A method to identify, dissect and stain equine neuromuscular junctions for morphological analysis

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Abstract

Morphological study of the neuromuscular junction (NMJ), a specialised peripheral synapse formed between a lower motor neuron and skeletal muscle fibre, has significantly contributed to the understanding of synaptic biology and neuromuscular disease pathogenesis. Rodent NMJs are readily accessible, and research into conditions such as amyotrophic lateral sclerosis (ALS), Charcot–Marie–Tooth disease (CMT), and spinal muscular atrophy (SMA) has relied heavily on experimental work in these small mammals. However, given that nerve length dependency is an important feature of many peripheral neuropathies, these rodent models have clear shortcomings; large animal models might be preferable, but their size presents novel anatomical challenges. Overcoming these constraints to study the NMJ morphology of large mammalian distal limb muscles is of prime importance to increase cross-species translational neuromuscular research potential, particularly in the study of long motor units. In the past, NMJ phenotype analysis of large muscle bodies within the equine distal pelvic limb, such as the tibialis cranialis, or within muscles of high fibrous content, such as the soleus, has posed a distinct experimental hurdle. We optimised a technique for NMJ location and dissection from equine pelvic limb muscles. Using a quantification method validated in smaller species, we demonstrate their morphology and show that equine NMJs can be reliably dissected, stained and analysed. We reveal that the NMJs within the equine soleus have distinctly different morphologies when compared to the extensor digitorum longus and tibialis cranialis muscles. Overall, we demonstrate that equine distal pelvic limb muscles can be regionally dissected, with samples whole-mounted and their innervation patterns visualised. These methods will allow the localisation and analysis of neuromuscular junctions within the muscle bodies of large mammals to identify neuroanatomical and neuropathological features.

KEYWORDS
comparative anatomy, equine, large mammal, muscle fibre teasing, neuromuscular junction, NMJ-morph, synapse
1 | INTRODUCTION

The neuromuscular junction (NMJ) is a specialised, excitatory, chemical synapse formed between a lower motor neuron and a skeletal muscle fibre. The NMJ connects a pre-synaptic axonal terminal to a post-synaptic region rich in acetylcholine receptors (AChRs), allowing for neuronal activation of muscle contraction (Rana et al., 2017). As NMJs are relatively large and readily accessible, they have been a mainstay of vertebrate research (Tokuyama et al., 2018; Bermedo-García et al., 2018; Desaki & Uehara, 1981; Heuser & Reese, 1973) and invertebrate (Keshishian et al., 1996) neuromuscular research from the 1800s (Kuhne, 1887) until the present day; rodent studies, in particular, continue to reveal the cellular changes involved in synaptic formation (Roche et al., 2014), degeneration (Gillingwater & Ribchester, 2001) and repair (Lawrence et al., 2008).

In contrast to the relative abundance of small mammalian NMJ research, there is a paucity of corresponding large mammalian data, and in humans, this work is restricted by ethical and logistical issues (Boehm, Miller, et al., 2020; Jones et al., 2017). Common to NMJ quantification across species, large muscles that cannot be prepared as wholemounts for microscopic examination have generally required cryosectioning prior to immunolabelling of NMJs, thereby generating sectioning and refraction-related imaging artefacts, which increase analytical subjectivity and limit possible analyses. Small rodent models present an obvious advantage in this regard: their muscles are more readily dissected and whole-mounted, and their innervation patterns are more easily identified along with fewer sectioning-related artefacts (McArdle et al., 1981; Murray et al., 2010; Sleigh, Burgess, et al., 2014). This allows for more accurate quantification of NMJ development (Mech et al., 2020), degeneration (Sleigh, Grice, et al., 2014) and repair (Martineau et al., 2018). The caveats inherent in using the (much smaller) rodents to model human NMJ pathobiology (these issues are expanded on in Cahalan et al., 2022) create a need for large mammal NMJ analysis (Pereira et al., 2020; Sullivan et al., 2020).

Recent comparative mammalian pelvic limb NMJ data have revealed marked morphological heterogeneity and suggest that large animal neuromuscular models have translational benefits in human NMJ pathobiology (these issues are expanded on in Cahalan et al., 2022) create a need for large mammal NMJ analysis (Pereira et al., 2020; Sullivan et al., 2020).

To our knowledge, there are no peer-reviewed data describing the morphological structure of equine NMJs.

Here, we describe a technique for processing equine pelvic limb muscles for NMJ analysis, identifying gross NMJ distribution, dissecting and teasing muscle fibres, and using immunofluorescent staining and microscopy to illustrate the neuromuscular phenotype. This technique can easily be applied to other large muscles to perform detailed neuromuscular analysis in horses and other large mammals.

2 | MATERIALS AND METHODS

2.1 | Animals

5 Welsh Mountain ponies (3 female, 2 neutered males) were used for all experiments (Table 1). Animals were kept outside at grass pasture with ad lib access to water. Euthanasia was conducted (for reasons unrelated to this study) via intravenous barbiturate overdose. All muscles were dissected immediately post mortem. Muscle samples (Table 2) were obtained with ethical approval of the Royal Veterinary College’s Clinical Research Ethical Review Board.

| Subject | Age (years) | Mass (kg) | Sex | Height (cm) | Breed |
|---------|-------------|-----------|-----|-------------|-------|
| Pony 1  | 4.5         | 180       | Female | 110  | Welsh Mountain Section A |
| Pony 2  | 7.5         | 230       | Male N | 120  | Welsh Mountain Section A |
| Pony 3  | 5           | 400       | Female | 140  | Welsh Mountain Section D |
| Pony 4  | 8           | 350       | Male N | 125  | Welsh Mountain Section A |
| Pony 5  | 8           | 350       | Female | 125  | Welsh Mountain Section A |
2.2 | Reagents

The reagents and quantities/concentrations used are listed in Table 3.

2.3 | Equipment and software

The equipment used is listed in Table 4. ImageJ version 2.1.0/1.53c (macOS v12.3.1) (http://rsb.info.nih.gov/ij/) was used for projecting Z-stack images and combined with the aNMJ-morph workflow (Jones et al., 2016; Minty et al., 2020) to quantify NMJ morphological variables.

2.4 | Acetylcholinesterase (AChE) staining

AChE staining, adapted from (Cheetham et al., 2008; Couteaux, 1998; Karnovsky & Roots, 1964), was used to visualise the macroscopic endplate distribution in pelvic limb muscles (Figure 1) so that NMJ-rich areas could be targeted for subsequent sampling and microscopy. 3L of 0.1 M Tris-buffered saline solution was made with 16% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) was diluted in PBS to a 4% working solution and used on the day of preparation. Permeabilisation, blocking, and antibody solutions were all made on the day of preparation (see section 2.7). Mowiol mounting medium was made by adding 2.4 g Mowiol 4–88 and 6 g glycerol to 6 ml distilled water in a 50 ml conical flask, mixing slowly overnight at room temperature on a magnetic stirrer. The following day, prewarmed—to 50°C in a water bath—12 ml 0.2 M Trizma hydrochloride (pH 8.5) was added, and the medium was heated in a water bath at 50°C for 1 h with regular mixing every 10 min. The medium was left to settle for 30 min at room temperature, the supernatant decanted into a graduated cylinder, and volume noted. Finally, 1.4-diazobicyclo-[2.2.2]-octane (DABCO) at 2.5 g per 100ml was added to reduce fading. The medium was aliquoted into 2 ml Eppendorf tubes to avoid repeated freeze–thaw cycles and stored at −20°C for up to a year.

2.5 | Teasing and staining reagent setup

Sylgard 184 silicone elastomer was prepared by combining the elastomer base with the curing agent at a 10:1 ratio, pouring it into 90mm Petri dishes, and then allowing it to set for at least 48 h. The mixture was stored at 4°C, and then allowing it to set for at least 48 h. 16% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) was diluted in PBS to a 4% working solution and used on the day of preparation. Permeabilisation, blocking, and antibody solutions were all made on the day of preparation (see section 2.7). Mowiol mounting medium was made by adding 2.4 g Mowiol 4–88 and 6 g glycerol to 6 ml distilled water in a 50 ml conical flask, mixing slowly overnight at room temperature on a magnetic stirrer. The following day, prewarmed—to 50°C in a water bath—12 ml 0.2 M Trizma hydrochloride (pH 8.5) was added, and the medium was heated in a water bath at 50°C for 1 h with regular mixing every 10 min. The medium was left to settle for 30 min at room temperature, the supernatant decanted into a graduated cylinder, and volume noted. Finally, 1,4-diazobicyclo-[2.2.2]-octane (DABCO) at 2.5 g per 100ml was added to reduce fading. The medium was aliquoted into 2 ml Eppendorf tubes to avoid repeated freeze–thaw cycles and stored at −20°C for up to a year.

2.6 | NMJ immunolabelling—muscle dissection and fibre teasing

For whole-mount muscle preparation of NMJ immunostaining, whole muscles were freshly dissected within 30 min post-euthanasia and divided into sections of, at a maximum, 4cm³ sized pieces and stored in 10 times the muscle volume of 4% PFA at 4°C for a maximum of 12h. Samples were always chosen from each muscle’s same distal superficial location, highlighted in Figure 1. Fixed muscles were then washed three times in TBS for 20 min on a rotator at room temperature, prior to long-term storage in 0.1 M Sodium Azide in TBS at 4°C. This allowed for repeated muscle fibre teasing and staining for up to a year. Upon dissection, muscles were pinned in a Sylgard silicone elastomer-covered petri dish using insect pins and immersed in TBS. A dissection microscope was used for all subsequent steps. Individual muscle bundles were identified, connective tissue was detached from between the muscle fibres, and individual fibre bundles were stripped away and carefully teased apart to facilitate antibody

TABLE 2 List of muscles sampled, including abbreviation, origin, insertion, action, mean and reported mean percentage fast twitch muscle fibre data, with references

| Muscle          | Abbreviation | Origin                  | Insertion                        | Action                               | Mean % fast twitch (SD) | Reported mean % fast twitch (SD) | Reference               |
|-----------------|--------------|-------------------------|----------------------------------|--------------------------------------|-------------------------|----------------------------------|--------------------------|
| Tibialis cranialis | TC           | Tibial lateral condyle and crest | Cuneiforms and metatarsal III | Flexion of hock (Tarsus)            | 58.5% (±14.46)          | 64.6% (±15.6)                   | Valberg et al. (2015)       |
| Extensor digitorum longus | EDL          | Femoral extensor fossa | Distal phalangeal extensor process | Extension of digits and flexion of hock (Tarsus) | 75% (±9.69)               | 89.4% (±13.4)                  | Kawai et al. (2009)         |
| Soleus | SOL | Fibular head | Gastrocnemius aponeurosis | Minimal hock (Tarsus) extension, many muscle spindles - proprioceptive function? | <1% | 1%–14% (±15) | Meyers and Hermanson (2006) Kawai et al. (2009) |
penetration, see Figures 2 and 3. See supplemental video for added instruction https://figshare.com/s/40926670e1fc65e038c1. Unwanted material such as fibro-vascular connective tissue and fat were identified and removed to minimise tissue autofluorescence.

2.7 | NMJ immunolabelling and mounting

Unless otherwise specified, all permeabilisation, incubation, and washing steps were performed on a rocker at room temperature. Solutions were changed by filling alternate wells of a 12-well plate, then transferring muscle fibres across, rather than replacing the solutions with fibres in situ, to minimise fibre losses. Each well contained between 40–50 teased fibres typically submerged in a volume of 500 μl. Teased fibres were transferred to a 12-well plate and washed with 1 ml of 0.1 M glycine in TBS pH 10.4 for 1 h. A 10 min TBS wash was followed by a 30min incubation with Alexa Fluor 594 α-bungarotoxin (αBTX 1/1000 in TBS). Fibres were permeabilised with 4% Triton X-100 in TBS for 90min and then blocked in 4% BSA and 2% Triton X-100 in TBS solution for 30min; samples were then incubated for 72 h at 4°C in this same blocking solution containing primary antibodies—mouse neurofilament-associated (3A10, 1/50) and mouse pan anti-synaptic vesicle 2 (SV2, 1/50)—labelling axons and nerve terminals, respectively. Samples were then washed four times for 20 min each in TBS. Alexa Fluor 488 goat anti-mouse IgG secondary (1/400 in TBS) was incubated overnight at 4°C. Samples were washed four more times for 20 min each in TBS. Approximately 0.25 ml of Mowiol mounting medium was dropped onto a glass slide and a bundle of 5–10 fibres, depending on length and diameter were placed within the medium. Using forceps, fibres were spread out and straightened to avoid overlap and curling. Fibres were cover-slipped and placed flat at 4°C for 24 h. Slides were then stored at −20°C for up to 2 years, without detectable loss of fluorescence whilst avoiding repeated freeze–thaw cycles.

2.8 | NMJ immunostaining assessment, image acquisition and analysis

NMJs were imaged on a Leica SP8 confocal microscope using established protocols reported by Jones et al., 2016, 2017. In brief, the following settings were used: 8-bit depth, 512x512 frame size, x63 magnification, x2 zoom and 1 μm z-stack interval with sequential image acquisition and a HC PL APO 63x/1.40 OIL CS2 oil
TABLE 4  Details of the equipment used in muscle fibre dissection and teasing, NMJ immunofluorescence and wholemount muscle fibre mounting and imaging

| Item                                | Manufacturer | Description                                                                 |
|-------------------------------------|--------------|-----------------------------------------------------------------------------|
| Dissection scope                    | Zeiss        | Zeiss Stemi DV4 Stereo Microscope 8x–32x                                    |
| SuperFine Vannas Scissors           | WPI          | 8 cm long, 3mm blades, 0.015 mm x 0.015 mm tips                             |
| Curved Iris Forceps                  | WPI          | Full Curve #4                                                               |
| Dumont Mini Forceps                 | Fine Science Tools | Item No. 11200-10 - Style #3                                              |
| Dumont Fine Forceps                 | Fine Science Tools | Item No. 11254-20 Straight/Inox/11 cm                                      |
| SYLGARD 184                         | Dow Corning  | 1.1KG—Silicone Elastomer, Flowable.                                        |
| 90 mm Petri Dishes                  | Thermo Scientific™ | Sterilin™ Standard                                                        |
| Minutiens insect pins               | Austerlitz 0.20 | ~ 0.2 mm                                                                  |
| Rocker                              | VWR          | 444–0116                                                                    |
| 2 ml Eppendorf tubes                | Eppendorf    | 3810X                                                                       |
| 15 ml and 50 ml Falcon tubes        | BD Falcon    | 352,097 and 352,070                                                        |
| Magnetic stirrer and stir bar       | VWR          | 442–0883 and 442–0272                                                       |
| 12-well tissue culture plates       | BD Falcon    | 353,047                                                                     |
| Magnetic stirrer and stir bar       | VWR          | 442–0883 and 442–0272                                                       |
| 50 ml conical flask                 | Corning      | 70,980                                                                      |
| Superfrost™ Ultra Plus Adhesion Slides | Thermofisher Scientific | J3800AMNT                                        |
| Rectangular coverslips             | Thermofisher Scientific | 22X30-1                                                                   |
| Leica ASP8 inverted scanning confocal microscope | Leica | SP8                                                                         |

FIGURE 1  Acetylcholinesterase staining of endplate distribution within cranialis tibialis, extensor digitorum longus and soleus muscles: (a) caudolateral aspect of dissected cranialis tibialis muscle, showing overlapping, white, dotted bands of endplates (arrows), repeating along the entire length of the muscle—inset: Boxed area is shown at a higher magnification, scale bar = 5 cm (b) lateral and distal aspect of extensor digitorum longus muscle (c) lateral and distal aspect of soleus muscle, scale bars = 1 cm.
immersion objective. On confocal microscopy, all available *en face* NMJs were imaged. See Figure 4 for common imaging pitfalls. The numbers of NMJ imaged were as follows: *N* = 5 ponies, *n* = 15 muscles, 194 *tibialis cranialis* NMJs, 169 *extensor digitorum longus* NMJs, 187 *soleus* NMJs. All imaged NMJs were analysed using ImageJ software (https://imagej.nih.gov/ij/) combined with the aNMJ-morph plugin, a semiautomated NMJ morphology analysis workflow (Minty et al., 2020). For the purposes of analysis, ‘morphological variables’ were defined as the 19 morphological variables that are generated using aNMJ-morph across the 15 muscles. A 20th variable of innervation-status was visually assessed. A 21st variable of muscle fibre diameter (MFD), assessed via light microscopy, is described in section 2.10. Means±SEM were plotted of values for all NMJs imaged within each muscle for each pony and analysed using repeated-measures one-way mixed model with a Bonferroni correction. An assessment, while blinded to muscle of origin, of whether nerve terminals conformed to *en grappe* or *en plaque* morphology, was completed from a Z-stack projection of each NMJ, once NMJ-morph analysis was completed. Assessment criteria were based on a published dataset (Hess, 1962) whereby nerve terminals were classed as either *en plaque*—a series of curled, irregular, pretzel-like branches, or clustered as bunches of *en grappe*, spherical boutons.

2.9 | Muscle fibre typing, immunostaining and analysis

Fresh skeletal muscle samples from muscle regions immediately adjacent to those sampled for NMJ analysis were snap-frozen in isopentane precooled in liquid nitrogen. Thereafter, 7 μm cryosections were air-dried onto glass slides (Superfrost plus) and stored at ~80°C. Multiple antibody fluorescence labelling using a Zenon Alexafluor labelling kit was carried out, as previously described (Tulloch et al., 2011). In brief, a goat polyclonal anti-collagen V IgG antibody (1:10 dilution) was applied for 1 h followed by PBS washes. Three separate mouse monoclonal antibodies that detect
type 1 (Slow myosin heavy chain [MHC] IgG antibody, MAB1628), type 2a (Type 2a MHC IgG antibody, A4.74) and both type 2a and 2x (MHCf IgG antibody) were individually labelled respectively with Alexa fluor-conjugated IgG1 Fab fragments designed for 3 different emitting wavelengths: 350 nm–Zenon Alexafluor 350 mouse IgG1 labelling kit; 488 nm–Zenon Alexafluor 488 mouse IgG1 labelling kit; and 594 nm–Zenon Alexafluor 594 mouse IgG1 labelling kit. The three labelled primary antibodies were mixed with a secondary Alexafluor 488 rabbit anti-goat IgG5 secondary antibody (1:500) and applied for 1 h to the cryosection, followed by rinsing in PBS. The section was postfixed with 4% PFA in PBS for 15 min at room temperature and then washed in PBS. Sections were subsequently mounted (VectorShield mounting medium) and examined using a digital scanning fluorescence microscope (Zeiss Axioscan) with filters designed for the different emitting wavelengths. Six 20X images were captured at random per muscle, and pseudo-coloured composites were generated for manual counting of fibres. Muscle fibre type percentages per section were calculated.

2.10 | Muscle fibre diameter analysis

After confocal imaging of NMJs, slides with labelled NMJs in fibre bundles were scanned using an Axioscan digital slide scanner with a 20x objective (Figure 5a) using opensource software NDP.view2, v2.9.29, provided by Hamamatsu, available at www.hamamatsu.com.

Individual muscle fibres were chosen randomly, and fibre diameters were determined by taking three width measurements at their beginning, mid-, and endpoint. Care was taken to avoid damaged or fused fibres. Forty fibres were measured per muscle per animal, frequency distributions assessed and means calculated and compared using repeated measures one-way ANOVA mixed-effects model analysis with Bonferroni’s multiple comparisons test. Means±SEM was plotted (n = 5) and individual data points were generated from each pony, i.e. the mean values from 40 fibres per muscle.

2.11 | NMJ morphology statistical analysis

GraphPad Prism 9 (version 9.3.1) for macOS was used for all statistical analyses. The means of each morphological variable, derived from all NMJs within each pony and muscle were calculated. The mean of means was compared across each muscle using a repeated-measures one-way analysis of variance (ANOVA), mixed-effects model, with pairwise comparisons compared with Bonferroni’s multiple comparisons test. Correlation against body weight, muscle fibre type and diameter were assessed using Pearson’s product–moment correlation (p<0.05).

2.12 | Principal component analysis

Principal component analysis (PCA) was used for dimensionality reduction of the large dataset of pony NMJ morphological variables, condensing the existing variables generated by aNMJ-morph into new principal components (PCs) created through linear combinations of the original dataset and plotted as PC scores. The variance between each PC is maximised, thus revealing additional information about the dataset by illustrating which variables contribute most. Loading analysis revealed how strongly each morphological value contributed to each PC. PCA plots and loading analysis were...
FIGURE 5  Muscle fibre diameter quantification following light microscopic imaging (following confocal imaging) of muscle fibre bundles reveals intermuscular differences. (a) Whole-mounted fibres scanned by light microscopy using an Axioscan digital slide scanner with a 20x objective. Teased muscle fibres from the wholemount muscles showing comparatively smaller diameter soleus muscle fibres. Scale bars: 100μm. (b) Mean muscle fibre diameters (MFDs) in the tibialis cranialis (TC) and extensor digitorum longus (EDL), datapoints representing mean±SD, compared to the soleus (SOL). Mixed model analysis with Bonferroni's multiple comparisons test revealed a significant difference between the MFDs of the SOL, compared to the TC and EDL (*p < 0.05).

created using GraphPad Prism 9 (version 9.3.1). Using the PC scores plot of all combined averaged data, the percentage of overlapping data points for individual NMJs per muscle were calculated.

3  | RESULTS

3.1  | NMJs in the equine soleus muscle are morphologically distinct from those in the extensor digitorum longus and tibialis cranialis muscle

AChE staining identified the typical gross distribution of endplate-rich areas as a series of overlapping bands on the muscle surface (Figure 1), repeating along the entire length of the muscle, indicating target regions for muscle fibre teasing in subsequent samples. Following immunolabelling of teased fibres, the equine NMJ revealed motor nerve terminals and endplates that were closely morphologically aligned across the three muscles: the distal nerve terminals were singly innervated, branched and varicose, and ACh receptors were highly concentrated at endplates, with striations revealing the orientations of postsynaptic folds (Figure 6). Intermuscular differences in distal nerve terminal morphologies were revealed, showing tibialis cranialis (TC, NMJs = 200) and extensor digitorum longus (EDL, NMJs = 168) muscles with, respectively, 91.2% [±4.7] and 91.7 [±9.1] (mean [SD]) curled branch or irregular pretzel-like en plaque terminals, while soleus (SOL, NMJs = 187) nerve terminals appeared exclusively in grape-like or en grappe clustered spherical boutons (Figure 7c). Mean percentages of muscle fibre types (Figure 7h), calculated per muscle per pony showed 58.5% [±14.4] and 75% [±9.6] type 2 muscle fibres within the TC and EDL, respectively. Whereas the SOL contained almost exclusively type 1 fibres (>99%), except for occasional type 2 labelled muscle spindles (Figure 7a). Type IIA fibres represented 37.3% [±7.9] and 43.5% [±14.1] of the TC and EDL type II fibre total, respectively, with 21.1% [±7.9] and 32.2% [±20.8] being IIX fibres respectively.

3.2  | Modest intermuscular NMJ morphological variability revealed by aNMJ-morph analysis

From a comparison of 21 variables (19 morphological variables generated through aNMJ-morph and the 2 associated variables of axonal input and MFD) and across the three muscles (see Table 5), significant differences ("*p < 0.01) were detected in the core post-synaptic variables of endplate diameter and perimeter and AChR perimeter, occurring between the EDL and SOL muscles only (Figure 8b). Of the rest, significant (*p < 0.05) differences were noted between either the EDL and the SOL and/or TC and the SOL for pre-synaptic and postsynaptic variables and ‘combined’ values derived from combinations of pre- and postsynaptic measurements (Figure 8a–c, respectively). As a direct measure of alignment, the lowest percentage overlap between endplate and nerve terminal in the ponies was 58.49% for the TC, and the highest was 67.33% for the SOL; no significant difference was noted between these or the remaining values (see Table 5).

Mean muscle fibre diameters (MFDs) in TC and EDL fibres, were 60.9 μm ±4.2 and 64.2 μm ±6.5 respectively (mean ±SEM), compared to 39.7 μm±2.3 in the SOL (Figure 5b). Mixed model analysis with Bonferroni’s multiple comparisons test revealed a significant difference between the MFDs of the SOL, compared to the TC and EDL (*p < 0.05). MFD values correlated with synaptic contact area (r = 0.79, p = 0.0004) and with AChR area (r = 0.772, p = 0.0007). No significant correlation was noted between aNMJ-morph–derived variables and body weight or muscle fibre type (data not shown).

3.3  | Principal component analysis separates morphologically distinct NMJs into muscle-associated groups

The complete dataset composed of 20 variables (19 morphological variables generated through aNMJ-morph and MFD, less the number...
of axonal inputs) was condensed into linear combinations and plotted along the first two most important principal components (PCs) (Figure 9a). PC1 accounted for 82.9% of the variation in the dataset, clearly separating most of the SOL NMJs from those of the TC and EDL. PC2 explained 8.72% of the variation within the dataset, yet it also separated SOL NMJs from the other two muscles. An even distribution of TC and EDL datapoints, plotted across PC 1 and 2, indicated a similarity between NMJ variables for those muscles, yet only 35% of TC and 17.5% of EDL datapoints overlapped with those of the SOL. Loadings plot analysis of the 20 variables showed that endplate area contributed most to PC1, with nerve terminal perimeter and AChR area and perimeter also contributing. Average area of AChR clusters contributed most to PC2 (Figure 9b).

4 | DISCUSSION

This paper presents NMJ morphology data from the equine pelvic limb and addresses the main challenges of working with large mammalian muscles: (1) to detect gross endplate distribution; (2) to reduce fixation- and muscle fibre teasing-associated artefacts; and (3) to obtain sufficient antibody penetration during immunolabelling, prior to high-resolution confocal imaging. We reveal the organisation of equine NMJ morphology to be similar to that of other vertebrates and report NMJ morphological variability between equine pelvic limb muscles with different muscle fibre type proportions.

Similar to many long muscles in humans and other vertebrates, multiple end-plate bands were evident in the equine EDL, TC and SOL, suggesting that most of the constituent fibres do not run from origin to insertion, given that NMJs are typically located in the middle of the muscle fibre (Bianchi & Bianchi, 2018; Patton & Burgess, 2005). This paper’s muscle fibre teasing method, adapted from studies in smaller mammals and humans (Boehm, Alhindi, et al., 2020), successfully yielded quantifiable NMJs for analysis with minimal staining artefacts, indicating its cross-species compatibility.

The equine NMJ has presynaptic vesicle clusters within terminal boutons that are well aligned to postsynaptic ACh receptors, regardless of the differences in nerve terminal shape between these muscles. Thus, highlighting a stereotypical organisation that also occurs across other vertebrate species (Boehm, Alhindi, et al., 2020; Patton & Burgess, 2005; Petralia et al., 2017). Soleus muscle nerve terminals had exclusively an en grappe morphology, in marked contrast to the TC and EDL, which were mostly en plaque. Distinct nerve terminal morphologies have been reported for different muscles across vertebrates—in general, smaller single boutons or en grappe-like terminals are noted on muscle fibres that undergo slow and sustained (tonic) contraction, whereas longer branched, en plaque-like nerve terminals are seen on fast twitch fibres (Patton & Burgess, 2005; Petralia et al., 2017). Exceptions to this generalisation exist, highlighted by the discrepancy between fibre type percentage and NMJ morphology in the TC and EDL muscles (Figure 7), indicating that not all type I fibres in these muscles have an en grappe shape.
NMJ morphological heterogeneity has been reported in select pelvic limb muscles (limited by availability of comparative human NMJ data (Jones et al., 2017)) within and across 6 other mammalian species in a recent NMJ study by Boehm, Alhindi, et al. (2020). Similar to their within-species findings, most core pre- and postsynaptic variables in the ponies showed either only limited or modest inter-muscular differences—the most significant of those, backed by mixed model and principal component analysis, occurred post-synaptically.
between the SOL and the EDL, the smallest and largest pony NMJs respectively. Boehm et al revealed that the mouse and human soleus contains the largest relative NMJs, whereas sheep and pig contain the smallest; a comparison of representative pony EDL NMJs (the only muscle present in all seven species) shows that, in general, the pony has a larger terminal axon than that of the human NMJ, yet its endplates are similarly fragmented. aNMJ-morph-generated variables did not significantly correlate with body weight or muscle fibre diameter in the pony, similar to the other mammalian species studied and to previous reports in humans (Jones et al., 2017)—the common factor that governs NMJ morphological variation across species is not thus far evident, highlighting a translational gap in the understanding of NMJ form, which perhaps can be explained by species-specific differences in biomechanics-driven motor unit function.

Differences in NMJ shape and postsynaptic morphology between the SOL and EDL are particularly mirrored in each muscle's size and function. Equine soleus muscles are relatively small, representing approximately 0.01% of total equine pelvic limb muscle mass—a figure approximately 0.01% of total equine pelvic limb muscle mass—a figure derived from data across two studies containing a combined 20 mix-breeds and Thoroughbred horses (Meyers & Hermanson, 2006; Payne et al., 2005). In contrast, TC and EDL muscles represent 0.6% and 0.93% respectively, and the gluteus medius at 17.33% is the largest relative muscle. Large differences also exist between the MFDs of the SOL compared to the TC and EDL—perhaps explained by muscle fibre type differences. The presumed fibre types reported for TC (Valberg

**Table 5: Average morphological data for pony pelvic limb muscles generated in aNMJ-morph**

| Variable | TC N = 5, n = 5200 NMJs | EDL N = 5, n = 568 NMJs | SOL N = 5, n = 5187 NMJs |
|----------|------------------------|------------------------|------------------------|
| Core variables |                        |                        |                        |
| Pre-synaptic |                       |                        |                        |
| (1) Nerve terminal area (μm²) | 183.7 ± 4.62 | 215.3 ± 6.46* | 150.9 ± 4.65 |
| (2) Nerve terminal perimeter (μm) | 227.1 ± 5.31 | 244.5 ± 7.54* | 158.2 ± 4.47 |
| (3) Number of terminal branches | 29.43 ± 1.10 | 31.57 ± 1.43 | 21.73 ± 0.89 |
| (4) Number of branch points | 22.57 ± 1.05* | 24.46 ± 1.08 | 12.67 ± 0.64 |
| (5) Total length of branches (μm) | 114.1 ± 3.06* | 124.7 ± 4.03* | 70.06 ± 2.24 |
| Post-synaptic |                       |                        |                        |
| (6) AChR area (μm²) | 248.6 ± 6.39* | 270.1 ± 7.34* | 131.5 ± 3.94 |
| (7) AChR perimeter (μm) | 211.2 ± 5.40* | 219.0 ± 6.9** | 114.9 ± 3.32 |
| (8) Endplate area (μm²) | 414.9 ± 11.61* | 460.4 ± 15.92* | 232.0 ± 7.84 |
| (9) Endplate perimeter (μm) | 95.08 ± 1.51 | 103.0 ± 2.33** | 74.77 ± 1.81 |
| (10) Endplate diameter (μm) | 35.00 ± 0.64 | 37.63 ± 0.8** | 27.44 ± 0.71 |
| (11) Number of AChR clusters | 2.49 ± 0.12 | 2.47 ± 0.16 | 3.01 ± 0.22 |
| Derived variables |                       |                        |                        |
| Pre-synaptic |                       |                        |                        |
| (12) Average length of branches (μm) | 4.56 ± 0.18 | 4.73 ± 0.18 | 3.72 ± 0.13 |
| (13) Complexity | 4.72 ± 0.03* | 4.77 ± 0.05* | 4.10 ± 0.04 |
| Post-synaptic |                       |                        |                        |
| (14) Average area of AChR clusters (μm²) | 151.2 ± 7.87 | 160.5 ± 8.37 | 62.96 ± 4.89 |
| (15) Fragmentation | 0.41 ± 0.02 | 0.39 ± 0.02 | 0.54 ± 0.03 |
| (16) Compactness (%) | 61.11 ± 0.6 | 61.58 ± 0.79 | 58.80 ± 0.80 |
| (17) Overlap (%) | 58.49 ± 0.77 | 62.97 ± 0.84 | 67.33 ± 0.75 |
| (18) Area of synaptic contact (μm²) | 142.7 ± 3.69* | 168.9 ± 4.93* | 88.41 ± 2.56 |
| Associated nerve & muscle variables |                       |                        |                        |
| (19) Axon diameter (μm) | 1.582 ± 0.05 | 1.65 ± 0.06 | 2.104 ± 0.05 |
| (20) Muscle fibre diameter | 60.90 ± 1.24* | 64.19 ± 1.49* | 39.72 ± 0.75 |
| (21) Number of axonal inputs | 1 ± 0.0 | 1 ± 0.0 | 1 ± 0.0 |

**Note:** Means ± SEM of each morphological variable per muscle: core variables (1-11), derived variables (12-19) and associated nerve and muscle variables (19-21). N = animals, n = muscles, and total NMJs analysed are listed: Tibialis cranialis (TC, 200 NMJs), extensor digitorum longus (EDL, 168 NMJs) and soleus (SOL, 187 NMJs), corresponding to the data shown in Figures 8 and 9. Statistical difference between the soleus and the other two muscles were compared using repeated-measures one-way mixed model analysis: *p < 0.05; **p < 0.01; ***p < 0.001; no asterisk = non-significant result.
et al., 2015) and EDL (Kawai et al., 2009) (see Table 2) are close to those noted in these ponies and as reported previously (Meyers & Hermanson, 2006), the pony SOL sections contained almost 100% slow-twitch fibres. Across mammals, the soleus muscle typically contains a majority of slow-twitch fibres (Schiaffino & Reggiani, 2011), with the predominant equine type I skew likely reflecting its purported

![Image](image-url)
minor role in hock extension (Payne et al., 2005), but also crucially, a postural or tonic contractile one (Meyers & Hermanson, 2006). This is in contrast to the TC and EDL’s function in hock flexion and, in the latter, digit extension and locomotion. Our findings thus suggest a relationship between pony muscle fibre type/function and NMJ morphology that is, smaller diameter SOL fibres accompanied by smaller *en grappe* NMJs—perhaps driven by species-specific functional demands for weakly sustained synaptic transmission/contraction, compared to the longer branched EDL and TC NMJs aiding more vigorous contraction. A similar relationship between MFD and NMJ size has been shown in a select range of muscles within multiple species (Ogata, 1988) and rodent diaphragm (Prakash et al., 1996); yet other rodent distal limb studies contrast this with larger NMJs observed in muscles with lower fast-twitch fibre percentages (Mech et al., 2020).

Recent comparative work highlights a clear disconnect between NMJ morphology and muscle fibre size/identity within select human and rodent pelvic limb muscles (Jones et al., 2017) and within the same muscle set across four other species (Boehm, Alhindi, et al., 2020). Given the interspecies fibre type variation reported within the SOL, with smaller species containing markedly more type II muscle fibres (Schiaffino & Reggiani, 2011), perhaps our equine NMJ findings highlight the difficulty in making translational cross-species comparisons, even within the same muscle, as motor neurons have evolved within each species to meet their specific biomechanical and functional demands at the nerve terminal. These likely contribute to cross-species variations in NMJ morphology, with some recent evidence to support this (Jones et al., 2017).

Caveats in our data include sample location, with EDL and TC samples, in particular, reflecting a small percentage of the overall muscle mass and an inability to directly correlate the MFD and fibre type to each respective NMJ. Differences in anatomic sample location and methodology likely also underpin some of the variations noted both within- and between species. An area for further cross-species study is the development of a method to co-label/identify NMJ and muscle fibres for concurrent morphological and volumetric analysis.

**5 | CONCLUSION**

Despite the technical difficulties inherent in large mammalian muscle experiments, the techniques outlined show that equine muscles can have their NMJ morphology visualised using immunofluorescence confocal microscopy. We have used pony tissue to demonstrate the cross-species translational potential of muscle preparation for highlighting NMJ phenotypes in large mammals and describe the detailed morphology of equine NMJs.

**AUTHOR CONTRIBUTIONS**

SDC, JDP, IB, RAJ, THG and RJP conceived and designed the study. IB and SDC performed experiments and analysed data. SDC, IB, JDP, RAJ, THG and RJP wrote the manuscript. All authors edited and approved the manuscript and have no conflict of interest to declare.

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**OPEN RESEARCH BADGES**

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [https://doi.org/10.5281/zenodo.6519637](https://doi.org/10.5281/zenodo.6519637).
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