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Motor Neuron Trophic Factors: Therapeutic Use in ALS?

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Abstract

The modest effects of neurotrophic factor (NTF) treatment on lifespan in both animal models and clinical studies of Amyotrophic Lateral Sclerosis (ALS) may result from any one or combination of the four following explanations: 1.) NTFs block cell death in some physiological contexts but not in ALS; 2.) NTFs do not rescue motoneurons (MNs) from death in any physiological context; 3.) NTFs block cell death in ALS but to no avail; 4.) NTFs are physiologically effective but limited by pharmacokinetic constraints. The object of this review is to critically evaluate the role of both NTFs and the intracellular cell death pathway itself in regulating the survival of spinal and cranial (lower) MNs during development, after injury and in response to disease. Because the role of molecules mediating MN survival has been most clearly resolved by the *in vivo* analysis of genetically engineered mice, this review will focus on studies of such mice expressing reporter, null or other mutant alleles of NTFs, NTF-receptors, cell death or ALS-associated genes.

2. INTRODUCTION

The discovery of nerve growth factor (NGF) by Levi-Montalcini and Cohen 50 years ago strikingly supported the nascent concept, originated by Hamburger (1), that secreted molecules produced by the target of a developing neuron are required for it to survive programmed cell death (PCD), an embryonic period during which roughly half of developing postmitotic neurons die by the morphological process of apoptosis. This neurotrophic hypothesis, coined by Purves (2), provided an intellectual background for the identification of subsequently identified neurotrophic factors (NTFs): a candidate NTF would be added to or removed from a specific neuronal population during PCD and its effects on survival recorded by measuring the number of healthy or dying neurons. Simultaneous progress in the field of intracellular cell death machinery, largely performed by Horvitz and colleagues, led to the identification of many key molecules whose activity was required for the passage of a cell through its apoptotic, programmed death states (3). Thus, whereas early work focused on the nature of NTFs as extracellular signals regulating PCD, subsequent studies established that a.) neurons dying during developmental PCD activate a program of genes and b.) NTFs rescue neurons from PCD by inhibiting this program (4). The robust effect of NTFs and anti-PCD molecules on the survival of MNs during development soon led to the idea that they might ameliorate neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) and thereby aid in the treatment for those suffering from these diseases (5).

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The intent of this review is to critically evaluate the validity of the notion that NTFs are physiological survival-promoting factors for spinal MNs during development, after injury or in MN diseases such as ALS. The almost complete lack of success exhibited by NTFs in human clinical trials of ALS, coupled with recent data suggesting that several prominent NTFs are in fact dispensable for the survival of skeletomotor (α) MNs during PCD, have questioned the idea that cell death in some of these contexts reflects inadequate trophic support, and have therefore made such an evaluation timely. On the other hand, several observations indicate that NTF dysfunction may represent key clues to or intermediates within the pathogenesis of ALS: 1.) mutations in at least 3 NTFs cause MN disease 2.) expression of several target-derived NTFs is reduced in ALS, 3.) downstream signaling initiated by other target-derived NTFs is blocked in ALS and 4.) centrally, intrathecally and/or virally delivered NTFs often exhibit superior neuroprotective effects when compared to systemically administered NTF (the route used for NTF delivery in ALS clinical trials). These findings suggest a more complicated neuromuscular scenario underlying ALS, in which NTFs and their receptors are dynamically expressed by different subcellular regions of MNs as well as by many other interacting cell types such as glia, muscle and endothelial cells. Therefore, before dismissing the efficacy of NTFs based solely on poor performance in human clinical trials, we intend to put forth in their defense a summary of the hurdles NTFs face as therapeutics for MN disease. Because the regulation of MN survival by NTFs has been most well characterized during developmental PCD, we begin with the analysis of NTF expression and signaling during this period. After presenting a review of ALS pathogenesis with a detailed emphasis on the role of cell death, we return to the function of NTFs in ALS transgenic and NTF KO mice, focusing on sites of action (i.e. soma vs. axons vs. synaptic terminals), modes of action (i.e., anti-apoptotic vs. anti-excitotoxic) and overall effects on motor function and lifespan.

3. NEUROTROPHINS AND NEUROTROPHIC HETEROGENEITY

The presence of dying neurons in the developing nervous system was noted over a century ago (6) but not appreciated until nearly half a century later, when Hamburger and Levi-Montalcini noted that the number of dying neurons during development varied depending upon the size of the target field (7). Further evidence of a relationship between factors produced by the periphery and the number of dying neurons was provided by Hamburger (1), who observed a large increase in the number of dying sensory neurons and MNs several days after the surgical removal of their target limb. Because the increase in dying MNs occurred during the same period that dying MNs had normally been found, these experiments indicated that neurons undergoing death during normal development might also depend on target-derived signals (8). The presence of such natural-occurring cell death is observed in many different neuronal subpopulations of the central and peripheral nervous systems (CNS, PNS), such as the lateral motor column of the spinal cord, where approximately 50–60% of the MNs produced die this way (9). The extent of cell death is temporally and spatially reproducible across many neuronal populations and species (10) suggesting it occurs under the environmental control of a genetic program (4,5,11). The idea that this process of programmed cell death (PCD) is not fixed but regulated by target-derived factors was further supported by experiments showing a decrease in normal MN death after transplantation of a supernumary limb (12). Evidence that skeletal muscle is the cell type mediating these effects on MN survival was provided by the finding that purified muscle extract reduces MN cell death *in vitro* (13) and *in vivo* (14,15). Together, these studies demonstrate that muscle-derived NTFs block the death of MNs normally fated to die during PCD. More recent work shows that elimination of muscle cells increases MN death (16–18), indicating that muscle-derived NTFs factors also block the death of MNs normally fated to live during this period. These studies also suggest that the concentration of target-derived NTFs is normally limiting and that an environment increasing the production of or

access to NTFs would lead to a commensurately larger rescue of MNs from PCD (19). In this way, PCD may provide an element of plasticity in controlling neuronal number and therefore have evolved as a mechanism to match the number of neurons and their targets (20,21).

These studies suggested that extracellular, target-derived molecules profoundly regulate MN survival during PCD, but the discovery of NGF as such a factor for sympathetic and a subset of sensory neurons provided more specific evidence that the neurotrophic hypothesis was valid. Moreover, the demonstration that NGF is produced by the target of these neurons (22), is retrogradely transported from the target to their cell bodies (23,24), and is necessary (25) and sufficient (26) for their survival implied that similar molecules might rescue other populations of developing neurons (27). The biochemical isolation and survival-promoting effects of a second trophic molecule structurally related to NGF, brain-derived neurotrophic factor (BDNF), supported this idea (28). The relatedness of these molecules combined with the technological advances of molecular cloning led to the identification of NT-3 and NT4/5, thus forming the neurotrophin family of molecules (29). Additional work demonstrated that each neurotrophin exerts its target-dependent, retrograde survival effect through binding and activation of high-affinity tyrosine kinase receptors: NGF with TrkA, BDNF and NT4/5 with TrkB and NT-3 with TrkC (30,31). Studies of mice lacking any of the neurotrophins or their high-affinity receptors, however, reveal that whereas some populations of neurons act in accordance with the neurotrophic hypothesis, others don't (32). For example, studies of NGF- and TrkA-deficient mice show a complete absence of sympathetic and nociceptive sensory neurons, as predicted from earlier reports, but no loss of TrkA-expressing basal forebrain cholinergic neurons (33,34). Similarly, while TrkB-expressing sensory neurons in the vestibular ganglion depend on BDNF for their survival during PCD, TrkB-expressing hippocampal granule neurons require neither BDNF nor TrkB (35,36). Finally, whereas TrkC-expressing proprioceptive sensory neurons require both NT-3 and TrkC for their survival (37,38), TrkC-expressing cerebellar granule neurons survive in the absence of NT-3 or TrkC (39). In contrast, CNS neurons require multiple neurotrophins for survival, as combined genetic elimination of TrkB and TrkC, but not of either factor alone, increases the number of dying granule neurons in the dentate gyrus and cerebellum (40). These *in vivo* studies indicate that neurotrophins act in a redundant manner during developmental PCD of CNS but not PNS neurons (41).

The discovery of multiple NTFs in addition to the neurotrophins allowed this idea to be further tested in MNs. In contrast to the results obtained with neurotrophins and CNS granule cells, the removal of single NTFs results in the loss of subpopulations of MNs (summarized in Table 1). Therefore, similar to PNS neurons, the loss of individual NTFs causes MN deficits, but in contrast to that of PNS neurons, the death of MNs appears less severe, with the greatest loss observed amounting to about 40%. The fact that the inactivation of different single NTFs each results in partial MN losses indicates that MNs may exhibit trophic heterogeneity during development; i.e., that different MN subtypes each respond to a distinct NTF or combination of NTFs (42–44). Analysis of mice deficient for some of these NTFs, which include glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factors I and II (IGF-I, II), hepatocyte growth factor (HGF), and vascular endothelial-derived growth factor (VEGF), supports this idea (Table 1 and see the section below under GDNF). Such trophic heterogeneity may reflect a changing trophic environment encountered by MNs as they navigate toward and penetrate their targets, as has been demonstrated for cranial PNS neurons (45). Alternatively, the trophic dependence of each MN subpopulation on a different NTF may reflect the specific environments these subpopulations encounter during development, such as limb vs. trunk mesoderm, forelimb- vs. hindlimb-derived neural crest cells, cranial vs. spinal descending inputs. Evidence consistent with the idea that different

cell types supply MNs with specific trophic support is provided by studies showing that selective elimination of supraspinal input, sensory afferent input, Schwann cells or astrocytes increase MN PCD (46–49). Moreover, pre-treatment with antibodies against GDNF or the cytokine cardiotrophin-1 (CT-1) abrogate the trophic effects of muscle and Schwann cells, respectively, on cultured MNs (50). Finally, MNs themselves express unique combinations of NTF receptors depending on position, target innervation and stage of maturation, suggesting that differential receptor and ligand distribution together contribute to MN neurotrophic heterogeneity (51). For example, exogenous BDNF only rescues lumbar MNs from the latter half of the PCD period because its receptor TrkB is only expressed at this time (52). Similarly restricted rescue effects are observed for HGF, whose receptor Met is expressed only by limb-innervating populations of spinal MNs (53,54), and for cardiotrophin-like cytokine/cytokine-like factor for lumbar and facial MNs (CLC/CLF; 55). Recent evidence has demonstrated that smaller MN subpopulations known as motor pools also express discrete patterns of NTFRs, indicating that the trophic heterogeneity exhibited by MNs may be related to transcriptionally encoded patterns of target selection (56). A more detailed review of the rescue-promoting effects of individual NTFs is presented in Table 1 and below.

APOPTOSIS DURING DEVELOPMENTAL AND NERVE INJURY-INDUCED PCD

Whereas the abundance of extracellular-derived, neurotrophic molecules illustrates the diversity of intercellular signaling mechanisms used to regulate neuronal PCD, the discovery of a relatively small group of evolutionarily conserved, intracellular death pathways activated by dying neurons indicates a common mien of cellular destruction during PCD (57). This interpretation is supported by the observation that NTF-deprived neurons during development typically die by a morphologically characteristic, physiologically efficient process known as apoptosis (58). Perhaps more importantly, the activation of this pathway and the presence of apoptosis in injured and degenerating neurons have fuelled the idea that the NTF regulation of apoptosis is operative or at least capable of exploitation in these pathological forms of cell death (5). While considerable support for this idea has been found in studies of experimental neuronal injury (59), the notion that either NTFs or apoptotic pathways of cell death underlie disease is less clear (60). In particular, recent observations suggest that caspase-deficient developing MNs, excitotoxically insulted MNs and degenerating MNs exhibit either necrosis or a third, autophagic form of PCD morphologically distinct from apoptosis and necrosis (61–64). In order to revisit this notion with respect to MN disease such as ALS, we will quickly review the basic biochemical pathways that mediate apoptotic and non-apoptotic death during mammalian developmental PCD or in response to injury. Invertebrate intracellular death pathways are not covered here but can be accessed in several excellent reviews (3,57).

4.1 Development: intrinsic pathway of apoptosis

In the vertebrate system, developing neurons activate phosphoinositide-3 kinase (PI-3K) in response to NTF stimulation, which stimulates Akt and largely mediates the inhibition of the two major intracellular pathways of apoptosis, known as the intrinsic and extrinsic pathways (Figure 1; 65,66; but see also 67). The intrinsic pathway involves the mitochondria and endoplasmic reticulum and is composed of a variety of anti- and pro-apoptotic members containing one or several copies of the Bcl-2 Homology (BH) (68). This family of proteins can be further subdivided into proteins with several BH domains, the so-called multidomain proteins (MDPs; 69) and those with only 1 BH3 domain, the BH3-only proteins (BOPs; 70). Pro-apoptotic MDPs include Bax and Bak whereas at least 18 members of pro-apoptotic BOPs have been identified (71). Anti-apoptotic members include only MDPs such as Bcl-2,

Bcl-X_L, Bcl-w and MCP-1 (72). MDPs directly regulate apoptosis, whereas BOPs stimulate Bax/Bak or inhibit Bcl-2/Bcl-X_L in response to NTF deprivation or other death signals (73,74). BOPs are themselves either transcriptionally or post-translationally regulated by NTF signaling; for example, Akt inhibits the pro-apoptotic activity of Bad by direct phosphorylation (75,76). The common result of NTF deprivation or many other forms of cell stress is the homo-oligomerization and translocation of Bax from cytosol to outer mitochondrial membrane (77,78) as well as Bak activation within the mitochondrial membrane. Oligomerized, membrane-targeted Bax forms a pore and permits flow of cytochrome *c* (cyt *c*) from mitochondria to cytosol (79–81), while activated Bak causes the fragmentation of mitochondria (82). Cytosolic cyt *c* binds the apoptosis protease-activating factor-1 (Apaf-1), which facilitates the binding and activation of caspase-9 in a tertiary complex called the apoptosome (83). Caspase-9 is a member of the cysteine proteases that reside in the cytosol as inactive zymogens and initiate a proteolytic cascade when activated (84). Initiator caspases such as caspase-9 cause the activation of effector caspases such as caspase-3, whose activity dismantles the cell and results in the phenotypic expression of apoptosis (85,86).

In addition to cyt *c*, several other apoptogenic molecules are released from the mitochondria, including Smac/Diablo, which activates caspases by inhibiting a family of caspase-inhibiting molecules known as the inhibitors of apoptosis (87–88), apoptosis-inhibiting factor (AIF) and the endonuclease EndoG, both of which cause apoptosis in a caspase-independent fashion (89–91). However, AIF and EndoG may exit mitochondria significantly later than cyt *c* and thus occur only after caspase activation (92). While these molecules form the bulk of the core pathway mediating PCD, additional inputs stimulated by pathological cell stress often stimulate the intrinsic pathway (93). For example, genotoxic, osmotic or oxidative stress activates the c-Jun N-terminal kinases (JNKs), which activate a wide variety of targets including the transcription factors c-Jun and p53 and the pro-apoptotic BOP BIM (94–96). p53 subsequently triggers apoptosis by transcriptionally activating Bax and Bax-stimulating BOPs (97,98). Similarly, protein misfolding activates an ER-dependent, Ca⁺⁺-sensitive pathway which stimulates mitochondrial permeability transition (mPT), resulting in the dissipation of the mitochondrial electrochemical gradient, liberation of cyt *c* and activation of caspases (99,100). The extent to which mPT also occurs in response to classic intrinsic pathway activation, i.e., Bax-dependent cyt *c* release and caspase activation, is still under debate (101).

While *in vitro* pharmacological studies of MNs have established a critical role for PI-3K- and Akt-mediated signaling in response to NTFs (102), analyses of neuronal PCD in mice genetically lacking these enzymes have not been performed on account of early embryonic lethality caused by constitutive inactivation (PI-3K; 103,104) or redundancy of multiple isoforms (Akt; 105). In contrast, genetic studies have shown that key regulators of post-mitotic neuronal PCD *in vivo* include MDPs such as Bax, Bcl-2 and Bcl-X_L, but not BOPs (106). Although caspase activation is a feature of post-mitotic neurons undergoing PCD, caspase-3 is required only for the death of cycling neuronal progenitors (107). Accordingly, post-mitotic spinal MNs still die but in a caspase-independent manner in caspase-3, caspase-9 and Apaf-1 null mutant mice (61,108). The morphological process by which caspase-deficient MNs die is similar to autophagic death, but this non-apoptotic form of death is unlikely to normally regulate MN survival because MN PCD is unaffected by deletion of ATG7, a gene required for autophagy (106). In marked contrast, overexpression of Bcl-2 and inactivation of Bax profoundly arrests neuronal PCD (109–111); MNs, for example, exhibit no developmental MN death whatsoever in Bax knockout (KO) mice (112). In fact, a recent analysis of total axons in the fourth lumbar ventral root (L4 VR) of Bax-deficient mice indicates that 2 of 3 rather than 1 of 2 MNs die during developmental PCD (113). MNs rescued from PCD by Bax-deficiency are smaller in diameter than wild-

type MNs, and many are unmyelinated, suggesting that Bax deletion alone (i.e., in the absence of elevated NTF signaling), does not increase the number of MNs that are competent to innervate extrafusal muscle targets (113).

4.2 Nerve injury: intrinsic pathway of apoptosis

MNs maintain dependence on their targets even after the period of developmental PCD, because rapid apoptosis occurs if an axon is transected and prevented from re-innervating its muscle during early postnatal development, (114–118). In contrast, axotomy in the adult causes a much slower degeneration characterized by neuronal atrophy and loss of neurotransmitter expression (119,120), as well as the increase in expression of factors involved in axonal regeneration (121). Apoptotic death of adult MNs is observed, however, in response to some types of axonal injury, such as proximal transection or avulsion of the sciatic nerve (122–124).

Viral-mediated expression of a constitutively active form of Akt prevents MNs from neonatal axotomy (125). Transgenic overexpression of Bcl-2 or Bcl-XL (126,127) as well as deletion of Bax (110,111) also inhibits this form of MN death. In contrast, inactivation of caspase-3 (128) or viral-mediated expression of caspase-inhibiting IAPs delays but does not block neonatal axotomy-induced MN cell death (129). The death of adult MNs in response to avulsion is also prevented by Bax deletion (130; but see 131) or viral-mediated Bcl-2 overexpression (132). Interestingly, whereas individual Bax-activating BOPs are dispensable for developmental PCD, their absence after nerve injury significantly attenuates MN death. For example, MNs lacking DR5/Harakiri or Noxa are protected from injury-induced death in adult mice (133,134). Finally, apoptotic death caused by nerve injury involves the activation of several pathways that are dispensable for programmed cell death, such as the stress-activated transcription factors p53 and c-Jun (130,135), suggesting that the intrinsic pathway of cell death, gated by Bcl-2, Bcl-X_L and Bax, is activated by specific sets of stimuli in MNs undergoing developmental or injury-induced apoptotic death.

4.3 Development and nerve injury: extrinsic pathway of apoptosis

An additional pathway leading to apoptotic death can be initiated even in the presence of NTFs (136). This extrinsic pathway of apoptosis is initiated by cell surface receptors such as Fas, tumor necrosis factor alpha receptor (TNFR) and the low-affinity neurotrophin receptor p75^{NTR}, which when activated by extracellular ligands exhibit a change in conformation that favors the assembly of a death-inducing signaling complex (DISC) on a region of their cytoplasmic tails known as the death domain (137). The association of activated “death receptors” and DISC causes the recruitment and activation of procaspase-8 (138), which leads to mitochondrial-independent activation caspase-3 and apoptotic death in so-called Type I cells or a mitochondrial-dependent, caspase-3 mediated apoptosis in Type II cells (139). This latter pathway is mediated by caspase-8-mediated proteolytic activation of BID, a BOP which activates the oligomerization and mitochondrial insertion of Bax, thus linking the extrinsic and intrinsic apoptotic pathways (140–143). Both Bcl-2 overexpression and Bax/Bak deficiency block this pathway (144,145).

Fas-mediated killing of MNs occurs in an *in vitro* model of PCD through this Type II pathway (136) as well as through another mitochondrial-dependent, caspase-8-independent pathway requiring the upregulation of neuronal nitric oxide synthase (nNOS; 146). However, Fas null mutant mice exhibit no difference in MN number at the end of the PCD (147). In contrast, MN survival after neonatal or adult nerve injury is robustly increased by Fas deletion (147,148). Mitochondrial-dependent, apoptotic death of injured MNs is also blocked in nNOS-deficient mice, suggesting that Fas and NO underlie MN death caused by pathological disconnection of MNs from their targets (148). Similarly, MN survival in mice

lacking p75^{NTR} or both TNFR1 and TNFR2 is unaffected during developmental PCD, but increased after nerve injury (135,149). Together, these studies show that injury-induced (but not developmental) apoptotic death of MNs occurs through an extrinsic, death receptor-mediated activation of the intrinsic pathway of apoptosis.

5. EFFECT OF NEUROTROPHIC FACTORS ON DEVELOPMENTAL AND NERVE INJURY-INDUCED PCD

5.1 GDNF

GDNF was first identified as a midbrain dopaminergic trophic factor produced by the supernatant of a clonal glial cell line (150). GDNF is expressed by Schwann cells and skeletal muscle during the period of MN PCD, and is upregulated after nerve injury (151,152). GDNF is also expressed in astrocytes after excitotoxic injury to the spinal cord (153). GDNF is transported to MNs in both retrograde and anterograde directions, indicating a wide variety of potential cellular sources (154–156). Treatment with exogenous GDNF prevents the apoptotic death of neonatal MNs in response to axotomy (151,157) and blocks MNs from undergoing PCD (158,159). GDNF also reduces the loss of ChAT expression and enhances the regeneration of adult MNs after axotomy (154,160). Deletion of GDNF in mice causes a 20–30% additional loss of spinal MNs during the process of PCD (161–163). GDNF acts via a multicomponent receptor composed of the tyrosine kinase Ret and the glycosylphosphatidylinositol- (GPI-) linked GDNF family receptor $\alpha 1$ (GFR $\alpha 1$; 164). Although GDNF can induce the activation of the RAS/ERK, p38 MAPK, JNK, PI-3K, PKA and Rho kinase signaling pathways (165,166), GDNF-mediated survival signaling in MNs requires the phosphorylation of Ret on tyrosine (Y) 1062, recruitment and activation of PI-3K signaling (51,167,168). Ret and GFR $\alpha 1$ are expressed by spinal MNs during MN PCD and both GFR $\alpha 1$ and Ret KO mice exhibit a 20–30% loss of spinal MNs, similar to GDNF KOs (51,113,163,169,170). These results suggest that a specific subset of spinal MNs require GDNF for survival during development. Some MNs in these KOs fail to project to several muscles in the trunk and hindlimb (171–173), but these losses are insufficient to explain the full extent of MN loss in GDNF KOs (51). Recent reports suggest that the remaining component of MN loss in GDNF ligand or GDNF receptor KO mice is restricted to fusimotor γ MNs (51,174). More than 90% of lumbar fusimotor axons are missing in adult mice lacking the GDNF receptors Ret or GFR $\alpha 1$ exclusively in MNs, and muscle spindles receive severely diminished fusimotor innervation in MN-specific Ret KO mice (51). In contrast, in the 50% of GDNF KO mice that fail to exhibit an early pathfinding deficit (causing peroneal MNs to select the tibial nerve and subsequently die; 173), skeletomotor (α) MNs are completely unaffected (51). Moreover, MN number is unaffected in the facial nucleus, a cranial MN subpopulation which contains only α MNs (161,162 but see 163).

In addition to the potent, restricted neurotrophic effect of GDNF on fusimotor neurons, overexpression of GDNF under either a muscle-specific (Myo-GDNF) or astrocyte-specific (GFAP-GDNF) promoter results in the hyper-innervation of both intra- and extrafusal motor fibers and a delay in the period of synapse elimination (175,176). Myo-GDNF mice also exhibit a doubling of motor axons in the ventral root, nearly all of which represents an increase in the number of small-diameter axons in the lumbar ventral roots (113,174). Therefore, the current data suggests that the trophic effects of GDNF during the developmental PCD period are restricted to muscle spindle-innervating γ MNs, despite the fact that all MNs express GDNF receptors and respond to exogenous GDNF. Moreover, these studies support the idea that specific populations of MNs require distinct, individual NTFs during this period.

5.2 BDNF

BDNF was originally isolated as a trophic factor for neurons from brain extract (177). Expression of BDNF (178) and the high-affinity BDNF receptor TrkB (179) is observed in skeletal muscle and MNs, respectively during MN PCD. BDNF and TrkB expression are increased in the peripheral nerve and MNs, respectively, after nerve injury (180,181). MNs retrogradely transport BDNF and exogenous BDNF rescues MNs from embryonic PCD, neonatal axotomy-induced death, and embryonic deafferentation-induced death (182–185). Although MN death is increased in TrkB KO mice in response to neonatal axotomy (186), neither BDNF nor TrkB null mutant mice exhibit a loss of MNs after the period of MN PCD (36,187–190). Therefore, the trophic effects of BDNF during development, in which a subpopulation of MNs is rescued from the second half of the cell death period (52), are restricted to MNs that would normally die rather than those that would normally live through the PCD period.

5.3 NT-3

The neurotrophin NT-3 and its high-affinity receptor TrkC are required for the survival during PCD of several populations of sensory neurons (37–39). In particular, proprioceptive sensory neurons of the dorsal root ganglia (DRG) depend on muscle-derived NT-3, which is expressed robustly by intrafusal muscle fibers (191–193). The loss of spinal proprioceptive afferent neurons prevents the induction of intrafusal muscle fibers, and the resulting absence of muscle spindles is believed to cause the death of fusimotor MNs, which are completely missing in NT-3 KO mice (51,194,195). In contrast, NT-3 is not required for the survival of skeletomotor MNs during PCD, because numbers of facial MNs, a population containing no fusimotor MNs, are unaffected in NT-3 KO mice (37,39). Additionally, the number of large-diameter α MNs appears normal in the lumbar spinal cord of NT-3 null mutants, although they exhibit a 15% reduction in somal diameter (195). It is worth noting that while single deficits in BDNF, NT-4/5 or NT-3 cause no loss of facial MNs during MN PCD, mice deficient in all three of these neurotrophins contain fewer facial MNs at birth (196). Interestingly, muscles of NT-3 KO mice lose neuromuscular innervation postnatally (197). This deficit likely reflects the requirement of NT-3 for early postnatal Schwann cell survival (198), as Schwann cells themselves are required for MN survival (48) and maintenance of neuromuscular innervation (199). Future experiments aimed at deleting the TrkC receptor in selective cell types (e.g., MNs, Schwann cells, perineurial and muscle fibroblasts) should help confirm that NT-3 exerts these neuropathic effects on MNs indirectly through its effects on Schwann cells. Although exogenous NT-3 is required for fusimotor MNs to survive during MN PCD, it is insufficient to rescue MNs that would normally die during this period (15). Exogenous NT-3, similar to BDNF, also promotes the survival of MNs after neonatal axotomy and embryonic deafferentation (183;200,201). Together, these studies show that NT-3 is indirectly required for the survival of fusimotor MNs during PCD and for the maintenance of neuromuscular innervation in the adult.

5.4 CNTF, LIF, CT-1, CLC/CLF

The interleukin-6 (IL-6) family of neurotrophic cytokines includes leukemia inhibitory factor (LIF), CNTF, CT-1 and cardiotrophin-like cytokine/cytokine-like factor 1 (CLC/CLF), and each of these neurotrophic factors act through a signal-activating heterodimeric receptor composed of the tyrosine kinases LIF receptor- β (LIFR- β) and glycoprotein130 (gp130; 202–205). Additionally, CNTF and CLC/CLF require a GPI-linked co-receptor, CNTF receptor- α (CNTFR α), and CT-1 requires a similar, as-yet unidentified co-receptor. CNTF binding to LIFR- β /gp130 in MNs activates the janus kinase / signal transducer and activator of transcription-3 (JAK/STAT) and PI-3K signalling pathways, thus leading to the inhibition of the intrinsic apoptosis pathway (206,207). CNTF was isolated as a neurotrophic factor for parasympathetic neurons of the ciliary ganglion (208,209), and exogenous CNTF

exerts trophic effects on dissociated embryonic MNs (210), neonatal MNs after axotomy (211) and MNs undergoing PCD (212). LIF also rescues embryonic MNs from PCD *in vivo* (213) or death *in vitro* (214). In contrast to other NTFs, CNTF is expressed only after MN PCD and in the nerve rather than muscle, and contains no leader sequence and hence is not classically secreted (215,216). However, CNTF is found in the medium of cultured Schwann cells and is retrogradely transported by spinal MNs (217,218), suggesting that CNTF is released in a non-classic manner. Inactivation of CNTF in mice has no effect on MN number during MN PCD, but leads to the atrophy and then loss of a subset of MNs postnatally, resulting in muscle weakness (219). Similarly, LIF null mutants fail to exhibit changes in the numbers of MNs after PCD (220,221), although combined deletion of LIF and CNTF exacerbates the postnatal loss of MNs observed in CNTF KO mice (222).

In contrast to CNTF or LIF null mutants, mice lacking the neurotrophic cytokine receptors CNTFR α (223), LIFR- β (224) or gp130 (225) all show a profound (~40%) loss of spinal and cranial MNs during PCD. These results suggest that cytokines other than CNTF and LIF must account for these effects. Mice deficient for one such cytokine, CT-1, accordingly exhibit a 20–30% loss of MNs after the period of MN PCD (226). CT-1, which was expression cloned as a factor inducing cardiac myocyte hypertrophy, also lacks a classical leader sequence but is found in embryonic skeletal muscle during MN PCD (227,228). CT-1 deletion is not required for the maintenance of MNs postnatally (226), and its absence does not potentiate the loss of MNs observed in CNTF- or LIF-deficient KO mice (229). Because the actions of CT-1 require gp130/LIFR- β but not CNTFR α (230) the loss of MNs observed in CNTFR KO mice cannot be accounted for by CT-1 deficiency. Accordingly, inactivation of the second CNTFR α ligand, CLC/CLF, also results in a 20–30% reduction of MNs during the PCD period (55). Interestingly, although CNTFR α , LIFR- β and gp130 are expressed in most murine MNs, the loss of spinal MNs in CLC/CLF KO mice is only observed in lumbar and not thoracic or brachial regions (55), suggesting that the subtype of MNs which is lost in these mice is different from fusimotor MNs. In the developing chicken spinal cord, lumbar MNs expressing CNTFR α are very well circumscribed and restricted to several motor pools, including those innervating the adductor muscles (56). However, due to the inability of CNTFR α and other cytokine KO mice to suckle, they fail to survive postnatally and thus a more detailed analysis on subtypes of murine MNs lost is still forthcoming.

5.5 HGF

Hepatocyte growth factor (HGF) / scatter factor (SF) was originally isolated as a mitogen for regenerating liver cells and a motogen for developing muscle cells, and HGF signals through the tyrosine kinase receptor Met (231–234). Mice deficient in either HGF or Met exhibit liver, muscle and nerve pathfinding deficits and die during embryogenesis before the completion of MN PCD (235). Nevertheless, the trophic effects of exogenous HGF on cultured MNs (53,236,237). MNs undergoing PCD *in vivo* (54) and neonatal MNs undergoing axotomy-induced death (238) indicate an important role for HGF in MN survival during development. Interestingly, the expression of Met is restricted to limb-innervating subtypes of MNs (53), and the expression of HGF in the limb mesenchyme is required for a subpopulation of MNs to innervate the LD and CM muscles of the forelimb, the same muscles which lack innervation in GDNF ligand/receptor KO mice (236,239,240). Recently, it was observed that these two NTFs collaborate to establish the transcriptional identity and thus target selection of MNs innervating these muscles (241). HGF-dependent limb innervation requires the presence of PI-3K-binding tyrosine residues on Met, emphasizing the central importance of this pathway in mediating both tropic and trophic effects of NTFs on developing MNs (240). A more detailed analysis of the effects of HGF / Met deletion on MN number has yet to be reported, but the recent generation of conditional Met mutants makes such a study possible (242).

5.6 IGFs

The family of insulin-like growth factor (IGF) includes IGF-I and IGF-II, two secreted proteins structurally similar to pro-insulin, the tetrameric tyrosine kinase receptors IGFR Type 1 and 2 (IGFR-1 and IGFR-2), and at least 6 IGF binding proteins (IGFBP); IGF-1 binds IGFR-1 as well as insulin receptor (IR), and IGF-II binds IGFR-1 and IGFR-2 (243,244). The expression of IGFs is elevated in skeletal muscle and Schwann cells during synaptogenesis and after peripheral denervation and exogenous IGFs induce motor axon sprouting and reinnervation (245–247). Furthermore, the increase in neuromuscular branching observed after paralysis is blocked by treatment with IGFBP (248). Consistent with these studies, exogenous IGFs enhance regeneration after nerve injury and blockade of endogenous IGFs reduces this effect (249), and exogenous insulin also enhances motor reinnervation after sciatic nerve injury (250). IGFs are also necessary and sufficient for MN survival after neonatal axotomy (200,251). During the process of developmental MN PCD, IGFs are expressed by muscle but not Schwann cells (245,252), and IGFRs are expressed by embryonic MNs (246). Accordingly, exogenous treatment with IGF-I and IGF-II reduces MN PCD (253), and treatment with exogenous IGFBP blocks the survival-promoting effects of activity blockade (254). Conversely, administration of function-blocking antibodies to IGF-1 and targeted deletion of IGF-1 both result in an enhanced loss of spinal MNs during PCD (254; RW Oppenheim, personal communication). Although a wide variety of reports have indicated that the neurotrophic effects of IGFs are mediated through PI-3K activation, it has not been established whether signaling through this pathway is required for MN survival during PCD. IGFs also activate the JAK/STAT pathway (255), suggesting a potential link between IGF and cytokine signaling, a hypothesis supported by the finding that LIF deficiency potentiates the loss of MNs in IGF-1 KO mice despite exerting no such effect alone (256). In contrast, because the number of facial MNs is reduced in IGF-1-deficient mice, the trophic effects and signaling pathways of IGF-1 in MNs are likely different than those of either NT-3 or GDNF (256). IGFR-1 null mutant mice have been generated as well, but a detailed analysis of MN defects has not been reported (257). A recent study reported the generation of conditional IGFR-1 mutant mice (258), which when crossed with HB9-Cre mice will begin to address this issue.

5.7 VEGF

In contrast to each of the previous NTFs discussed, whose role in ALS has been extrapolated based on its developmental trophic effects, the discovery of VEGF as a candidate gene for sporadic ALS (see below) has reversed the equation and stimulated studies to elucidate its role in MN development. VEGF regulates the growth of blood vessels during development and after hypoxic injury (259), but has recently been shown to exert direct trophic effects on neurons (260). VEGF is a homodimeric glycoprotein that activates a variety of homodimeric tyrosine kinase receptors including VEGFR1 (FMS-like tyrosine kinase-1; Flt-1), VEGFR2 (Fetal liver kinase-1; Flk-1) VEGFR3 (FMS-like tyrosine kinase-4; Flt-4), as well as the neuropilin coreceptors Nrp1 and Nrp2, although the majority of effects on neurons are believed to occur through VEGFR2 (261). VEGF exerts trophic effects on MNs in culture and in spinal cord explants by activating the PI-3K signaling pathway and Bcl-2 (262–263). VEGF also enhances the regeneration of adult MNs after axotomy, but whether these effects are direct or indirect through an increase in angiogenesis are unclear (264). Although VEGF-KO and VEGFR-KO mice have been generated, the effect of VEGF on MN PCD has not been examined, because even haplo-insufficient VEGF mice die early during embryogenesis due to defective blood vessel formation (265,266). Similarly, the expression of VEGF and VEGFR has not yet been thoroughly analyzed in the developing neuromuscular system, although a recent report demonstrated that VEGF is essential for the developmental migration of facial MNs (267). Conditional mutant mice lacking VEGF and VEGFR have been generated and will hopefully soon be crossed to mice expressing Cre

recombinase in specific neuromuscular tissues in order to explore the role of this growth factor during development.

5.8 Summary of NTFs during MN developmental PCD

Although the first NTF/NTFR KO mice were generated nearly 15 years ago, the precise MN subtypes lost in each of them remains largely unknown. This is partially because constitutive NTF/NTFR null mutants often exhibit embryonic or neonatal lethality, thus allowing for gross but not precise analysis of missing MNs. Nevertheless, these studies demonstrate that the absence of an individual NTF nearly always enhances MN PCD, suggesting that like PNS neurons, MNs are sensitive to the removal of single NTFs. In the most thoroughly characterized example, such as conditional GDNF receptor KO mice, the near complete loss of γ MNs may indicate that NTFs are required for the complete survival of MN subtypes rather than for partial survival across many subtypes. Although this finding is consistent with the hypothesis that MNs which fail to gain access to individual NTFs are eliminated during PCD (268), it also suggests that different MN populations exhibit complete dependence on single and non-overlapping NTFs. In contrast, nearly all NTFs are effective in preventing the death of axotomized MNs. Finally, the inactivation of CNTF, NT-3 or VEGF causes a loss of MNs postnatally (see below), indicating that trophic deprivation may at least partially contribute to MN disease. However, the notion that all MNs exhibit a universal response to NTF deprivation caused by developmental, injury-induced or degenerative stimuli is not supported by these studies. Indeed, the idea that MN death during diseases such as ALS is similar to that during developmental PCD is itself questionable and is the subject of the following section of this review.

6. PATHOPHYSIOLOGY OF ALS

The death of MNs occurs and may play a pathogenetic role in MN diseases such as Amyotrophic Lateral Sclerosis (ALS). ALS causes death of both lower MNs (i.e., cranial and spinal MNs that innervate skeletal muscle) upper MNs (e.g., corticospinal neurons projecting to the spinal cord; 269), spinal interneurons (270,271) and sensory neurons (272), and results in muscle weakness, paralysis and death by respiratory failure within 3–5 years of symptom onset. 90–95% of ALS occurs with unknown etiology but increasing subsets of sporadic ALS (sALS) cases are associated with mutations in a diverse set of genes, including dynactin, Neurofilament-H, Peripherin, Angiogenin and VEGF (273). The remaining 5–10% of ALS cases are inherited and approximately one-fifth of these familial ALS (fALS) mutations are found within the gene encoding superoxide dismutase 1 (SOD1; 273). SOD1, also known as Cu/ZnSOD in humans, encodes a dimeric metalloenzyme which catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide (275). Transgenic mice containing various human SOD1 mutations develop progressive neurodegeneration and MN death, providing an animal model that has greatly contributed to the understanding of fALS pathogenesis (276). SOD1 mutations cause fALS by exerting a dominant gain of function, because the loss of SOD1 does not cause disease (277) and because disease-causing SOD1 mutations often fail to affect the antioxidant activity of SOD1 (278).

Wild-type (wt) mammalian SOD1 is robustly expressed by MNs (279). The 32-kD SOD1 homodimer is composed of two 16-kD subunits, each of which bind one zinc and one copper ion and contain one intrasubunit disulfide bond. After receiving zinc, SOD1 monomer forms a heterodimeric complex with the copper chaperone for SOD1 (CCS), which in the presence of oxygen results in the loading of copper onto SOD1, the formation of the disulfide, the disassociation of SOD1 from CCS, and the subsequent formation of dimeric SOD1 (280). The quaternary structure of dimeric SOD1 is very stable and the disulfide linkage is unusual for a protein present within the reducing environment of the cytosol (281,282). Demetallated

forms of SOD1 (apo-SOD1) are destabilized (283,284) and demetallated, sulfide-reduced forms of SOD1 prevent the formation of dimeric SOD1 (281,285). Interestingly, only this reduced, apo-form of SOD1 can be transported into mitochondria (286). The biological activity of dimeric SOD1 occurs in two half-reactions requiring the copper ion cofactor, which oxidizes one superoxide anion to form oxygen and then is re-oxidized by a second superoxide anion, creating hydrogen peroxide (275).

6.1 Molecular mechanisms of ALS

Two molecular mechanisms have been postulated to explain how mutant SOD1 causes ALS. The oxidative hypothesis proposes that mutant SOD1 generates the formation of toxic reactive oxygen species (ROS) through altered redox activity of the copper ion. These alterations in oxidative activity are believed to be caused by the reduced affinity for zinc exhibited by mutant SOD1 (287). One of these species, peroxynitrite, which also depends on the presence of the intercellular messenger nitric oxide (NO), causes the nitration of tyrosine residues on proteins (288). Elevated 3-nitrotyrosine products are detected within the spinal cord of both human ALS and mice models of fALS (289,290). Nitrated proteins may cause degeneration of MNs by a variety of mechanisms including interference with cell signaling, protein degradation, axonal transport or activation of cell death pathways (291). The role of zinc in SOD1 oxidative dysfunction is also supported by the finding that WT SOD1 lacking zinc causes the death of MNs *in vitro* through a NO-dependent mechanism (292). Further evidence supporting the role of NO in MN degeneration is supported by the finding that inactivation of the inducible form of nitric oxide synthase (iNOS) significantly extends lifespan of mutant SOD1 mice (271). However, mice expressing mutant SOD1 in which the four copper-binding residues are also mutated nevertheless develop MN disease, suggesting that copper-mediated redox activity at the active site is not pathogenic (293). Nevertheless, the potential for mutant SOD1 to bind copper through non-native interactions and thereby cause aberrant oxidative activity remains unexplored.

The second possible molecular mechanism is based on the observation of SOD1-immunoreactive aggregates in neurons and glia in patients with fALS and in transgenic mouse models (294–297). SOD1 immunoreactive aggregates are composed of high molecular weight, detergent-insoluble macromolecules of SOD1 present only in CNS tissue at about the time of symptom onset in fALS mice (298). These insoluble aggregates are composed of intersubunit disulfide-linked multimers of SOD1, suggesting that immature disulfide-reduced forms of SOD1 monomer are the precursor for SOD1 aggregates (299–301). Together with reports that intrasubunit disulfides are more likely to be reduced in mutant SOD1 (302) and that protecting these bonds from reduction reduces the formation of aggregates (303), these studies indicate that both nascent SOD1 monomers and SOD1 monomers formed from the dissociation of unstable dimers may contribute to the disulfide cross-linked SOD1 multimers found within aggregates (304). However, it was recently demonstrated that fALS mutant SOD1 species lacking all four cysteine residues, and thus unable to form disulfide linkages, nevertheless retain the ability to form aggregates *in vitro* (305). Conversely, mice in which mutant SOD1-mediated disease is accelerated by overexpression of the copper chaperone CCS (306) exhibit increased disulfide reduction without the formation of aggregates (307). Aberrantly reduced and/or cross-linked, aggregated SOD1 may cause MN degeneration by a variety of mechanisms. Because only the demetallated, reduced form of SOD1 enters the mitochondria, mutant SOD1 expression within this organelle may increase to pathological levels. Alternatively, aggregates may represent the binding of mutant SOD1 to non-native substrates. Finally, the presence of aggregates in vesicles of the ER-Golgi system indicates that a percentage of mutant SOD1 is secreted and thus capable of exerting toxicity non-cell autonomously (308,309). Together,

these studies suggest that oxidative dysfunction and aggregation represent the major modes of molecular pathogenesis.

6.2 Cellular mechanisms of ALS

Several cellular mechanisms have been proposed to mediate the neuronal dysfunction caused by mutant SOD1 oxidative or aggregative properties (Figure 2). The first and perhaps most prevailing is that mutant SOD alters mitochondrial structure and/or function (310). Morphologically altered mitochondria are found in the spinal cord of human sALS and fALS patients (311,312) and of mutant SOD1 transgenic mice (313–315). SOD1 immunoreactivity is found in a rimlike pattern around these vacuoles in histological sections of the spinal cord (316). Mitochondrial abnormalities are observed as early as P14 in the spinal cord of SOD1 G93A mutant mice (317), which exhibit symptom onset near P90 (318). Electron transport chain activity and ATP production are also reduced in mutant SOD1 mice, but not until symptom onset (319,320). Although small amounts of cytosolic WT SOD1 are detected within the intramembrane space of mitochondria (321), a significantly larger percentage of mutant SOD1 is found within various regions of this organelle (322–324). Importantly, whereas fALS mutants can exhibit normal or severely deficient metal content and thus biophysical activity (325), both of these classes share a pathologically enhanced association with spinal cord mitochondria (326). Enhancing the levels of mitochondrial-associated mutant SOD1 by overexpressing the CCS chaperone accelerates the death of mutant SOD1 transgenic mice, further implicating mitochondria as an important locus of toxicity (306). Furthermore, overexpressing WT SOD1 in a mutant SOD1 background accelerates death and increases the percentage of mitochondrial WT SOD1, suggesting that the toxic effects of SOD1 mutations are mediated by enhanced distribution of SOD1 to this pool (327). Because demetallated, disulfide-reduced forms of SOD1 monomer are exclusively imported into mitochondria (328) and because mutant SOD1 exhibits altered antigenic accessibility (326) these data suggest that destabilized SOD1 species are tracked to mitochondria, where they misfold to form cytotoxic, non-native intersubunit-linked multimers (327,329,330). Interestingly, a recent study found that a novel, disulfide-independent non-native dimer of mutant SOD1 occurs in both familial and sporadic ALS, suggesting a common mechanism through which mutant and WT SOD1 cause ALS (331). Although the precise pathway by which mitochondrial dysfunction causes MN disease is unclear, several important possibilities include dysregulation of mitochondrial motility or transport (332), reduced energy production (333), altered ionic homeostasis (334) and/or activation of apoptosis (335). Interestingly, mutations preventing the movement of mitochondria to axons and presynaptic terminals are sufficient to cause defective neuromuscular transmission and death (336). Reduced numbers of mitochondria in MN presynaptic terminals was also recently demonstrated in transgenic mice expressing human TAR DNA-binding protein-43 (TDP-43), a recently generated ALS animal model, suggesting that mitochondrial distribution rather than function is affected in MN disease (337).

The second potential cellular mechanism by which mutant SOD1 causes MN degeneration involves axonal transport, which as just mentioned may also overlap with perturbations in mitochondrial function and motility. A possible role for impaired axonal transport in ALS pathogenesis was suggested by the presence of neurofilamentous inclusions in the MN perikarya of human sALS patients and transgenic fALS mice (311,338). Additionally, mutations in the heavy neurofilament gene (NF-H) are associated with a small number of sALS cases (339) and cause MN disease in mice (340). Slow anterograde axonal transport is impaired early in fALS mice (340,341), as is retrograde transport (342) and fast anterograde transport (332). Additionally, mutations within members of the retrograde motor system, including dynein, dynactin and dynamitin produce are associated with sALS (343–345) and

produce MN disease in mice (63,344,347,348). Impaired axonal transport may result in MN degeneration by a variety of mechanisms, including focal damage to axons (349), alterations of SOD1 secretion (308,350), interruption of vesicle trafficking and/or increases in protein aggregation (351,352) impairment of retrograde neurotrophic signaling (353), activation of autophagic death (63) and impaired delivery of mitochondria to distal axons and synaptic terminals (332,354). Together, these studies suggest mutant SOD1 is toxic to neurons by impairing several mutually non-exclusive cellular processes, including mitochondrial distribution and axonal transport.

6.3 Tissue mechanisms of ALS

Because both the pathophysiology (e.g., motor weakness and paralysis) and pathology (e.g., degeneration of ventral root axons and ventral horn somata) of ALS indicate a disease whose primary locus is MNs, the role of other cells in mediating ALS has (until recently) been ignored, with the exception of defective astrocytic glutamate handling observed in human sALS patients (355). The findings that neuronal-specific overexpression of fALS-associated SOD1 mutants is insufficient to cause disease in mice (356,357; but see 358), and that expression of mutant SOD1 in non-neuronal cells is sufficient to cause MN disease (359) has suddenly cast light on other cell types as candidates mediating ALS to MNs in a non cell-autonomous manner (360). These include, in principle, any endocrine cell in the body, suraspinal, propriospinal or sensory ganglia neurons sending afferent input to MNs, multiple classes of interneurons mediating spinal reflexes, astrocytes in the ventral horns, oligodendrocytes in the ventrolateral funiculus, Schwann cells in the ventral root, peripheral nerve and neuromuscular junction, microglia in the spinal cord or nerve, skeletal muscle at the periphery, vascular smooth muscle in the arterioles irrigating the spinal cord, and vascular endothelial cells lining the walls of spinal, nerve and muscle capillaries (Figure 2). For the heroic efforts required to identify the offending cell type(s), conditional or excisable alleles of mutant SOD1 are proving indispensable. Conditional or floxed mutant SOD1 mice, crossed to mice expressing Cre-recombinase behind a promoter robustly expressed by microglia, delays the onset and progression of MN disease (361), suggesting that mutant SOD1 toxicity to microglia contributes to MN disease. Similarly, the removal of mutant SOD1-expressing microglia and replacement with wild-type (WT) SOD1-expressing microglia delays progression (but not onset) of fALS mice (362). Eliminating mutant SOD1 from astrocytes also delays progression but not onset of disease (363), consistent with a report showing that astrocytes expressing mutant SOD1 secrete toxic substances to MNs (354,365). However, overexpression of mutant SOD1 in astrocytes alone is not sufficient to cause MN disease in mice (366). Removal of mutant SOD1 from skeletal muscle or endothelial cells fails to affect either disease onset or progression (367,368). Interestingly, a recent study showed that expression mutant SOD1 in muscle cells is sufficient to cause MN disease (369). This study is in accord with the finding that muscle-restricted mitochondrial uncoupling causes mild, late-onset MN degeneration and pathology (370). However, because WT SOD1 also causes disease in this paradigm (369), the physiological significance of this finding remains unclear.

7. MN DEATH IN ALS: PASSERBY, PARTICIPATORY OR PARAMOUNT

Although degeneration of MNs is a prominent feature of ALS, whether activation of the cell death machinery represents a pathogenetic mechanism or a downstream effector of MN degeneration remains unclear. Four types of studies have helped clarify this issue. (1) Because certain forms of death have been historically more associated with active vs. passive killing of neurons (e.g., apoptosis vs. necrosis), the structural and molecular heterogeneity underlying these forms of cell death have been characterized in human cases and animal models of ALS. (2) The temporal correlation between cell death activation and other aspects of MN dysfunction such as neuromuscular denervation has been established in

fALS mutant mice. (3) The extent to which cell death inhibition delays onset vs. progression of disease has been examined in transgenic fALS mice. (4) The correlation between MN death and disease has been investigated in which one or the other has been experimentally changed. A summary of the effects of these studies is presented in Table 2.

MN death underlies the pathology of both human cases and mouse models of familial and sporadic ALS (63,276,311,371). Post-mortem studies of human ALS patients and end-stage fALS mice estimate the loss of spinal MNs to be near 60%, and morphometric analyses of both ventral root axons and ventral horn MN somata indicate that this loss is restricted to large-diameter, skeletomotor α MNs (271,315,318,372–374). However, because MNs undergo atrophy in ALS (375), and muscle spindle innervation has only been systematically investigated in neonatal and not adult or fALS mice (51), it remains possible that a subset of dying MNs are spindle-innervating, fusimotor γ MNs and that a subset of remaining MNs are atrophied α MNs. Indeed, specific subsets of α MNs are retained even at advanced stages of disease in fALS mice (376).

In human ALS post-mortem tissue, dying MNs exhibit some but not all of the morphological features of apoptosis, such as cell shrinking and nuclear condensation, but not formation of apoptotic bodies or DNA fragmentation (371). Additionally, remaining MNs in human ALS spinal tissue exhibit prominent intracellular inclusions (377), damage to organelles such as Golgi (378) and mitochondria (379) and cytoskeletal alterations (311), none of which are observed in classical apoptosis. Similarly, Guégan and Przedborski (2003; 380) observed apoptosis in some but not all dying MNs within the spinal cord of symptomatic fALS mice. Other studies have completely failed to detect evidence for apoptosis in either human cases or mouse models of ALS (381,382). One recent report demonstrated MN swelling and a reduction of membrane-associated Na^+ , K^+ ATPase immunoreactivity in high-expressing G93A SOD1 mutant mice, reminiscent of necrosis (271). Another recent report described evidence for Type II autophagic cell death in dynactin sALS mutant mice (63) and autophagic dysfunction in SOD1 fALS mutant mice (64). Similarly, in a chick model of MN disease displaying tubulovesicular inclusions and neuromuscular dysfunction, autophagic but not necrotic or apoptotic cell death was observed (62,383,384). Together, these morphological studies do not support a role for widespread apoptosis in ALS.

Similarly, biochemical studies have supported a limited role for apoptosis in ALS. $\text{TNF}\alpha$ and Fas are extracellular activators of the extrinsic pathway of Type I cell death or apoptosis. Although $\text{TNF}\alpha$ is elevated in multiple cell types within the spinal cord of fALS mice (385,386), genetic deletion of $\text{TNF}\alpha$ exerts no effect on their lifespan or MN number (387). Fas and FasL appear to exhibit enhanced MN-associated immunoreactivity in presymptomatic fALS mice (388), and the reduction of FasL expression modestly extends lifespan of SOD1 mutant mice. In contrast, intrathecal injection of Fas siRNA significantly lengthens the lifespan of fALS mice (389), but the more convincing study of crossing Fas KO to fALS mice has yet to be reported, despite the availability of both mice for over 15 years. Fas-mediated death of mutant SOD1-expressing embryonic MNs *in vitro* proceeds through a pathway including Daxx, ASK1 and p38 and NO (146), and the elevation of p38 MAPK and iNOS is increased in MNs of presymptomatic fALS mice (271,390). Pharmacological inhibition of p38 MAPK increases lifespan of DO1 mutant mice by only 6 days, however (391). Inactivation of iNOS extends the lifespan of high- but not low-expressing G93A SOD1 mutant mice (271,392), but because NO is provided by multiple sources and cytotoxic by multiple mechanisms its participation cannot by itself be taken to indicate a specific death pathway in ALS (393; see below). Finally, although the expression of p53 is upregulated in fALS mice, its import into the nucleus is blocked (271), a finding which may explain the failure for inactivation of p53 to extend the lifespan of fALS mice

(394,395). Together, these studies argue that the extrinsic pathway of apoptosis plays at most a minor role in ALS.

Similar studies have shown that upregulation of Bax, and Caspases-1 and -3 are observed in presymptomatic fALS mice, suggesting that the intrinsic apoptotic machinery operates during ALS (396–398). However, inhibition of upstream and downstream members of this pathway has yielded contradictory results: whereas Bcl-2 overexpression and Bax deletion extends the lifespan of fALS mice (375,399) inactivation of Caspase-1 or Caspase-11, whose own activity is required for Caspase-3 activation, fails to increase lifespan (271,400,401 but see 402). Together with the finding that activated Caspase-3 immunoreactivity is predominantly associated with astrocytes (381), these findings suggest that although Bax translocation to mitochondria occurs, caspases are not activated in MNs of fALS mice. Bcl-2 overexpression may inhibit MN death by other mechanisms independent of apoptosis: (1) dilution of the toxic effects of direct physical association of mutant SOD1 with Bcl-2 (403); (2) inhibition of necrotic cell death (404); (3) prevention of autophagic death (405); (4) enhancement of axonal regeneration (406). Finally, the contribution of caspase-independent apoptosis to MN degeneration in ALS has recently been investigated. While genetic inactivation studies of either AIF or EndoG have yet to be performed, the expression of AIF is increased in MNs of SOD1 G93A mutant mice, but this elevation appears restricted to mitochondrial-associated vacuoles and not the nucleus (271), where AIF causes DNA fragmentation and apoptotic death (407). Together with the finding that Bax deletion completely prevents MN death in fALS mice (375), these results suggest that MNs die by a Bax-dependent, Bcl-2-sensitive pathway that differs from caspase-dependent apoptosis.

The occurrence of autophagic death has recently been shown to occur in a variety of neurodegenerative disorders (408), and the inhibition of autophagy is sufficient to cause neurodegeneration in mice (409,410). Conversely, induction of autophagy by deletion of a gene required for the unfolded protein response pathway (UPR) enhances the survival of fALS mice (411). Autophagic clearance of both protein aggregates and damaged organelles depends on the activity of the retrograde motor complex (351), and mutations in dynactin that cause ALS in humans and mice lead to a pathological accumulation of membrane vesicles and autophagic death (63). Similarly, embryonic chick MNs subjected *in vivo* to sublethal excitotoxicity exhibit elevated protein retention in the ER and autophagic degeneration rather than apoptosis or necrosis (384,412). A similar deficit in autophagy could either cause or result from mutant SOD1-containing protein aggregates in the ER (309) and in the dysfunctional, hypertrophic mitochondria observed in fALS. Together, these studies warrant further exploration of the autophagic death pathway in ALS.

Necrosis but not apoptosis or autophagic death triggers local inflammation, and the observation of increased levels of activated microglia in the spinal cord of fALS mice near the onset of MN death lends additional support to the notion that such cell death is necrotic (413). Activated microglia release pro-inflammatory cytokines, ROS, NO and glutamate (414,415), and treatment of fALS mice with minocycline, a potent inhibitor of microglial activation, prolongs their lifespan (416,417). Similarly, inhibition of NADPH oxidase diminishes the production of microglial-derived ROS and accordingly extends the lifespan of SOD1 mutant mice (418). Microglia may represent the source of elevated CNS glutamate in human ALS cases and fALS mice (176,355). Alternatively, excessive extracellular glutamate in ALS may reflect dysfunction of the astroglial glutamate transporter EAAT2 (419–420). Glutamate-triggered depolarization allows Ca^{++} to enter MNs through voltage-sensitive and ligand-dependent, voltage-sensitive Ca^{++} channels, and excessive activation causes excitotoxic elevations of intracellular Ca^{++} , which may perturb cellular viability by increasing neuronal NOS activity, stimulating the ER stress response, promoting

mitochondrial permeability transition (mPT) and activating Ca^{++} -sensitive enzymes such as calcineurin and calpain (393). Interestingly, sustained administration of NMDA to chick embryos causes a sublethal excitotoxicity that exhibits considerable morphological similarity to ALS, suggesting that elevated glutamate receptor-mediated signaling is sufficient to trigger these pathological alterations (383). Together, these studies provide evidence that the MN death observed in ALS contains features of necrosis.

Although these studies indicate the importance of microglia as important sources of excitotoxic signals to MNs in ALS, they fail to indicate by themselves which death pathway MNs adopt in the end. For example, NO activates the DNA repair enzyme PARP1, the excessive activity of which during stroke leads to an energy-depleting form of death most similar to necrosis (421). In contrast, NO mediates apoptosis after cerebral ischemia by catalyzing the S-nitrosylation of matrix metalloproteinase 9 (422). Similarly, excessive intracellular Ca^{++} can cause the death of neurons through apoptosis or autophagy triggered by ER stress (423–425), or necrosis triggered by mPT (426,427). Interestingly, PUMA, a BOP activator of Bax/Bak, is required for apoptosis caused by ER stress in cultured MNs and is elevated in the spinal cord of presymptomatic fALS mice (428). Furthermore, elevations of misfolded proteins in the ER, which stimulate ER stress-mediated apoptosis through the UPR (429), have recently been reported in SOD1 mutant mice (309). However, inactivation of PUMA fails to extend the lifespan of fALS mice (428), suggesting that the mechanism by which high intracellular Ca^{++} kills cells is not ER stress-mediated apoptosis. Together, these studies suggest that dying MNs use upstream biochemical pathways such as Bax which are common to apoptosis, necrosis and autophagy, fail to either activate downstream caspases or to die by apoptosis, and instead exhibit a hybrid morphological character of necrotic and autophagic death. Most importantly, they suggest that cell death in ALS can be effectively blocked and that, in conjunction with therapies aimed at reversing more proximal molecular disease mechanisms such as protein oxidation or aggregation, therapies aimed at inhibiting Bax may protect and rejuvenate diseased MNs.

While it is reasonably straightforward to characterize the cell death pathway selected by MNs dying in ALS, providing data to argue for or against the cell death pathways as a principal pathogenetic mechanism in ALS or merely downstream executor is more complicated. One obvious strategy is to compare the temporal activation of cell death components in fALS mice with other measurements of disease progression. In high-expressing G93A SOD1 mutant fALS mice, cell death is initially observed at P65–P90, close to the onset of symptom onset, which occurs near P100 (271,276,318). Cell death pathway activation, measured by upregulation of Bax protein (375,396), occurs significantly later than deficits in mitochondrial morphology (317), axonal transport (343) and neuromuscular innervation (430,375), suggesting it acts as a downstream effector of MN degeneration. The coincident upregulation of cell death proteins and frank MN degeneration, between P60–P75, generally holds true for the measurement of most cell death proteins believed to mediate cell death. The implication of these results is that the core components of the cell death pathway are not the preponderant site of molecular damage underlying ALS, and that a key feature mediating MN dysfunction, namely progressive neuromuscular withdrawal, occurs long before this pathway is activated. Whether regressive events at the neuromuscular junction (NMJ) represent local, presynaptic toxicity or represent a response by MNs to toxicity initiated elsewhere is unclear (431). However, a recent study has challenged the interpretation that cell death in fALS is downstream of denervation by demonstrating that Bcl-2 represents an early, direct pathological target of mutant SOD1 (335). Future studies aimed at corroborating these data and elucidating its significance should prove interesting.

Another method to distinguish whether MN death is a disease target or a terminal process comes from studies in which particular experimental manipulations result in the extension of fALS lifespan. If cell death plays a principal role in mediating MN degeneration, then factors extending lifespan by blocking the cell death pathway might be expected to delay disease onset rather than progression, because inhibition of cell death would precede inhibition of denervation and motor weakness. In contrast, treatments resulting in the extension of disease progression imply that the cell death inhibition is downstream of denervation and weakness and therefore a downstream process of MN disease. With respect to members of the extrinsic apoptotic pathway, deletion of FasL and p38 MAPK both delayed progression but not onset of disease (391,432), whereas this distinction was not reported after iNOS deletion (271). In contrast, overexpression of Bcl-2 or deletion of Bax, BIM or PUMA all extend disease onset, suggesting that the intrinsic pathway may be involved in disease pathogenesis (375,399,428,433). However, the extent to which proteins in this pathway cause disease by promoting death or some other aspect of degeneration is not clear; for example, the ability of Bcl-2 to promote re-innervation after injury suggests a regenerative as well as anti-death role for this protein (406 but see 434).

In fact, the complete inhibition of MN death in SOD1 mutant, Bax-deficient mice supports the idea that these “pro-apoptotic” proteins delay disease onset through cell death-independent mechanisms: if the delay in disease onset by Bax deletion reflects the inhibition of Bax-mediated death, then indeed Bax-deficient SOD1 mutant mice should never die, as Bax-mediated MN death is completely inhibited. Instead, SOD1 mutant, Bax KO mice die despite retaining all MNs (375), suggesting that MN death itself is not required for MN dysfunction. This profound dissociation between MN survival and organism death has been reported in other models of MN disease (435). Moreover, recent studies show a similar discrepancy between MN survival and lifespan extension in fALS mice (391,428). This likely represents a differential capacity for the specific regional compartments of the MN such as the soma, axon and synaptic terminals to withstand cellular stresses (431). Supporting this idea, transplantation of GDNF-overexpressing neural progenitors into the spinal cord of fALS rats robustly enhances the survival of MN cell bodies but fails to impede neuromuscular denervation and thus degeneration (436).

8. NTFs, MN DEATH AND ALS

Because MN death in ALS occurs late in disease, proceeds through a hybrid of necrotic-autophagic morphologies and is dispensable for disease, therapies aimed exclusively at inhibiting developmental or axotomy-induced apoptotic cell death pathways may not succeed in ameliorating symptoms. Nevertheless, because NTFs regulate maintenance and regeneration as well as developmental apoptotic death of MNs, their use as therapies for ALS has been extensively studied in animal models of disease and in human clinical trials. Additionally, a.) the degree to which NTF expression or signaling is affected in MN disease and b.) the ability of NTF treatment methodologies to restore some aspects of MN function and c.) the knowledge that specific NTFs and NTFRs are expressed in discrete cell types, has provided some insight into the functional status of different inter- and intracellular pathways (Figure 3). For example, the loss of GDNF expression in human ALS muscle tissue, together with the lifespan-extending effects of muscle- but not astrocyte-derived overexpression of GDNF, suggest that MN presynaptic terminals but not cell bodies are deprived of and maintain responsiveness to this NTF (see below). The following section aims to summarize these studies, with an emphasis again being placed on transgenic animal studies employing fALS mutants.

8.1 GDNF

Exogenous GDNF and the autophagy inhibitor 3-methyladenine prevent the death of excitotoxically injured MNs in spinal cord explants and promote motor axon outgrowth, suggesting that GDNF may be protective against oxidative stress (437,438). Interestingly, the growth but not neuroprotective effects of GDNF are abolished by inhibitors of PI-3K, suggesting that GDNF inhibits developmental apoptotic death and excitotoxic necrotic-autophagic death by different mechanisms (437). Because GDNF also protects against the death-inducing effects of proteasomal inhibition in cultured dopaminergic (DAergic) neurons (439), the ability of GDNF to ameliorate autophagic dysfunction may result from its ability to attenuate aggregation. Together with the finding that GDNF blocks DAergic neuronal neurodegeneration caused by the mitochondrial toxin 3-nitropropionic acid (440), these studies suggest that exogenous GDNF protects neurons from cell death induced by a variety of oxidative/aggregative stimuli that involve the mitochondria. In contrast, endogenous GDNF appears dispensable for these neurotoxic effects, because deletion of *Ret* fails to increase the death of DAergic neurons in mice treated with the neurotoxin MPTP (441). In addition to disturbing mitochondrial morphology and function, ALS causes an early disturbance in axonal transport (see above). Although GDNF regulates axonal growth and synaptic maturation of MNs during development (175,442), GDNF does not appear to enhance the retrograde transport of molecules within degenerating MNs (443). Finally, because ALS may result from disease to MN neighboring cells such as afferent neurons, astrocytes, Schwann cells or microglia, it is noteworthy that the expression of both GDNF and *GFR α 1* is increased in injured peripheral nerves (444). Exogenous GDNF induces the proliferation of Schwann cells and stimulates the myelination of unmyelinated axons in adult rats, indicating a non-cell-autonomous manner by which GDNF may influence motor function during disease (445).

The effects of GDNF on innervation, cell death and motor function have been studied in a variety of neurodegenerative conditions (446). In contrast to various animal models of Parkinson's disease, where treatment with GDNF rescues DAergic neurons, prevents presynaptic terminal withdrawal and delays locomotor decline (447–449), studies of mice with MN disease reveal that GDNF preferentially protects the cell body rather than presynaptic terminals of MNs, with modest or no beneficial effects on disease onset and variable effects on disease progression (436,450–453; see Table 3). Interestingly, when GDNF overexpression is targeted to the muscle rather than to the CNS, muscle innervation is maintained longer and disease progression is extended (454–456). In human ALS patients, GDNF is upregulated in muscle (457), *Ret* phosphorylation of Y1062 is detected (458), suggesting that MNs maintain responsiveness to GDNF. These studies indicate that treatment with GDNF, by attenuating necrotic-autophagic MN death, represents a useful component of a multi-factor therapeutic approach to treat ALS, but that by itself is insufficient to arrest the motor axon withdrawal that precedes and likely drives this MN death.

The prospect of GDNF as a therapeutic factor for ALS is not only limited by these concerns of biological efficacy, but also those of pharmacological delivery. Like all peptide neurotrophic factors, GDNF exhibits pharmacokinetic constraints, including the inability to cross the blood-brain barrier (BBB) and the regulation by molecules in the plasma, CSF or local extracellular matrix such as heparan sulfate (459). Because GDNF receptors are expressed throughout the body and brain, delivery of this NTF outside of its immediate target region may also produce unwanted side effects (460). Additionally, prolonged GDNF treatment may reduce endogenous GDNF signaling by causing receptor downregulation or stimulating the production of GDNF antibodies (461). Some of these issues are encountered even when NTF delivery is optimized by surgical implantation of a catheter within the CNS proximal to the target. For example, despite the successful results of intrastriatal infusions of recombinant GDNF in both animal studies and Phase 1 clinical trials of Parkinson's disease,

a larger, double-blind Phase 2 trial failed as a result of poor penetration distal to the delivery site (462,463). Moreover, GDNF activation of Purkinje cells in the cerebellum was observed to result from the contamination of CSF with GDNF from the catheter (463). Therefore, a variety of techniques to maximize the specificity and control of GDNF delivery are currently being evaluated, including surgical infusion of recombinant GDNF, transplantation of encapsulated, GDNF-overexpressing cells (464), infection of various types of viral vectors expressing GDNF (465,466), administration of small lipophilic compounds capable of upregulating GDNF expression (467), chemical modification of GDNF to enhance its passage through the BBB (468) or increase its internalization by neurons (469,470). Because one recent study demonstrated that GDNF overexpression in muscle cells but not astrocytes extends the survival of fALS mice (454), systemic or intramuscular delivery of viral or modified GDNF, rather than CNS-targeted delivery, may be a preferred therapeutic approach to achieve MN neuroprotection in ALS (Figure 3).

8.2 BDNF and NT-3

In contrast to their ability to protect striatal neurons from excitotoxicity (471–473), BDNF and NT-3 signaling through the high-affinity receptors TrkB or TrkC exerts no effect on MN survival in response of excessive glutamate receptor stimulation (474,438). In some studies, BDNF actually increases the vulnerability of MNs to excitotoxic stress (475), consistent with findings that BDNF and TrkB increase the production of ROS and cause necrotic death (476,477). Although the effects of BDNF and NT-3 on protein degradation or aggregation have not been examined, BDNF increases the oxidative efficiency of brain-derived mitochondria, suggesting a potential role for this NTF in ameliorating mitochondrial dysfunction in ALS (478), and NT-3 reduces diabetes-induced deficits in the mitochondrial membrane potential of sensory neurons (479). In contrast to GDNF, both BDNF and NT-3 increase retrograde transport within degenerating MNs (443). Interestingly, NT-3 regulates the survival of developing Schwann cells (480), and NT-3-deficient mice exhibit a postnatal loss of Schwann cells and motor neuropathy (197). These results suggest that alterations in NT-3 signaling may play a cell non-autonomous role in the degeneration of MNs.

Exogenous BDNF and NT-3 slow MN degeneration and increase lifespan in two models of inherited MN disease, the *wobbler* and progressive motor neuropathy (*pnn*) mice, respectively (481,482). Similar to GDNF, the expression of neurotrophins and phosphorylated TrkB are detected in the muscle and spinal cord, respectively, of human ALS patients, suggesting that MNs can access and respond to these factors during disease (483). However, in both fALS mice and Phase 3 human clinical ALS trials, treatment with subcutaneous recombinant BDNF fails to affect motor decline or lifespan (484,485).

Crossing fALS mice to mice lacking the low-affinity neurotrophin receptor p75 also fails to significantly extend lifespan (483). Although BDNF is able to cross the endothelial cells of the BBB by a saturable transport mechanism, significant penetration into the CNS is limited by the parenchyma, to which most systemically BDNF is bound (486). Animal and human studies of intrathecally administered BDNF also show the trapping of BDNF by spinal parenchyma, but a significant portion of BDNF also binds to motor axons coursing through the subarachnoid space and is retrogradely transported to MN perikarya (487,488).

Nevertheless, this method of delivery still fails to increase motor performance or lifespan in clinical trials (489). Finally, because tyrosine kinase receptors are transactivated by G-protein-coupled receptors (GPCRs) such as the 2A adenosine receptor (Ad_{2A}-R), therapies with small-molecule agonists of this receptor are being explored (490). The sum of these studies indicate that although neurotrophins appear unable to ameliorate ALS, they may retain use as therapies for MN diseases such as spinal motor atrophy (SMA), whose early onset and quick progression more closely resemble that of the *pnn* and *wobbler* mutant mice.

8.3 CNTF

Although CNTF reduces excitotoxicity in animal models of Huntington's Disease (491,492), exogenous CNTF, LIF or CT-1 fails to promote the long-term survival of MNs in spinal explants after exposure to excitotoxic stress (438,474). Because oxidative stress inhibits signaling induced by CNTF in a MN cell line (493), ROS produced during ALS may prevent exogenous CNTF from rescuing excitotoxically injured MNs. Furthermore, oxidative stress may cause ALS in part by impairing endogenous CNTF signaling, because deletion of CNTF in mice causes the loss of MNs and a modest motor weakness (219). This idea is also supported by the findings that CNTF expression is reduced (494) and CNTFR 1 expression is increased (495) in the spinal cord of human ALS patients (but see 496). Similarly, after sciatic nerve axotomy, CNTF expression in nerve-derived Schwann cells is dramatically reduced (497) and CNTFR 1 expression in MNs is increased (498). CNTFR α 1 is expressed by astrocytes and activation of these cells by CNTF mediates the neuroprotective effects observed in the striatum (491). CNTFR α is also expressed by Schwann cells (499) and CNTF is required for the proper maintenance of axon-Schwann cell networks (ASNs; 500). Together, these studies suggest that disruption of cytokine signaling in spinal or nerve-derived glia may contribute to non-cell autonomous MN disease.

Although systemic administration of CNTF to both *pnm* and *wobbler* mutant mice increases MN survival, improves motor performance and lengthens lifespan (183,501), subcutaneously injected CNTF causes severe side effects (502–504) and exerted no effect on motor function or lifespan in human Phase 3 clinical trials of ALS (505). Whereas systemically administered CNTF induces MN sprouting in muscles of wild-type mice (506), it has yet to be shown whether diseased MNs, Schwann cells or astrocytes respond or gain access to systemic CNTF. Intrathecal implantation of encapsulated cells engineered to express CNTF results in long-term expression and the absence of major side effects in Phase 1 studies, providing hope that this route of CNTF may be better tolerated and more likely to activate CNTFR α expressed by MNs and Schwann cells (487,507).

8.4 HGF

Although the trophic effects of HGF on developing MNs are well established, few studies have examined the role of this factor in ALS. Exogenous HGF prevents the decline in ChAT expression observed after adult hypoglossal axotomy (508), indicating that MNs retain responsivity to this NTF despite downregulating its receptor Met shortly after developmental PCD (53). HGF also reduces MN death in response to excitotoxic stress (508). Consistent with these studies, crossing fALS mice to transgenic mice overexpressing HGF extends the lifespan of fALS mice exclusively by delaying disease onset (509). In this study, HGF overexpression delayed the induction of iNOS but exerted no effect on mutant SOD1-mediated accumulation, suggesting that HGF interferes with oxidative but not aggregative processes associated with ALS (509). Astrocytes in fALS mice upregulate the expression of Met and HGF overexpression retards reactive gliosis in the spinal cord, suggesting that HGF, like CNTF, may also signal through astrocytes to ameliorate disease (509,510). Intrathecally injected HGF also extends lifespan of fALS rats when given at symptom onset (511). Met is also expressed by endothelial cells, whose response to VEGF during hypoxia may contribute to ALS (see below). Similar to expression of receptors for GDNF and BDNF, Met is expressed in spinal cord MNs in human ALS (512). Levels of phosphorylated Met are strongly reduced in fALS rats (511), indicating that HGF levels decline during disease. HGF has not been developed as a therapy for human ALS, in part because of its association with tumor invasion and metastasis (513). However, HGF-expressing plasmid DNA injected intramuscularly promotes angiogenesis in patients with limb ischemia and neither leaks into blood nor influences the growth of an innoculated cancerous cell line, suggesting that this methodology of HGF expression may be viable for ALS (514). Because

viral vectors encoding HGF retrogradely transduce neurons and promote recovery after nerve injury (512), intramuscular or intrathecal injection of such vectors might provide a safe way to target MNs in ALS.

8.5 IGF-1

Excess IGF-1 modestly protects MNs from excitotoxicity (473;492). Oxidative stress directly carbonylates IGF-1 receptors (418) and antagonizes IGF-1-induced activation of Akt, suggesting that intracellular trophic and oxidative pathways intersect to govern neuronal survival (515–517). IGF-1 also induces sprouting in uninjured muscle and accelerates MN regeneration after axotomy (246,518), indicating an important role for IGF-1 in the response to ALS-mediated denervation. IGF-1 receptors are expressed not only by MNs but also by oligodendrocytes, Schwann cells, astrocytes, smooth and skeletal muscle cells and endothelial cells, indicating a plethora of potential cellular non-autonomous mechanisms by which IGF-1 could regulate MN disease. Evidence for such signaling is provided by the finding that although the serum or spinal cord levels of IGF-1 are normal in ALS patients (519,520), increased astrocytic IGF-1 receptor expression is observed in the spinal cord of human ALS patients and fALS mice (520–522).

When administered at symptom onset, exogenous IGF-1 attenuates the loss of muscle weight and decline of behavioural performance in *wobbler* mutant mice (523). Intramuscular injection of adeno-associated viral IGF-1 (AAV-IGF-1) also delays disease onset and progression of fALS mice (453). Similar treatment with lentiviral-IGF-1 exerts significant but less pronounced effects in fALS mice, demonstrating that the transduction of both muscle cells and MNs by AAV is more protective than the transduction of muscle cells alone by lentiviral-IGF-1 (453). Indeed, postnatally restricted, muscle-specific overexpression of IGF-1 fails to prolong the survival of fALS mice (524). Interestingly, however, embryonic and postnatal overexpression of IGF-1 in muscle delays denervation, disease onset and progression of fALS mice (525). These results may result from the myotrophic and neurotrophic effects of IGF-1 during embryonic development, and are consistent with embryonic deficits of retrograde transport reported in fALS MNs (343).

Although IGF-1, HGF and VEGF (below) all prolong survival of fALS animals when administered at symptom onset, clinical trials with subcutaneously delivered IGF-1 have produced mixed effects, with a large-scale Phase III study recently failing to show benefit (526–528). Although IGF-1 readily crosses the BBB (529), the disparate results may reflect diminished responsivity of MN terminals to subcutaneously delivered IGF-1 in MN disease or to the presence of IGF binding proteins or other factors that affect the bio-availability of this NTF when injected systemically. On the other hand, IGF-1 serum half-life is significantly increased when administered as a complex with IGF binding protein 3 (IGFBP-3), and approval was recently granted by the FDA for an early-phase clinical study of this IGF-1/IGFBP-3 complex, called IPLEX, in human ALS patients (530). Other strategies to elude such pharmacokinetic hurdles include direct intrathecal or intraparenchymal delivery of either recombinant IGF-1 or AAV-IGF-1 (531). However, human clinical studies with spinal-targeted AAV-IGF-1 have recently been suspended on account of AAV serotype complications as well as pre-existing axonal and neuromuscular degeneration that is observed in symptomatic patients (RT Bartus, personal communication).

8.6 VEGF

MN disease rather than MN PCD established the connection between VEGF and MNs; mice deficient for the hypoxia response element (HRE) in the VEGF promoter develop late-onset, paralytic MN disease (371). VEGF has subsequently been demonstrated to strongly protect MNs from excitotoxicity (263,438). The protective effect of exogenous VEGF after

axotomy is mediated by enhanced angiogenesis (264), and MNs are sensitive to ischemic hypoxia (532), suggesting that endothelial- or smooth muscle-derived VEGF receptors contribute non-cell autonomously to MN disease. On the other hand, VEGF receptors are expressed by MNs and VEGF directly rescues MNs *in vitro*, suggesting a more direct effect (262).

Consistent with the reduction of VEGF expression observed in the spinal cord of HRE-VEGF-deficient mice, VEGF promoter haplotypes resulting in diminished VEGF levels are correlated with human sporadic ALS (533). Other types of MN disease, including spinal and bulbar spinal muscular atrophy (SBMA), are also associated with a decrease in VEGF levels (534). Deletion of the HRE-VEGF in fALS (SOD1 mutant) mice accelerates disease, suggesting an overlap in pathogenesis between these two forms of ALS (534). The induction of VEGF after hypoxia is impaired early in fALS mice, further indicating such an interaction (535). Intramuscular injection of a retrogradely transportable VEGF delays disease onset when injected early postnatally into fALS mice and extends lifespan when injected at disease onset (536). Systemic and intraventricular injection of VEGF into fALS mice or rats, respectively, also delays disease onset and progression (537,538). Overexpression of VEGF in neurons also delays disease onset and prolongs survival of fALS mice (539), suggesting that impaired production rather than reception of VEGF by MNs is associated with disease in fALS mice. Clinical studies studying the effects of VEGF are still forthcoming.

9. SUMMARY AND PERSPECTIVE

In an effort to discern which of 4 explanations might most likely explain the meager effects of NTFs in ALS clinical trials, we have reviewed studies which demonstrate that NTFs are bona fide regulators of MN survival during development and after injury (1,2), and in some cases block or delay MN death in ALS despite exerting only modest effects on motor function and lifespan (3). Because NTF receptors are expressed by a diverse number of cell types known to interact with MNs, it also appears that systemically administered recombinant NTFs are severely limited by pharmacokinetic constraints (4). Together with the evidence suggesting that cell death in ALS is quite distinct from that in development or after injury, these data suggest that NTFs are unlikely to significantly affect MN degeneration in most forms of ALS. One exception to this conclusion is the spontaneous *wobbler* mutant, in which the disease onset and progression are significantly ameliorated by treatment with a variety of NTFs. Because this mutant predominantly affects the central soma rather than peripheral nerve terminal of affected cervical MNs, it may serve as a less valuable model of human ALS than the SOD1 transgenic mouse or even the *p^{mn}* spontaneous mutant, in which dysfunction appears first in the distal nerve terminal and then later in the axon and soma.

Nevertheless, these studies provide astonishingly useful information on the inter- and intracellular mechanisms of MN degeneration in ALS. Moreover, their results when taken together are more insightful than when considered individually, and help identify common and selective NTF-resistant pathways of degeneration. For example, VEGF and GDNF provide strong protection against glutamate excitotoxicity in spinal explants, but CNTF has no effect (438). Furthermore, the location and propagation of MN disease subtypes can be hypothesized based on the interpretation of the a.) effects of different types of NTF delivery, b.) subcellular timecourse of degeneration observed in different ALS mutants missing or supplemented with NTFs, and c.) expression of NTFs and NTF receptors on different cell types in proximity to MNs (Figure 4). For example, the lack of effect of CNTF in human clinical trials of sALS, the proximal-distal gradient in MN dysfunction observed in CNTF-deficient mice (219), and the expression of CNTFR α in astrocytes and proximal MNs suggest that this form of MN disease is initiated centrally rather than peripherally. In

contrast, MN degeneration in fALS mice exhibits a distal-proximal gradient (374) and treatment with GDNF peripherally but not centrally (via transgenic overexpression) increases lifespan (454).

In conclusion, although the evidence cited herein suggests that subcutaneously delivered NTFs are ineffective therapies for most forms of human ALS, several opportunities for specialized applications remain. First, as a general rule, it appears that NTF treatment via gene- or cell-based approaches are more likely to avoid the pharmacokinetic limitations that often reduce bio-availability and increase side effects. Second, it appears that NTFs are more effective in MN disease subtypes that exhibit central before peripheral dysfunction, such as the *wobbler* mouse mutant. However, even when NTFs fail to affect disease onset or lifespan, such as in SOD1 mutants, their potent effects on MN somal size may justify their inclusion in a cocktail of anti-ALS pharmacotherapies that also include axon-targeted and NMJ-targeted molecules. Therefore, while there is reason to question the validity of launching another clinical trial featuring the subcutaneous delivery of an NTF that has been identified on its ability to arrest developmental cell death, we still feel cautiously optimistic that when applied to the appropriate MN disease subtype with the appropriate delivery system, NTFs will “live up” to their therapeutic expectations in ALS.

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Figure 1.

Basic intracellular molecular pathways regulating MN survival during development. Cells die through apoptosis (Type I), autophagic cell death (Type II) or necrosis (not pictured). Two different pathways mediate apoptosis, called the intrinsic and extrinsic pathways. The intrinsic pathway involves activation of the pro-apoptotic multi-BH-domain proteins (MDP) Bak and Bax and inhibition of the anti-apoptotic MDPs Bcl-2 and Bcl-X_L by the pro-apoptotic BH3-domain-only proteins (BOPs). Bak/Bax activation promote mitochondrial permeability transition (mPT), loss of mitochondrial membrane potential (ψ_m), release of mitochondrial cytochrome *c* (cyt *c*) and activation of caspases (casp3, casp9), which dismantle the cell (lightening strike). Bak/Bax activation also depletes (ψ_m) by causing the transfer of Ca^{++} from the endoplasmic reticulum (ER) to the mitochondrion (M). The extrinsic apoptotic pathway directly activates caspases (red arrow) or activates the intrinsic death pathway through the BOP Bid (purple arrow). Autophagic death (lightening strike) occurs independently of apoptosis and is characterized by the presence of abundant autophagic vacuoles (AV) and the activation of autophagic genes (ATGs) such as Beclin 1 (*atg6*). Cell death is regulated by extracellular anti-apoptotic and pro-apoptotic stimuli that operate through neurotrophic factor receptors (NTFRs) or death receptors (DRs). Activated DRs activate the extrinsic death pathway by assembling a death-inducing signaling complex (DISC). Activated NTFRs antagonize apoptotic, autophagic and necrotic death by activating cytosolic kinases such as phosphoinositide-3 kinase, Akt and B-Raf, inhibiting pro-apoptotic BOP activity, activating anti-apoptotic and inhibiting pro-apoptotic gene expression in the nucleus (N). Blue lines indicate anti-apoptotic function and black lines depict pro-apoptotic function; arrows indicate activation and flat lines indicate inhibition. For details see text.

Cellular mechanisms of neurodegeneration in fALS mutant SOD1 mice. MNs may succumb by intrinsic (cell autonomous) or extrinsic (cell non-autonomous) signaling changes in ALS. Intrinsic mechanisms include defects in transport and/or mitochondrial function, and extrinsic signaling changes include altered trophic, excitotoxic (reactive oxygen species (ROS) or glutamate) or death receptor (DR) signaling from other cell types. A MN is depicted in yellow, and cell types known to positively and negatively regulate MN survival are shown in purple and orange, respectively, including muscle cells (1), Schwann cells (2) endothelial cells in spinal, nerve or muscle tissue (3), descending supraspinal neurons (4), local interneurons (5), spinal sensory ganglia (6), endocrine glands (7), nerve- or muscle-derived fibroblasts (8), microglia (9) or astrocytes. See text for further details.

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Figure 3.

Neurotrophic signaling between MNs and other neuromuscular cell types in the context of ALS. (A) Expression of NTFs and NTFRs in cell types which may regulate MN survival during disease. MNs (in yellow) express nearly all NTFRs as well as several NTFs such as VEGF and NT-3. Expression of NTFs (blue type) and NTFRs (black type) by cell types known to positively and negatively regulate MN survival (purple and orange, respectively). Other NTF or NTFR sources not shown include oligodendrocytes in the ventrolateral funiculus and smooth muscle cells of spinal, neural and muscular arterioles. Exogenously administered NTFs may bind NTFRs expressed by any of these cell types (B) Results of studies with transgenic or null mutant mice expressing elevated or reduced amounts of NTFs in specific cell types. Arrows indicate likely direction of signaling based on NTFR expression analysis. VEGF "KO" refers to HRE-VEGF mutant (see text). SC refers to Schwann cells. Two small arrows indicate a positive effect of NTF overexpression on lifespan of FALS mice; two dashes signify no such effect.

Figure 4.

Subcellular degeneration of MNs in fALS and NTF KO mice. Cell types and colors are the same as those in figures 2 and 3. fALS mice exhibit degenerative changes (indicated by broken lines) at the neuromuscular junction (NMJ) and axon (1,2) before the soma (3). CNTF KO mice, similar to fALS mice, display reduced CNTF in the nerve and increased CNTFR α in the spinal cord, and a loss of countable MN soma (1) before neuromuscular changes at the axon and NMJ (2,3). Consistent with this scenario, systemic (subq=subcutaneous) CNTF treatment fails to ameliorate human ALS. NT-3 KO mice display a neuropathy that likely results from Schwann cell death and subsequent loss of axons and NMJs (1,2) before loss of MNs. Finally, HRE-VEGF mice (VEGF "KO" mice)

exhibit a reduction in VEGF upon ischemic stress, which could arise from impaired vascular support of muscle, nerve or spinal tissue. Although the sequence of MN degeneration has not been elucidated, treatment with VEGF reduces both astrogliosis centrally and loss of NMJs peripherally.

Table 1

NTF	NTFR(s)	Motoneuron Subtype Affected during Developmental PCD in KO mice
GDNF	<i>GFRa1; Ret</i>	Brachial: LD,CM - Ingrowth 171,172; Lumbar: Peroneal – Choicepoint173; sGM / TFL, iPsM - Ingrowth51 All spinal levels: 20–30% loss51,113,161–163; γ MNs (~90% loss)51; Facial: ~20% loss: Yes163; No161,162,174
Neurturin	<i>GFRa2, Ret</i>	Spinal and cranial levels: No deficits noted in Neurturin KO or GFRa2 KO mice*
BDNF, NT-4/5	<i>TrkB; p75</i>	Spinal and cranial levels: No deficits noted in BDNF KO36,187–189, NT-4/5 KO187–189, TrkB KO190; p75 KO149
NT-3	<i>TrkC; p75</i>	NT-3 KOs: All spinal levels: γ MNs (complete loss)51,192,195; Facial MNs: No deficits37,39
CNTF	<i>CNTFRa; LIFRβ; gp130</i>	CNTF or LIF KOs: Spinal and cranial levels: No deficits; CNTFRa223; LIFR β 224; gp130225 KOs: ~40% loss
LIF	<i>LIFRβ; gp130</i>	Same as above
CT-1	<i>LIFRβ; gp130</i>	CT-1 KO: All spinal levels: 20–30% loss226; Facial: ~20% loss,226; Hypoglossal: ~20% loss226
CLC/CLF	<i>CNTFRa; LIFRβ; gp130</i>	CLC/CLF KO: Spinal levels: Lumbar (25% loss); Brachial55, Thoracic (No loss); Facial: - ~30% loss55; Hypoglossal: No loss55
HGF	<i>Met</i>	Met KO: Brachial: LD,CM - Ingrowth239; Spinal and cranial levels: MN counts NE (embryonic lethality)
IGF-I, IGF-II	<i>IGFR-1,IGFR-2</i>	IGF-I KO: Spinal levels: ~30 loss**; Facial: ~25% loss256; Trigeminal: ~30% loss256; IGR-1,2 KOs NE ^{but see} 59
VEGF	<i>Flk-1;Flt-1;Flt-4</i>	VEGF KO: Facial: Migration267; Spinal and cranial levels: MN counts NE (embryonic lethality)

Table 2

Gene	Neurons - Knockout (KO)	Effect on MN death during PCD, after axotomy, or in response to ALS		
		PCD <i>In vivo</i>	Injury <i>In vivo</i>	ALS <i>In vivo</i>
p75-NGFR	Sensory, symp neurons ↑↑540	KO --149	KO ↓↓149	KO --395
kRas	Central neurons ↑↑541	KO ↑↑541	OE ↓↓542	ND
B-Raf	Sensory neurons --543	ND*	ND	ND
PI3-K	ND*	ND	ND	ND
PTEN	ND*	ND	ND	ND
Akt	Reduced brain & body size544,545	ND	DN ↑↑125 CA ↓↓125	ND
Bcl-2, Bcl-X _L	Postnatal neurons ↑↑546	KO ↑↑547 OE ↓↓109	KO--548; OE ↓↓126	OE ↓↓399
BH3-Only	Mild, no obvious defects106	KO -- 106	KO ↓↓133,134	KO ↓↓433
Bax/Bak	Postmitotic neurons ↓↓110,549	KO ↓↓112 OE ↑↑550	KO ↓↓130,551	KO ↓↓375
IAPs	No Effect552,553	ND	ND	ND
Apaf-1	Neuronal precursors ↓↓554,555	KO ↓↓108	ND	ND
Caspase3	Neuronal precursors ↓↓107	KO ↓↓61	ND	ND
Caspase9	Neuronal precursors ↓↓556,557	KO ↓↓557	ND	ND
AIF	No Effect558	KO -- 108	ND*	ND
p53	Sympathetic neurons ↓↓559	KO --130	KO ↓↓130,134	KO --395
Fas, FasL	KO -- 147	KO --147	KO ↓↓147	KO ↓↓432
TNF/TNFR	Sensory neurons -- ↓↓560	KO --135	KO ↓↓135	KO -- 561
iNOS	ND	ND	KO ↓↓148	KO ↓↓271

MN=motoneuron; PCD=programmed cell death; KO = knockout; OE = overexpression; DN,CA=overexpression of constitutively or dominant negative activated alleles

* Conditional mutants generated but not determined (ND); Red, Blue = Pro-, Anti-apoptotic

Table 3

NTF	Familial ALS Manipulation	Onset	Duration	Treatment Initiation ^{Ref}
GDNF	<i>OE Myoblasts in SOD1 Mouse Muscle</i> <i>Tg OE in SOD1 Mouse CNS</i> <i>Tg OE in SOD1 Mouse Muscle</i> <i>OE Neural Progenitors in SOD1 Rat CNS</i> <i>OE Mesenchymal SC in SOD1 Rat Muscle</i> <i>AdV-GDNF in SOD1 Mouse: Muscle to CNS</i> <i>AAV-GDNF in SOD1 Mouse: Muscle to CNS</i> <i>AAV-GDNF in SOD1 Mouse: Muscle to CNS</i> <i>GDNF Phase I in human ALS – ICV</i>	87 vs. 102 days No change 74 vs. 84 days No change No change 106 vs. 116 days 101 vs. 114 days 91 vs. 107 days NA	Not examined No change 43 vs. 50 days No change 10 vs. 28 days 10 vs. 18 days 21 vs. 24 days 32 vs. 27 days No change	Presymptomatic Injection 454 Embryonic OE 455 Embryonic OE 455 Presymptomatic Injection 436 Presymptomatic Injection 456 Young postnatal OE 451 Presymptomatic OE452 Presymptomatic OE453 Symptom Onset 562
IGF-1	<i>AAV-IGF1 in SOD1 Mouse: Muscle to CNS</i> <i>AAV-IGF1 in SOD1 Mouse: Muscle to CNS</i> <i>Tg OE in SOD1 Mouse CNS</i> <i>Tg OE in SOD1 Mouse Muscle</i> <i>Tg OE in SOD1 Mouse Muscle</i> <i>AAV-IGF1 in SOD1 Mouse: Intraparenchymal</i> <i>IGF1 Phase III in human ALS - Subcutaneous</i>	91 vs. 122 days NA No change No change 111 vs. 121 days 103 vs. 115 days* NA	32 vs. 38 days 56 vs. 34 days No change No change 12 vs. 32 days No change No change	Presymptomatic OE453 Symptomatic OE453 Presymptomatic OE524 Presymptomatic OE524 Embryonic OE525 Presymptomatic OE531 Symptom Onset 528
VEGF	<i>EIAV-VEGF in SOD1 Mouse CNS</i> <i>Recombinant VEGF in SOD1 Mouse - IP</i> <i>Recombinant VEGF in SOD1 Mouse – ICV</i> <i>Tg OE in SOD1 Mouse CNS</i>	95 vs. 123 days 127 vs. 139 days 107 vs. 120 days 95 vs. 115 days	30 vs. 40 days 5 vs. 4 days 8 vs. 19 days No change	Presymptomatic 536 Presymptomatic 537 Presymptomatic 538 Young postnatal 538
BDNF	<i>OE Neural Progenitors in SOD1 Mouse CNS</i> <i>BDNF Phase III in human ALS - Subcutaneous</i>	No change NA	No change No change	Presymptomatic 563 Symptom Onset 485
NT-3	<i>OE Neural Progenitors in SOD1 Mouse CNS</i>	No change	No change	Presymptomatic 563
HGF	<i>Tg OE in SOD1 Mouse CNS</i>	138 vs. 162 days	No change	Young postnatal 509
CNTF	<i>CNTF Phase III in human ALS - Subcutaneous</i>	NA	No change	Symptom Onset 505

* Male only

OE – Overexpressing

Tg – Transgenic

SC – Stem cells

NA – Not applicable

AdV – Adenovirus

AAV-Adeno-associated Virus

EIAV – Equine Infectious Anaemia Virus

IP – Intraperitoneal injection

ICV – Intracerebroventricular injection