IMMUNOLESIONING OF IDENTIFIED MOTONEURON POOLS BY THE INTRAMUSCULAR INJECTION OF THE IMMUNOTOXINS, 192-IGG-SAPORIN AND OX7-SAPORIN, IN RATS

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William E. Peterson
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ABBREVIATIONS

AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
DiI	1,1-dioctadecyl-3,3,3,3-tetramethyl-indocarbocyanine perchlorate
DRG	Dorsal Root Ganglia
E	Embryonic Day
EDL	Extensor Digitum Longus
FG	Fluoro-Gold
GDNF	Glial-Derived Neurotrophic Factor
H&E	Hematoxylin and Eosin
i.c.v.	Intracerebroventricular
I.M.	Intramuscular (injection)
IGF-I	Insulin-like Growth Factor-I
I-NGF	^{125}Iodinated Nerve Growth Factor
-ir	Immuno-reactive
KA	Kainic Acid
LD_{50}	Lethal Dose at which 50% of animals die
LG	Lateral Gastrocnemius
MG	Medial Gastrocnemius
mnd	Motor Neuron Degenerative
MNs	Motoneurons
NF-L	Neurofilament Light-Chain
NGF	Nerve Growth Factor
NT-4/5	Neurotrophin-4/5
p75	Low-Affinity Nerve Growth Factor Receptor
PBS	Phosphate Buffered Saline
PMA	Progressive Muscular Atrophy
PD	Post-Natal Day
pmn	Progressive muscular neuropathy
RIP	Ribosome-Inactivating Protein
SCG	Superior Cervical Ganglia
SOD1	Super-Oxide Dismutase 1
SMA	Spinal Muscular Atrophy
TA	Tibialis Anterior

ABSTRACT

Many animal models have been developed to study motoneuron diseases, including amyotrophic lateral sclerosis (ALS), which involves the selective degeneration of upper and/or lower motoneurons (MNs). Unfortunately, the animal models developed, to date, have characteristics that limit their utility for assessing the therapeutic value of cell replacement strategies, including stem cells, for the treatment of MN diseases. Recently, immunotoxins have been developed which may allow for a more selective lesion of cells not previously obtaind with other techniques. Immunotoxins combine the specificity of a monoclonal antibody with the cytotoxicity of a ribosome-inactivating protein.

In this study, two immunotoxins, 192-IgG-saporin and OX7-saporin, have been used to lesion motoneurons in the neonatal and adult rat respectively. Moreover, this study demonstrates for the first time that MNs in the neonatal rat can interalize the monoclonal antibody, 192-IgG conjugated to Cy3 or saporoin, by receptor-mediated endocytosis. Once internalized, 192-IgG-Cy3/-saporin are retrogradely transported to the soma of MNs within the spinal cord. The novel finding that the immunotoxin, 192-IgG-saporin, can be used to lesion MNs following an intramuscular (I.M.) injection in neonatal rats has not previously been demonstrated. In addition, this study provides strong evidence that the observed muscular atrophy following an I.M. injection of 192-IgG-saporin in neonatal rats is likely due to direct necrotic effects on the muscle. Finally, this study also demonstrates that the immunotoxin, OX7-saporin, can produce MN loss following I.M. injection in adult rats, but unlike 192-IgG-saporin, does not result in severe muscular atrophy. This may serve as a useful model for studying cell replacement strategies for motoneuron diseases like ALS.

INTRODUCTION & LITERATURE REVIEW

Animal Models

Many animal models have been produced for studying motoneuron diseases (MND), such as progressive muscular atrophy (PMA), spinal muscular atrophy (SMA), and amyotrophic lateral sclerosis (ALS) which involve the selective death of upper and/or lower motoneurons. These animal models can be divided into two broad categories, **i) genetic models** (which includes spontaneously occurring mutations and transgenic animals that leead to a gain or loss of function of specific genes) and **ii) experimentally induced models** (which includes peripheral nerve injury of adult and neonatal animals, intraspinal injections of kainate, and injections of cytotoxic plant lectins). Many of these models have been used to study various pharmacological treatments for motoneuron diseases. For example, studies have found that the administration of ciliary nerotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4/5), glial-derived neurotrophic factor (GDNF), and/or insulin-like growth factor I (IGF-I), could prevent the massive death of motoneurons typical of neonatal axotomy (Sendtner et al., 1990,1992a; Yan et al., 1992; Li et al., 1994, 1995; Oppenheim et al., 1995; for review see Elliott, 1999). This has lead to clinical trials of some neurotrophic factors for the treatment of ALS.

However, the treatment of motoneuron diseases (with neurotrophic factors) has been met with limited success. This is in part because the etiology of MNDs, such as ALS, is unknown and perhaps multifactoral (Siddique et al., 1991; Appel et al., 1993; Couratier et al., 1993; Rosen et al., 1993; Rothstein 1993; for reviews see Eisen, 1995; Gutmann and Mitsumoto, 1996). However, it is known about MNDs is that by the time of onset of

symptoms and/or diagnosis there is often extensive motoneuron cell loss and atrophy of the denervated muscles. The successful replacement and integration of motoneurons may prove to be a useful strategy for ameliorating the symptoms of motoneuron diseases like ALS. However, the animal models to date (both genetic and experimentally induced) have characteristics that limit their utility for assessing the benefits that cell replacement therapies may offer (see **Table 1** for details).

There are several characteristics that a good animal model should possess for studying the possible therapeutic value of motoneuron replacement. i) As in most motoneuron diseases, there should be a loss of motoneurons (neuronopathy and not just axonopathy). **ii)** The integrity of the ventral roots should be maintained. **iii)** The animal should be able to survive for long periods of time. **iv)** The model should be reproducible. v) The temporal and spatial course of motoneuron loss should be known (to know when and where transplanted cells are needed). A more detailed explanation of the above criteria and the shortcomings of other animal models of motoneuron disease, as well as the rational for producing the new model, follows.

i) ***Motoneuron Death.***

While the etiopathology of motoneuron diseases may differ, a common feature of these diseases is the selective involvement and death of motoneurons. Thus, an animal model should exhibit neuronopathy (death and loss of the cell body) of motoneurons and not just axonopathy (degeneration of axons with soma preserved). In addition, experiments have shown that cells transplanted into a non-depleted CNS tend not to survive, rather it appears some cell loss is required for the graft to integrate into the host's tissue. Moreover, as long as a muscle in normally innervated, it is unresponsive to additional innervation (Frank et al., 1975). Thus, experimental animal models, such as the *progressive muscular atrophy (pmn)*

mouse mutant (Schmalbruch, 1991) or the adult rat sciatic nerve transection model (Schmalbruch, 1984a; Friedman, et al., 1995), are not useful for assessing motoneuron replacement because even though these models demonstrate motor impairment, they do not result in any significant loss of motoneurons within the spinal cord.

ii) ***Peripheral Nerve & Ventral Root integrity.***

In the developing animal, motoneurons migrate and settle in the ventral horn of the spinal cord. Soon thereafter, axonogenesis takes place and the motor axons leave the spinal cord through their segmental ventral roots (Landmesser, 1980). Motor axons then follow a specific path (guided by intrinsic and extrinsic cues) through the plexus, fasciculate, and travel through the peripheral nerve to the appropriate muscle (Landmesser, 1984, Meier and Walace, 1998).

Cellular replacement and integration of transplanted motoneurons requires that the axons exit the spinal cord (via the ventral root) and travel through the corresponding peripheral nerve to the denervated muscle. Therefore, the induction of motoneuron cell death should not result in the destruction of the ventral root or peripheral nerve. Thus, while neonatal (rat) nerve transection (Schmalbruch, 1984) and adult (rat) ventral root avulsion (Li, et al., 1995) can result in significant motoneuron loss, these methods are highly invasive and result in the destruction of the ventral root or peripheral nerve. In addition, these experimentally induced methods of motoneuron death also result in severe sensory cell loss. In human motoneuron diseases, such as ALS, there is relatively little involvement or death of sensory neurons. However, it may be that the successful transplantation and integration of motoneurons is not compromised by the lack of sensory fibres in the peripheral nerve.

iii) ***The animal model should be able to survive for long periods of time.***

In the developing rat, embryonic motoneurons migrate to the ventral horn where they begin axonogenesis (Altman and Bayer, 1984). At approximately embryonic-day 12 (E12), motor axons reach their muscle targets; however, it takes several weeks before the mature neuromuscular junction is formed (Lomo and Slater, 1980, Denzer, et al. 1997). Therefore, an animal model for motoneuron replacement should be able to survive for at least several weeks, if not many months, to be able to assess the long-term stability of newly formed neuromuscular junctions.

This shortened life-span/survival is where all the null mutants for motoneuron diseases fall short. For example, knockout mice with a ciliary neurotrophic factor – alpha receptor (CNTF-alpha) deletion display fairly selective motoneuron death, but die before or soon after birth (DeChiara et al., 1995). In mice with spontaneous mutations that result in motoneuron loss, such as the *wasted* mutant, a life span of 3 weeks also limits its usefulness as a motoneuron replacement model (Shultz, et al., 1982).

iv) ***Reproducibility.***

Obviously, the animal model should be reproducible. Genetic models are easily reproducible and do not require highly invasive methods of induction. However, other methods resulting in motoneuron loss are not reproducible. For example, intraspinal injections of kainic acid are not limited to motoneurons (produce non-specific cell death) and result in the general disruption of the cytoarchitecture of the spinal cord (Nothias, et al., 1990, Larson and Sun, 1992).

More recently, cytotoxins such as ricin, abrin, modeccin, and volkensin, have been used to lesion motoneurons (Wiley et al., 1982; Wiley and Oeltmann, 1986; Streit et al., 1988; Nogradi and Vrbova, 1992; Leanza and Stanzani, 1998). These cytotoxins (known as

ribosome-inactivating proteins or RIPs) are extracted from plants and once internalised, inactivate ribosomes (by depurinating the 28s subunit of ribosomal RNA) and inhibit protein synthesis (Olsnes et al., 1974; Eiklid et al., 1980). The arrest in protein synthesis eventually results in cell death.

Typically, these cytotoxins are injected into a peripheral nerve, and are retrogradely transported to the cell body where they inhibit protein synthesis, which results in cell death. However, this involves major surgical procedures to expose the nerve, and thus, is highly invasive. In addition, these cytotoxins are not motoneuron specific, and result in the severe loss of sensory fibres (Wiley et al., 1982; Wiley and Stirpe 1987; Wiley and Stirpe 1988). There have also been conflicting reports of the completeness of the lesion produced and whether there is transneuronal spreading of the toxins to cells outside the intended lesion area (Wiley and Stirpe, 1987, Wiley and Oeltmann, 1989; Leanza and Stanzani, 1998). These neurotoxins also produce local necrosis at the injection site (Wiley et al., 1982; Leanza and Stanzani, 1998). Moreover, ricin, and especially volkensin, are among the most toxic of the plant lectins, and often the dose required to lesion a motoneuron pool approaches the toxic limits for the animal. Indeed, numerous studies have lost animals due to systemic toxicity (Nogradi and Vrbova, 1992; Leanza, and Stanzani, 1998). Unlike ricin, in which the effects of systemic toxicity can be reduced with anti-ricin antiserum (Wiley and Oeltmann, 1989), there is no known countermeasure for volkensin, which is ten times more toxic than ricin (Barbier et al., 1984).

More recently, other lesioning tools, called **immunotoxins**, have been developed to combine the specificity of monoclonal antibodies with the toxicity of RIPs. Immunotoxins may allow for cell-specific lesioning *in vivo* which has not been previously obtained with genetic mutations or other lesioning methods. However, few antibodies that recognise only

one specific type of neuron are available, and many neurons express similar epitopes to which antibodies are produced. For example, the immunotoxin **OX-7-Saporin** recognises the Thy-1 antigen (an abundant cell surface glycoprotein) which is present on virtually all murine neurons (Morris et al ., 1980; Morris, 1985). Because Thy-1 is expressed by all murine neurons it cannot be used to differentiate between the various neural sub-types. One means of overcoming this problem is by the route of administration. That is, depending on whether the injection is made in peripheral tissue, intraspinally, or intracerebroventricularly, the anatomical region exposed (and therefore affected) by the toxin can be made more focal (Wiley and Oeltmann, 1986).

v) ***Temporal and Spatial loss of Motoneurons.***

In order to know the optimal time to transplant cells, it is important to know when motoneuron degeneration has occurred. The specific location of motoneuron depletion is also important to assure that transplanted cells are injected into an area requiring cellular replacement. Thus, the temporal and spatial degeneration of endogenous motoneurons within the model should be known. However, genetic models such as the mnd (Messer, et al., 1987), SOD1 (Gurney et al., 1994; Ripps et al., 1995), and NF-L mouse mutant (elevated expression of the neurofilament light chain subunit) (Lee et al., 1994) result in an unpredictable pattern of motoneuron death throughout the spinal cord, making it difficult to predict when and where transplantation of motoneurons should occur.

In summary, current animal models of motoneuron disease lack certain characteristics and are unfavourable for assessing the therapeutic benefit of cell replacement strategies. However, the possible clinical value of this therapy is promising for the treatment of motoneuron diseases, like ALS. Thus, the need for a more optimal animal model has been established.

192-IgG-SAPORIN

An immunotoxin, called 192-IgG-Saporin, has been used to lesion cholinergic neurons (which express the low affinity nerve growth factor receptor, p75) in the basal forebrain of adult rats following intraventricular administration, while sparing neighbouring neurons of the striatum which do not express the p75 receptor (Wiley et al., 1991; Book et al., 1992; Waite et al., 1994; Book et al., 1995; Seeger et al., 1997).

192-IgG-saporin is an immunotoxin comprised of a monoclonal antibody, called 192-IgG, which binds the (rat) low affinity nerve growth factor receptor ($p75^{LNGFR}$) (Chandler et al., 1984) and has been well characterised (See "192-IgG" below for details). Conjugated to 192-IgG is a ribosome inactivating protein called saporin, which is similar to ricin, abrin, volkensin, and, modeccin. Like other ribosome-inactivating proteins, saporin prevents protein synthesis by depurinating the 28S subunit of ribosomal RNA (Olsnes, et al., 1974). Eiklid, et al. (1980) found that the inhibition of protein synthesis by these toxins is consistent with single hit (first order) kinetics, and suggests that just one molecule of cytotoxin within the cytosol may be sufficient to cause cell death. This is possible because the A-chains of the toxins are capable of inactivating a large number of ribosomes per minute. Thus, these cytotoxin appear to act in an all-or-non fashion. That is, either one or more molecules of toxin is present in the cytosol and the cell dies, or there is no toxin present in the cytosol and the cell survives (Eiklid, et al., 1980).

Many studies have shown proof of concept for 192-IgG-Saporin as an immunolesioning tool (Wiley et al., 1991; Book et al., 1992, 1995; Waite et al., 1994; Seeger, et al., 1997). This has served as a model for studying various therapeutic strategies for the treatment of Alzheimer's Disease. However, this immunotoxin has not been used as a motoneuron lesioning tool.

192-IgG: This monoclonal antibody recognises a moiety of the low-affinity nerve growth factor receptor ($p75^{LNGFR}$) that is specific to rats (Chandler et al., 1984). Moreover, studies have shown that 192-IgG does not bind NGF, nor does it compete with NGF for binding to the $p75^{LNGFR}$ receptor, but instead it facilitates the binding of NGF to $p75^{LNGFR}$ (Chandler et al., 1984; Taniuchi and Johnson, 1985). Studies have also shown that following intraventricular injection or intra-ocular injection [^{125}I]-192-IgG is internalized by receptor-mediated endocytosis and retrogradely transported to the soma of cholinergic neurons of the basal forebrain (Taniuchi, and Johnson, 1985; Schweitzer, 1989). In addition, the mechanism and rate of transport of [^{125}I]-192-IgG was similar to that for [^{125}I]-NGF (Taniuchi and Johnson, 1985).

Saporin: Saporin is a RIP isolated from the seeds of *Saponaria officinalis* and has some properties distinct from the other cytotoxins. For example, saporin (LD_{50} = 20mg/kg) is not as toxic to the animal as ricin or volkensin (LD_{50} = 1.38μg/kg). Moreover, saporin, unlike other cytotoxins which bind to common glycoprotein receptors on the surface of cells, has no method of cell entry on its own (Lappi, et al., 1985). Thus, pure saporin can be used as a control in studies using 192-IgG-saporin. These unique features of saporin make it an ideal cytotoxin, since systemic toxicity should also not be a problem, and transneuronal transport is unlikely. Moreover, when ricin is conjugated to the 192-IgG antibody, the resulting immunotoxin is not active *in vivo*, whereas 192-IgG-Saporin is active *in vivo* (Pubols and Foglesong, 1988).

$p75^{LNGFR}$

The functional role of the low-affinity nerve growth factor receptor (LNGFR) [so named because of its binding/dissociation constant of $K_d \sim 10^{-9}$ M], also known as **p75** (according to its molecular weight) is still unclear. The p75 receptor can bind the neurotrophins NGF, BDNF, NT-3 and NT-4/5, with similar affinities (Rodrequez-Tebar et al., 1990; Hallbook, et al., 1991; Hempstead, et al. 1991; Kaplan and miller, 2000). However, the p75 receptor does not itself transduce the intracellular signals resulting from antigen binding to the receptor, but is thought to be important for the functioning of the trk (high-affinity tyrosine receptor kinase) receptors (Hempstead, et al., 1991).

Of interest, over expression of the cytoplasmic domain of p75 enhanced axotomy-induced cell death of facial motoneurons in adult mice (Majdan et al., 1997). Furthermore, in knockout mice (p75 -/-), survival and regeneration of axotomized motoneurons was improved compared to control animals suggesting a protective effect for the absence of p75 against post-axotomy of motoneurons (Ferri et al., 1998). Collectively, studies to date suggest that p75 may play a role as a cell-death receptor for MNs. Important to our studies, 192-IgG-saporin targets cells which express the p75 receptor.

Adult motoneurons do not express $p75^{LNGFR}$ (Ernfors et al., 1989; Koliatsos, et al., 1991b; Paqueron, et al., 2001), which may explain why 192-IgG-saporin has not been used to lesion MNs (Wiley et al., 1991). However, adult motoneurons can upregulate and re-express p75 following crush or transection of peripheral nerves (Ernfors et al, 1989; Koliatsos et al., 1991a; Paqueron et al, 2001). Nevertheless, using these methods to upregulate the p75 receptor to allow for the immunotoxin to bind would not be helpful, since peripheral nerve injury alone is sufficient to produce a motoneuron deficit (Schmalbruch, 1984). As noted

before, however, mechanical injuries to the peripheral nerves disrupt the integrity of the nerve and therefore are not desirable methods of inducing motoneuron loss within the spinal cord.

An immunohistochemical study of $p75^{LNGFR}$ in developing rats showed intense labelling of spinal motoneurons with 192-IgG at postnatal day 0 (Yan and Johnson, 1988). This staining progressively declined over several days until it was no longer detectable (Yan and Johnson, 1988). Thus, there is a period when motoneurons are still immunoreactive to 192-IgG and should internalize the 192-IgG-saporin by receptor-mediated endocytosis. However, since muscles also expresses p75 at this time in development, muscle cells may also internalize the immunotoxin and be lesioned.

OX7-SAPORIN

OX7-saporin is an immunotoxin similar to 192-IgG-saporin, but consists of a different monoclonal antibody, OX7, which has a high affinity to the Thy-1 antigen (Mason and Williams, 1980). Thy-1 is an abundant cell surface glycoprotein present on virtually all murine neurons (Morris et al., 1980; Morris, 1985). Like p75, Thy-1 is expressed on muscle during development *in vitro* (Lesley, and Lennon, 1977) *and in vivo* (Booth et al. 1984) but lacks expression in adult muscle (Morris et al., 1983). Since Thy-1 is expressed by all murine neurons it cannot, therefore, differentiate between the various neural sub-types, but "anatomical selectivity" may be achieved depending on the site of administration. For example, the intraventricular injection of OX7-saporoin in adult rats results in destruction of Purkinje cells and some cells of the molecular layer of the cerebellum (Davis and Wiley, 1989). The apparent selective targeting of OX7-saporin to Purkinje cells may be due to differences in access to the various neuronal cell-types. That is, Purkinje cells have large

amounts of Thy-1 on their surface compared to other cell-types (Borges et al., 1985) and readily take up substances present in the CSF (Morris, 1985).

In addition, Morris et al (1983) reported the appearance of Thy-1 labelling on hypoglossal motor axons, terminal branches, and the motor end plate. This suggests that an intramuscular injection of OX7 should result in the internalization of the antibody by the motoneurons innervating that muscle. In support of this hypothesis, LaRocca and Wiley (1988) were able to use immunoperoxidase staining to detect the presence of OX7 in hypoglossal motoneurons following injection of the monoclonal antibody into the tongue muscles of adult rats. Moreover, Wiley et al (1989) already demonstrated that OX7-saporin can lesion motoneurons following an intraneural injection. Specifically, the injection of OX7-saporin into the cervical vagus nerve resulted in the anatomically selective destruction of the nodose ganglion (NG) and dorsal motor nucleus of the vagus (DMNX). These results strongly suggest that the intramuscular injection of OX7-saporin should result in the internalization of the immunotoxin by motoneurons, and subsequent death of those motoneurons.

RATIONALE & HYPOTHESES

192-IgG-saporin:

The specificity of the 192-IgG monoclonal antibody to $p75^{LNGFR}$ is well established (Chandler et al., 1984). In addition, it has been shown that the 192-IgG antibody does not bind to NGF, nor compete with NGF for binding to the $p75^{LNGFR}$ receptor (Chandler et al., 1984). Other studies using rats have demonstrated that the 192-IgG antibody is internalised by receptor mediated endocytosis and is transported at a similar rate to NGF (Taniuchi and Johnson, 1985). Studies also show that, motoneurons in the neonatal rat are p75+ unlike adult rats (Yan and Johnson, 1988). Saporin, a relatively new ribosome-inactivating protein, is not taken up into the cell on its own, and has been shown to prevent protein synthesis in a manner similar to ricin, volkensin, and other RIPs (Olsnes et al., 1974; Lappi et al., 1985). Numerous studies have demonstrated that 192-IgG-saporin is an effective immunotoxin for selectively lesioning $p75^{+}$-cholinergic neurons of the basal forebrain, while sparing other neighbouring neurons lacking the p75 receptor (Wiley et al, 1991; Book et al., 1992, 1995, Seeger et al., 1997).

Based upon the above findings, we hypothesize that **the intramuscular injection of 192-IgG-saporin in neonatal rats should result in the internalization of the immunotoxin (by receptor-mediated endocytosis), retrograde transport (to the soma of motoneurons), and subsequent motoneuron death (by preventing protein synthesis).** Since skeletal muscle in neonatal rats is $p75^{+}$ (Yan and Johnson, 1988), they too may be lesioned by the I.M. injection of 192-IgG-saporin. However, the intramuscular injection of 192-IgG-saporin to induce motoneuron loss has not been previously reported. To test whether motoneurons internalize 192-IgG, we used 192-IgG conjugated to the fluorophore

Cy3 (Advance Targeting Systems, San Diego, CA). The resulting compound, 192-IgG-Cy3, was intramuscularly injected into the MG and LG muscles of adult mice, neonatal mice, adult rats, and neonatal rats. Although motoneurons in adult animals are known not to express the p75 receptor, adult animals were injected to indicate whether general endocytosis is a possible mechanism of uptake for the 192-IgG antibody. These experiments demonstrated that motoneurons of only neonatal rats internalized and retrogradely transported 192-IgG-Cy3. Based upon these results, we proceeded to inject neonatal rats with the immunotoxin, 192-IgG-saporin, in an attempt to induce motoneuron loss.

OX7-saporin:

OX7 is a monoclonal antibody directed against the Thy-1 surface antigen (Mason and Williams, 1980). Thy-1 is found on most murine neurons, including motoneurons (Morris, 1980, Morris et al. 1985). As mentioned earlier, studies have demonstrated the presence of Thy-1 on motor end plates of adult rats (Morris, 1983). Furthermore, studies have shown that OX7 accumulates in motoneurons following injection into the vagus nerve (Wiley et al., 1989). Finally, Wiley et al. (1989) demonstrated that the immunotoxin, OX7-saporin, can lesion both sensory neurons and motoneurons following injection into the vagus nerve. However, OX7-saporin has not previously been shown to lesion motoneurons following intramuscular administration. Thus, my hypothesis **was that the I.M. injection of OX7-saporin in adult rats would result in MN loss.** This agent may possibly have the advantage over 192-IgG-Saporoin by not directly lesioning the muscle into which it was injected.

MATERIALS & METHODS

Experimental Animals

Both male and female animals were used in this study. Animals were housed under a 12/12 hour light/dark cycle with *ad libitum* access to food and water and treated in accordance with the Canadian Council of Animal Care (CCAC) guidelines. All procedures used protocols that were approved by the University of Manitoba Animal Care Committee. Depending on the experimental paradigm, either adult mice (CD1, > 2 weeks of age), neonatal mice (CD1, post-natal day 4-5), adult rats (Sprague-Dawley, S-D, > 3 weeks of age), and/or neonatal rats (S-D, post-natal day 4-5) were used in these studies.

Surgeries

All animals were initially anesthetized by inhalation of methoxyflorane in a bell jar for approximately 5 minutes. For neonatal animals, a microvial with a cotton swab soaked with methoxyflourane was used to maintain the surgical level of anaesthesia, while adult animals received an intraperitoneal (IP) injection of ketamine and xylazine (80mg/kg and 10mg/kg, respectively). Hind legs of both neonates and adults were washed with betadine and/or alcohol (70% ethanol).

For I.M. injections, an incision was made around the entire ankle with microsurgical scissors, so the skin could be pulled up to expose all the lower leg muscles. Intramuscular injections (as described elsewhere in "methods") were made into the medial and lateral gastrocnemius and tibialis anterior muscles (MG, LG, and TA respectively) using a Hamilton syringe (30 gauge needle) attached to a micromanipulator.

For sciatic transections (neonatal rats only), a small epidermal incision was made parallel to the thigh with microsurgical scissors, and the underlying musculature exposed. The thigh muscles were then either teased apart with No. 5 forceps to expose the sciatic nerve, which was freed from surrounding connective tissue with a curved-tip glass rod. The sciatic nerve was then transected with microsurgical scissors or freed from surrounding tissue and not transected to serve as a sham-operated control.

Following surgical treatments, sutures were used to close the incision and animals received an I.M. injection of the analgesic, ketoprofen (2.5mg/kg and 5.0 mg/kg for neonatal and adults respectively) prior to fully recovering from the anaesthetics (i.e. when the animals were able to ambulate around the cage). Following recovery, adult animals were housed separately, while neonatal animals were returned to the dam within 2 hours.

Animal & Tissue Processing

All animals were sacrificed with an i.p. injection of ketamine/xylazine (300/30 mg/kg). Thereafter, a 30cc syringe (with 21 gauge needle) was used to transcardially perfused animals with heparinized-PBS (30 mls for mice or neonatal rats and 90mls for adult rats) followed by 4% paraformaldehyde at 4 °C. All tissue was post-fixed for 24 hrs in 4% paraformadehyde and cryoprotected in 15% sucrose in PBS. Coronal sections (10 or 25μm) of the lumbar spinal cord were obtained with a Cryotome (Shandon Scientific Limited) and stained with cresyl violet using standard Nissl-staining techniques. In some experiments, the MG and LG muscles were dissected out at the level of the tendon using microsurgical forceps and scissors, weighed immediately, sectioned (5μm), and stained using standard hematoxylin and eosin (H&E) techniques.

Motoneuron Counts

Motoneuron counts were done on twenty-five Neurolucida tracings of Nissl-stained (transverse) sections (25μm) from the lumbar spinal cord (segments L_4 - L_5) of neonatal or adult rats 3 weeks following I.M. injection into several lower limb muscles with 192-IgG-saporin or OX7-saporin, respectively. Only every third serial section was used in the counts, providing 50μm distance between sections. A "marker" (diamond (♦) profile) was placed bilaterally in the location of the tracing where MNs were found, and counts of MN profiles were done using NeuroExplorer software. To be considered (and therefore counted) as a "MN", cells had to meet three criteria: **i.** contain darkly stained Nissl bodies; **ii.** be located in the spinal cord grey matter where MNs are known to reside (i.e. lamina IX of the ventral horn) and finally, **iii.** have a well-defined nucleolus and/or be at least 25μm in size (i.e. fill a calibrated 25μm circle when placed over the cell).

Imaging

Sections were viewed with a Nikon Eclipse E600 for brightfield microscopy of Nissl-stained sections or by fluorescence (cube: Cy3 excitation λ546 nM; FG excitation λ323 nM). Images were obtained using CoolSnap-Pro™ (Media Cybernetics, Inc.) digital camera and Image-Pro® Plus (Media Cybernetics, Inc.) software. Tracings of coronal sections were done using Neurolucida (MicroBrightField, Inc.) and processed with NeuroExplorer (MicroBrightField, Inc.).

Statistics

Results are expressed as the mean ± standard deviation (SD). Comparisons between the control and experimental treatments were done with Student's t-Tests and a p value of $\leq$ 0.05 was taken as the level of significance.

I. Uptake & Retrograde Transport: *192-IgG-Cy3*

To test whether 192-IgG could serve as a carrier to deliver substances such as saporin to motoneurons following an I. M. injection, 192-IgG conjugated with the fluorophore Cy3 (Advanced Targeting Systems; San Diego, CA) was used. Adult mice, neonatal mice, adult rats, and neonatal rats all received an intramuscular injection with 0.5 μg (in 2.0μl of PBS) of 192-IgG-Cy3 into the left medial and/or lateral gastrocnemius (MG, LG) muscles (**Fig. 1A**). DiI or FG were injected into the right MG and LG muscles as a positive control and to locate the level of the motoneuron pools of interest (**Fig. 1A**). Animals were sacrificed 3 days post-injection. The spinal cord was harvested, sectioned, and viewed under fluorescence microscopy.

II. Motoneuron Lesioning: *192-IgG-saporin & OX7-saporin*

To test the ability of the immunotoxin, 192-IgG-saporin, to induce MN loss, neonatal rats (PD 4-5) were anesthetized with methoxyflurane, and with the aid of a dissecting microscope, the MG, LG, and TA muscles exposed. For OX7-saporin injections, adult rats were anesthetized by an i.p. injection of ketamine and xylazine (80mg/kg, 10mg/kg respectively) and the MG, LG, and TA muscles exposed as in neonates. A micromanipulator was used to inject 0.5μg of 192-IgG-saporin or OX7-saporin (Chemicon) into each of the

MG, LG, and TA muscles (**Fig. 1B**). The equivalent volume of pure saporin was I.M. injected into the contralateral MG, LG, and TA muscles as a control (**Fig. 1B**). Animals were sacrificed 3 weeks post-injection. Sections were Nissl-stained and viewed under brightfield microscopy. Twenty-five sections (50μm apart) were traced using Neurolucida and cell counts performed using NeuroExplorer.

III. <u>Muscle Atrophy:</u> ***Sciatic Nerve Transection Vs. 192-IgG-saporin***

To test whether the muscle atrophy seen in a pilot study with 192-IgG-saporin was secondary (due to denervation) or primary (due to direct effects of 192-IgG-saporin) eight rats from the same litter were divided into two groups; **Group 1:** had their left sciatic nerve transected at mid-thigh level, with sham-operated contralateral control (**Fig. 1C**). **Group 2:** received I.M. injections of 192-IgG-saporin (MG and LG, 0.5μg each) (**Fig. 1C**). Contralateral I.M. injections into the MG and LG muscles with a similar volume of saporin (1.1μg in 2.0μl of PBS) or DiI served as controls (**Fig. 1C**). Animals were sacrificed 3 days post-treatment. A 3-day survival time was chosen to allow maximum muscle effects (from both acute denervation and injection of immunotoxin) and to ensure that the loss of MNs by uptake of the immunotoxin would not take place. Thus, changes in muscle weight following treatment with 192-IgG-saporin would not be due to denervation atrophy as the animals would be sacrificed before this could take place. The MG and LG muscles of both hind limbs for all animals were dissected and weighed on an Ohaus analytical scale. The MG and LG muscles of adult rats used in the OX7-saporin experiments were weighed 3 weeks post-injection.

RESULTS

I. Uptake & Retrograde Transport: *192-IgG-Cy3*

The I.M. injection of 192-IgG-Cy3 resulted in large (~25-45μm) ventral horn cells being intensely labelled with Cy3, but only in the neonatal rats (**Fig. 2 D, d**). Based on their large size (25-45μm), location within the ventral grey matter (lamina IX), and retrograde labelling following I.M. injection, these cells were considered to be alpha MNs. Moreover, Cy3 labelling was restricted to motoneuron pools which correspond/innervate the muscles injected, indicating that labelling with 192-IgG-Cy3 is focal, and that transneural spreading does not occur following I.M. injection (**Fig. 2D**). Cy3 labelling was not seen in the spinal cord of adult mice, neonatal mice, or adult rats, following I.M. injection of 192-IgG-Cy3 (**Figs. 2 A-c** respectively, n= 3). The fact that both adult and neonatal mice did not have Cy3-labelled MNs is consistent with the known species specificity (to rats) of the 192-IgG antibody and suggests that the uptake was due to a specific interaction between the antibody (192-IgG) and receptors (p75). The fact that MNs of adult rats lacked Cy3-labelling shows that a more general and non-specific uptake of 192-IgG-Cy3 does not occur.

Furthermore, sections containing Cy3-labelled cells that had unintentionally faded could be re-visualized by immunohistochemistry using antibodies against the mouse 192-IgG and conjugated with Cy3 (i.e. donkey anti-mouse secondary antibodies conjugated with Cy3). This indicated that both the Cy3 fluorophore and 192-IgG monoclonal antibody are retrogradely transported to the soma following an I. M. injection with 192-IgG-Cy3.

Interestingly, there were approximately 3.5 times as many MNs observed with a Nissl stain then that labelled with Cy3 (**Fig 11**). For example, a series of 22 sections yielded 154

Cy3-labelled MNs and 546 MNs when the same sections were Nissl stained. That is, there was approximately 72% more MNs observed with a Nissl stain than labelled with Cy3 (n=3). This suggests that cell counts from Nissl-stained sections do not accurately reflect the number of cells actually exposed to 192-IgG (and its conjugates) following I. M. injection.

II. Motoneuron Lesioning: *192-IgG-saporin & OX7-saporin*

192-IgG-saporin

Neonatal rats survived the I.M. injections with 192-IgG-saporin and displayed no signs of general toxicity throughout the experiment. However, by two weeks post-injection, animals showed signs of a locomotor deficit characterized by a dorsi-flexion of the foot at rest, and absent dorsi-flexion of the foot during locomotion (n=3, data not shown). These observed functional deficits were limited to the immunotoxin-treated limb and were not observed in the control limb. Three weeks post-injection, hind-limb muscles injected with 192-IgG-saporin, but not with pure saporin, showed severe muscle wasting (**Fig. 3**). Interestingly, despite severe muscle wasting, the sciatic nerve was still present and appeared somewhat translucent.

Significant MN loss was evident in transverse sections stained with cresyl-violet (Nissl stain) (**Fig. 4 A-C**). To estimate the extent of MN loss within the lumbar spinal cord, cell counts of MN profiles were made from 25 neurolucida tracings of Nissl-stained sections (**Fig. 5**). Only every third (25μ) serial section was used, this provided a 50μm distance between sections used in the cell counts, thereby avoiding counting cells more than once. There was a significant deficit of MNs in lamina IX of the spinal cord 3 weeks following I.M. injection with 192-IgG-saporin (**Fig. 5**). Specifically, the immunotoxin-treated side

contained 129 MNs compared to the control side which contained 381 MNs (**Fig. 6**, n=1). This corresponds to a 67% decrease on the 192-IgG-saporin treated side and supports the hypothesis that 192-IgG-saporin can be used to lesion MNs following an I. M. injection in neonatal rats.

OX7-saporin

No signs of general toxicity were seen in any animals at any time throughout the experiment; however, one adult rat died 8 days post-injection of unknown causes. No obvious locomotor deficit was seen in any animals treated with OX7-saporin. Three weeks post-injection, muscles injected with OX7-saporin appeared only slightly atrophic compared to controls. Although a MN deficit was not apparent in individual Nissl sections, MN cell counts from three rats indicated an average of deficit of 49 $\pm$ 18 cells on the OX7-saporin side versus control side (p=0.02). This corresponded to a 12 % difference between the two sides (n=3). Specifically, there was an average of 403 $\pm$ 93 MNs on the control side and 353 $\pm$ 94 MNs on the OX7-treated side (**Fig. 7**, n=3, p= 0.02). This supports the hypothesis that OX7-saporin can lesion MNs following an I.M. injection, and is consistent with the known ability of OX7-saporin to lesion MNs following intra-neural administration.

III. Muscle Atrophy: *Sciatic Nerve Transection Vs. 192-IgG-saporin*

Although it is well established that denervation results in muscle atrophy, the large degree of atrophy seen just three weeks post-injection with 192-IgG-saporin suggested that it was not likely to be due exclusively to MN death (see **Fig. 3**). To assess the degree to which the observed muscle atrophy was due to denervation alone, neonatal rats either received a sciatic nerve transection (sham-operated contralateral control) or an I.M. injection of immunotoxin, 192-IgG-saporin (saporin-injected contralateral control)[see **Fig. 1C**]. A

three-day survival time was chosen to ensure that denervation by immunolesioning of MNs with 192-IgG-saporin did not occur prior to sacrifice.

Animals that received a sciatic nerve transection lost the use of the injected hind leg, but the hind legs of the sham-operated controls were unaffected. Animals that received I.M. injections with 192-IgG-saporin did not display any functional deficits over the three day time period.

The MG and LG muscles of all animals in the two groups were weighed, and the mean muscle weights (± SD) were plotted as a histogram (**Fig. 8A**, n= 4). There was a significant decrease in the mean muscle weight of the MG, but not the LG, muscles treated with 192-IgG-saporin compared to control-injected muscles (**Fig. 8A**, n= 4, p= 0.008 and 0.12 respectively). More specifically, the MG and LG muscles injected with 192-IgG-saporin had a mean muscle weight that was 78.0% and 83.6% that of controls (**Fig. 8B**, n=4). There was no significant difference between the sham-operated control limb compared to the transected limb with respect to the mean muscle weight (± SD) for the MG or LG muscles (**Fig. 8A**, n= 4, p= 0.49, p= 0.21respectively). Stated another way, the mean muscle weights for the MG and LG muscles of the transected group were 99.1% and 88.1%, respectively, that of controls (**Fig. 8B**). These results suggest that 192-IgG-saporin plays a more direct role in the observed muscle atrophy, other than just MN death-induced denervation atrophy.

To further investigate the effects of 192-IgG-saporin on muscle, longitudinal sections (5μ) of the MG and LG muscles for the two groups of animal were dissected, sectioned, and stained with H&E. The MG and LG muscles of both the sham-operated and transected limbs had no signs of inflammation or necrosis (**Figs. 9A** and a respectively). Muscles injected with non-conjugated saporin showed signs of inflammation characterized by infiltration of

mononuclear cells (**Fig. 9 B**). Muscles treated with 192-IgG-saporin showed signs of severe inflammation and necrosis (characterised by necrotic muscle fibres and macrophages three days post-injection with 192-IgG-saporin (**Fig. 9 b**, n=4).

The results obtained by measuring muscle weights and histology suggest that 192-IgG-saporin directly leads to necrosis of skeletal muscles which subsequently results in acute muscle atrophy.

<u>OX7-saporin</u>

There was little, if any, observable difference in the size of the MG or LG muscles injected with OX7-saporin when compared to controls. Student's t-tests of MG and LG muscle weights three weeks post injection with OX7-saporin indicated that there was no difference between the OX7-saporin treated muscles versus controls (**Fig. 10 A**, p=0.27 and 0.09 respectively, n= 3). Compared to controls, the mean muscle weights of the OX7-saporin treated MG and LG muscles three weeks post-treatment were 84.5.0 % and 76.8%, respectively, compared to controls (**Fig. 10 B**). These findings suggest that OX7-saporin does not lead to muscle necrosis in this adult rat model system.

Table 1. Summary of the "characteristics" and "limitations" of some **genetic** and **experimentaly induced** animal models of MND. The term "Limitations" is meant in the context of evaluating the utility of the animal model's suitability for assessing cell replacement strategies for the treatment of MNDs. References for the models are also provided.

Table 1

Characteristics and limitations of some animal models of MND.

Models *(Genetic)*	Characteristics	Limitations
Mnd *Motor Neuron Degenerative*	onset: 5-11 months course: 9-14 months	very late onset
	wide-spread vulnerability	More than MNs affected
	chromosome 8p	requires Inter-animal controls
	Model for cerebral ceroid lipofuscinosis	Temporal and spatial course of motoneuron degeneration uncertain
	First described in 1987 Messer et al. 1987.	
PMN *Progressive Muscular Nuropathy*	Onset: 3 weeks Course: 3-4 weeks	Premature death
	Distal axonopathy with preservation of proximal axons and cell bodies	No motoneuron degeneration (neuromopathy)
	First described in 1991 Schmalbruch, 1991.	Requires inter-animal controls
Wobbler	Onset: 3-5 weeks Course: 3 weeks, 3-12months	Primarily restricted to cervical level
	Vacolar degeneration of cervical motoneurons	Requires Interanimal controls
	Chromosome location, 11	Gonadal dysfunction (of unknown etiology)
	First described in 1956. Falconer 1956.	

Models *(Genetic) Continued...*	Characteristics	Limitations
SOD1	Various mutants with different onsets, course, and severity of disease (4-14 months)	Spatial and temporal pattern of MN degeneration unpredictable.
	Gain-of - function mutation	Requires inter-animal controls.
	Vacuolation and death of anterior horn cells.	
	Ch 12q	
	Autosomal dominant	
	Gurney et al. 1994.	
NF-L	Onset: 18days Course: 28 days	Short life-span (premature death)
	Increased expression of light-chain neurofilament subunit	Spatial course of MN degeneration unknown.
	Large accumulation of NF in motoneurons and sensory neurons.	Requires inter-animal controls.
	MN degeneration and muscular atrophy	
	Lee et al. 1994.	
Knock-out mice	Die before or soon after birth.	Premature death
	Not limited to MNs.	Not MN specific.
		Requires inter-animal controls.

Models *(Experimentally Induced)*	Characteristics	Limitations
Neonatal Rat Nerve Transection (peripheral)	90-100% MN degeneration in neonatal rats; very little MN degeneration in adults.	Kills many motoneuron pools.
	Onset: at time of injury Course: several days Apoptosis model of MN death	Severe sensory loss.
	Schmalbruch, 1984.	Integrity of peripheral nerve disrupted.
		Highly invasive.
Adult Ventral Root Avulsion	Similar to above	Similar to above
Intraspinal injection of Kainate	Excitotoxicity of Cells	Not specific to motoneurons.
	Nothias et al, 1990.	General disruption of cytoarchitecture of spinal cord.
Cytotoxins *(ricin,volkensin,abrin, modeccin)*	Irreversibly inactivate ribosomal subunit.	Not MN specific (sensory also affected).
	Bind to surface glycoproteins or carbohydrate receptors	Transneural spreading reported.
	Some only retrogradely transported in PNS, not CNS.	Systemic toxicity.
		Local necrosis at injection site.
	Both MNs and Sensory fibers degenerate.	Severe Muscle Wasting.
	Wiley and Stirpe, 1987.	

Models *(Exp. Induced) Continued....*	Characteristics	Limitations
192-IgG-saporin	Binds to p75 receptor. Targets cells with p75 receptors.	Not VN specific
	Specific to Rats	DRG may also be affected.
	Onset: 3-4 days post injection Course: 1-2 weeks.	Severe muscle wasting (in neonatal rats).
	Inhibits protein synthesis.	
	Does not bind with NGF, nor compete with neurotrophins for binding site.	
	Not as toxic as pure cytotoxins.	
	Wiley et al., 1991	
OX7-saporin	Binds to murine Thy-1 cell surface antigen.	Not specific to VNs DRG also affected.
	Onset: 2-5 days post injection. Course: 1-2 weeks.	
	Inhibits protein synthesis	
	Not as toxic as pure cytotoxins.	
	Wiley et al., 1989.	

Figure 1(A-C). Schematic diagrams depicting the three experimental paradigms used in this study. **(A)** I.M. injection of 192-IgG-Cy3 and the lipophilic fluorescent dye, DiI (control), into muscles of the left and right hind limbs, respectively. **(B)** I.M. injection of the immunotoxin, 192-IgG-saporin or OX7-saporin, and pure saporin (control), into the MG, LG, and TA muscles of the left and right hind limbs, respectively. **(C)** Comparison of two treatment groups, sciatic nerve transection (with sham-operated contralateral control), and I.M. injection with 192-IgG-saporin (with saporin-injection contralateral control), respectively.

Figure 1A

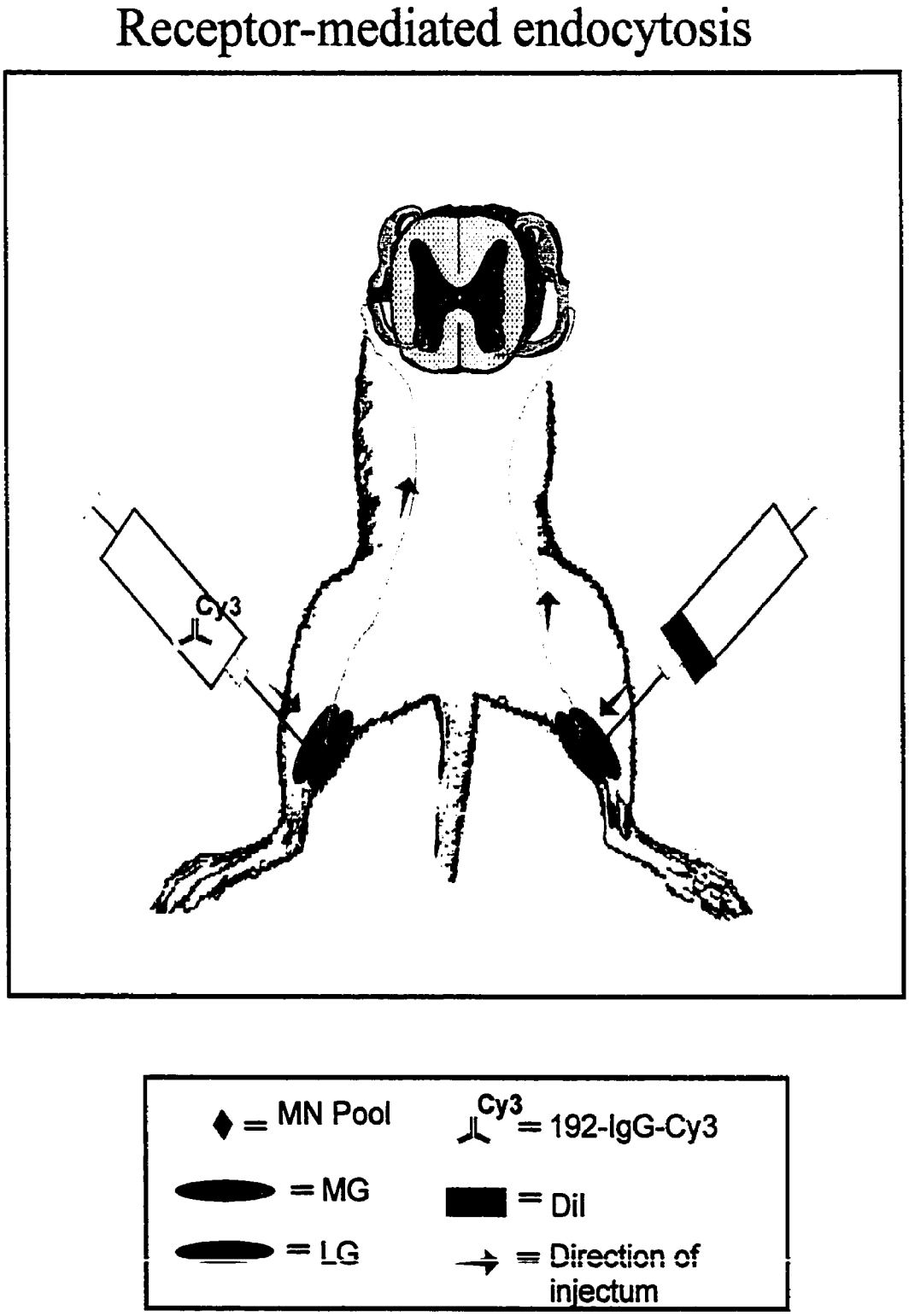

Receptor-mediated endocytosis
Cy3
MN Pool
Cy3 = 192-IgG-Cy3
= MG
= DiI
= LG
= Direction of injectum

Figure 1B

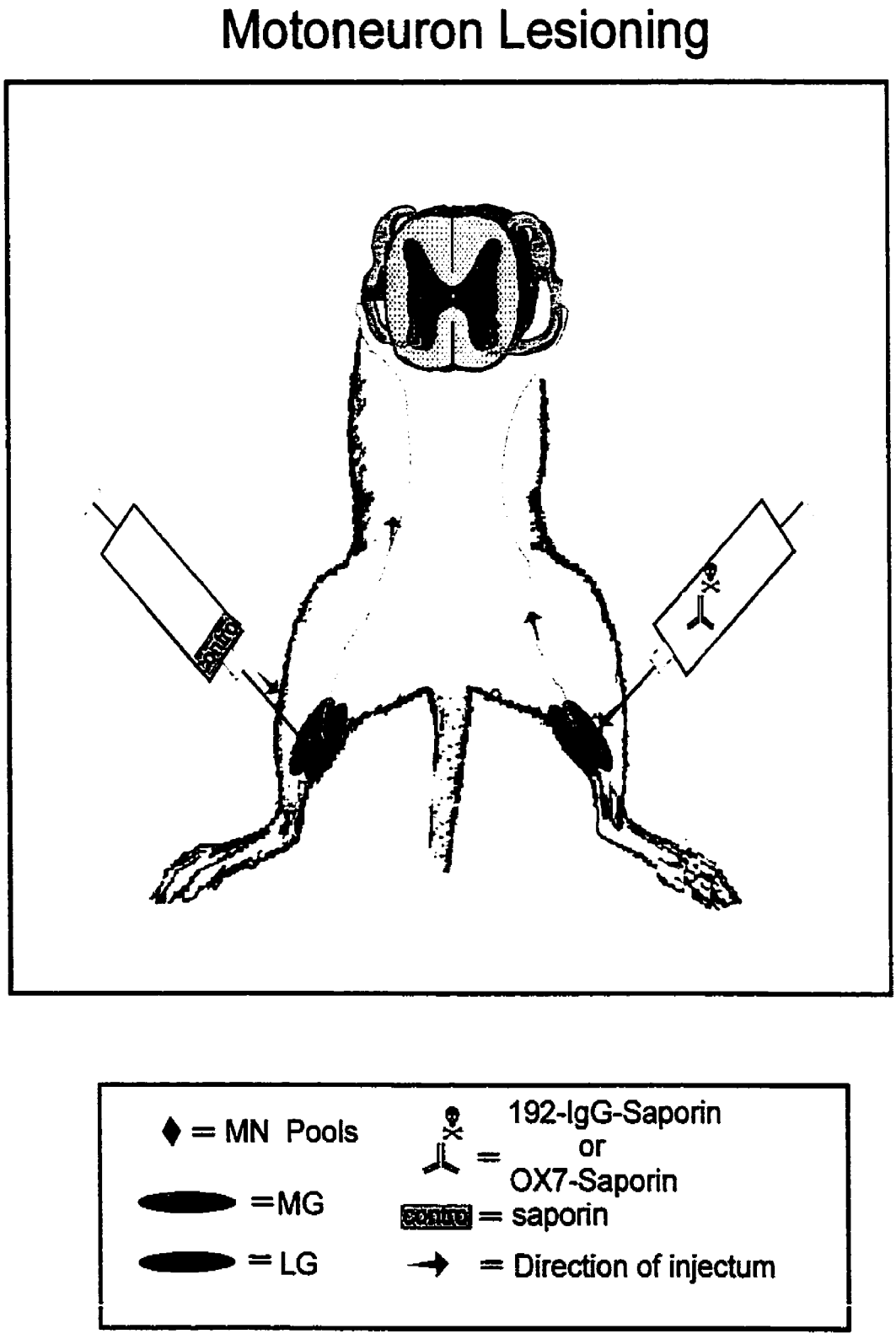
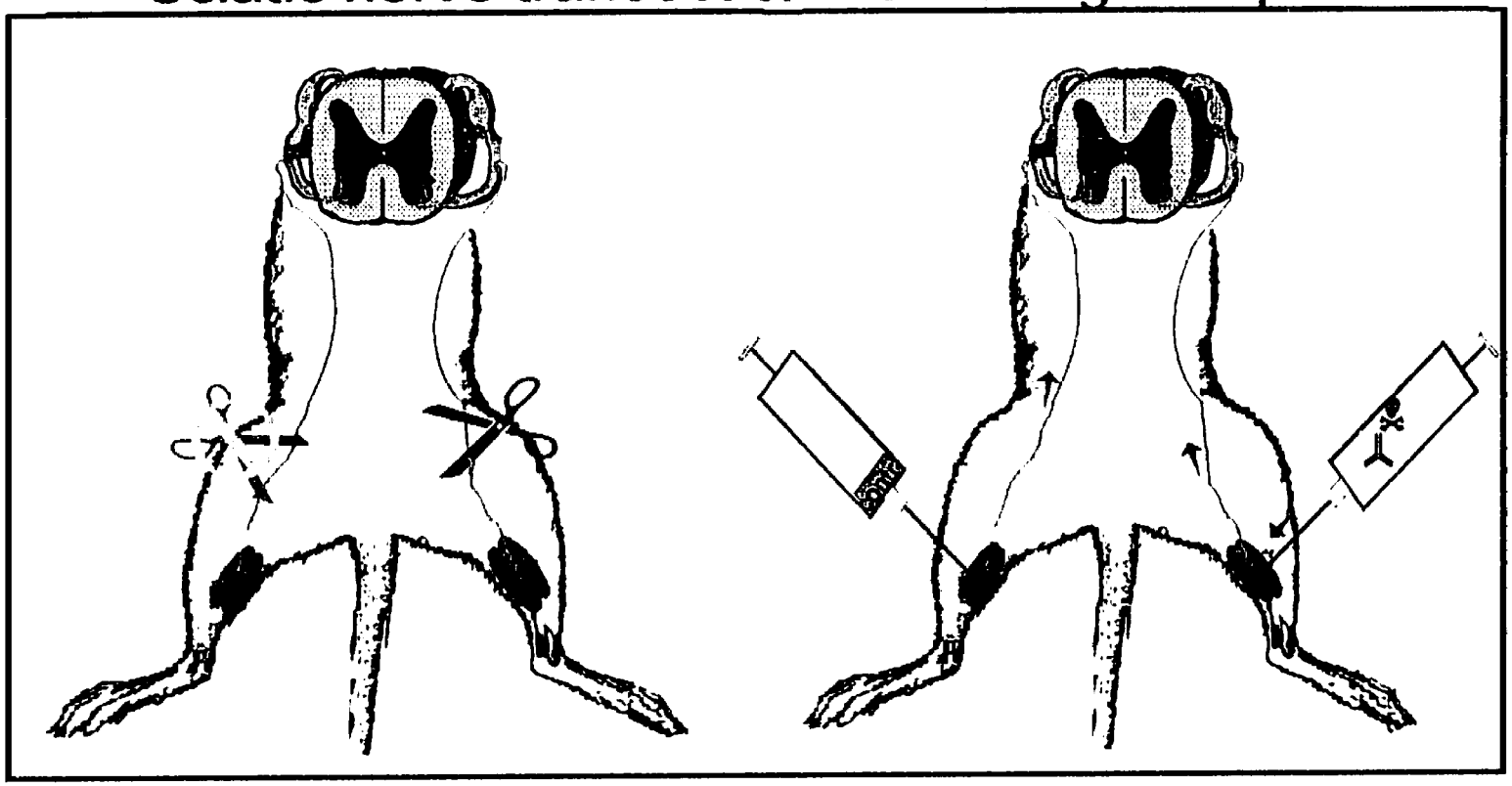

Motoneuron Lesioning
saporin
♦ = MN Pools
=MG
=LG
192-IgG-Saporin
or
OX7-Saporin
= saporin
= Direction of injectum

Figure 1C

Sciatic nerve transection Vs. 192-IgG-Saporin



♦ = MN Pool

= MG

= LG

= Sham-operated control

= Sciatic transection

= 192-IgG-Saporin

contrl = saporin

= Direction of injectum

Figure 2. Low (10x) and High (40x) magnification photomicrographs of transverse sections from the lumbar spinal cord (lamina IX) of an adult mouse (A, a; n=4), neonatal mouse (B, b; n=3), adult rat (C, c; n=3), and neonatal rat (D, d; n=4) three days post-injection with 192-IgG-Cy3 into the MG and LG muscles. Note the intense Cy3-labelling of the neonatal rat MNs (D, d) while labelling was absent in the neonatal mouse, adult mouse, and adult rat (A-c respectively).

Figure 2

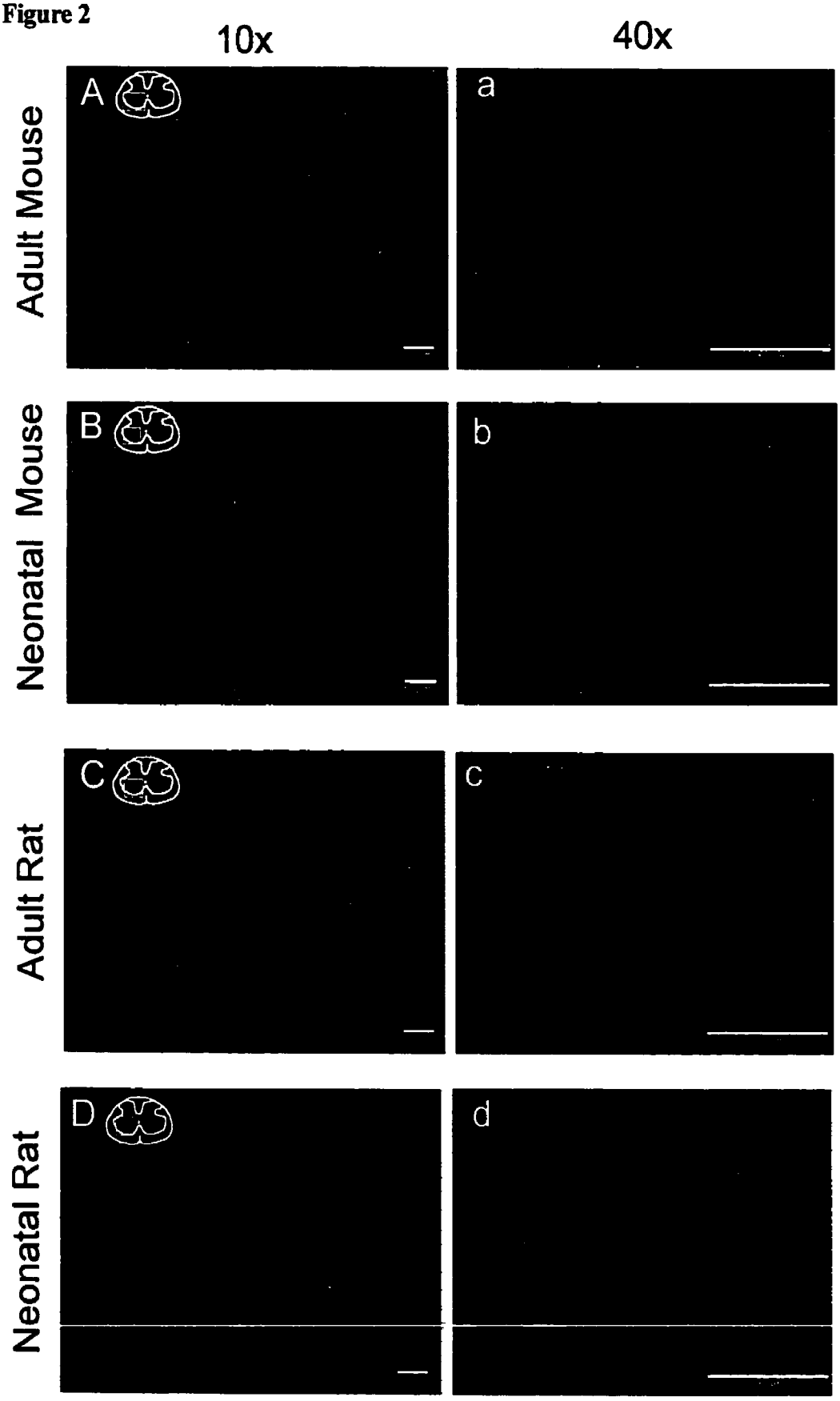

10x
40x
A
a
Adult Mouse
B
b
Neonatal Mouse
C
c
Adult Rat
D
d
Neonatal Rat

Figure 3. Digital photographs of the hind limbs of a neonatal rat 3 weeks following I.M. injections with 0.5 μg of 192-IgG-saporin into each the MG, LG, and TA (PD 4-5). Saporin or DiI was injected as control in contralateral muscles. (A) Dorsal and (B) ventral views are shown. Note the severe atrophy of muscles following treatment with 192-IgG-saporin.

Figure 3

Muscle wasting following intramuscular injection of 192-IgG-Saporin

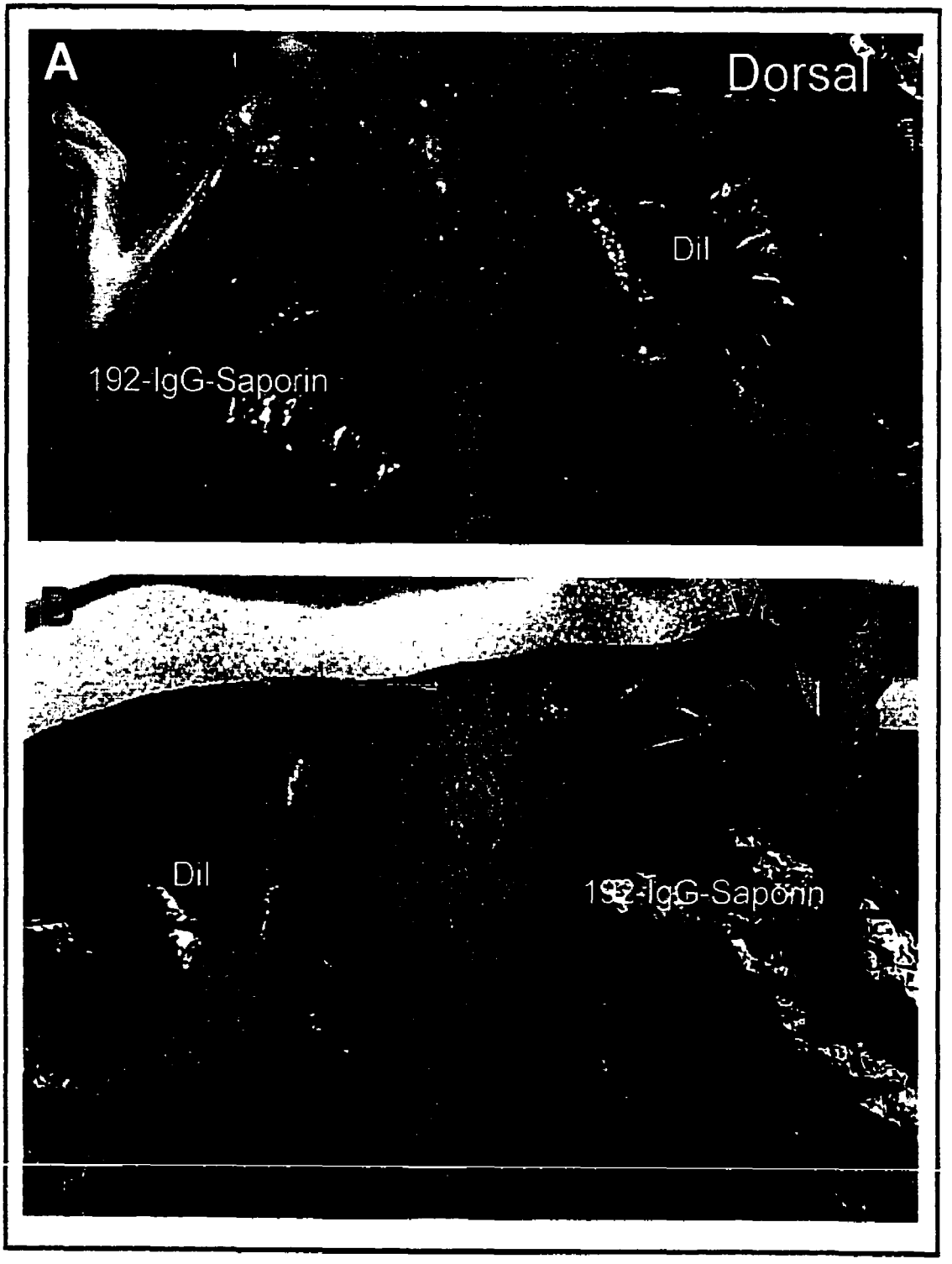


Figure 4. A representative example of a 25μm transverse section stained with cresyl violet used to generate the cell maps (Neurolucida tracings) in Figure 5. The section shown is taken from the lumbar spinal cord (L_4-L_5) of a neonatal rat three weeks post-injection of multiple hind-limb muscles with saporin or 192-IgG-saporin (PD 4). Note the lack of large ventral horn cells in lamina IX on the 192-IgG-saporin injected side. The two bottom panels are enlargements (20x) of lamina IX from the section above showing a deficit of large ventral horn cells corresponding to MNs (n=3).

Figure 4

Nissl-stained section showing motoneuron loss

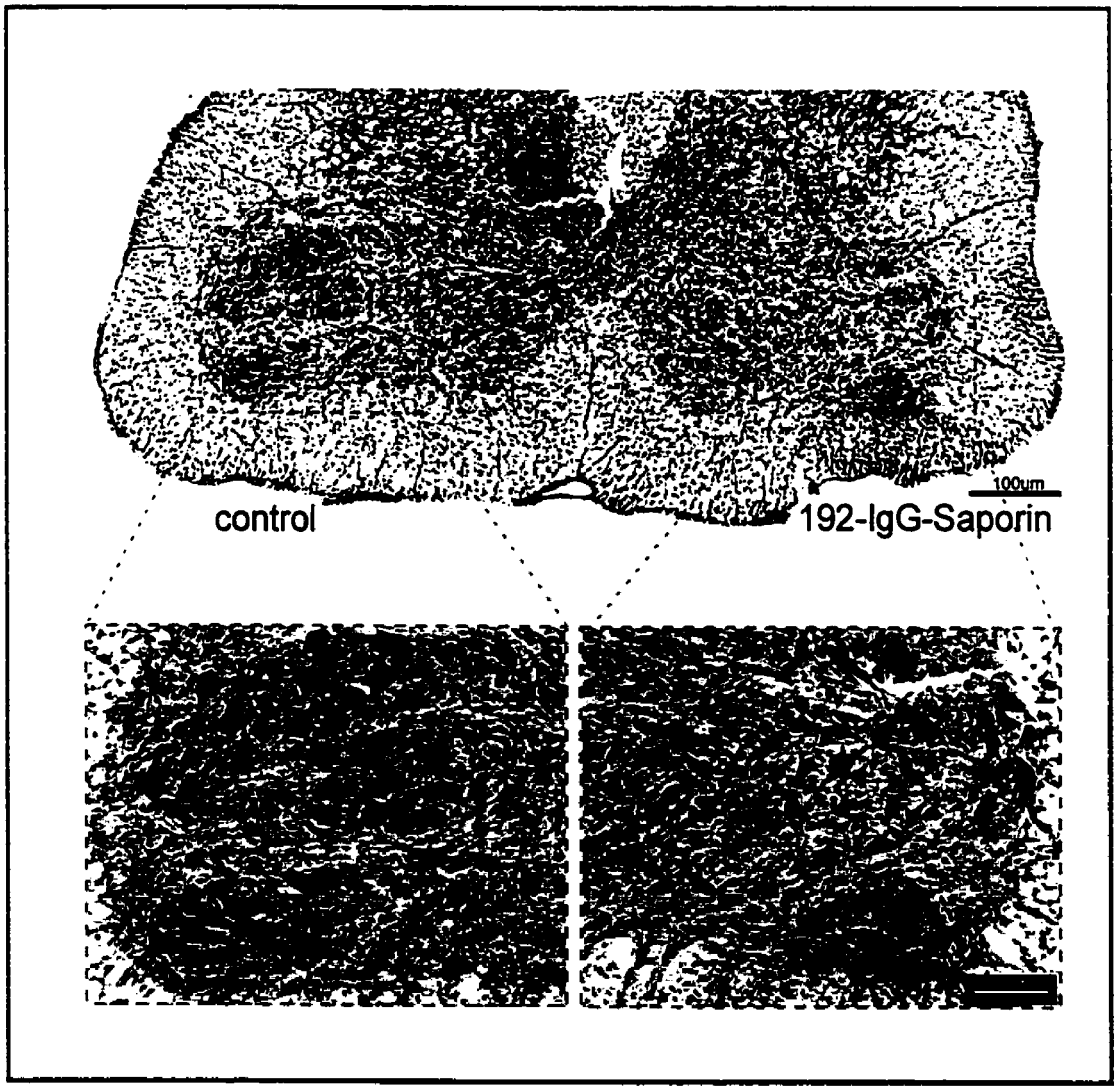


Figure 5. Overlay of 25 Neurolucida tracings (cell maps) of Nissl-stained sections shown in Figure 4, used to generate counts of MNs. Red diamonds (♦) represent MNs. The number of MNs for saporin and 192-IgG-saporin treatments were 381 and 129, respectively. Sections represent approximately 2 mm of the lumbar spinal cord corresponding to L_4-L_5 (n=1).

Figure 5

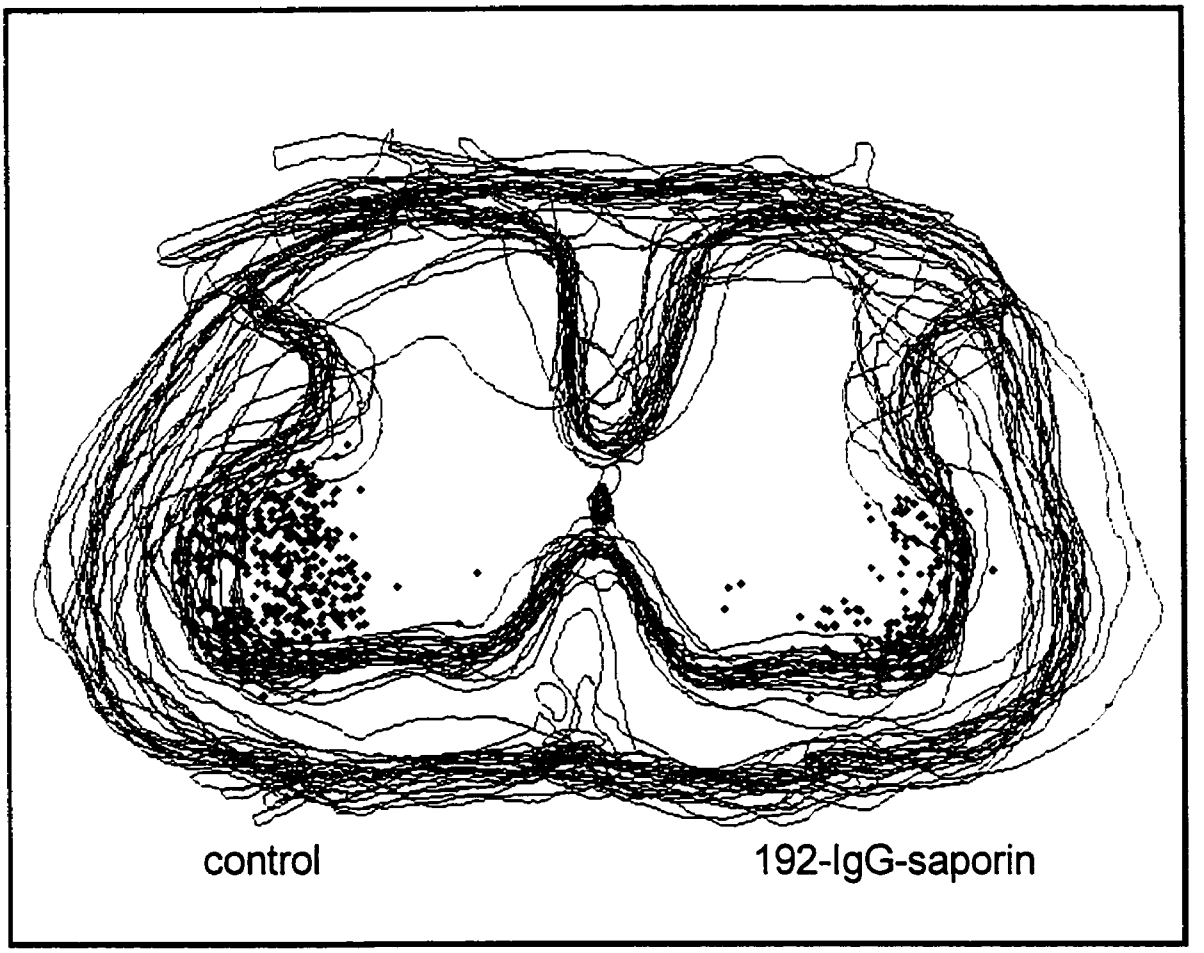


Figure 6. Summary histogram of the MN profiles counted from 25 Neurolucida tracings (Figure 5) from the lumbar spinal cord of a neonatal rat three weeks following I.M injection with saporin (control) and 192-IgG-saporin. The number of MN profiles for the two treatments was 381 and 129 respectively (representing a 67% MN loss, n=1).

Figure 6

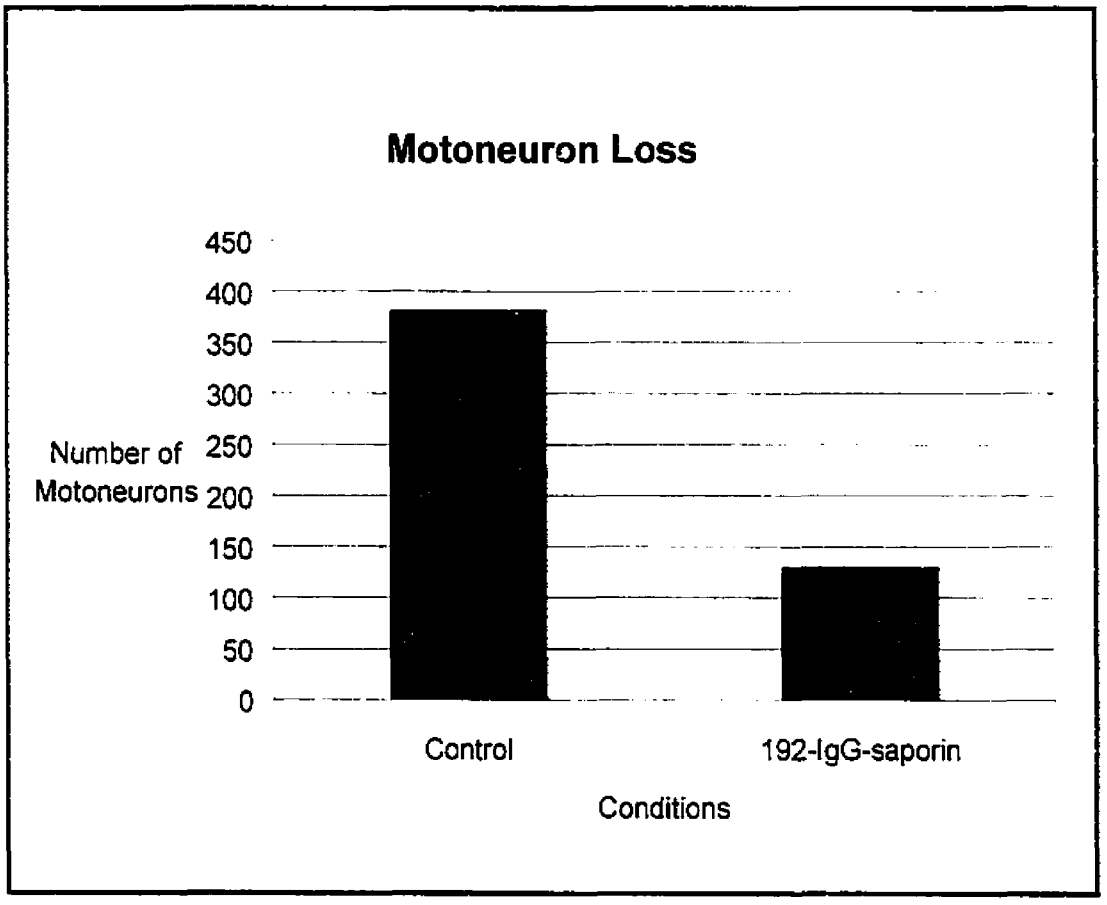

Motoneuron Loss
450
400
350
300
250
200
150
100
50
0
Number of Motoneurons
Control
192-IgG-saporin
Conditions

Figure 7. Histogram showing the mean number of MNs from the lumbar spinal cord of adult rats three weeks following an I. M. injection of 0.5 μg of OX7-saporin (non-conjugated saporin is control) into each the MG, LG, and TA muscles. Specifically, there was an average of 403 ± 94 MNs on the control side compared to 354 ± 95 MNs on the OX7-saporin treated side ($p = 0.078$, $n=3$). This corresponds to a 12.4% decrease.

Figure 7

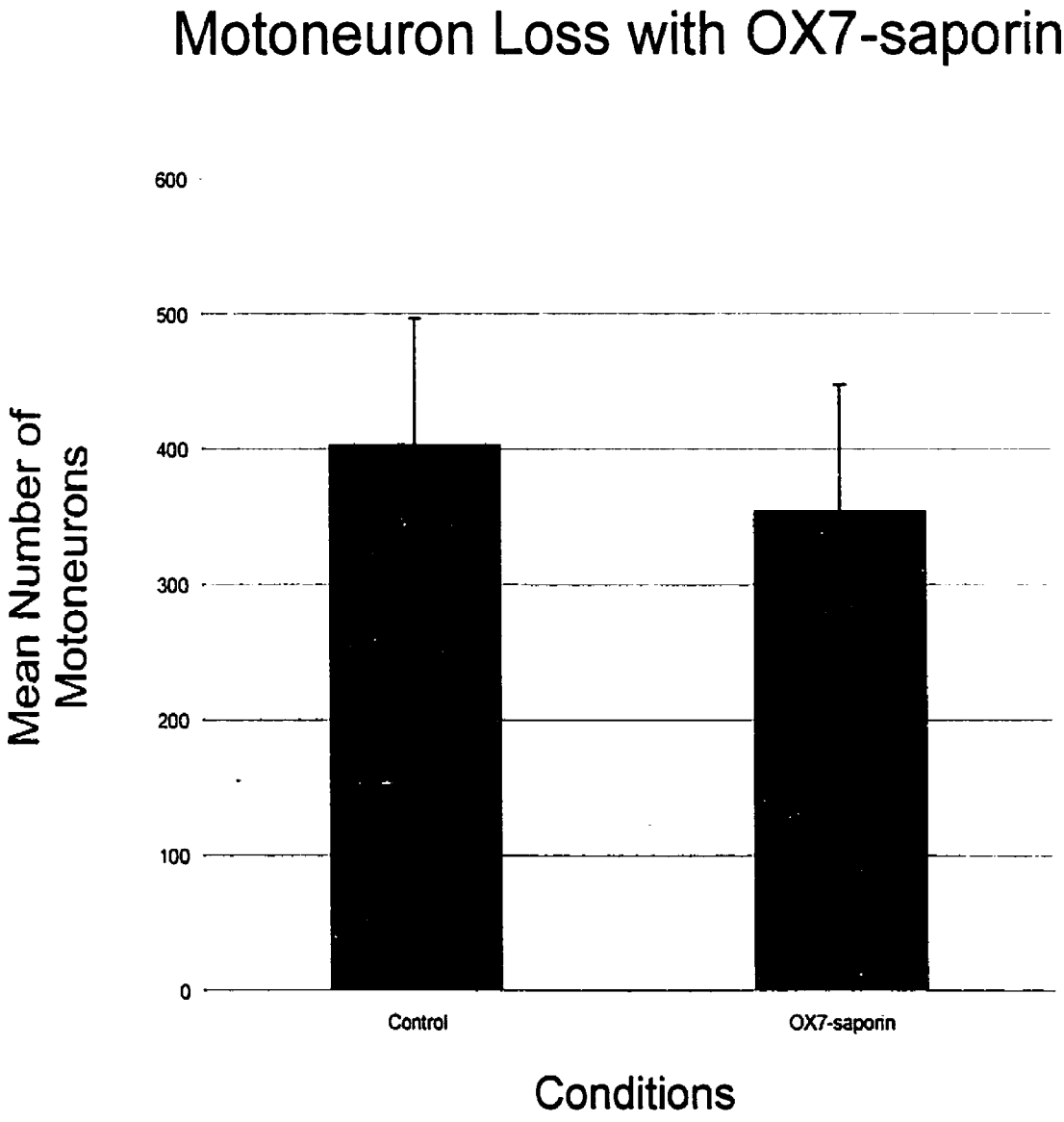

Motoneuron Loss with OX7-saporin
Mean Number of Motoneurons
600
500
400
300
200
100
0
Control
OX7-saporin
Conditions

Figure 8. **(A)** Histogram of mean muscle weight (± SD) for the MG (light blue bars) and LG (dark blue bars) muscles of neonatal rats 3 days post-treatment (sham-operated control/sciatic nerve transection and I.M. injection with 192-IgG-saporin/saporin control, respectively). n=4.

Student's t-test indicated a **significant difference** in the MG muscle weights between the 192-IgG-saporin and saporin conditions three days post-treatment ($p= 0.01$). **No significant difference** was found for the LG muscle weights of the two treatments ($p= 0.12$). There was **no significant difference** between the MG or LG muscle weights of the sham-operated control and transected treatments ($p= 0.49$, and $p= 0.21$, respectively). Level of significance defined as $p \leq 0.05$.

(B) Histogram of mean muscle weight, expressed as a percent (%) of control, for the same data set as in (**A**). Mean muscle weights as percent control for the MG and LG muscles were 99.1 % and 88.1 % (following sciatic transection treatment) and 78.0 % and 83.6 % (after 192-IgG-saporin treatment), respectively.

Figure 8 A

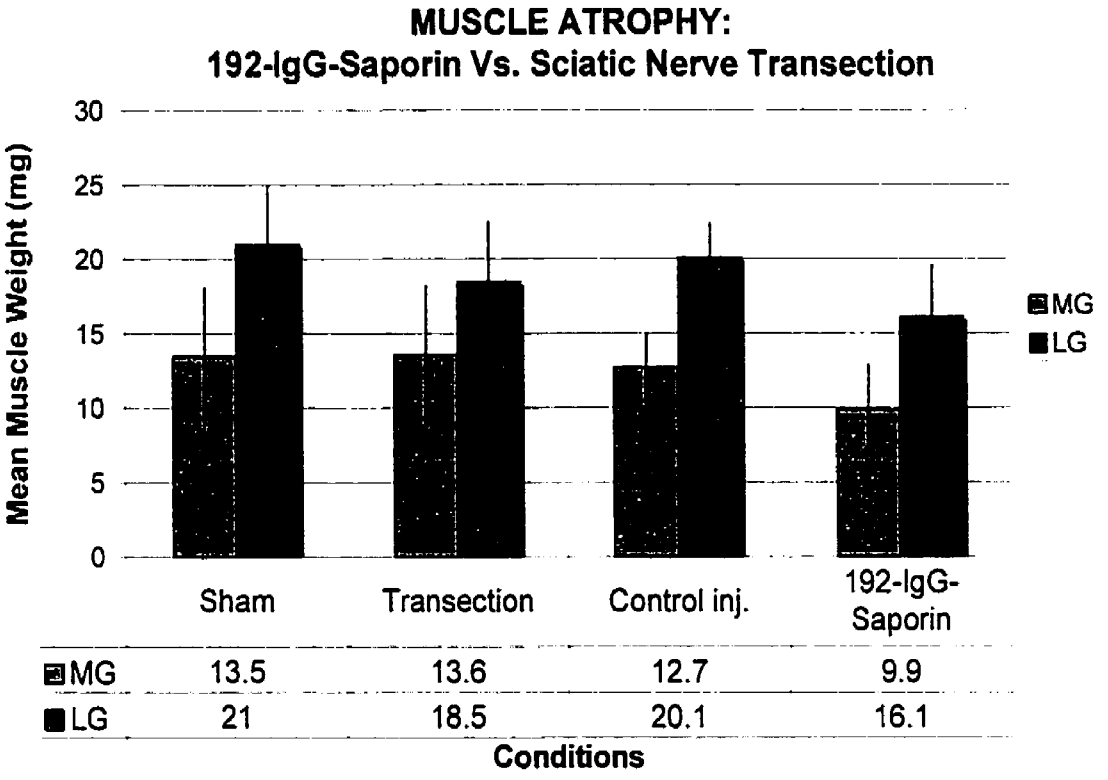

MUSCLE ATROPHY:
192-IgG-Saporin Vs. Sciatic Nerve Transection
Mean Muscle Weight (mg)
30
25
20
15
10
5
0
MG
LG
Sham
Transection
Control inj.
192-IgG-Saporin
MG 13.5 13.6 12.7 9.9
LG 21 18.5 20.1 16.1
Conditions

Figure 8 B

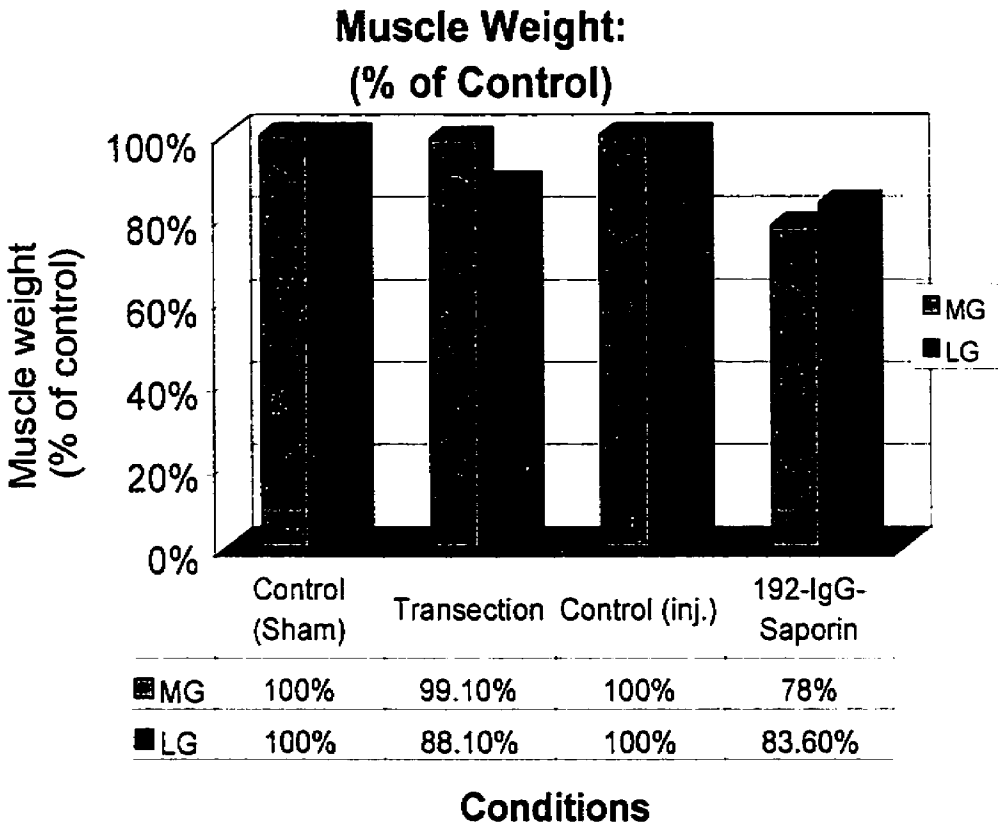

Muscle Weight:
(% of Control)
Muscle weight
(% of control)
100%
80%
60%
40%
20%
0%
MG
LG
Control (Sham)
Transection
Control (inj.)
192-IgG-Saporin
MG 100% 99.10% 100% 78%
LG 100% 88.10% 100% 83.60%
Conditions

Figure 9. Photomicrographs of sections of the medial gastrocnemius muscle from neonatal rats 3 days post-treatment after H&E staining: (**A**) sham-operated control, (**B**) sciatic nerve transection, (**C**) saporin-injected control, and (**D**) 192-IgG-saporin. Muscle cells (**mc**) of the sham-operated control and Sciatic nerve transection group appear "normal". saporin injected muscles display edema and infiltration of mononuclear cells, such as lymphocytes (**L**) and polymorphonuclear (**PMN**) cells characteristic of an inflammatory response. Injection with 192-IgG-saporin resulted in necrosis of muscle cells characterized by edema, necrotic muscle (**nm**) cells, and the presence of macrophage infiltration (**M**). Blood vessels (**bv**), fibroblasts (**fb**), and nuclei (**N**) of muscle cells are also shown. Scale bars are 10 um.

Figure 9

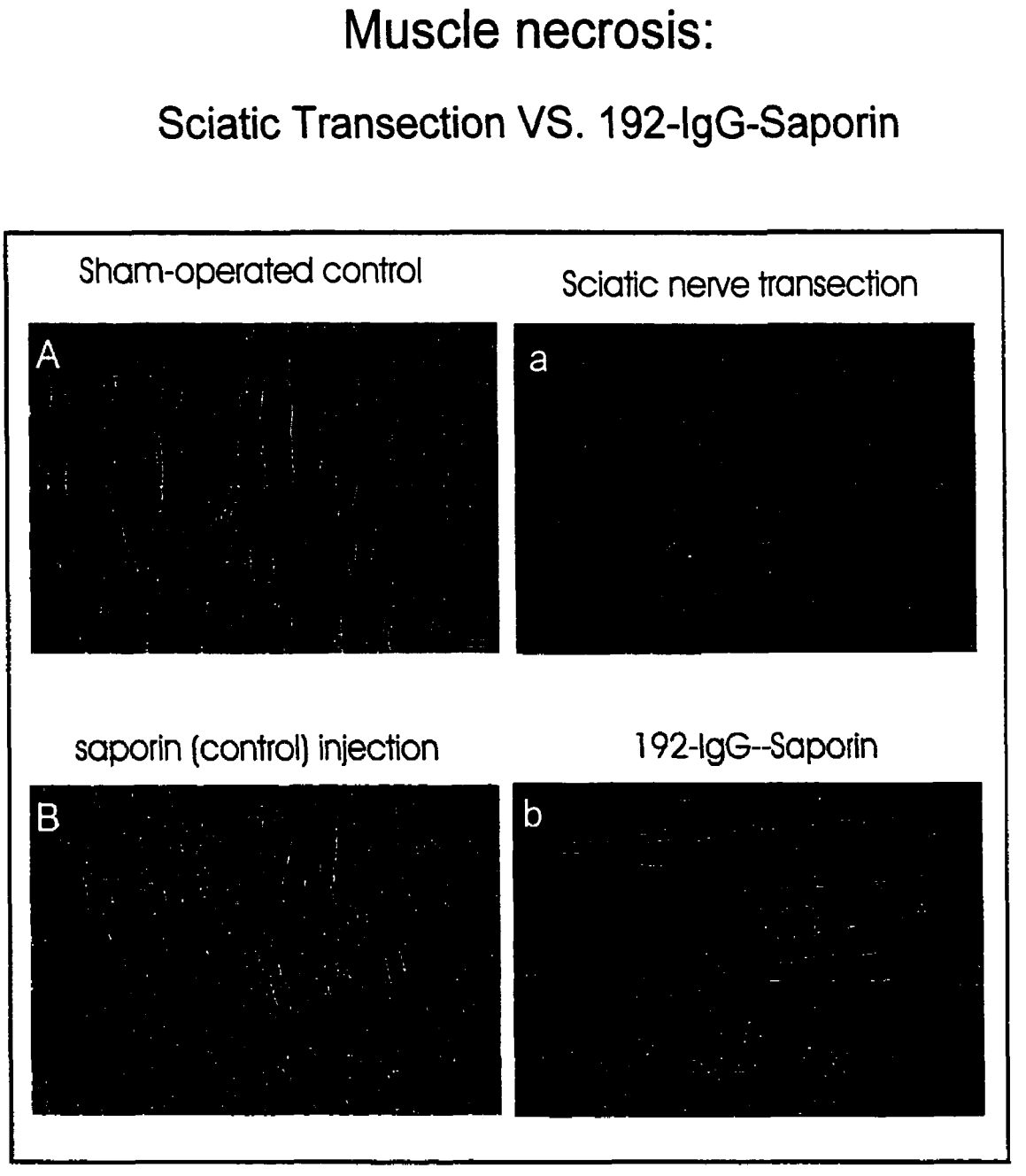

Muscle necrosis:
Sciatic Transection VS. 192-IgG-Saporin
Sham-operated control
Sciatic nerve transection
A
a
saporin (control) injection
192-IgG--Saporin
B
b

Figure 10. (A) Histogram of mean muscle weight (± SD) for the MG (light blue bars) and LG (dark Blue Bars) muscles of adult rats 3 weeks post-treatment (Control injection with non-conjugated saporin and OX7-saporin, respectively). n=3. Student's t-test indicated that there was no significant difference in both the MG (p=0.23) and LG (p=0.91) muscles three weeks post treatment with OX7-saporin compared to controls (n=3).

(B) Histogram of mean muscle weight, expressed as a percent (%) of control, for the same data as in (A). The MG and LG muscles treated with OX7-saporin were 84.5% and 77% that of controls, respectively.

Figure 10 A

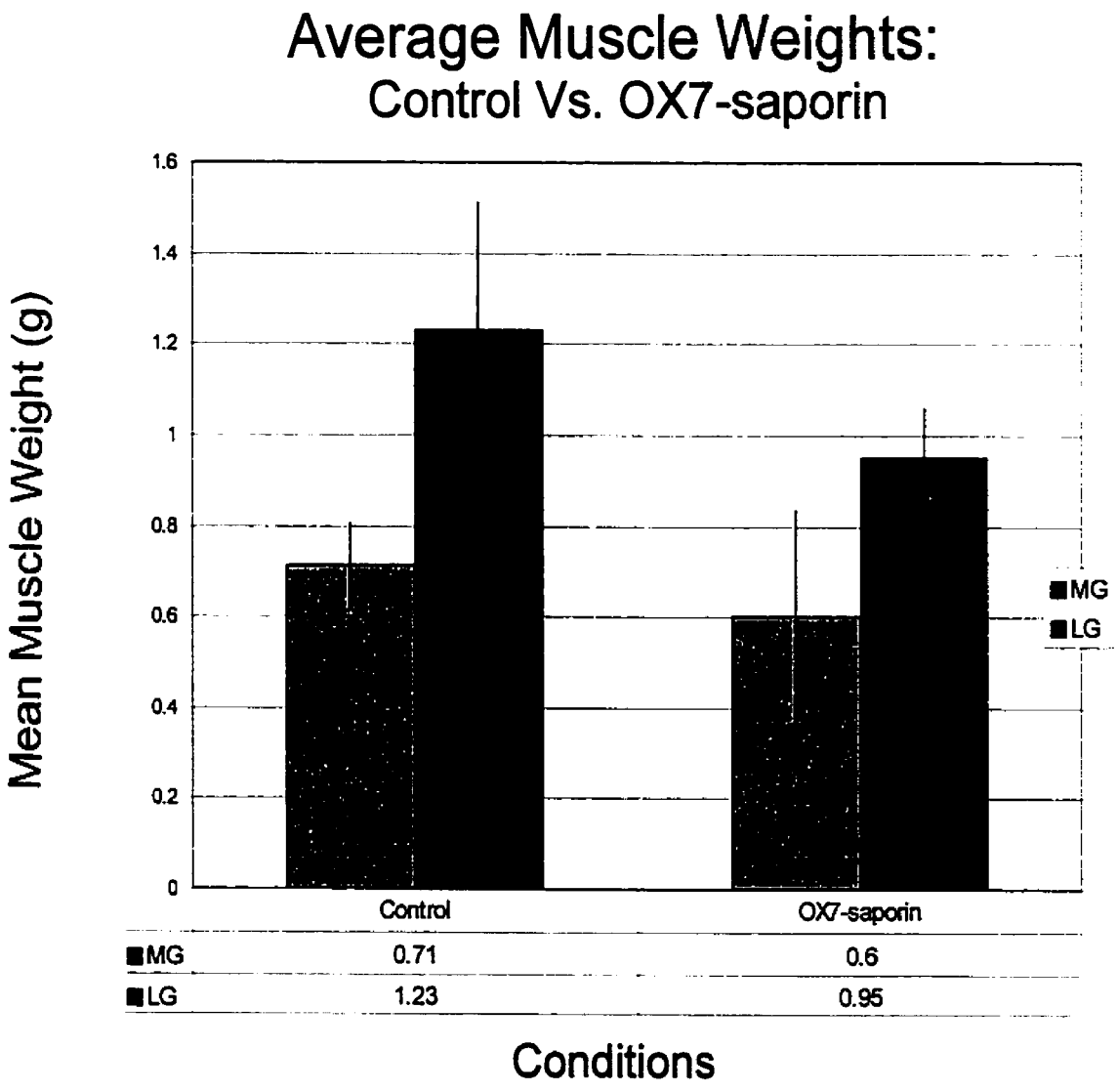

Average Muscle Weights:
Control Vs. OX7-saporin
Mean Muscle Weight (g)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MG
LG
Control
OX7-saporin
MG 0.71 0.6
LG 1.23 0.95
Conditions

Figure 10B

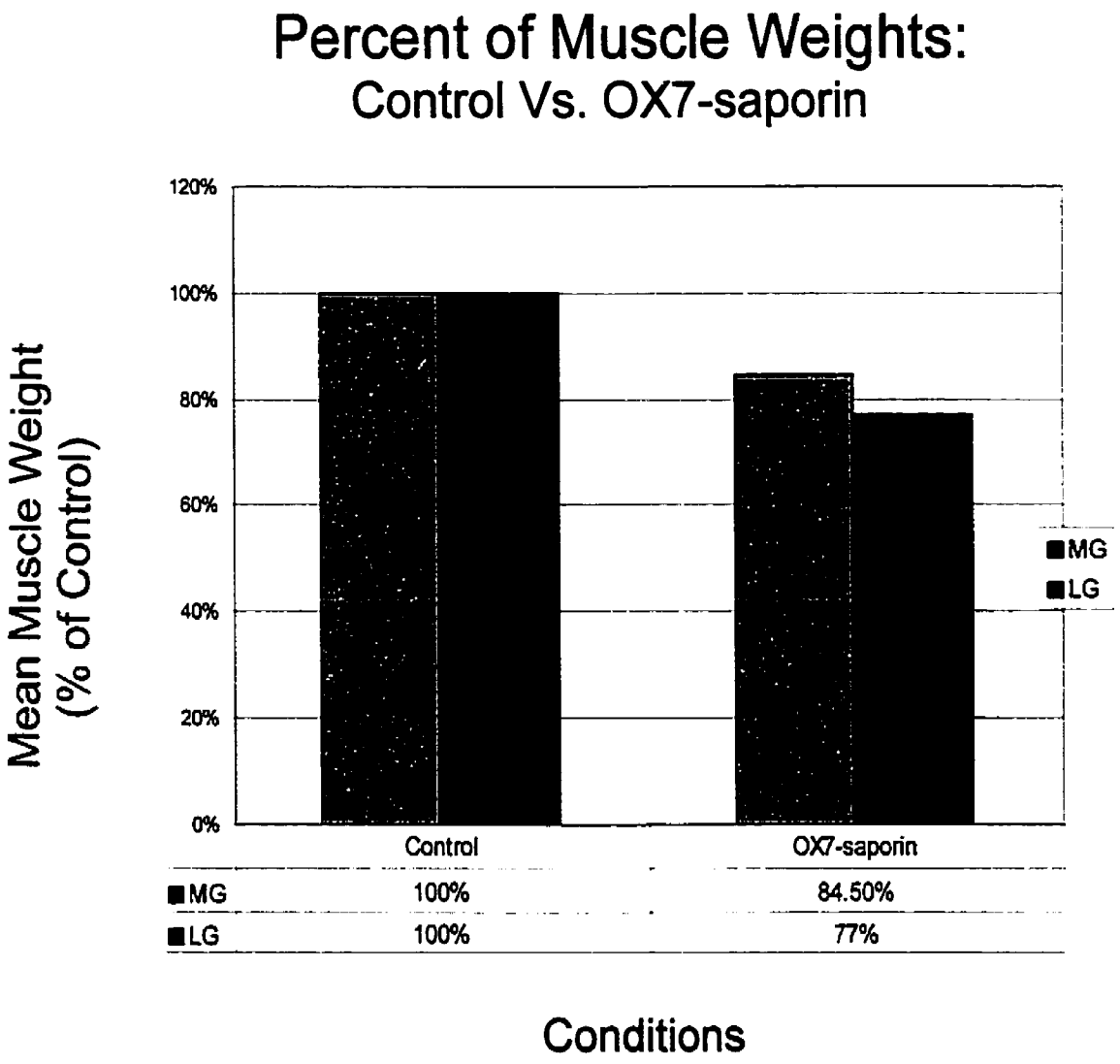
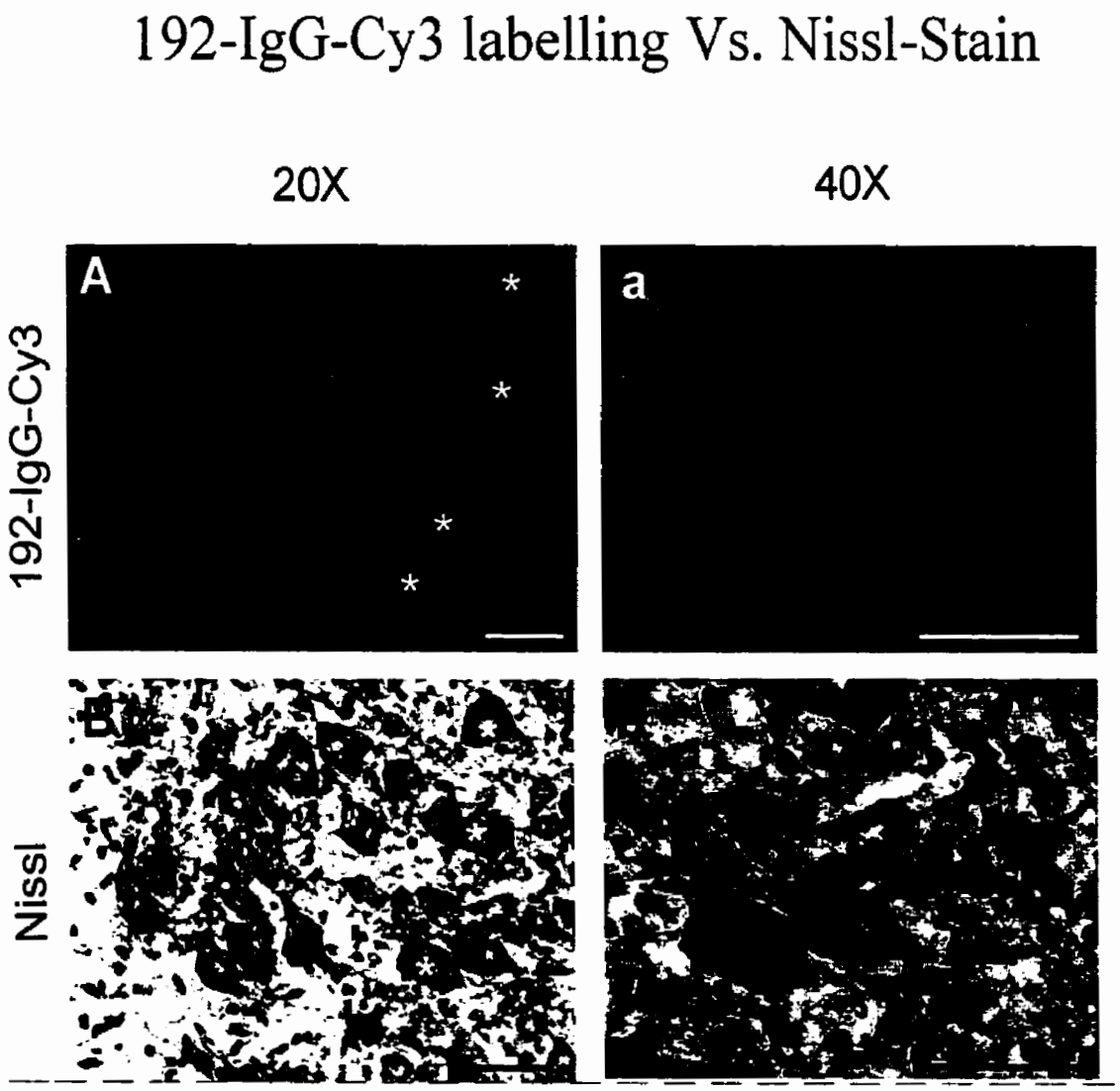

Percent of Muscle Weights:
Control Vs. OX7-saporin
Mean Muscle Weight
(% of Control)
120%
100%
80%
60%
40%
20%
0%
MG
LG
Control
OX7-saporin
MG 100% 84.50%
LG 100% 77%
Conditions

Figure 11. Low (20x) and high (40x) power photomicrographs of transverse sections from the lumbar spinal cord (lamina IX) of a neonatal rat depicting Cy3-labelled MNs [white (*) **A, a**]. Same sections as above were stained with cresyl-violet (**B, b**). Note that only a small number of MNs are labelled with Cy3 following an I. M. injection of 192-IgG-Cy3 when compared to the number of MNs observed with cresyl violet staining. Scale bars are 50 microns.

Figure 11


192-IgG-Cy3 labelling Vs. Nissl-Stain
20X
40X
192-IgG-Cy3
Nissl
A
a
B
b

DISCUSSION

There are four main findings in this study. i) MNs in the neonatal rat take up the monoclonal antibody 192-IgG (and conjugates Cy3 and saporin) and retrogradely transport them to their soma within the spinal cord following intramuscular injection; the . ii) The I.M. injection of 192-IgG-saporin can be used to lesion MNs in the neonatal rat. iii) The I.M. injection of 192-IgG-saporin results in myo-necrosis and severe muscle wasting. iv) The intramuscular injection of the immunotoxin, OX7-saporin, can be used to lesion MNs in adult rats and does not produce severe muscle necrosis/atrophy.

i) Uptake & Retrograde transport

This study demonstrates for the first time that MNs in the neonatal rat can internalize the 192-IgG monoclonal antibody and conjugates of Cy3 and saporin. Moreover, the results suggest that the internalization was by receptor-mediated endocytosis (via p75 receptor). This supposition can be drawn by the finding that only neonatal rats had Cy3-labelled MNs following an intramuscular injection of 192-IgG-Cy3 (**Fig. 2**). Studies have shown that MNs in neonatal animals, but not adults, express the p75 receptor (Yan and Johnson, 1988). If the internalization of the 192-IgG-Cy3 were not p75 receptor-mediated, but by non-specific endocytosis, then one may expect MNs in all animal groups to be labelled with Cy3. However, our observations revealed this was not the case (**Fig. 2**). In fact, even when a larger dose of 192-IgG-Cy3 with a survival time of 6 days was given, adult rat MNs were not labelled with Cy3. The fact that adult mice did not have Cy3-labelled MNs is not surprising on two accounts; first, adult murine MNs lack the p75 receptor (Enofors et al., 1989; Paqueron et al., 2001) and second, 192-IgG is known to recognize a rat-specific antigen

(Chandler et al., 1984). The fact that MNs of neonatal mice express p75, yet were not labelled with Cy3, is also consistent with the known species specificity (to rats) of the 192-IgG monoclonal antibody and further supports our findings.

Our studies also addressed the of whether both the 192-IgG monoclonal antibody and Cy3 fluorophore conjugate were retrogradely transported to the soma. Since the soma of MNs in the neonatal rat spinal cord were labelled with Cy3, the Cy3 component was transported, but was the 192-IgG? It is important to emphasize that no further processing was required to visualize the Cy3-labelled MNs following the I.M. injection of 192-IgG-Cy3 in neonatal rats. I coincidentally noticed that several months later the Cy3 had faded and the once-labelled MNs were no longer visible. Knowing which sections were previously labelled, I set about to re-detect the presence of 192-IgG-Cy3 in the MNs. This was accomplished by immunohistochemistry using donkey anti-mouse antibodies conjugated with Cy3. Since we had MNs from a rat spinal cord section, these cells should contain the mouse-anti-rat monoclonal antibody (192-IgG). Thus, a donkey anti-mouse secondary (conjugated with Cy3) should allow for the re-visualisation of the now faded 192-IgG-Cy3. In fact, we were able to re-detect the presence of mouse 192-IgG in the rat motoneurons. Furthermore, only those MNs previously labelled with 192-IgG-Cy3 were labelled with the secondary. The contralateral side served as control and did not contain any labelling (data not shown). This suggests that both components of the 192-IgG-Cy3 are retrogradely transported to the soma following an I.M. injection.

Labelling of MNs with 192-IgG-Cy3 was not wide-spread and was restricted to focal MN pools, suggesting that only MNs innervating the injected muscles were labelled (**Fig. 2**). MNs of other pools (innervating non-injected muscles) were not labelled, suggesting that Cy3 labelling was focal and transneuronal spreading did not occur.

A key technical issue with this study was our ability to identify MNs over other neural types. This was previously addressed in the "methods" section (refer to "motoneuron counts"). In this study, cells were considered to be MNs based on their large ($\geq$25 μm) soma, general morphology, their position within the ventral horn (e.g. lamina IX), and whether or not they are retrogradely labelled following an I.M. injection. However, there is a further division that should also be considered; whether MNs are of a gamma MNs (γ-MNs) or alpha MNs (α-MNs) subtype. Gamma MNs are relatively smaller (15-25 μm) (Swett et al. 1986) and innervate intrafusal muscle fibers. In contrast, extrafusal muscle fibers are innervated by the larger (30-50 μm) α-MNs (Swett et al. 1986). Although the focus of this study was directed toward large α-MNs, this study does not attempt to distinguish between the two, other than that based on size. Thus, there is no reason to believe that γ-MNs are unaffected from these treatments but they were not the intended focus of this study.

In summary, the results obtained strongly support the hypothesis that MNs in the neonatal rat internalize 192-IgG-Cy3, likely by receptor-mediated endocytosis. Furthermore, following retrograde transport, both 192-IgG and Cy3 are present in the soma of MNs innervating the injected muscles.

ii) 192-IgG-saporin and MN lesioning

This study also demonstrates that the I.M. injection of 192-IgG-saporin can result in significant MN loss in the neonatal rat (**Fig. 5**). The neonatal rat was used due to the species-specificity of the 192-IgG monoclonal antibody and developmental constraints of the animal studied (i.e. MNs in the neonatal but not the adult rat, express the p75 receptor).

These findings are consistent with the known ability of 192-IgG-saporin to lesion $p75^{+}$-cholinergic neurons of the basal forebrain following i.c.v. injection (Wiley et al., 1991; Book

et al, 1992, 1995; and Seeger et al., 1997). However, the I.M. administration of this immunotoxin with subsequent MN loss has not been previously demonstrated. Nissl-stained sections from the lumbar spinal cord (L_4-L_5) of a neonatal rat indicated a loss of 67% of MNs compared to the control side three weeks following injections with 0.5 μg of 192-IgG-saporin into the hind limb muscles (MG, LG, TA) (**Fig. 6**). Specifically, there were 381 MNs on the control side and 129 found on the immunotoxin-treated side. Importantly, developmental MN death is completed by birth in both mice and rats, and therefore, can be eliminated as a possible source of MN loss (Lance-Jones, 1982; Schmalbruch, 1984; Oppenheim, 1986).

The accuracy of cell counts is usually contested. In this study, an absolute count of motoneurons in the rat lumbar region was not deemed necessary; instead, the goal was to accurately compare the two sides. To accomplish this, alternate 25 μm serial (cryostat) sections were placed on three slides. This allowed for 50 μm between the sections on any one slide. Thus, in order for a MN to be counted more than once on any one slide, a MN would not only have to exceed 50 μm, but also satisfy the "at least 25 μm in size" criteria. This was thought to be a very rare occurrence. Using these criteria, an undercount may be more likely as Swett et al (1986) found that α-MNs of the MG and LG motoneuron pool ranged from 32 μm to 45 μm in size. However, the criteria used in this study are comparable to those used by Oppenhiem et al (1989) to count embryonic chick MNs. Again, it should be emphasised that over and under counts were not critical, since both sides would experience similar counting errors, and the relative number of cells are the focus of this report.

This study makes one assumption important to determining the accuracy of the cell counts. Specifically, that the number of MNs on one side of the spinal cord is similar to that

of the other. Quadrupeds, including mice and rats, have a medial body symmetry in which the two sides are similar. However, there will be some variability in the number of MNs on one side versus the other, but how much variability? Swett et al. (1986) performed an extensive study on the number and location of MNs of the rat sciatic nerve using HRP labelling. From 6 rats, they obtained a combined MN count for the MG and LG pools of 323 ± 13. When labelled individually, the number of MG and LG motoneurons was 145 ± 11 (n=5) and 187 ± 20 (n=3) respectively. Thus, even between animals the number of MNs appears to be well conserved. Moreover, Leanza and Stanzani (1998), as part of their study, compared left and right MG motoneurons within neonatal rats following an I.M. injection with fluoro-gold (FG) and reported a count of 112 ± 7 and 107 ± 2 respectively (n=6). Thus, the assumption that there are similar numbers of MNs on the left and right sides does not seem unreasonable. The 252 cell deficit obtained in this study with 192-IgG-saporin is, therefore, well beyond the innate variation existing between the two sides (**Fig. 6**).

iii) **192-IgG-saporin and muscle necrosis**

There was a 22% ($p= 0.008$) and 16.4% ($p= 0.12$) difference in the weight of the MG and LG muscles, respectively, just three days following injection with 192-IgG-saporin in neonatal rats (**Fig. 8B**, n= 4). In contrast, following nerve transection, there was only a 0.9% ($p= 0.49$) and 11.9% ($p= 0.21$) difference in the weight of the MG and LG muscles of neonatal rats respectively (**Fig. 8B**, n= 4). The LG muscles treated with 192-IgG-saporin was not statistically significant when compared to the control injection ($p= 0.12$, n=4). However, the decrease in weight for the LG muscles treated with 192-IgG-saporin may be biologically significant, as it has a lower mean value than the saporin-injection, sciatic nerve transection, and sham-operated controls. Of more importance, we have already observed that two weeks post-injection with 192-IgG-saporin there is little muscle to sample (**Fig. 3**). One

should recall that these results were obtained just three days post-treatment and that a three-day survival time was chosen to ensure that denervation of the muscles from MN lesioning with 192-IgG-saporin did not contribute to the observed muscle wasting. This was confirmed by the lesioning results. Thus, any observed weight loss in this group would lend support to the theory that 192-IgG-saporin plays a more direct role in the muscle atrophy observed.

In one study, Nogradi and Vrbova (1992) injected the cytotoxin Volkensin into the sciatic nerve of new-born rat pups, resulting in 80-100% MN loss. Moreover, they found that four weeks post-treatment, the extensor digitum longus (EDL) and tibialis anterior (TA) muscles lost 55.9 $\pm$ 1.5 % and 62.4 $\pm$ 2.9 % of their mean weight, respectively (Norgradi and Vrbova, 1992). This suggests that even four weeks following dervervation in adult animals, a little less than half of the muscle is still present. This is in contrast to our results obtained from the I.M. injection with 192-IgG-saporin in neonatal rats, in which practically all of the muscle tissue had atrophied in just 2-3 weeks. Interestingly, Schmalbruch (1984) found that denervation in neonatal rats at birth resulted in atrophic muscles, which were eventually replaced with fat cells. Although these results may have similarities to the 3-week survival animal treated with 192-IgG-saporin, it is not consistent with the acute muscle atrophy seen in the 3-day survival experiments.

Thus, the results obtained in this study suggest that 192-IgG-saporin has a more direct effect on muscle causing atrophy in addition to inducing denervation. Histology on the muscles illustrate this point and shows that treatment with 192-IgG-saporin resulted in necrosis of the muscle (**Fig. 9 b**). Thus, it is likely that muscle cells also internalize 192-IgG-saporin at this age, resulting in myo-necrosis. This is supported by the fact that muscle is also $p75^{+}$ at the time the immunotoxin is injected (Yan and Johnson, 1988). Blood vessels

also express p75 at this developmental time, and it may be that a combination of denervation, direct myo-necrosis, and reduced blood supply all contribute to the severe muscle atrophy observed (Yan and Johnson, 1988).

The finding that 192-IgG-saporin results in myo-necrosis requires a re-examination of the observed MN depletion. Specifically, it may be that the loss of the peripheral target (i.e. muscle) in the neonatal rat significantly contributed to the MN loss. Studies have shown that MN survival in the neonatal animal are highly dependent on contact with their peripheral target (Schmalbruch, 1984; Snider et al., 1992). That is, removal of the peripheral target in embryonic or neonatal animal results in MN degeneration/death (Oppenheim, et al., 1978; Crews and Wigston, 1990). In the adult, however, MNs are less dependent on targets in the periphery and can survive even following axotomy (Koliatsos et al., 1991a, 1991b, 1994). Thus, a possible explanation may be that the I.M. injection of 192-IgG-saporin results in MN death by directly preventing protein synthesis within the MNs which had taken it up, as well as by toxin-induced disruption of the peripheral target (muscle).

To estimate the contribution each of these processes may play in the observed MN loss, the number of Cy3-labelled MNs following I.M. injection with 192-IgG-Cy3 was compared those obtained when the same sections were Nissl stained (**Fig. 11**). An average of 28% of the MNs seen with Nissl stain were labelled with Cy3 (n=3). This suggests that less than half of the MNs are exposed to compounds like 192-IgG-Cy3 (or 192-IgG-saporin) following a single I.M. injection. However, this estimate does not indicate the percentage of Nissl-stained cells that would be expected to innervate the injected muscles. One study which used HRP labelling and acetylcholinesterase activity to estimate the number of motoneurons innervating the hind limb muscles, found the MG, LG, and TA muscles to be

innervated by 438 MNs (Nicolopoulos-Stournaras and Iles, 1983). Thus, it may be estimated that the I.M. injection of 192-IgG-saporin directly contributes to approximately 30% of MN loss and the remaining 40% of the loss is derived from destruction of the target muscle (assuming that all MNs exposed to the toxin died). Therefore, while the i.c.v. injection of 192-IgG-saporin produces almost complete loss of $p75^{+}$-cholinergic neurons of the basal forebrain, there is limited exposure of the immunotoxin to $p75^{+}$-cholinergic MNs following an I.M. injection.

iv) OX7-saporin

Studies have already shown that OX7-saporin can lesion MNs in the adult rat following injection into the vagus nerve (Wiley et al., 1989). Our results extend these findings to include the I.M. application of the immunotoxin in adult rats. As mentioned above, the I.M. route of administration limits the number of MNs exposed to the toxin and may explain the relatively small (12.4%) MN loss obtained (**Fig. 7A**, $p= 0.02$). Interestingly, OX7-saporin did not result in severe muscle atrophy even after three weeks post-injection (Figs. 10A and 10B, $p= 0.21$ and 0.08, MG and LG respectively). This is consistent with the fact that there is a lack of the Thy-1 antigen on muscle cells to which OX7 binds (Morris, 1983). In addition, these counts were done on Nissl-stained sections and would include MNs not exposed to the toxin. This has the effect of under-estimating the percent of MNs killed from particular pools. Thus, the 12.4% MN loss seen with OX7-saporin would in fact be higher if only MNs of the MG and LG pools were counted. The percent of MN loss would also appear higher if the number of MNs exposed to the immunotoxin could be determined. While the I.M. injection of a retrograde tracer, like FG, may help limit cell counts to the MN pools of interest, it does not label the entire MN pool or indicate which MNs were exposed to the toxin. The animal model produced in this study

has characteristics that are lacking in other animal models produced to date. Specifically, this model has a focal MN deficit, which does not negatively effect the survival of the animal. Moreover, the peripheral nerves and ventral root remain intact, as they are anatomically isolated from the toxin when it is injected intramuscularly. In addition, the temporal and spatial course of MN loss is known therefore, the optimal time and location of the transplanted cells can be determined. However, the I. M. administration of 192-IgG-saporin in neonatal rats also results in severe muscle wasting which is an undesirable feature in an animal model for stem cell transplantation (as the peripheral target of newly formed MNs has been destroyed).

Future avenues to explore may be to conjugate a fluorophore, like Cy3, with the OX7-saporin and may allow for the precise localization of the immunotoxin and the cells that have taken it up. In addition, this may yield a precise account of the number of MNs killed by the immunotoxin. Other methods of counting fluorescent-labelled motoneurons, such as flow cytometry, may help establish a more precise account of the number of motoneurons exposed to toxins/fluorophores. In addition, multiple injections into any one muscle may prove useful in distributing the toxin throughout a muscle, thereby affecting more MNs and yielding a higher deficit. In addition, experiments involving the specific inhibition of the p75 receptor may unequivocally show that the mechanism of uptake of 192-IgG-Cy3/saporin is indeed receptor-mediated. Combined with multiple injections into a muscle, this method of MN lesioning may produce a valuable animal model for studying cell replacement strategies This would produce an animal model with characteristics important to studying the possible therapeutic value of cell replacement strategies (such as that which may be obtained with the use of stem cells) and lead to a more promising treatment for motoneuron diseases, like ALS, in the clinical setting.

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