but is thought to be closely associated with the pre-synaptic calcium channels suggesting that α4 interaction with such a receptor could promote increases in transmitter release (Sunderland et al., 2000). Finally, laminins 4, 9 and 11 could also interact with pre-synaptic integrin receptors to promote transmitter release (Wong et al., 1999). Together, these observations suggest that the stabilisation and presentation of synaptic laminins through agrin play a key role in survival of the motoneuron.
Cell-Matrix Interactions regulate Cell Survival: Anoikis

Anoikis is “the subset of apoptosis triggered by inadequate or inappropriate cell-matrix contacts” (Frisch and Screaton, 2001). Anoikis was first found to occur in epithelial cells so correct cell numbers are maintained in an environment where constant turnover of cells is required. Anoikis is currently a major area of research due to its role in the spread of cancer. Prevention of anoikis is required for neoplasia as a cell must detach from the matrix, translocate to a different site within the body and then re-attach to spread tumours (Ruoslahti, 1999). From this recent literature, many similarities between matrix molecules that regulate anoikis of epithelial cells and those matrix molecules in the synaptic basal lamina that regulate motor neurite terminal adhesion to their target muscle fibres are becoming apparent.

Cell-matrix adhesion dependent death is thought to be primarily dependent on integrins via activation of integrin-linked kinase (Delcommenne et al., 1998; Attwell et al., 2000). Integrins are transmembrane heterodimers that function as low affinity receptors for fibronectin and laminin to attach a cell to the surrounding extracellular matrix. They also directly associate with receptor tyrosine kinases (Sundberg and Rubin, 1996; Falcioni et al., 1997; Schneller et al., 1997; Moro et al., 1998) and activate signalling cascades that regulate gene expression (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). The laminins and integrins that are implicated in anoikis of epithelial cells are those that are also found at the neuromuscular junction. By drawing correlations between these laminins and integrins we can begin to test whether motoneuron survival during embryonic development is dependent on its interactions with the skeletal muscle basal lamina.

The α1, α3-7, αv and β1 integrins are found at the presynaptic terminal and α1, α3, α7, α9, αv and β1 are localised to the postsynaptic membrane (Martin et al., 1996). Integrins containing αv and β1 subunits are involved in agrin-induced AChR clusters as blockade of αv, β1 integrins inhibit agrin induced AChR clusters (Martin and Sanes, 1997). Blocking antibodies to β1 integrin increase the number of dying motoneurons after nerve crush injury (Wong et al., 1999). Thus, integrin dimers containing αv and β1 subunits may have an altered distribution in agrin-deficient mice but not in rapsyn-deficient mice and may account for the sudden drop in motoneuron death when the basal lamina becomes patchy.

Integrin dimers containing αv and β1 play major roles in anoikis. Anoikis of human colon cancer cells and differentiated human neuroblastoma cells is dependent on β1 integrin/matrix interactions (Strater et al., 1996; Bonfoco et al., 2000). Human carcinoma intestinal cells (Caco-2) are resistant to anoikis due to a low expression of αv integrin (Kozlova et al., 2001). Expression of αvβ3 integrin in Caco-2 cells makes them much more susceptible to anoikis (Kozlova et al., 2001). The αv integrin regulates angiogenesis of endothelial cells in carcinomas (Ruoslahti, 1999).

**Fig. 2. A proposed model of the synaptic domains at stable and unstable neuromuscular junctions.** At stable synapses, agrin (A, red) is deposited into the synaptic cleft where it can bind to synaptic laminins 4, 9 and/or 11 (L). This allows for laminins to interact with pre-synaptic receptors at the nerve terminal. These receptor interactions include α5 laminin binding to a receptor complex associated with calcium channels (CCAR), α4 laminin binding to SV receptor at sites of synaptic vesicle (SV) release at active zones (*), and possible (?) interactions of all three laminins with pre-synaptic integrin receptors (Int, yellow). In addition, these laminins and agrin can bind to postsynaptic receptor complexes as well. These include the following: laminin and agrin binding to α-dystroglycan (α), part of the dystrophin associated transmembrane complex (α±DG) that links to the muscle’s cytoskeleton through utrophin (Ut) and F-actin; agrin binding to the muscle specific kinase (MuSK) complex that is composed of a muscle associated specific component (MASC) and transmembrane linker (R) to rapsyn (Rap), which upon agrin binding induces phosphorylation of MuSK (red P) which causes acetylcholine receptor (AChR) clustering under nerve terminals via rapsyn; and potential (?) interactions of laminins with integrin receptors in the postsynaptic membrane. Together these molecular interactions bring about stable adhesion of pre- and postsynaptic elements to promote motoneuron viability. At unstable synapses, when molecules such as agrin are missing the presentation of laminins to both pre- and postsynaptic receptors are lost and/or are less stable (dashed arrows). In the case of missing agrin, induction of postsynaptic specialisations are not maintained. Together, this could result in unstable adhesion interactions between pre and postsynaptic elements that result in loss of these nerve terminals, and death of the motoneuron. This could be triggered by a number of mechanisms such as: a drop in transmitter release through a loss of laminin pre-synaptic receptor interaction; a loss of laminin presynaptic integrin interaction that could trigger anoikis; a failure to induce postsynaptic specialisations due to a loss agrin-MuSK interactions; and/or by a loss of laminin and agrin interacting with α-dystroglycan.
However, the induction of cell death using αvβ3 antagonists may occur just prior to detachment (Brassard et al., 1999). Integrins αv and β1 are likely candidates to function in regulating motoneuron survival during embryonic development through anoikis. Besides integrin αv and β1, localising laminins and integrins to the neuromuscular synapse, and finding laminins and integrins that function in anoikis is still in its infancy leaving open many other possibilities, the most promising include integrin α7 and laminins α5 and β2.

**Trophic Factors which Promote Motoneuron Survival and Synaptic Plasticity may Inhibit Anoikis**

The proposal that motoneurons undergo anoikis implicates that adhesion between the motor nerve terminal and the synaptic basal lamina is required for cell survival. However, neuromuscular synapse formation is highly plastic during the coincident period of motoneuron PCD leaving open the question as to how motor units are able to survive while they are establishing synaptic connections.

Trophic factors and integrins that promote motor neurite terminal plasticity in vivo include glial derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF) (D’Costa et al., 1998; Ivankovic-Dikic et al., 2000; Keller-Peck et al., 2001). Administration of IGF into developing chick embryos promotes motor terminal branching and synapse formation and ultimately motoneuron survival (D’Costa et al., 1998). It is thought that through increased terminal endings, motoneurons have greater access to muscle derived trophic factors allowing more motoneurons to survive (D’Costa et al., 1998). IGF may also function to inhibit anoikis by activating the Akt pathway when cells are detached from matrix molecules (Prisco et al., 1999; Yu et al., 2001). It is not yet known whether GDNF functions to inhibit anoikis. Trophic factors such as IGF and GDNF promote motor neurite terminal plasticity and may function to inhibit motoneurons form undergoing anoikis while final innervation patterns are decided.

The hypothesis that a motoneurons fate is dependent on its survival through increased terminal endings indicate that trophic factors and their receptors are involved in synapse formation (Oppenheim, 1991). Neurotrophin 4 potentiates pre-synaptic activity at the neuromuscular junction (Wang et al., 1998). Neurotrophin 4 and brain-derived neurotrophic factor (BDNF) destabilise AChR clusters at the neuromuscular synapse by activating their tyrosine kinase B receptor (TrkB; Wells et al., 1999). Moreover, disruption of TrkB-mediated signalling disrupts the formation of the neuromuscular synapse (Gonzalez et al., 1999). Conversely, neutralisation of the TrkB receptor results in increases in AChR clustering (Wells et al., 1999). These studies indicate that trophic factors have local actions at the neuromuscular synapse supporting the hypothesis that neuromuscular synapse formation is required for motoneurons to access muscle derived trophic factors.

We therefore propose a model that trophic factors inhibit anoikis while motoneurons are finding their innervation patterns and once neuromuscular synapses become established, other trophic factors act to potentiate the presynaptic activity and make a stronger connection through deposition of matrix molecules. In this light, motoneurons that do not have a local expression of IGF or GDNF and are unable to compete effectively for muscle derived trophic factors at neuromuscular synapses detach from the muscle fibre and undergo anoikis.

**Links between Apoptosis Pathways in Motoneurons and those involved in Anoikis**

A key problem in the study of anoikis lies in characterising the initiating death pathway (Frisch and Screaton, 2001). Recently it was found that the Fas pathway is induced by detachment from matrix (Aoudjit and Vuori, 2001). Fas is activated by its ligand FasL (Fas ligand) to induce apoptosis through caspase 8. FLIP, a negative regulator of caspase 8 can inhibit this pathway (Irmler et al., 1997).

A recent study showed that embryonic motoneurons express both Fas and its ligand FasL at the beginning of PCD and is induced by trophic factor deprivation (Raoul et al., 1999). Blocking the Fas activation by FasL saves motoneurons which would otherwise die in culture (Raoul et al., 1999). Furthermore, trophic factors BDNF, GDNF and cardiotrophin 1 are able to inhibit the Fas induced death by inducing the expression of FLIP (Raoul et al., 1999). In addition, trophic factor deprivation increases the FasL expression (Raoul et al., 1999). As motoneurons and epithelial cells that undergo anoikis use the same Fas/FasL pathway, we postulate that without proper nerve-muscle connections, motoneurons do not obtain the correct trophic support and are induced to die by anoikis.

**Conclusion**

In this review we provide correlative evidence to suggest that a motoneuron’s dependence on its muscle fibre during the period of PCD may be regulated by its interactions with the muscle’s basal lamina. The most convincing evidence stems from a large drop in motoneuron survival in agrin-deficient mice at the same time the basal lamina becomes less stable (Noakes et al., 2001). Given these correlations, we propose that motoneuron survival during embryonic development is in part regulated by anoikis. During the early period of synapse formation, trophic expression in muscle fibres is low (Griesbeck et al., 1995; D’Costa et al., 1998), making it necessary for motoneurons to make connections with the muscle to survive. Agrin deposited by the developing motor nerve terminal into the synaptic basal lamina may act to promote motoneuron survival by concentrating target secreted trophic factors to the neuromuscular junction, as well as promoting the structural development of basal lamina and the anchoring of it to the muscle’s cytoskeleton. These concentrated trophic factors in turn promote the functional and molecular matura-

**Acknowledgments**

We thank Ms Mary White for proof reading the manuscript, and our lab members for general support. PGN is supported by grants from National Health & Medical Research Council of Australia, Motor Neuron Disease Institute Australia, and Ramaciotti Medical Research Foundation. GBB is supported by a University of Queensland Postgraduate fellowship.

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