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Review

Role of skeletal muscle in the epigenetic shaping of motor neuron fate choices

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Summary. We study the role of muscle in the epigenetic (N.B., we use this term with the broader and more integrative meaning) shaping of developing motor neuron fate choices employing an approach based on mouse mutagenesis and pathology. The developmental role of skeletal muscle is studied in the whole mouse embryo by knocking out myogenic regulatory factors Myf5 and MyoD, to obtain an embryo without any skeletal musculature (Rudnicki et al., 1993). Our goal is to find muscle-provided trigger(s) of motor neuron death relevant to motor neuron diseases such as amyotrophic lateral sclerosis. The reason for this kind of thinking is the fact that a complete absence of lower and upper motor neurons, which is the pathological definition of amyotrophic lateral sclerosis, is only achieved in the complete absence of the muscle (Kablar and Rudnicki, 1999). Mutual embryonic inductive interactions between different tissue types and organs, between individual cell types belonging to the same or different lineages, and between various kinds of molecular players, are only some examples of the complex machinery that operates to connect genotype and phenotype. So far, our studies indicate that some aspects of this interplay can indeed be studied as proposed in this review article, suggesting the role of skeletal muscle in the epigenetic shaping of motor neuron fate choices. We will therefore continue this investigation as outlined to gain more insight into the nature of the epigenetic events that lead to the emergent properties of a phenotype.

Key words: Mouse muscle development, Myf5 and MyoD, Neurotrophic factors, Motor neurons, Microarrays

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Introduction

The current review focuses on the prenatal period of development that includes the formation of synaptic contacts between the developing muscles and muscle spindles and their innervating neurons, and the period of neuronal cell death that follows for a large percentage of the neuronal cell populations. The development of skeletal muscle and the development of the nervous system have been studied in great detail, both individually and with respect to each other. Though there remain a variety of unknowns, the availability of mouse gene mutations, molecular markers, and imaging techniques has enabled significant advances in the depth and breadth of our understanding of the cellular and molecular processes involved in these dynamic events. In the paragraphs to follow, we will discuss the subsequent topics: a) developmental relationships between neurons and the muscles they innervate, including ways to test the neurotrophic hypothesis; b) *in utero* treatments of muscleless (or *Myf5-/-:MyoD-/-*) embryos; c) systematic subtractive microarray approach (SSMAA) to discover novel muscle-derived (or contained/provided) motor neuron survival factors.

Developmental relationships between neurons and the muscles they innervate

In this section, both motor and proprioceptive neurons are considered. Of particular interest are three populations of motor neurons located in the facial motor nucleus (FMN) of the brainstem, the medial motor column (MMC) and the lateral motor column (LMC) of the spinal cord. Mature motor neurons stimulate skeletal muscle fibers directly to contract via the transmission of the neurotransmitter acetylcholine at the neuromuscular junction. Also examined are three populations of proprioceptive neurons located in the mesencephalic nucleus (Me5) of the brainstem, the thoracic dorsal root ganglia (T-DRG), and the lumbar DRG (L-DRG). These

Ia afferents are pseudounipolar and are in direct and indirect contact with skeletal muscle structures because they synapse with muscle proprioceptors (i.e., muscle spindles) in the periphery and with motor neurons (within the brainstem and spinal cord) that in turn innervate skeletal muscle.

In the mouse, starting at embryonic day (E) 9.5, motor neurons of the FMN leave the mitotic cycle and migrate to their final location in the ventrolateral margin of the pons, within the fourth and fifth rhombomeres, between the pons and medulla oblongata border (Ashwell and Watson, 1983; Pfaff and Kintner, 1998; Kablar and Rudnicki, 1999). The muscles innervated by the neurons of the FMN are associated with external ear movement, facial expression, and vibrissae movement in rodents, and are derived from late-differentiating second branchial arch myoblasts that disperse to periauricular, periorbital, and perioral locations (Noden and Francis-West, 2006). The neurons of the FMN are first distinguishable at E13.5 and naturally occurring programmed cell death (NPCD) normally begins around E16.5 (Ashwell and Watson, 1983). As revealed by observations in rats and human fetuses, there is a relatively late emergence of reflexes and spontaneous movement of facial musculature as compared to body musculature indicating delayed development of facial neuromuscular connections relative to neuromuscular connections in the trunk and limb (Ashwell and Watson, 1983) (as illustrated in Fig. 1A).

The neurons of the Me5, on the other hand, are the first evident in the mesencephalon (Hunter et al., 2001) and neuromuscular connections occur much sooner in development than in the FMN (as illustrated in Fig. 1B). It is commonly believed that mesencephalic proprioceptive neurons arise from neural crest cells of the dorsal neural fold (Widmer et al., 1998). There is also evidence to suggest that the neurons in this nucleus arise from the dorsal neural tube and develop as an integral part of the mesencephalon (Hunter et al., 2001). The proprioceptive neurons of the Me5 innervate proprioceptors (i.e., muscle spindles) of jaw closing muscles that develop from migratory precursor cells of the first (mandibular) branchial arch (Noden and Francis-West, 2006) and make an appearance as condensations of myoblasts at E13 (Widmer et al., 1998). The Me5 nucleus is also known to send projections to the periodontal ligament (in animals with teeth) (Hunter et al., 2001) starting on postnatal day (P) 2 (Widmer et al., 1998).

In the spinal cord, the wall of the neural tube is initially composed of thick, pseudostratified, columnar neuroepithelium (constituting the ventricular zone), that gives rise to all neurons and macroglial cells in the spinal cord (O'Shea, 1986; Bayer, 1989). As proliferation and differentiation in the neural tube proceed, a longitudinal groove called the *sulcus limitans* appears that demarcates the alar plate and the basal plate. The cell bodies of these two plates develop to form the dorsal (sensory) gray columns and the ventral and lateral columns, respectively (Zimmerman et al., 1993).

Included in the cell populations of the ventral horn are the motor neurons of the MMC and LMC. The neurons of the lateral half of the MMC (MMCl) send projections to the musculature of the body wall, while the neurons of the medial half of the MMC (MMCm) send projections to the musculature of the dermomyotome (Pfaff and Kintner, 1998). As revealed by studies investigating motor neuron retrograde transport of horseradish peroxidase (HRP) injected into muscles, all motoneurons that innervate the limb muscles are located within the LMC of the spinal cord (Hollyday, 1980a,b). The LMC neurons in the cervical spinal cord innervate the muscles of the forelimbs and the LMC neurons in the lumbosacral spinal cord innervate the muscles of the hindlimbs; the cell bodies in the dorsolateral half of the LMC (LMCl) innervate the dorsal limb bud, while cell bodies in the ventromedial half of the LMC (LMCm) innervate the ventral limb bud (Pfaff and Kintner, 1998). The limb bud is relatively undifferentiated when motor axons first grow into it (Hollyday, 1980a). Motor neurons are among the first formed in the spinal cord (Hollyday, 1980a) and Islet-1 (Isl-1) is the first LIM-HD to be expressed in a motor neuron upon exiting the cell cycle; the expression of Isl-2 follows that of Isl-1 (Ericson et al., 1992; Pfaff et al., 1996; Jessell, 2000). The neurons in the MMCm, LMCm, MMCl all express both Isl-1 and Isl-2, whereas the LMCl neurons express Isl-2 along with Lim-1 (Pfaff and Kintner, 1998). Developmental cues and differential expression of transcription factors are not the only differences between these two populations of cells. Spatial and temporal gradients of development, the induction of neuronal and muscle development, the timing of innervation, and the expression of target-derived neurotrophic factors and their respective receptors also show slight variations when comparing MMCs to LMCs (Figs. 2A, 3A, respectively).

The cells of the DRG arise from neural crest cells (Hamburger and Levi-Montalcini, 1949; Carr, 1984). Muscle spindles, innervated by proprioceptive neurons of the DRG, undergo morphogenesis around E15.5 when sensory Ia afferents reaching the muscle first contact myotubes (Leu et al., 2003). The neurons of the T-DRG innervate the muscle spindles located in the axial skeleton and body wall while the neurons of the L-DRG innervate the muscle spindles located in the hindlimbs. In part due to later development of their peripheral target, the period of NPCD for the cells of the DRG occurs at a slightly later stage in development than that of the motor neurons of the spinal cord (compare Figs. 2B and Fig. 3B). The DRG afferents and motor neuron dendrites begin to form contacts in the spinal cord around E17 of gestation (Kablar and Rudnicki, 1999). In wild-type (WT) embryos, the neurons of the six populations of muscle-associated neurons (MANs) of interest express the relevant neurotrophic factor receptors (with regards to the neurotrophic factors being investigated), and their respective target tissues are

known to normally express the neurotrophic factors of interest during the period of developmental NPCD, as illustrated in Figs. 1 to 3.

The neurotrophic hypothesis was born out of pioneering studies, that ultimately led to the identification of nerve growth factor (NGF) by Hamburger and Levi-Montalcini in the 1940s and 1950s (Oppenheim, 1989). Some time after the discovery of NGF it was realized that NPCD and neurotrophic factors are closely related, in that the former occurs due to a

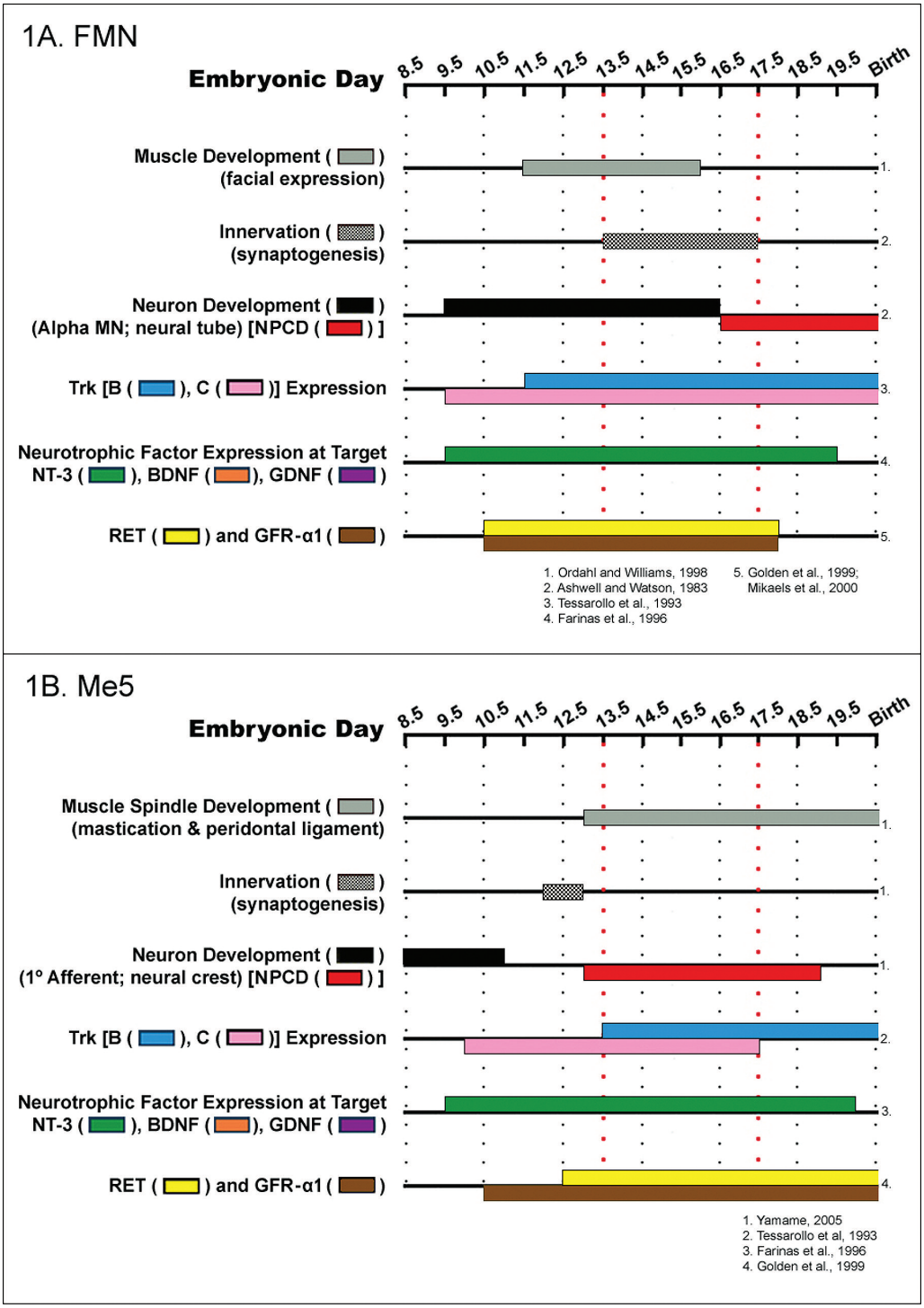

Fig. 1. Timeline of developmental events of cranial muscle associated neurons in the mouse embryo. **A.** The events related to the development of the facial motor nucleus (FMN). Muscle development indicates the onset of myocyte differentiation. **B.** The events related to the development of the mesencephalic nucleus (Me5). Muscle spindle development indicates the onset of recognized condensations of myoblasts. In both **A and B**. innervation indicates the extension of the innervating axon into the target field and initiation of contact with target. Neuron development starts when cells leave the mitotic cycle to migrate. The red dotted line at embryonic day (E) 13.5 indicates administration of neurotrophic factor into the amniotic cavity. The red dotted line at E17.5 indicates harvesting of embryos. The day of vaginal plug in each case is E0.5. MN, motor neuron; NPCD, naturally occurring programmed cell death; Trk, tropomysin-related kinase; NT-3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFR-α1, GDNF family receptor α1.

lack of the latter (Hamburger, 1992). The classic studies in which the developing chick has sustained a reduction in peripheral fields (ablation of wing or limb bud) or an enlargement of peripheral fields (transplantation) have demonstrated that the extent of cell death of motor neurons is regulated by the target and its secreted trophic factors (Hollyday and Hamburger, 1976; Oppenheim, 1991). Subsequent research has demonstrated the ability of members of the neurotrophin family of neurotrophic factors (Johnson et al., 1986) and of glial cell line-

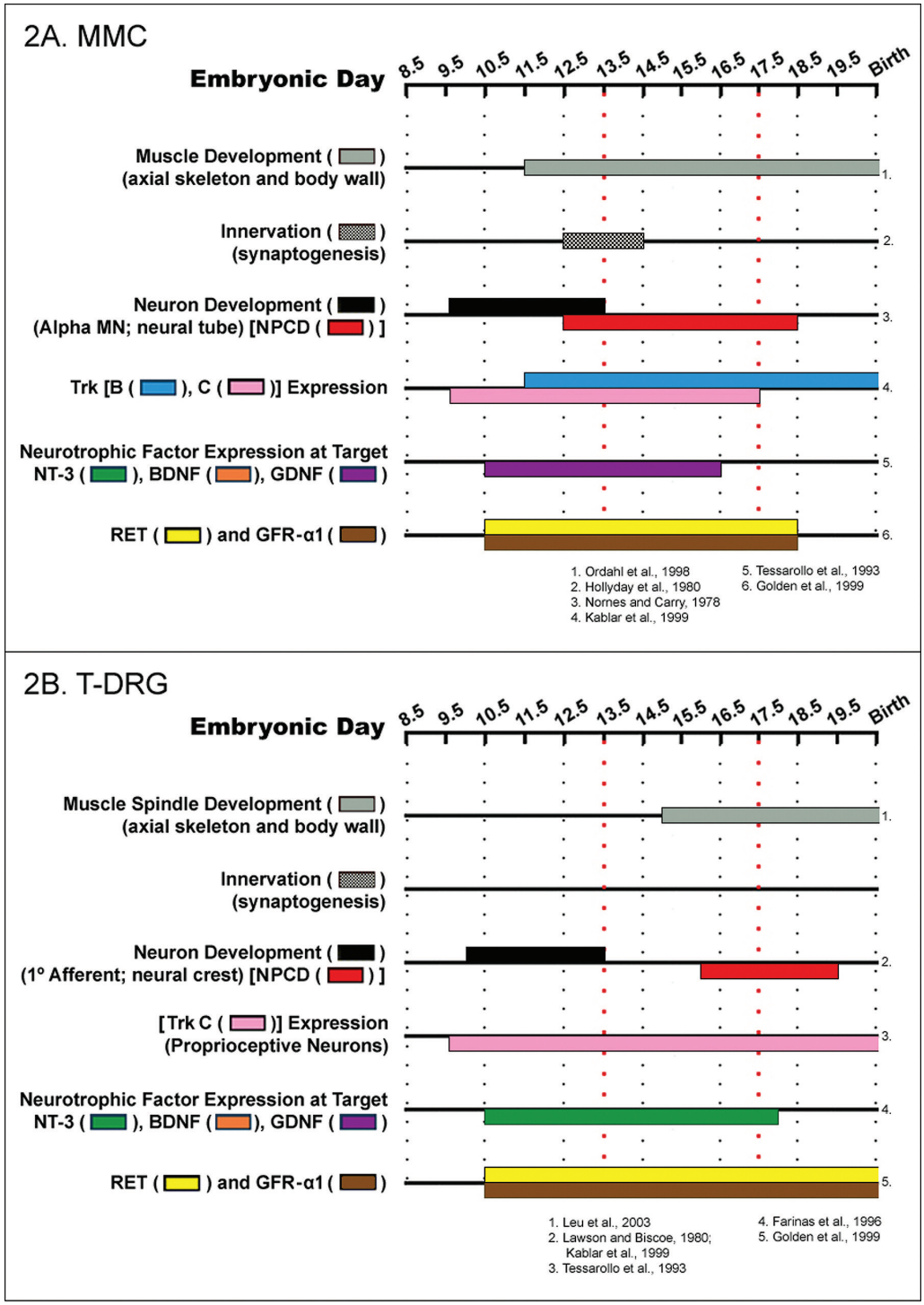

Fig. 2. Timeline of developmental events of thoracic level muscle associated neurons in the mouse embryo. **A.** The events related to the development of the medial motor column (MMC). Muscle development indicates the onset of myocyte differentiation. **B.** The events related to the development of the proprioceptive neurons in the thoracic dorsal root ganglion (T-DRG). In both **A and B.** innervation indicates the extension of the innervating axon into the target field and initiation of contact with target. Neuron development starts when cells leave the mitotic cycle to migrate. The red dotted line at embryonic day (E) 13.5 indicates administration of neurotrophic factor into the amniotic cavity. The red dotted line at E17.5 indicates harvesting of embryos. The day of vaginal plug in each case is E0.5. MN, motor neuron; NPCD, naturally occurring programmed cell death; Trk, tropomysin-related kinase; NT-3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFR-α1, GDNF family receptor α1.

derived neurotrophic factor (GDNF) (Lin et al., 1993; Oppenheim et al., 1995) to rescue neurons from cell death *in vitro*. Additionally, *in vivo* experiments have also revealed the ability of these factors to rescue both healthy (Oppenheim et al., 1993; Houenou et al., 1994) and injured (Oppenheim et al., 1993; Houenou et al., 1994; Li et al., 1994; Yin et al., 1994) neurons from NPCD and injury-induced (deafferentation or axotomy-induced) cell death, respectively.

Genetic manipulations have permitted alternative

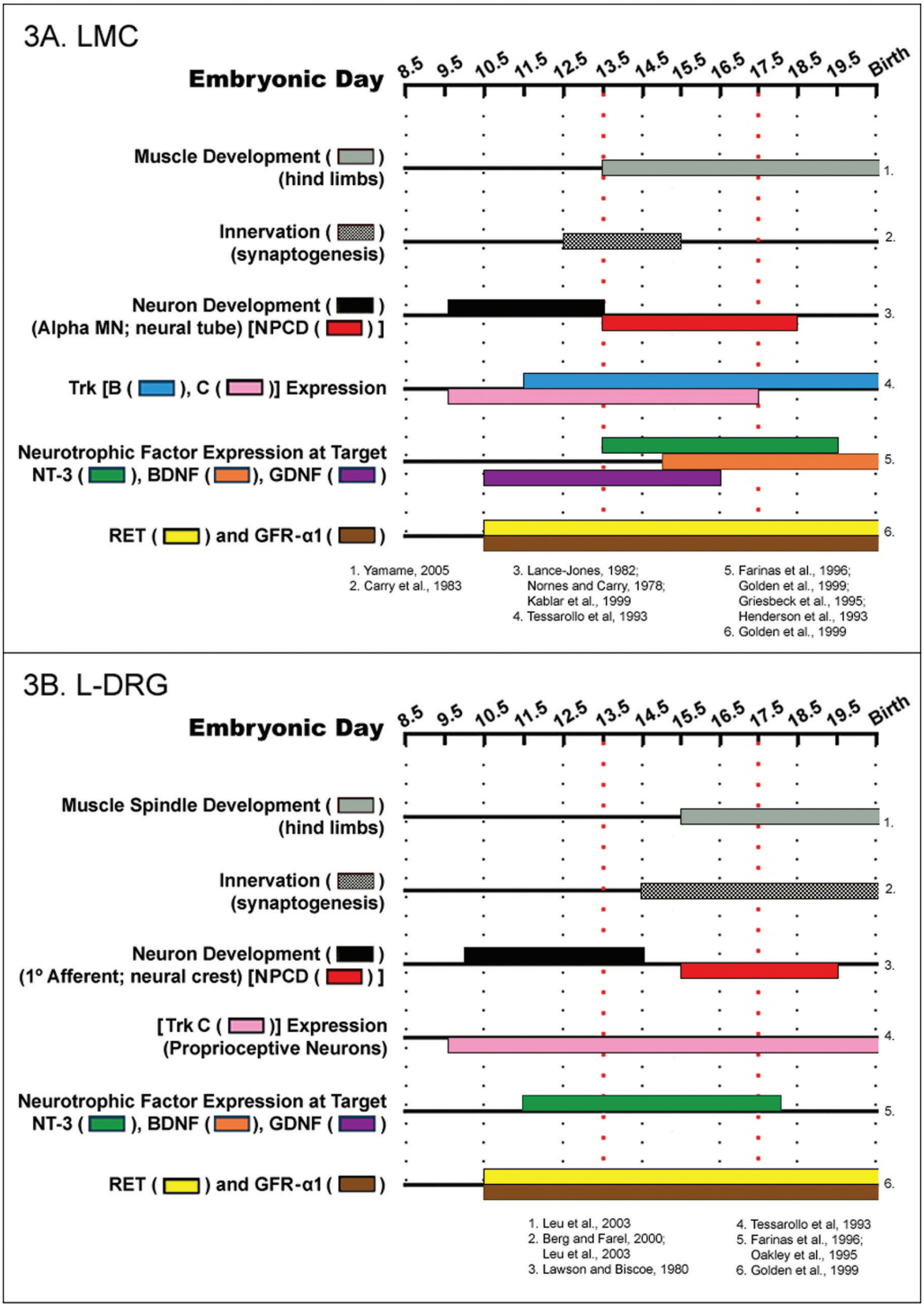

Fig. 3. Timeline of developmental events of lumbar level muscle associated neurons in the mouse embryo. **A.** The events related to the development of the lateral motor column (LMC). Muscle development indicates the onset of myocyte differentiation. BDNF expression information is from investigations in rat embryos. **B.** The events related to the development of the proprioceptive neurons in the lumbar dorsal root ganglion (L-DRG). In both **A and B.** innervation indicates the extension of the innervating axon into the target field and initiation of contact with target. Neuron development starts when cells leave the mitotic cycle to migrate. The red dotted line at embryonic day (E) 13.5 indicates administration of neurotrophic factor into the amniotic cavity. The red dotted line at E17.5 indicates harvesting and sacrifice of embryos. The day of vaginal plug in each case is E0.5. MN, motor neuron; NPCD, naturally occurring programmed cell death; Trk, tropomysin-related kinase; NT-3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; GFR-α1, GDNF family receptor α1.

means to investigate the role of particular neurotrophic factors *in vivo*. Analyses of knockout mice that lack the genes for a variety of either single or double neurotrophic factors, or their respective receptors, have revealed incomplete losses of motor and sensory neurons (as illustrated in Tables 1, 2). However, the precise role of neurotrophic factors in the survival of specific populations of motor and sensory neurons remains unclear and due to the invasive nature of manipulating target tissues in developing mammals, the lack of an adequate mammalian model has presented limitations in the extent to which the role of neurotrophic factors can be fully elucidated.

In utero treatments of muscleless (or Myf5-/-:MyoD-/-) embryos

It appears that matching the number of neurons to

Table 1. Neuronal loss observed in neurotrophic factor-deficient mice.

	NT-3	GDNF	BDNF	NT-3/BDNF	NT-3/BDNF/NT4	NT-4/BDNF	NT4
FMN	N.S. (1; 2)	18 (3); N.S. (4; 5)	N.S. (2; 6; 7)	N.S. (2)	20-22 (1)	N.S. (8)	N.S. (8; 9)
Me5	68 (2); 60 (5); 56 (10; 11); 65 (12)	N.S. (4; 5)	21 (2); 30 (6); 39 (7); 44 (13)	74 (14)	88 (1); 95 (11)	30 (8); 46 (11)	N.S. (8); 8 (11)
MMC	N.A.	22 (3)	N.A.	N.A.	N.A.	N.A.	N.A.
T-DRG	T1: E13: 53; E17: 62.8; P0: 71.6 T6: E13: 37.8 (15)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
LMC	N.S. (10; 16); 28.8 (15) (96.8% of gamma MNs)	**L:** 37 (3); **L1-L6:** 22 (4; 5); **L:** 25 (18)	**L1-L6:** N.S. (1; 7)	**L1-L2:** N.S. (2)	**L3-L5:** N.S. (1)	N.A.	**L:** N.S. (9)
L-DRG	**L4-L5:** E13: 36; P0: 78 (2; 10; 12) **L1:** E13: 52.6; E17: 56.2; P0: 56.9 **L4:** E13: 60.6; P0: 73.4 (13) Complete loss of Ia afferents (19)	**L4-L5:** E18.5: N.S. (3) **L5:** 23 (4; 5)	**L4:** 35 (2; 7) **L4:** 30 (10; 12; 13; 20)	**L4:** 83 (2) **L4:** E13: 46; P0: 84 (13)	**L4:** 92 (1)	**L4:** N.S. (13)	**L4:** N.S. (7; 8)

Values represent the percent of neurons lost in embryonic and postnatal mutants compared with wild type controls. The observed sensory losses in the dorsal root ganglion (DRG) represent counts from the whole DRG, except where indicated. NT-3, neurotrophin-3; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; NT-4, neurotrophin-4; FMN, facial motor nucleus; Me5, mesencephalic nucleus; MMC, medial motor column; T-DRG, thoracic dorsal root ganglion; LMC, lateral motor column; L-DRG, lumbar dorsal root ganglion; N.S., no significant difference from wild type; N.A., data not available; T, thoracic level of spinal cord; E, embryonic day; P, postnatal day; L, lumbar level of spinal cord; MNs, motor neurons. References are in parentheses. 1. (Liu and Jaenisch, 2000); 2. (Liebl et al., 1997); 3. (Oppenheim et al., 2000); 4. (Moore et al., 1996); 5. (Cacalano et al., 1998); 6. (Huang and Reichardt, 2001); 7. (Jones et al., 1994); 8. (Liu et al., 1995); 9. (Conover et al., 1995); 10. (Farinas et al., 1994); 11. (Fan et al., 2000); 12. (Snider, 1994); 13. (Ernfors et al., 1994a); 14. (Liebl et al., 2000); 15. (Farinas et al., 1996); 16. (Ernfors et al., 1994b); 17. (Woolley et al., 1999); 18. (Garces et al., 2000); 19. (Klein et al., 1994); 20. (Snider and Silos-Santiago, 1996).

Table 2. Neuronal losses observed in neurotrophic factor receptor-deficient mice.

	TrkA	TrkB	TrkC	TrkB/TrkC	TrkB/TrkA	TrkC/TrkA	GRF-α1	RET
FMN	N.A.	~70 (1)	N.S. (2)	5 (3; 4)	N.A.	N.A.	N.S. (5)	N.A.
Me5	70 (6)	30 (6; 7)	21 (6)	N.A.	N.A.	N.A.	N.S. (5)	N.A.
LMC	N.A.	**L1-L5, S1:** 35 (1) **L2-L5:** 30 (4)	N.S. (2)	N.S. (8)	N.A.	N.A.	**L1-L6:** 24 (5; 9)	**L:** 25 (10)
L-DRG	**L4-L5:** 73-82 (1) (small/medium lost)	**T12-L3:** 30-50 (1) **L4:** ~25 (8; 11)	**L4:** 20 (3; 11) (100% Ia afferent lost)	**L4:** 41 (1; 3)	**L4:** 78 (11)	**L4:** 93 (11)	**L5:** N.S. (5)	**L4:** 14 (2)

Values represent the percent of neurons lost in embryonic and postnatal mutants compared with wild type controls. The observed sensory losses in the dorsal root ganglion (DRG) represent counts from the whole DRG, except where indicated. The thoracic dorsal root ganglion (T-DRG) and medial motor column (MMC) were omitted due to unavailability of data in the literature. Trk, tropomysin-related kinase; GFR, GDNF family receptor; RET, rearranged during transfection FMN, facial motor nucleus; Me5, mesencephalic nucleus; LMC, lateral motor column; L-DRG, lumbar dorsal root ganglion; N.S., no significant difference from wild type; N.A., data not available; L, lumbar level of spinal cord; S, sacral level of spinal cord; E, embryonic day; P, postnatal day. References are in parentheses. 1. (Klein et al., 1993); 2. (Barbacid, 1995); 3. (Liu and Jaenisch, 2000); 4. (Conover et al., 1995); 5. (Cacalano et al., 1998); 6. (Huang and Reichardt, 2001); 7. (Snider and Silos-Santiago, 1996); 8. (Minichiello and Klein, 1996); 9. (Garces et al., 2000); 10. (Gould et al., 2008); 11. (Minichiello and Klein, 1996); 12. (Farinas et al., 1994)

the number of targets during synaptogenesis is the major reason for NPCD. Using the muscleless or *Myf5-/-:MyoD-/-* or double-mutant (DM) embryos takes advantage of a mouse model in which all target tissue (i.e., skeletal muscle) and associated neurotrophic support has been genetically excised. Since the neurons in DM embryos are in a compromised state, this model not only allows a comparison of the response of six MANs, that are undergoing either excessive PCD (EPCD) or NPCD, to each other, but also allows a comparison of the responses of MANs to neurotrophic factors in healthy versus compromised neurons, *in vivo*. The comparison of the relative effects of a particular neurotrophic factor in the presence or absence of muscle allows further clarification of the precise role of these neurotrophic factors in the survival of developing neurons. Injections with the putative neurotrophic factor were made on E13.5, one day sooner than what has previously been reported in the literature (Houenou et al., 1994; Oppenheim et al., 2000). Embryos were harvested on E17.5 and the survival of MANs was assessed.

Assessment of neuron numbers on E17.5 revealed that BDNF was capable of rescuing a significant number of motor neurons from NPCD and EPCD in the spinal cord and brain stem (Geddes et al., 2006). A single *in utero* treatment with NT-3 on E13.5 was sufficient to increase neuron numbers in the T-DRG during NPCD, and in the LMC, MMC, FMN, and Me5 during EPCD (Angka and Kablar, 2007). Additionally, NT-3 was shown to rescue MMC, FMN, and L-DRG neurons from EPCD (Angka and Kablar, 2007; Fig. 4). Interestingly, NT-3 was also shown to decrease neuron numbers in the Me5 during NPCD. Treatment with GDNF on E13.5 was shown to increase neuron numbers in the MMC, FMN, and Me5 during EPCD, and to rescue FMN and L-DRG neurons from EPCD (Angka et al., 2008; Fig. 5). Finally, assessment of neuron numbers on E17.5 revealed that two combination treatments, BDNF/NT-3/GDNF and BDNF/GDNF, resulted in an increase of neuron numbers in the FMN during NPCD. Also shown was the ability of BDNF/NT-3 to increase neuron numbers in the LMC during NPCD (Figs. 6-8).

When interpreting the results of this work, a number

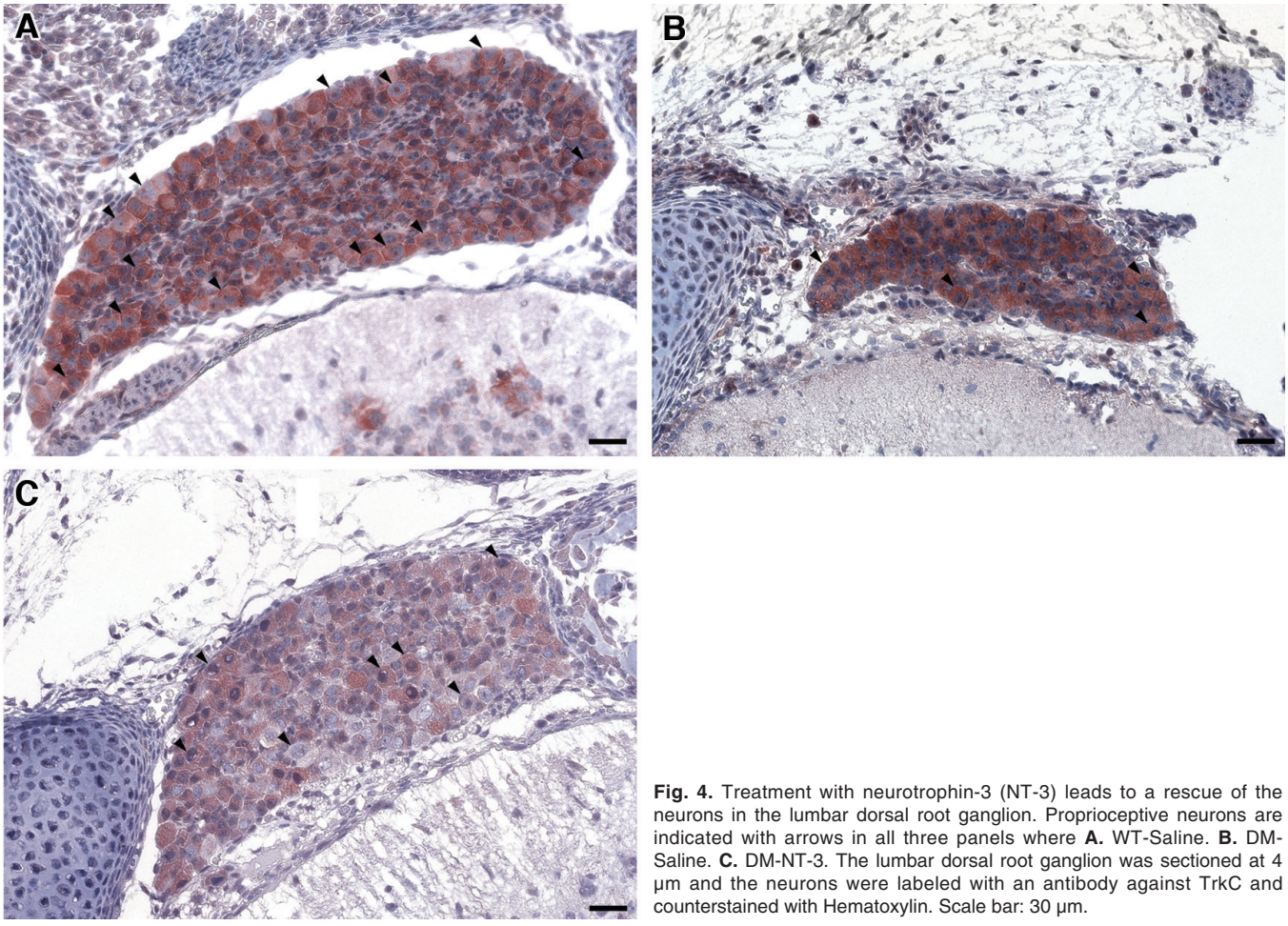

Fig. 4. Treatment with neurotrophin-3 (NT-3) leads to a rescue of the neurons in the lumbar dorsal root ganglion. Proprioceptive neurons are indicated with arrows in all three panels where **A.** WT-Saline. **B.** DM-Saline. **C.** DM-NT-3. The lumbar dorsal root ganglion was sectioned at 4 µm and the neurons were labeled with an antibody against TrkC and counterstained with Hematoxylin. Scale bar: 30 µm.

of factors must be kept in mind. To start, a direct assessment of neuron survival *per se* was not determined. This is an important distinction since, along with promoting cell survival and inducing cell death, the neurotrophic factors are also known to influence neuronal differentiation, proliferation, synapse formation and plasticity, target selection, and gene abundance (Huang and Reichardt, 2001; Reichardt, 2006). In order to assess specifically the action of neurotrophic factors on MAN populations it would be necessary to run separate experiments to test each of these possibilities. For example, in order to determine if cell death levels were altered, a label against either terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or caspase-3 could be used. Similarly, in order to determine whether the changes in neuron number were due to proliferation, an investigation of proliferating cell nuclear antigen or bromodeoxyuridine (5-bromo-2-deoxyuridine or BrdU) incorporation to determine DNA replication could be completed.

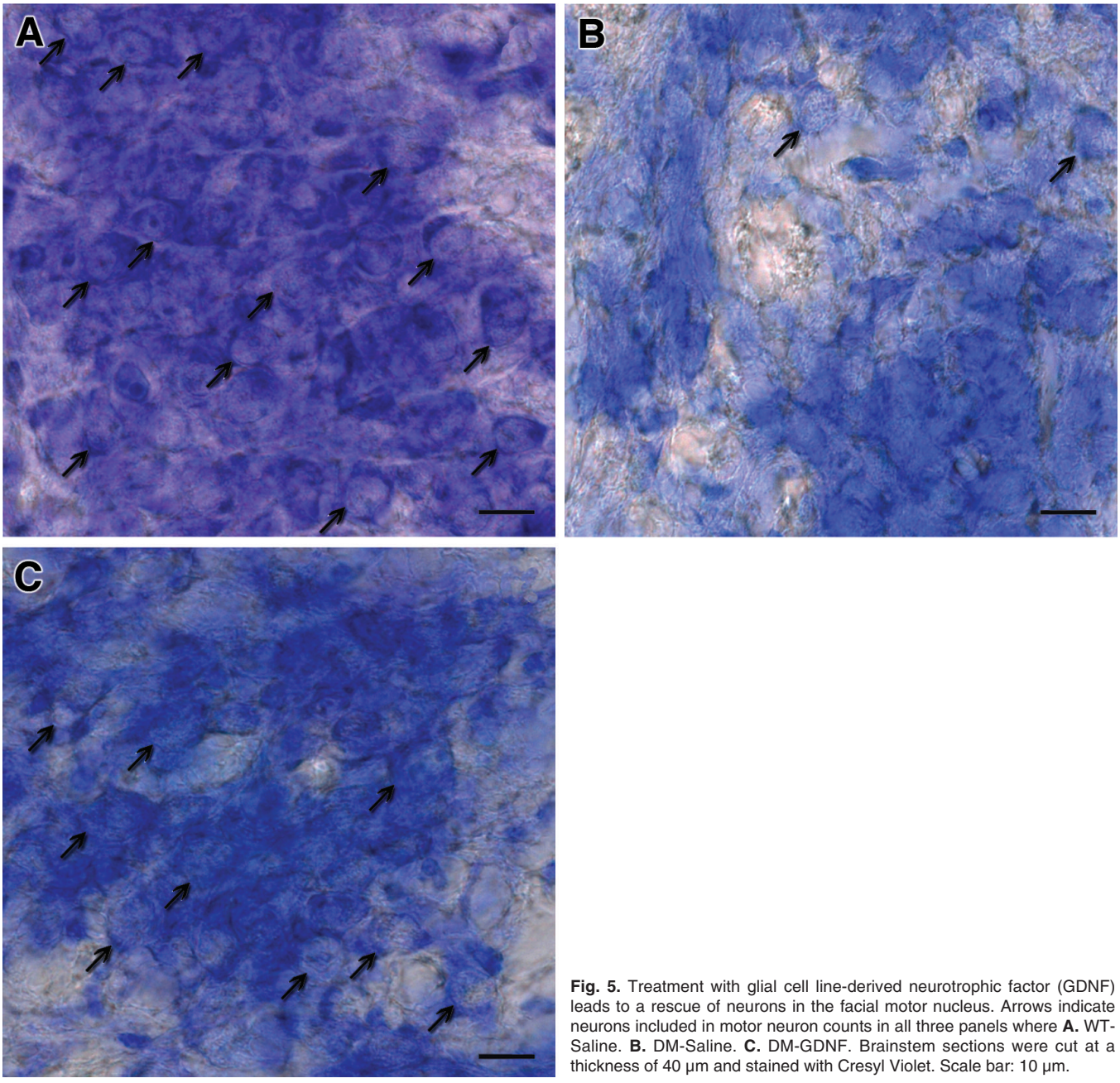

Fig. 5. Treatment with glial cell line-derived neurotrophic factor (GDNF) leads to a rescue of neurons in the facial motor nucleus. Arrows indicate neurons included in motor neuron counts in all three panels where **A.** WT-Saline. **B.** DM-Saline. **C.** DM-GDNF. Brainstem sections were cut at a thickness of 40 µm and stained with Cresyl Violet. Scale bar: 10 µm.

Another consideration is that the analyses of cell survival in all of these experiments were conducted only at one timeline. In each case, the neurotrophic factor treatment was administered on E13.5 and neuron numbers were assessed on E17.5. Assessment of knockout mice (partially illustrated in Tables 1, 2) reveals that many populations of neurons have specific requirements at different developmental stages. In fact, investigation of the progression of mutant phenotypes suggests that ligand activation of tyrosine kinases is important for survival of neural precursors and immature neurons, as well as of mature neurons in contact with their target; in some instances the same factor is required

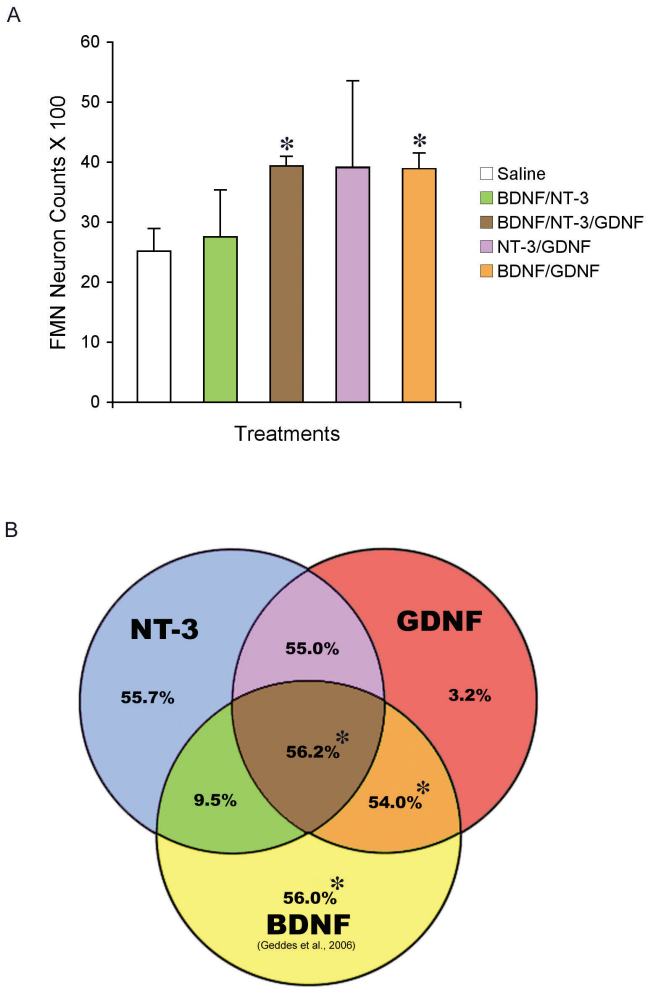

Fig. 6. An increase in facial motor nucleus (FMN) neuron numbers is observed with treatment from two combinations of neurotrophic factors. Both combinations included glial cell line-derived neurotrophic factor (GDNF). **A.** Neuron counts (estimated total neurons/nucleus) of coronal serial sections through the region of the brainstem containing the FMN as assessed on E17.5. Sample sizes: Saline (n=6); BDNF/NT-3 (n=3); BDNF/NT-3/GDNF (n=4); NT-3/GDNF (n=2); BDNF/GDNF (n=2). **B.** Percent change (PC) = [(NF-treated WT ÷ Saline-treated WT) x 100%] – 100% values based on neuronal cell counts in the FMN for combination neurotrophic factor or single neurotrophic factor treatments. Statistical significance (P≤ 0.05) was determined by non-parametric Mann-Whitney U-test (†) and T-test on log transformed data (*). BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3. Note: Percent change value for BDNF is based on values obtained in a previous study in our lab (Geddes et al., 2006).

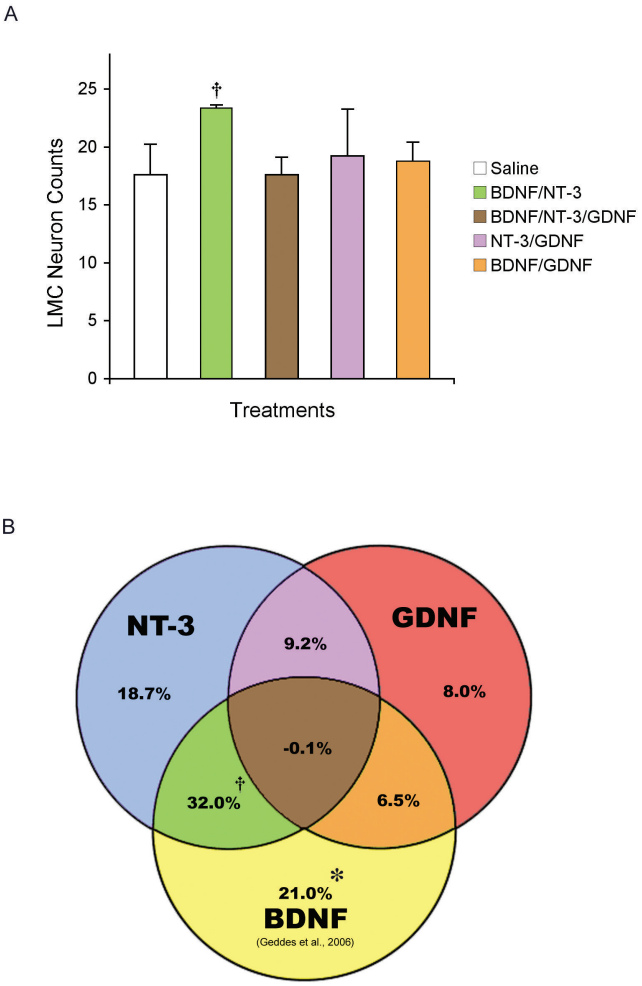

Fig. 7. In the lateral motor column (LMC), treatment with brain-derived neurotrophic factor and neurotrophin-3 (BDNF/NT-3) is capable of rescuing spinal cord motor neurons in wild-type (WT) embryos. In addition, this treatment has an additive effect on increasing neuronal survival compared with treatment with either NT-3 or BDNF alone. Treatment with combinations including glial cell line-derived neurotrophic factor (GDNF) did not have an effect on neuronal numbers in the LMC. **A.** Direct neuron counts of transverse serial sections through the lateral motor column (LMC, L3-L4) as assessed on E17.5 in WT embryos. Sample sizes: Saline (n=3); BDNF/NT-3 (n=3); BDNF/NT-3/GDNF (n=3); NT-3/GDNF (n=2); BDNF/GDNF (n=2). **B.** Percent Change (PC) = [(NF-treated WT ÷ Saline-treated WT) x 100%] – 100% based on neuronal cell counts in the LMC for all treatment groups including single neurotrophic factor treatments. Statistical significance (P ≤ 0.05) was determined by non-parametric Mann-Whitney U-test (†) and T-test on log transformed data (*). Note: Percent change value for BDNF is based on values obtained in a previous study in our lab (Geddes et al., 2006).

for many stages of development and, in others, different factors become important as development proceeds (Huang and Reichardt, 2001). In order to understand the effects of the examined neurotrophic factors on neuron number during the period of NPCD, a full time series analysis could be performed. In other words, treatment could be made at a variety of different times and then assessed later. There are some serious limitations to this proposal since amniotic sac puncture can have devastating consequences on the viability of the embryo (MacIntyre et al., 1995) making treatments at earlier time points essentially impossible. In fact, treatment with neurotrophic factors on E13.5 is the earliest time that neurotrophic factors have been injected into the mouse amniotic sac (Geddes et al., 2006).

A third consideration, in the case of the motor neurons investigated in the current study, is that specific subpopulations were not assessed. Various pools of motor neurons express different combinations of transcription factors (Tanabe and Jessell, 1996; Pfaff and Kintner, 1998) and various neurotrophic factor receptors are found in specific subsets of cranial and spinal cord motor neuron populations (Garces et al., 2000; Oppenheim et al., 2000). Moreover, examining specific subsets of neurons (e.g., within the ventral spinal column) in knockout mice suggests specific requirements of specific motor pools. One example is the specific loss of fusimotor neurons in newborn mice lacking NT-3 (Kucera et al., 1995; Woolley et al., 1999). The effects of GDNF on spinal cord motor neurons may also be restricted to fusimotor subtypes (Gould et al., 2008).

Finally, in this study, 10 µl of neurotrophic factor (1 µg/µl) was injected into the amniotic sac. Though this concentration is consistent with that reported in the literature for administration of BDNF and GDNF (Houenou et al., 1994; Oppenheim et al., 2000), a precise assessment of optimal concentration was not completed. In developing rats, the highest level of neurotrophin family of neurotrophic factor mRNA occurs at E15.5 (NPCD of neurons starts on E15.5), when relatively low concentrations are present (on the order of pg specific mRNA per µg polyA$^+$ RNA). Further, the expression of NT-3 is at least 15 times higher than expression of BDNF or NT-4 (Griesbeck et al., 1995). In the postnatal rat muscle (P6 to 3 months; hindlimb), GDNF protein is observed to range from 97.2 pg/mg to 157.9 pg/mg of total protein (depending on the muscle) and has a relatively high concentration (compared to BDNF, NT-3, and NT-4 levels) during this period (Nagano and Suzuki, 2003). On the other hand, NT-3 is the most abundant with levels of 4000 to 6000 pg/mg of total protein (Nagano and Suzuki, 2003). It would be of interest, given an adequate supply of neurotrophic factor, to conduct an assessment of dose response in order to ascertain minimal requirements and lethal doses in the systems used in the current study.

Along the same lines, another consideration is the

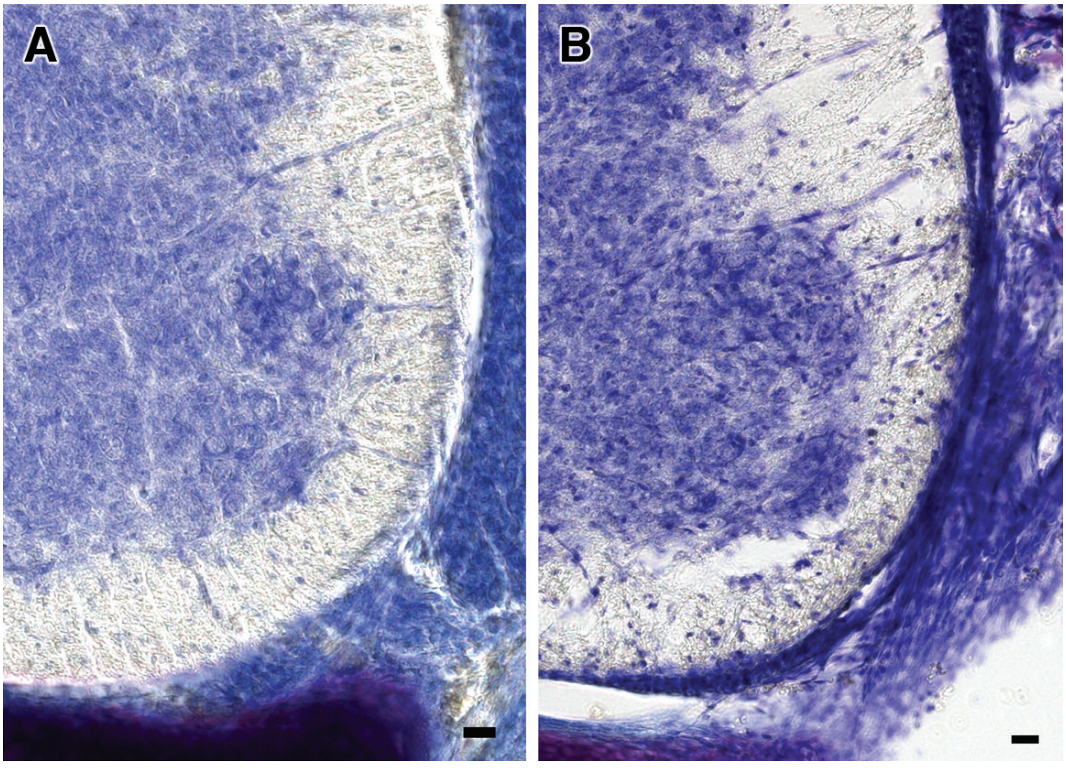

Fig. 8. Treatment with brain-derived neurotrophic factor and neurotrophin-3 (BDNF/NT-3) leads to an increase in the number of neurons in the lateral motor column (LMC) in wild-type embryos. The lumbar spinal cord sections were cut at 50 µm and stained with Cresyl violet. The panels represent treatment groups with saline **(A)** and BDNF/NT-3 **(B)**. Scale bar: 10 µm.

lack of a means to determine how much of the neurotrophic factor is arriving at the desired location once administered into the amniotic cavity. The use of radiolabeled neurotrophic factors (e.g., with ^{125}I) would allow an assessment of the concentration of the neurotrophic factor within the spinal cord or brainstem of the embryo. However, it would be necessary to inject with the radiolabeled factor only one embryo per uterus, to avoid confounding radioactivity from other embryos. In the case of DMs, which occur at a proportion of 1 in 16 embryos, together with the already high mortality rate due to the amniotic puncture, the probability of obtaining the DM might be close to zero. These experiments could be performed with WT embryos only. However, in the case of WT embryos the most informative application of this approach would be to inject the radiolabeled factor into the anatomical location of interest (e.g., LMC or MMC, etc.), which is at this time also impossible, because the ultrasound-guided techniques are limited by the size of mouse embryos.

SSMAA to discover novel muscle-provided motor neuron survival factors

It is thought that the observed discrepancy between the survival of motor neurons *in vitro* and in knockout mice was a reflection of functional redundancy in the natural system (i.e., the notion that any motor neuron has access to multiple trophic factors *in vivo*). It is becoming clear that subsets of motor neurons likely are dependent on specific neurotrophic factors and that the neuronal losses observed in mutants are small because of the diversity in trophic factor requirements of different motor pools (Mikaels et al., 2000; Oppenheim et al., 2000; Gould et al., 2008). In fact, it has been proposed that developing neurons likely switch (perhaps several times during development) from one source of trophic support to another. For example, the effects due to axotomy are less severe in adult compared to developing mammals, and might be explained by a switch from paracrine- to autocrine-derived trophic support at a later stage in development (Acheson and Lindsay, 1996).

Because of all these reasons, we believe it is crucial to know as many as possible (if not all) of the neurotrophic factors regulating the numbers of different MANs. To that end, we are currently performing a large scale functional analysis of the mammalian genome to understand the molecular basis of skeletal muscle's role in the survival and maintenance of MANs, and in turn in the ethiopathogenesis of motor neuron diseases (MNDs), such as amyotrophic lateral sclerosis (ALS), so that more appropriate treatment strategies of MNDs can be developed. It is important to realize that by employing the approach explained below, it will be possible to double or triple, in a short period of time, the number of factors known to enhance motor neuron survival from the skeletal muscle. It took more than 60 years for approximately 15 currently known factors to be discovered.

During its first phase, this project is profoundly based on the knowledge of muscle developmental biology. It has been established that during embryonic development motor neurons survive to a large extent only because of the presence of the skeletal muscle. Applying the analogy from the embryonic development, we hypothesize that adult neurons also depend on the factor availability from adjacent structures and in particular on the skeletal muscle neuronal survival factors. Neuroscientists normally study the aspects of the neuronal and, less commonly, axonal and junctional (neuromuscular) role in the ethiopathogenesis of MNDs, while muscle biologists concentrate on the aspects of muscle development and diseases, rarely thinking of the muscle as a factor in adult neuronal survival and maintenance. By contrast, we are coupling two processes that are happening at the same time, but have never been considered concomitantly before, mostly because of the existence of a natural split between the fields and also because of a lack of knowledge that we only recently provided (Kablar and Belliveau, 2005). One of the processes is the commitment of the myogenic precursor cells to the myogenic lineage, while the other process is the emergent ability of myogenic precursor cells to express/contain factors essential for neuronal survival. Using a number of sophisticated analyses and a unique opportunity provided by special mouse knock-out embryos, we were able to perform SSMAA, and out of almost 30,000 genes, we were able to identify a profile of only approximately 80 genes that are potentially regulators of motor neuron numbers in the Central Nervous System (CNS) (Baguma-Nibasheka and Kablar, unpublished data).

During its second phase, this project aims at collecting and producing information that is necessary for further narrowing of the molecular profile obtained in the first phase. Extensive literature reviews have been performed and it is now known that out of these 80 genes, approximately 40 have been studied and mouse mutants already exist harbouring the mutation in the gene of interest. However, these 40 candidates and their knockouts are not studied with the current hypothesis in mind. As a consequence of the availability of the new knowledge, these 40 knockout mice will be analysed with the current hypothesis in mind and with special attention to the number of motor neurons in different CNS locations, such as spinal cord, brain stem and motor cortex. According to the phenotypes obtained, further physiological, electrophysiological, behavioural and molecular analyses will be performed. The results of these analyses will attribute a function in motor neuron number regulation to each of these 40 candidates.

During the third phase of this proposal, conditional mouse mutants will be generated using only the molecules whose knockout mice have a clear motor neuronal phenotype. In addition, the other 40 candidates that have not been studied before and whose knockouts do not exist, will be generated in this phase of the project using the availability of the three international consortia

(EUCOMM/KOMP/NORCOMM). The international consortia will provide embryonic stem cells harbouring the mutation in the gene of interest and a conditional mouse mutant will be generated. According to the phenotypes obtained, further physiological, electrophysiological, behavioural and molecular analyses will be performed. The experiments performed in this phase of the project will result in answering the question of which genes from the total list of 80 are responsible for the regulation of motor neuron numbers from the skeletal muscle.

During the fourth phase of this proposal, we will use the most promising of the identified genes, develop their proteins for use as treatments in mice and perform both *in utero* and adult treatments. In other words, we will test *in vivo* the ability of some of the most promising factors to rescue neurons in mice with a MND. That way, the newly discovered players will become muscle-derived motor neuron survival factors. From here, the project will be taken up by experts who will be able to translate this data into clinical trials and ultimately usage in humans.

In conclusion, the results of our large scale genomics analysis will provide animal models and genotype/phenotype correlations that will not only attribute a function to the series of genes of interest, but will also assess the role of muscle in the determination of neuronal numbers (i.e., neuronal survival and maintenance), which is an important biological question directly relevant to the understanding of the basis of MNDs. In turn, these factors will ultimately serve as treatment agents on humans.

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