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TARGETING THE MOTOR END PLATES IN THE MOUSE HINDLIMB GIVES ACCESS TO A GREATER NUMBER OF SPINAL CORD MOTOR NEURONS: AN APPROACH TO MAXIMIZE RETROGRADE TRANSPORT

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Abstract—Lower motor neuron dysfunction is one of the most debilitating neurological conditions and, as such, significantly impacts on the quality of life of affected individuals. Within the last decade, the engineering of mouse models of lower motor neuron diseases has facilitated the development of new therapeutic scenarios aimed at delaying or reversing the progression of these conditions. In this context, motor end plates (MEPs) are highly specialized regions on the skeletal musculature that offer minimally invasive access to the pre-synaptic nerve terminals, henceforth to the spinal cord motor neurons. Transgenic technologies can take advantage of the relationship between the MEP regions on the skeletal muscles and the corresponding motor neurons to shuttle therapeutic genes into specific compartments within the ventral horn of the spinal cord. The first aim of this neuroanatomical investigation was to map the details of the organization of the MEP zones for the main muscles of the mouse hindlimb. The hindlimb was selected for the present work, as it is currently a common target to challenge the efficacy of therapies aimed at alleviating neuromuscular dysfunction. This MEP map was then used to guide series of intramuscular injections of Fluoro-Gold (FG) along the muscles' MEP zones, therefore revealing the distribution of the motor neurons that supply them. Targeting the entire MEP regions with FG increased the somatic availability of the retrograde tracer and, consequently, gave rise to FG-positive motor neurons that are organized into rostro-caudal columns spanning more spinal cord segments than previously reported. The results of this investigation will have positive implications for future studies involving the somatic delivery and retrograde transport of therapeutic transgenes into affected motor neurons. These data will also provide a framework for transgenic technologies aiming at maintaining the integrity of the neuromuscular junction for the treatment of lower motor neuron dysfunctions.

Key words: Motor neurons, Motor end plates, Retrograde transport, Muscles, Fluoro-Gold, Mouse hindlimb.

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Abbreviations: AChE, acetylcholinesterase; ALS, amyotrophic lateral sclerosis; FG, Fluoro-Gold; GDNF, Glial-derived Neurotrophic Factor; MEPs, motor end plates.

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INTRODUCTION

Several neurological diseases can affect the control of movement and thus significantly impact on the quality of life of affected individuals. Such debilitating conditions include amyotrophic lateral sclerosis (ALS), spinal muscular atrophy and Duchenne muscular dystrophy. Throughout the recent years, the control of movement has received considerable attention from clinicians as well as scientists working on several models of these diseases in pre-clinical settings. In this regard, the last decade has seen the development of a large number of transgenic mouse models of neuromuscular dysfunctions (Hsieh-Li et al., 2000; Kaspar et al., 2003; Ishiyama et al., 2004; Turner et al., 2009; Wegorzewska et al., 2009; Kimura et al., 2010; Pratt et al., 2013). Such transgenic animal models are widely used to investigate the mechanisms underlying the development and progression of diseases affecting the control of movement and to develop therapeutic strategies aimed at delaying or reversing their progression. These strategies often involve the intramuscular targeting (and subsequent gene transfer to the corresponding spinal cord motor neuron) of viral vectors coding for therapeutic compounds such as neurotrophic factors (Acsadi et al., 2002; Kaspar et al., 2003; Azzouz et al., 2004; Nakajima et al. 2008; Wu et al. 2009; Benkhelifa-Ziyyat et al., 2013; for reviews see Gould and Oppenheim, 2011; Federici and Boulis, 2012). For instance, Acsadi and co-workers (2002) have demonstrated that intramuscular delivery of adenoviral vectors carrying the gene sequence for Glial-derived Neurotrophic Factor (GDNF) delays the onset of the disease symptoms in a transgenic mouse model of ALS. On the other hand, evidence obtained over the last years have revealed that degeneration of skeletal muscles and their neuromuscular junctions significantly contributes to the development and progression of ALS (for reviews see Kanning et al., 2010; Krakora et al., 2012). In mouse models of ALS, intramuscular delivery of GDNF to the muscle has proved to strengthen the neuromuscular junction (Suzuki et al., 2008) and delay the onset of the disease phenotype (Li et al., 2007).

Intramuscular delivery of therapeutic molecules therefore offers a promising approach for the treatment of neuromuscular disease as it has the potential to target both the muscles and the spinal cord motor neurons that supply them. However, the outcomes of pre-clinical trials involving muscle targeting for neuromuscular conditions have proved to

be sub-optimal in rescuing the disease phenotype arguably due, at least in part, to the limited protective capacity of these therapeutic compounds. A factor that could also significantly impede on the success of such therapies could be sub-optimal intramuscular delivery methods. Motor end plates (MEPs) are highly specialized regions of muscles that offer direct access to the pre-synaptic peripheral nerve terminals and, consequently, to the spinal cord motor neurons. It has been demonstrated that the efficacy of substance uptake is dependent upon the exact location of the injections, within the muscle, with regard to the motor end plate (MEP) region (Shaari and Sanders, 1993; Chin et al., 2005; Gracies et al. 2009; Van Campenhout and Molenaers, 2010). We have recently detailed the organization of the MEP zone for several forelimb muscles in the rat and mouse (Tosolini and Morris, 2012; Tosolini et al., 2013). In the diverse mouse models of neuromuscular dysfunction mentioned above, the hindlimb is often the target of investigation (Flood et al., 1999; Turner et al., 2009; Wegorzewska et al., 2009; Kimura et al., 2010; Henriques et al., 2011; Ngo et al., 2012; Pratt et al., 2013). The aim of the present investigation was therefore to characterize the muscle–motor neuron organization in the mouse hindlimb.

EXPERIMENTAL PROCEDURES

Subjects and housing

All experimental procedures complied with the Animal Care and Ethics Committee of the University of New South Wales and were performed in accordance with the National Health and Medical Research Council of Australia regulations for animal experimentation. The Animal Care and Ethics Committee of the University of New South Wales has approved the conduct of this study. A total of 34 naïve adult male C57BL/6 mice (Animal Resource Centre, WA, Australia) weighing between 20 and 30 g at the time of surgery were used in this study. The animals were housed in groups of 5 in an animal-holding room under a 12-h light–dark cycle. Food and water were provided *ad libitum* throughout the course of the experiment.

Acetylcholinesterase histochemistry

To minimize the use of animals, perfused mouse bodies ($n = 4$) were obtained through tissue sharing. Acetylcholinesterase histochemistry (AChE) was performed on these mouse carcasses as per Tosolini et al. (2013). The skin was removed from the carcasses and the entire bodies were immersed for 4 h at 4 °C in a solution containing 200 ml of phosphate buffer (PB), 290 mg of acetylthiocholine iodide, 600 mg of glycine, and 420 mg of copper sulfate (all reagents from Sigma–Aldrich, Castlehill, NSW, Australia). The carcasses were subsequently washed for 2 min in distilled water and developed by rapid immersion (i.e., 5–10 s) in a 10% ammonium sulfide solution.

Surgical procedures

Anesthesia was induced with isoflurane (Provet, Sydney, NSW, Australia; 1–2% in O_2). The fur covering the areas of interest was shaved and cleaned with 70% ethanol. For each muscle under investigation, a small incision was made in the skin to expose the muscle. Series of Fluoro-Gold (FG, 5% in distilled water; Fluorochrome, Denver, CO, USA) injections were manually performed through graded glass micropipettes (DKSH, Zurich, Switzerland) along the entire motor end plate (MEP) zone. The volume of FG varied between 2 and 4 µl depending on the size of the muscle. For example, Gluteus Maximus received four 1-µl injections of FG along its entire motor end plate region. Great care was taken to preserve the fasciae covering the targeted muscles as well as those surrounding it. Special care was also taken to ensure that the blood vessels surrounding the muscles were left intact. After the injections, the muscles were wiped with gauze to remove any tracer that may have inadvertently seeped from the injected muscle. A total of 43 series of intramuscular injections were performed into the following muscles: Gracilis ($n = 6$), Biceps Femoris ($n = 6$), Gluteus Maximus ($n = 4$), Vastus Medialis ($n = 8$), Semitendinosus ($n = 5$), Vastus Lateralis ($n = 6$), Gastrocnemius ($n = 4$) and Tibialis Anterior (n = 4). Gluteus Maximus was also targeted with either a 4-µl bolus injection of FG into the thickest part of the muscle ($n = 6$) or with 2-µl injections restricted to the antero-lateral or postero-medial portion of its MEP region ($n = 6$). In additional animals, 4 µl of FG was applied directly onto the intact fasciae covering Gluteus Maximus ($n = 4$). Moreover Gracilis, on which two distinct MEP zones are present, was targeted with 4 µl of FG or Fluorescein (Life Technologies Australia Pty., Mulgrave, Vic, Australia 3000 MW) either along the MEPs near the proximal ($n = 2$) or distal attachment ($n = 2$) of the muscle or along both MEP regions together ($n = 3$). One additional animal was also administered with a series of Fluoro-Gold injections along the entire MEP region of Gluteus Maximus to demonstrate the localization of the injections on a representative muscle. In all cases, the skin was subsequently closed with surgical clips (Texas Scientific Instruments LLC, Boerne, TX, USA) and the mice were monitored post-operatively until they recovered from the anesthesia.

Dissection and histology

After the intramuscular injections of FG, the mice were kept for 7 days to allow for optimal retrograde transport of the neuronal tracer. After this period of time, the mice received a lethal dose of Lethabarb (150 mg/kg; Virbac, Sydney, New South Wales, Australia) and were perfused intra-cardially with 0.1 M PB followed by 4% paraformaldehyde in 0.1 M PB. A midline incision was made along the gut of the perfused animals to remove the viscera and the muscles of the posterior abdominal wall and to identify the caudal-most rib, T13 and its adjoining vertebra (see Fig. 1A). The vertebra rostral to