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Imaging neuromuscular junction pathology in mouse models of SMA

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1. OBJECTIVE

The objective of this SOP is to provide guidelines for performing morphological investigations of neuromuscular junctions (NMJs) in mouse models of SMA. We describe the procedures for immunohistochemical labeling of NMJs in nerve/muscle preparations that can be applied to mice of any age or genotype.

2. SCOPE AND APPLICABILITY

NMJs are early and important pathological targets in SMA (for review see Murray et al., 2010). Several studies, utilising a range of different mouse models of SMA, have identified key morphological correlates of NMJ breakdown, including loss of pre-synaptic motor nerve terminals and abnormal accumulations of cytoskeletal proteins (Cifuentes-Diaz et al., 2002; Murray et al., 2008; Kong et al., 2009; Simon et al., 2010; Michaud et al., 2010). The morphological techniques described in this SOP are presented with the aim of providing a resource for researchers who wish to examine NMJ pathology in mouse models of SMA, likely to be of particular relevance when assessing new therapeutic approaches (e.g. gene therapy or pharmacological agents). Whilst specific muscles and muscle groups are mentioned, it is intended that this SOP could be applied to any muscle or muscle group from SMA mouse models.

3. CAUTIONS

The most common problems with NMJ immunohistochemistry arise from issues surrounding dissection and fixation of nerve/muscle preparations as well as penetration of antibodies targeted to axons (e.g. against neurofilament proteins). In particular, many of the antibodies used to label NMJs (e.g. primary antibodies against neurofilaments) are sensitive to levels of fixation (resulting in poor penetration and a “blebby” appearance) and don’t work well if the muscle fibres have been damaged during the dissection process. This can result in a misleading morphological assessment of pre-synaptic axons and motor nerve terminals which can easily be mistaken for the presence of neurodegeneration. Likewise, it can also be important to label synaptic vesicles, in addition to neurofilaments, to reveal the entire nerve terminal morphology and avoid potential mis-interpretation of innervation patterns by labeling neurofilaments alone. An alternative to immunostaining of nerve fibres in muscle is the use of mouse lines expressing GFP as a transgene in motor neurons, but these mouse lines need to be crossed with Smn deficient mouse lines. Moreover, not all innervating motor nerve fibres are GFP positive in some of these mouse lines and

fluorescent proteins have been shown to modify neuronal form and function at the molecular and cellular level (Comley et al., 2011)

Where possible, thin muscles that can be stained and analysed in whole-mount preparations should be used, to avoid the requirement for sectioning and improve antibody penetration. It is also important to compare disease mice with litter-mate controls wherever possible. It is always beneficial to use confocal microscopy, rather than standard epifluorescence microscopy, to assess and quantify NMJ staining wherever possible.

The following protocol describes the use of 4% paraformaldehyde as a fixative. If this is proving problematic, other fixation options are available and can work for NMJ immuno, including ice-cold, absolute methanol. Where there are problems with antibody penetration, additional modifications can be incorporated (e.g. increase amount of detergent used [Triton-X] and/or time in detergent, or add a freeze-thaw step after fixation etc).

4. MATERIALS

The following specific equipment will be required (assuming that standard lab equipment such as measuring cylinders and pipettes are already available):

- Microdissection tools (scissors and forceps; can be purchased from Fine Science Tools [FST])
- Sylgard (available from Dow Corning) lined-petri dish with insect pins (or equivalent for pinning out tissue for dissection)
- Dissecting microscope with good quality illumination
- Multi-well plates
- Glass slides and coverslips
- Fluorescence microscope (preferably confocal) for analysis of immuno-labeled preparations
- Rocking platform

The following supplies will be required:

- Oxygenated mammalian physiological saline (mM: NaCl, 120; KCl, 5; CaCl2, 2; MgCl2, 1; NaH2PO4, 0.4; NaHCO3, 23.8; D-glucose, 5.6) bubbled to equilibrium with a 5% CO2/95% O2 mixture. If not available, it is possible to dissect in 1xPBS, although this can compromise the quality of the immunostaining

- 4% paraformaldehyde in 1xPBS
- α-bungarotoxin (conjugated to a fluorescent probe such as tetramethylrhodamine isothiocyanate [TRITC-α-bungarotoxin; 5µg ml-1, available from Molecular Probes]) for labeling post-synaptic acetylcholine receptors
- Bovine serum albumin (available from Sigma)
- Triton-X (available from Sigma)
- Primary antibodies for labeling axons and motor nerve terminals (e.g. 165kDa neurofilament (2H3) and synaptic vesicle protein SV2 antibodies from the Developmental Studies Hybridoma Bank, Iowa; **NOTE**: 150kDa neurofilament antibodies from Chemicon International previously recommended for this purpose no longer seem to work in several laboratories). Since SV2 antibody has to be prepared from hybridoma cell culture or mouse ascites, the quality control of each batch can be variable. Alternatively, a commercially available antibody against synaptophysin (a rabbit polyclonal antibody, Invitrogen) is recommended.
- Secondary antibodies appropriate for the primary antibodies used (e.g. FITC-conjugated swine anti-rabbit secondary antibody from Dako or DyLight 488 Donkey anti-Mouse secondary antibody from Jackson)
- Anti-fade mounting medium (e.g. Mowoil® from Calbiochem)

5. METHODS

Neonatal SMA mice and control littermates should be killed using the appropriate approved methodology dictated by the investigator's location (in the UK using chilling on ice and decapitation). Older mice should also be killed using the appropriate approved methodology for mice of a given age, dictated by the investigator's location. The muscle group to be dissected should then rapidly removed from the mouse in a gross fashion (e.g. skinned whole hind-limb if using gastrocnemius or tibialis anterior; cervical muscle group removed from the back of the head with the pinna attached if using levator auris longus; rib cage with anterior abdominal wall musculature attached if using transversus abdominis), pinned out in a sylgard lined-petri dish and immersed in oxygenated mammalian physiological saline before further microdissection is performed. The muscle or muscle group to be examined is then isolated using a dissection microscope, making sure to keep the whole muscle intact and not damage any muscle fibres. A preliminary attempt should be made to remove excess fat and connective tissue at this stage.

Muscles should then exposed to α-bungarotoxin (BTX; conjugated to a fluorescent probe such as TRITC; 5mg/ml in oxygenated mammalian physiological saline) for 10 minutes on a rocking platform before a quick wash in fresh oxygenated mammalian physiological saline. Muscles should then be fixed in 1xPBS containing 4% paraformaldehyde for 15 minutes at room temperature on a rocking platform. Muscle preparations should then be washed thoroughly in 1xPBS before a further stage of cleaning up under the dissection microscope (removing all excess fat and connective tissue). It is possible to be a bit more aggressive in cleaning the muscles once they are fixed. However, care should still be taken not to damage the muscle or tear incoming nerves. At this stage, clean micro-dissected muscles can be transferred to a multi-well plate, reducing the volume of liquid and antibodies required for subsequent immuno-labeling stages of the protocol.

Muscles should be blocked in 4% bovine serum albumin (BSA) and 1% TritonX in 1xPBS for 30min on a rocking platform at room temperature before overnight incubation in primary antibodies diluted in BSA/Triton-X containing blocking solution on a rocking platform in a cold room. After several rapid washes in 1x PBS, muscles should then be incubated in secondary antibodies made up in 1xPBS for 4h on a rocking platform at room temperature. Muscles should then be vigorously washed in 1xPBS before being whole-mounted in Mowoil® on glass slides and cover-slipped for subsequent imaging. Mowoil offers the advantage of being an anti-fade medium that also seals the cover-slip onto the slide, negating the requirement for other sealants (e.g. nail varnish). Thin muscles (e.g. levator auris longus or transversus abdominis) can be whole-mounted, but larger muscles (e.g. gastrocnemius) may require sectioning or further dissection to allow good quality mounting. Slides can be stored in the fridge or freezer until ready to be imaged. Experience suggests that the optimum time to image slides is within the first 2 weeks following preparation.

Immunostained nerve/muscle preparations can be imaged on a standard epi-fluorescence microscope or a confocal microscope. The latter is to be preferred as z-stack project images can be obtained that provide better high-resolution images of NMJs, allowing more accurate quantification. A minimum of 30 NMJs should be quantified per muscle (it is ideal to aim to quantify 100-200 NMJs per muscle). Non-SMA mouse controls should be examined to check for the quality of staining before commencing quantification of SMA tissue. One standard approach to quantify NMJ pathology is to classify each NMJ as either fully-occupied, partially-occupied or denervated, and then also make a note of the presence/absence of abnormal morphology (relative to non-SMA littermate control tissue) including, abnormal neurofilament accumulations, axonal and/or nerve terminal swelling or shrinkage, and disruption of axonal/or nerve terminal continuity. It is also possible to quantify morphological features such as motor endplate size and maturation from these preparations.

6. EVALUATION AND INTERPRETATION OF RESULTS

It is recommended that all quantification of NMJs is performed with the investigator blind to the genotype and/or treatment status of the tissue, and on randomized slides. It is possible to perform quantitative analysis away from the microscope if required, by taking micrographs of randomly selected regions of each muscle and then quantifying from the micrographs at a later date. This approach is often to be preferred if total randomization of images is required for unbiased analysis. Data from these analyses can be subject to standard statistical tests (e.g. t-test or Mann Whitney test).

Details regarding the time-course of NMJ pathology in SMA mice and muscles/muscle groups where pathology is particularly prevalent can be found in the references below (e.g. Murray et al., 2008; Ling et al., 2012). Of particular note, the levator auris longus muscle (see Murray et al., 2008) allows the investigator to examine two neighbouring muscle bands in the same mouse, one with severe NMJ pathology and the other with very little neuromuscular denervation. It is important that the investigator is aware of any selective NMJ vulnerability occurring with different regions of the same muscle or a group of muscles. If selectively vulnerable regions are present, then it is very important that any quantitative NMJ analysis takes this into account and samples from the same region from one mouse to the next, or equally across multiple regions if required.

It is also possible to validate and extent immunohistochemical observations of NMJs using electron microscopy. As this is a complex technique, we would suggest the reader contacts either Tilman Voigt or Tom Gillingwater from the Working Group for further information.

Examples of NMJs (showing affected and non-affected morphology) labeled with this protocol, or a similar protocol, can be found in the references below.

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