Arginine Methyltransferases in Motor Neuron Circuit Development and Maintenance

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ABSTRACT

The development and physiology of limb-innervating spinal motor neurons have been studied extensively for decades, both as a fascinating model for neural circuit development and because of their pertinence to human disease. The molecular mechanisms guiding circuit development - the processes of axon outgrowth and guidance, synaptogenesis, and synapse maturation - are clinically relevant for improving regeneration and preserving axon health, as well as understanding the remarkable development of the nervous system.

Protein arginine methlytransferases (PRMTs), a family of 9 enzymes, have been implicated in a considerable number of relevant processes, from controlling neuronal progenitor proliferation and differentiation, to cell motility, to regulating key genes in neurodegenerative and -developmental disorders. Despite this and the fact that arginine methylation is among the most widely-employed post-translational modifications, these enzymes have barely been studied in the context of circuit development and maintenance. To assess whether arginine methylation is implicated in axon outgrowth and pathfinding, I employed conditional knockouts of PRMT5 and PRMT1 in mice. I also used a pharmacological inhibitor of PRMT5 *ex vivo*. In addition to this, I attempted to characterize a motor phenotype in *Prmt7* knockout mice using immunofluorescence. While preliminary, results point to the main PRMTs 1 and 5 being dispensable for motor neuron development *in vivo*, an intriguing negative regulation of *ex vivo* embryonic motor axon outgrowth by *Prmt5*, as well as a role in maintaining neuromuscular junction integrity in adult mice by Prmt7.

RÉSUMÉ

Le développement et la physiologie des motoneurones spinaux innervant les membres ont été largement étudiés durant des dizaines d'années, à la fois en tant que modèle fascinant du développement des circuits neuronaux, et du fait de leurs implications dans les pathologies humaines. Les mécanismes moléculaires qui guident le développement des circuits -les processus de croissance et de guidage axonal, la synaptogenèse et la maturation des synapses- représentent un enjeu clinique majeur afin d'améliorer la régénération et la protection des axones, ainsi que pour comprendre le développement du système nerveux.

Les Protéines Arginine Méthyltransferases (PRMTs), une famille de 9 enzymes, ont été montrées comme étant impliquées dans un nombre considérable de processus importants, depuis le contrôle de la prolifération et de la différenciation des progéniteurs neuronaux à la régulation des gènes clefs dans les pathologies neurodégénératives et développementales en passant par la motilité cellulaire. Malgré cela et alors que la méthylation de l'arginine est l'une des modifications post-traductionnelles les plus largement employées, ces enzymes n'ont été que peu étudiées dans le contexte du développement et du maintien du circuit. Afin de déterminer si la méthylation de l'arginine est impliquée dans la croissance et le guidage des axones j'ai utilisé des knockouts conditionnels de *Prmt5* et *Prmt1* chez la souris. J'ai également utilisé un inhibiteur pharmacologique de PRMT5 *ex vivo*. De plus, j'ai tenté de caractériser par immunofluorescence les phénotypes moteurs de souris knockout pour *Prmt7* générées dans mon laboratoire. Ainsi, bien que préliminaires, les résultats suggèrent que les PRMTs principaux, 1 et 5, ne sont pas indispensables au développement des motoneurones *in vivo*, que, de manière intéressante, PRMT5 régule négativement la croissance des axones moteurs embryonnaires *ex*

vivo, et, enfin, que PRMT7 joue potentiellement un rôle dans le maintien de l'intégrité des jonctions neuromusculaires chez les souris adultes.

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PREFACE AND CONTRIBUTIONS

All experimental designs and thesis writing was performed by the M.Sc candidate DC. Stéphane Richard (SR) aided in editing. DC performed all necessary mouse breeding except for experiments done with *Prmt7*$^{-/-}$ mice, done by Gillian Vogel and Romeo Blanc. DC optimized and performed the qPCR, dissections, immunofluorescence, and writing and testing of image analysis software (Figs. 1bc, 2, 3, 4b, 5). Western blotting was performed by Sadig Niftullayev (Fig. 1a), and Romeo Blanc and Sara Calabretta helped DC with mouse behaviour (Fig 4a) and weighing (Fig S2), respectively.

Original contributions to knowledge:

- PRMT5 inhibitor EPZ-015666 considerably increases axon outgrowth from explanted embryonic ventral nerve cord tissue
- *Prmt5*- and *Prmt1*-null mice with motor neuron-specific *Hb9-Cre* fail to affect weight gain or motility of pups up to at least 12-weeks of age
- *Prmt7*$^{-/-}$ mice display aberrant limb extension reflexes and degenerating neuromuscular junction morphologies akin to neurodegenerative disease models

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ABBREVIATIONS

AChR - Acetylcholine Receptor

a/sDMA - Asymmetrical/Symmetrical Di-methylated Arginine

ALS - Amyotrophic Lateral Sclerosis

BTX - Bungarotoxin

CC3 - Cleaved Caspase-3

CNS - Central Nervous System

DCC - Deleted in Colorectal Cancer

FTLD - Frontotemporal Lobar Degeneration

GC - Growth Cone

LMC - Lateral Motor Column

MMA - Mono-methylated Arginine

MN - lower α-Motor Neuron

MuSK - Muscle-Specific Kinase

NMJ - Neuromuscular Junction

NPC - Neural Progenitor Cell

PNS - Peripheral Nervous System

PRMT - Protein Arginine Methyltransferase

PTM - Post-Translational Modification

SC - Satellite Cell

SMA - Spinal Muscular Atrophy

TUJ1 - neuronal β(III)-Tubulin

1) INTRODUCTION AND LITERATURE REVIEW

1a) Rationale and Objectives

The overarching goal of this project was to assess the roles of protein arginine methyltransferases (PRMTs) in neuronal circuit development. Here, neuronal circuit development is defined as the process by which neurons extend projections to form connections and establish functional networks, and extends to the reparation of these networks. Given the association of PRMTs with motor neuron diseases such as amyotrophic lateral sclerosis (PRMT1 and -8) and spinal muscular atrophy (PRMT5), as well as the *Prmt7*$^{-/-}$ motor phenotype discussed below, I focused on lower motor neuron (MN) circuit development and maintenance (Blanc and Richard, 2017a; Chittka et al., 2012; Cuscó et al., 2004; Yamaguchi and Kitajo, 2012). Conditional motor neuron knockout mice were generated for the main type I and II arginine methyltransferases – PRMT1 and -5, respectively – to evaluate whether they are required *in vivo*. As a highly specific pharmacological inhibitor of PRMT5 was recently made available, I also tested its effects on axonal outgrowth and survival of *ex vivo* models of motor neuron circuit development.

In a related second project, I attempted to characterize potential motor defects in a full-body *Prmt7* knockout mouse generated by my lab. Our recent work showed it to maintain satellite cell (SC; the adult muscle stem cell population) self-renewal by antagonizing senescence (Blanc et al., 2016). Interestingly, however, these mice exhibit phenotypes which cannot be explained by this muscle regeneration defect – a progressive loss of myofibers in the absence of muscle damage, reminiscent of neurogenic cachexia; and a “hind-limb clasping” behavior when

they attempt to extend their limbs when lifted by their tails. Despite these associations with neural development and dysfunction, to our knowledge, none of PRMT1, -5, or -7 have been investigated in the specific context of neuronal circuit development or regeneration. Using knockout mice and the aforementioned pharmacological inhibitor of PRMT5 *in vitro*, I sought to evaluate whether and how these enzymes are involved in motor circuit development and maintenance.

1b) Peripheral Motor Nerve Development and Regeneration

1b.1) Principles of motor neuron physiology and anatomy: Motor neurons provide the interface between the central nervous system (CNS) and skeletal muscles, making them the conduit for all voluntary motor control. Motor commands generated in the brain are relayed from upper motor neurons down the spinal cord along corticospinal axon tracts where they form synapses with lower motor neurons (or, for muscles in the face and neck, to brachial motor neurons in the cranial nerve nuclei of the brainstem**;** Lemon, 2008). These lower motor neurons which innervate muscle fibers are known as α-motor neurons. There are also ß- and γ-MNs which control muscle spindles, sensory organs within muscles important for proprioception and reflexes which regulate muscle tension (Manuel and Zytnicki, 2011). All subtypes are located within the gray matter of the ventral horn (Rexed lamina IX; Rexed, 1954), and are modulated by the brain as well as local circuits within the spinal cord. In addition to these excitatory and inhibitory connections, diverse populations of astrocytes and oligodendrocytes in the ventral horn play integral roles in motor neuron development and physiology (Miller et al., 1994). Within each

spinal level, motor neurons are also organized along the mediolateral axis such that medial neurons innervate proximal muscles such as those of the trunk, while lateral neurons innervate distal muscles (Landmesser, 1978). A subset of motor neurons reside in the lateral motor columns (LMCs), enlargements of the ventral horn found at cervical and lumbar enlargements, which innervate limb muscles; this population is the focus of this study and will hereafter be referred to as MNs.

Lower motor neurons are considered part of the peripheral nervous system (PNS), as their axons project from the spinal cord throughout the body. Motor axons leave the spinal cord in bundles known as ventral roots and converge with dorsal and other ventral projections from adjacent spinal levels to form nerve plexus, which then branch into peripheral nerves. Unlike some sensory neuron projections of the PNS, MN axons are always myelinated. Myelination (the process by which axons are ensheathed by layers of sphingolipid-rich membrane) is performed by Schwann cells in the PNS, with individual axons being myelinated by several Schwann cells; this is in contrast with the CNS where a single oligodendrocyte can myelinate multiple axons. Peripheral nerves are encapsulated within layers of connective tissue formed by endoneural cells, isolating them from the surrounding tissue and allowing them to maintain a more CNS-like environment conducive to their relatively extreme metabolic and electrophysiological needs (Jessen and Mirsky, 2005; Joseph et al., 2004).

Motor neurons can be categorized into “pools” – groups of adjacent MNs which innervate the same muscle. A single MN and all the myofibers (polynucleated muscle cells which make up skeletal muscle) it innervates are called a “motor unit”, as it represents the quantal unit of muscle contraction. MNs always innervate myofibers of the same type (slow type I, or fast type IIa or

IIb), and have specific morphologies and electrophysiological properties based on it, reflecting the close coupling of muscle and MN physiology (Tintignac et al., 2015). Because a depolarized MN will signal all connected myofibers to contract, muscles with smaller motor units (*ie.* a higher ratio of MNs to myofibers) can be more finely controlled; for this reason, small muscles where precision is important such as fingers can have bigger motor pools than much larger muscles such as triceps (Widmaier et al., 2014). This size-proportional-to-sensitivity relationship is preserved in the motor cortex of the brain, where relatively larger regions are devoted to muscles which require greater precision. As with so many other aspects of the nervous system, motor units and pools are plastic – if an MN dies, nearby motor axons from adjacent MNs in the same pool are able to branch and innervate its myofibers at the same site (Gordon et al., 2004).

MNs can extend axons to lengths greater than 1 meter in humans (such as those innervating feet muscles from the lumbar spinal cord) and conduct dozens of action potentials per second at velocities exceeding 100m/s, making them among the largest and most metabolically active cells in the body (Burke et al., 1973). No MNs are generated after birth, meaning they must also maintain these axons for the entire lifespan in spite of considerable oxidative and mechanical stress. Altogether, it is unsurprising that they need so many associated cells to function, and that they are disproportionately affected in many neurological diseases. In the contexts of development, regeneration, and maintenance, MNs engage in constant bidirectional communication with the cells of their environment. With this in mind, this section will outline the key steps of this system's development and function, with focus on the relevant processes of motor axon guidance, neuromuscular synaptogenesis, and neuromuscular junction maintenance and disease.

1b.2) Lateral motor column neurogenesis and axogenesis: Limb-innervating lower motor neurons, which reside in the LMC in brachial and lumber levels of the spinal cord's ventral horn, are among the first neurons to be generated. During prenatal development, motor neuronal progenitor cells (NPCs) lining the embryonic spinal canal give rise to distinct MN pools fated to innervate a common target muscle (Stifani, 2014; Tsuchida et al., 1994). For the LMC, for example, NPCs are specified to become limb-innervating MNs by factors, notably including retinoic acid prior to their terminal divisions (Novitch et al., 2003). These induce the unique expression of several transcription factors including the basic helix-loop-helix transcription factor *Olig1* and *-2*, which upregulates *Neurog2* – together, these promote expression of the commonly-used motor neuron marker, *Motor Neuron and Pancreas Homeobox 1* (*Mnx1*, or *Hb9*) – when *Neurog2* is downregulated after MN generation, the *Olig1* and *-2* instead promote oligodendrocyte generation from the same progenitor domain (Novitch et al., 2001; Tanabe et al., 1998). The combinatorial activity of specifying factors such as these across progenitor domains establishes a diverse number of MN subtypes in a highly temporally and spatially restricted manner (Song and Pfaff, 2005).

As newly-born MNs migrate ventrolaterally, guided by chemotaxic factors to populate the LMC, they must extend axons out of the developing spinal cord (Kania, 2014; Kim et al., 2015b). This begins with axon specification – immature neurons produce a number of projections known as neurites, one of which must be chosen as an axon while the others become dendrites. Dendrites for most neuronal subtypes remain fairly close to their cell bodies, while axons often must grow over long distances before branching and forming synapses with their targets. Axons

must also have the capacity to form presynaptic terminals, meaning they must have the molecular machinery required to produce and release neurotransmitters, while dendrites specialize as postsynaptic terminals which receive these transmitters. Thus, distinct factors must be localized to each compartment, including proteins as well as transcripts and regulatory RNA (as both types of processes are capable of local translation; Holt and Schuman, 2013). The exact mechanism by which an immature neuron choses which process to become an axon is not known, but it seems to involve a combination of extracellular cues, intracellular signalling, and organelle localization (Lewis et al., 2013). As newly specified axons grow out of the spinal cord to form the ventral roots (grouped with those of adjacent MNs), dendrites are contacted by interneurons and sensory neurons within the local spinal circuitry, as well as descending cortical projections. These connections will influence their dendritic development including which neurotransmitters they respond to, and allow immature MNs to coordinate their activity. While they will not be discussed in detail here, these early spinal networks are instrumental in regulating activity-dependent processes, such as electrical coupling for synapse maturation, later in the system's development (Bravo-Ambrosio and Kaprielian, 2011; Law et al., 2014).

1b.3) Motor axon pathfinding: Newly-specified motor axons must grow out of the spinal cord and through the rapidly-developing embryo to their target muscle via a process known as axon guidance. Highly specialized sensorimotor structures are formed at the tips of their axons, called growth cones (GCs), which respond to chemical and mechanical cues from their target and other tissues. Growth cones are produced by regenerating axons as well, where many of the same signalling pathways as in development are utilized (Twiss and van Minnen, 2006). LMC motor

axons of each spinal segment initially grow in tight fascicles, due to attractive axon-axon signalling and repulsive cues from somites, and then all converge at the base of the limbs. At this point they must choose where to project next. Different neuronal subtypes express different receptors at their GCs, allowing them to respond differentially to guidance cues which mark their paths. In this classical example, Isl1-expressing MNs in the medial LMC express receptors for the repulsive cue ephrinB2 at their GCs, which is secreted from the dorsal limb thereby driving axons ventrally; Lim1-expressing lateral LMC MNs express ephrinA receptors which guides axons dorsally (Luria et al., 2008). Synergistic signalling between these and other factors – classical guidance cues such as semaphorins and netrins, as well as growth factors such as GDNF – help fine-tune axon guidance, as the travelling axons de-fasciculate and project into different muscles (Kramer et al., 2006; Morales et al., 2015; Poliak et al., 2015). As in other neuronal systems such as the well-characterized developing optic nerve's projections, the combinatorial expression of guidance cue gradients and their corresponding receptors in GCs yields impressive accuracy; MN position along the spinal cord corresponds nearly perfectly to their target muscle's position in the rostrocaudal axis, belying the robustly deterministic rules governing axon guidance (Bonanomi and Pfaff, 2010).

Around E13-14 in mice, LMC axons reach their target limb muscles, consisting largely of newly-formed primary myofibers. At this terminal stage of axon guidance, signals from the muscle are relayed back to MN cell bodies to prepare for synaptogenesis, while axons are instructed to branch and contact the muscle. The specialized synapses formed between neurons and muscles – neuromuscular junctions (NMJs) – gradually mature over the next 3-4 weeks. These pioneering motor axon tracts also serve as guides for sensory axons, which innervate

proprioceptive organs in the muscle as well as other sensory receptors in the limb, and neural crest-derived progenitors. These crest-derived cells will become Schwann (the myelinating glia of the peripheral nervous system) and endoneural cells, eventually forming the myelin and connective tissue sheath which envelopes peripheral nerves (Bonanomi and Pfaff, 2010; Joseph et al., 2004). A subset of Schwann cells localized to the interface between neurons and muscles (dubbed perisynaptic Schwann cells) play a significant role in both NMJ formation (in development and regeneration contexts) and physiology (Barik et al., 2016; Feng and Ko, 2008; Reddy et al., 2003).

1b.4) Molecular mechanisms in axon guidance and outgrowth: As the axons of many neuronal subtypes (particularly MNs) can extend across distances thousands of times that of their cell bodies, much of the complex signal integration and response must be carried out locally at the level of the growth cone, independently of transcriptional mechanisms. Signalling across the axon to the nucleus and back (retrograde and anterograde transport, respectively) is essential, but ultimately is too slow and inefficient to control all of axon guidance and growth at such distances. GCs face a series of "choice points", such as at the aforementioned dorsal-ventral limb decision made by LMC axons, where which the balance of guidance cue signalling pathways determine their behavior. Mechanisms which control receptor trafficking and exocytosis, degradation, and expression (mRNAs of receptors are known to be translated at GCs in response to various signals) are employed to modulate their sensitivity (Jung and Holt, 2011; O'Donnell et al., 2009). Signals from these receptor complexes are thought to generally converge on the Rho GTPases *RhoA*, *Rac,* and *Cdc42*, which are strongly implicated in the motility of other cell types

and have well-characterized effects on cytoskeletal dynamics (Hanna and El-Sibai, 2013). By keeping these signalling cascades compartmentalized, GCs can respond to minute differences in concentrations of guidance cue gradients in a variety of overlapping ways. For example, the classical guidance cue Netrin binding to one of its receptors, Deleted in Colorectal Cancer (DCC), can promote attractive turning via several mechanisms that depend on spatially restricted processes: 1) a ZBP1-dependent local translation of ß-actin, favoring filipodia extension (Welshhans and Bassell, 2011), 2) inhibition of Ras via p120RasGAP to antagonize its constitutive actin-filament collapsing activity (Antoine-Bertrand et al., 2016), and 3) engage a "pulling" mechanism, dependent on myosin-II and the mechanosensor FAK, whereby substrate-bound netrin is thought to be used as an anchor (Moore et al., 2012).

GCs also signal retrogradely via exosomes, permitting the cell body to alter the profile of transcripts, miRNAs, and proteins it shuttles back to the GC (Jung et al., 2012). Many transcription factors and epigenetic regulators have been reported to be retrogradely transported, triggering changes in expression at the level of the nucleus, allowing it to mediate global changes in GC behavior (Puttagunta et al., 2014; Shin and Cho, 2017; Shin et al., 2012; Smith and Skene, 1997). As for PNS axons which must re-grow to their targets after injury, though the environment they must navigate through is very different, many of the same guidance cue pathways are thought to be utilized. Depending on the nature of the injury, Schwann cells are generally activated to assume a development-like "guidance" phenotype which includes mechanically relaxing and expressing numerous classical guidance cues to direct regenerating axons (Painter et al., 2014; Van Battum et al., 2015). Altogether, there are a plethora of coordinated extrinsic and intrinsic mechanisms employed to guide axons during both neuronal

circuit development and regeneration, which is unsurprising given the incredible diversity of targets they must grow to in the PNS alone.

Axon guidance mechanisms described above provide a steering mechanism for growth cones, but they must of course have a motor as well. Axons can grow at impressive speeds, having been observed extending by up to 8mm/day to at least 10cm for sensory projections *in vitro,* and are theorized to grow >2cm/day in developing large animals such as giraffes. Adult axons notably grow more slowly, at a maximum of 2mm/day *in vitro* when stretched (Geoffroy et al.; Loverde and Pfister, 2015). As with guidance, there are a number of distinct molecular mechanisms underlying axon growth, but all are dependent on the synthesis of new cytoskeletal proteins to elongate the axon. Axons are highly enriched for neurofilaments, actin, and microtubules, which are organized as parallel fibers with the plus end at the growth cone (Baas, 1997; Mitchison and Kirschner, 1984). Molecular motors such as kinesin and dynein move anterogradely and retrogradely along them, respectively, shuttling new actin and microtubule polymers to the growth cone along with many other factors (Bouquet and Nothias, 2007). Live imaging experiments have shown that while cytoskeletal polymers, monomers, and adaptor proteins are highly dynamic within growth cones, where they participate in guidance and branching, those of axons are relatively stable. When GCs shift their mass towards attractive cues, these more stable microtubule bundles polymerize into their central domains, and then are bundled and stabilized. In addition to this "push" from polymerizing filaments behind them in the axon, growth cones can propel themselves by actively "pulling". They are enriched for non-muscle myosin which generates force via actin filaments – a GC which encounters an attractive substrate with a filipodia can thus adhere to it (for example, through cadherins) and pull its mass

towards it (Bouquet and Nothias, 2007). Growth cones thus integrate numerous factors to effect changes in their cytoskeletons and adhesions, allowing them to navigate to their targets.

1c) Neuromuscular junction assembly

1c.1) Skeletal muscle development and regeneration: Skeletal muscle development begins as early as E9 in mice, as muscle progenitors produced in the embryonic myotome proliferate, migrate, and begin fusing into primary muscle fibers; a second wave of progenitors continue to proliferate to produce a subsequent wave of secondary muscle fibers around E15-17. Muscle lineages are thought to be specified in the somites prior to migration by *Wnts* and *Shh* signalling, which triggers the expression of myogenic differentiation markers *Myf5* and *MyoD* (Cooper et al., 1999; Cossu and Borello, 1999). Some of these progenitors settle in the basal lamina and enter a slowly-dividing state instead, with their progenitors fusing with existing muscle fibers to aid their growth, throughout postnatal development. These become the resident muscle stem cell population, satellite cells (SCs), which stop cycling in adulthood unless they are activated by injury (Cooper et al., 1999).

Quiescent SCs in adults are marked by the Pair-ruled transcription factor *Pax7*, and their characteristic localization around muscle fibers beneath the basal lamina. Following muscle injury, inflammatory cells residing within the muscle (as well as circulating inflammatory cells which are recruited) where they phagocytose necrotic tissue, and release factors thought to activate SCs (Merly et al., 1999). SCs rapidly proliferate, producing myoblasts which both fuse with existing fibers and create entirely new ones (Cooper et al., 1999). Interestingly, they appear

to undergo a different genetic program as they proliferate and differentiate into new myoblasts than during embryogenesis, though the differentiating markers more closely resemble that of secondary muscle fiber development (Cossu and Biressi, 2005; Tajbakhsh, 2009). Some progeny return to their niche under the basal lamina to renew the quiescent SC pool, as these newly formed myofibers increase in size and mature, resulting in morphologically and molecularly indistinguishable muscle tissue in roughly 3 weeks in mice (depending on the nature and extent of the injury (Karalaki et al., 2009; Mahdy et al., 2015). SCs also contribute to NMJ regeneration: Lui *et al*. showed that their depletion via an inducible diphtheria toxin resulted in heavily impaired NMJ regeneration, affecting both the pre- and postsynaptic compartments (Liu et al., 2015). This may be done through *Sema3A*, a classical guidance cue, again recapitulating developmental processes. Semaphorins are known to be secreted by activated SCs to also aid in myogenic differentiation and, interestingly, bone remodelling and inflammation. Taken together, SC's many roles in coordinating the response to skeletal muscle injury have lead them to be viewed as "regeneration switchboards" (Anderson et al., 2016; Suzuki et al., 2013).

1c.2) Neuromuscular Synaptogenesis: Neuromuscular transmission is achieved via acetylcholine release from the MN's presynaptic terminal, which diffuses to muscarinic acetylcholine receptors (AChRs) on the muscle. This produces an inward current which triggers a depolarization-dependant calcium release within the muscle, causing it to contract. This specialized synapse at the interface between MNs and muscles, the neuromuscular junction, is thus indispensable for the control of all skeletal muscle. NMJ development is thought to begin when motor axon terminals release the heparin sulphate proteoglycan Agrin, which binds the postsynaptic muscle-

specific kinase receptor (MuSK) in an LRP4-dependent manner, along with several other transmembrane and extracellular matrix factors (Glass et al., 1996; Weatherbee et al., 2006). MuSK then elicits AChR clustering as well as cytoskeletal changes to stabilize the postsynaptic terminal via adaptor proteins and scaffolds such as *Dok* and *Rapsyn*. As the NMJ matures, these postsynaptic complexes facilitate microtubule-dependent focal insertion of more AChRs to produce remarkably dense clusters – up to 20 000/um^2 have been reported, with less than 10/um^2 outside the NMJ (Hughes et al., 2006). MuSK can also initiate AChR clustering prior to contact by motor axons, and some evidence suggests that motor axons preferentially contact these clusters (the "myogenic" view of NMJ development which views muscles as the starting point; Darabid et al., 2014). In any case, aneural muscle AChR clusters disappear by E18.5, while innervated clusters mature over the next 3-4 weeks in mice. This process too appears to be agrin (and therefore neuron) dependent – while agrin-deficient motor axons can contact AChR clusters, the postsynaptic side fails to mature and non-innervated clusters are not cleared (Lin et al., 2001). In addition to the deeply intertwined processes going on between motor axons and the muscle, perisynaptic Schwann cells and the basal lamina surrounding the NMJ are also essential. Artificially placing agrin on the basal lamina of cultured myofibers is sufficient to induce AChR clustering, and regenerating axons are induced to differentiate into presynaptic terminals when they contact it even in the absence of muscle tissue (Burgess et al., 1999; Sanes et al., 1978). The contribution of perisynaptic Schwann cells is poorly understood, but several mouse mutants for *Neureg1* or its receptors *ErbB2* or *-3* have none at the NMJ, which results in degenerating axons and retracting presynaptic terminals (Lin et al., 2000). When ablated in

Xenopus nuclei, Schwann-cell-less NMJs were smaller and less convoluted, and became deinnervated with time (Reddy et al., 2003).

1c.3) Synapse elimination and maturation: Motor axons branch and innervate multiple overlapping myofibers initially; in extreme cases, every motor neuron in a particular pool innervates every fiber of the corresponding muscle (Tapia et al., 2012). Efficient muscle control however necessitates only one MN per muscle fiber, presumably because if one motor axon is stimulating the fiber at a slightly different latency than another it could interrupt the wave of depolarization necessary for fiber contraction. Thus, from late embryogenesis to approximately P14, NMJs are pruned in a complex activity-dependent mechanism – presynaptic terminals from the axon with the most connections to a given fiber are protected and can even expand along the fiber, while supernumerary terminals from other MNs retract and are eliminated by Schwann cells (Bishop et al., 2004). While molecular mechanisms are not fully characterized, this process is thought to be mediated by the secretion of "punishment-and-reward" signals from the postsynaptic muscle fiber in response to presynaptic activity (Darabid et al., 2014). During the first 3-4 postnatal weeks, to maximize the surface area between pre- and postsynaptic sides and optimize synaptic transmission, NMJs also mature into a characteristic convoluted "pretzel-like" morphology. This maturation is also dependent on acetylcholine release by the motor axon, likely through calcium signalling in the sarcolemma, and unsurprisingly involves perisynaptic Schwann cells as well (Darabid et al., 2013; Smith et al., 2013; Tintignac et al., 2015).

1c.4) NMJs in health and disease: Many of the same factors required for NMJ assembly are also essential for its maintenance (Barik et al., 2014; Hesser et al., 2006). This illustrates the importance of developmental studies which first elucidated these pathways, as the key genes of *Agrin*, *MuSK*, *Lrp4*, and others have been implicated in the NMJ-affecting myasthenia gravis and associated human disorders (Engel et al., 2003). Neuromuscular junction morphology disruptions have been described in a plethora of disorders – some muscular, some neuronal, and some systemic such as aging (Kariya et al., 2008; Sleigh et al., 2014). As NMJs lie at the interface between 3 distinct cell types (MNs, myofibers, and perisynaptic Schwann cells), defects in any could be the cause of their dysfunction. Impaired neuromuscular transmission resulting from a loss of NMJ integrity presumably underlies the weakness and muscle atrophy seen in a variety of disorders. Intriguingly, some evidence suggests NMJ defects could precede symptoms in diseases such as ALS or spinal muscular atrophy (SMA) – mouse models for both display defective NMJ transmission and morphology (Kariya et al., 2008; Lee et al., 2011; Moloney et al., 2014; Rocha et al., 2013).

If axons or muscle fibers are damaged (or simply hypo-active) and their contact is lost or weakened, NMJs will gradually degenerate into a "fractured" morphology – this can be rescued (to an extent) if the nerve regenerates or a nearby axon branches to reinnervate it. In both development and regeneration, however, there is thought to be a "critical period" of several days or weeks – past this point, though new synapses may still form, they are non-functional (Sakuma et al., 2016). Even after development, NMJ maintenance is affected by a diverse range of factors – abnormal morphologies and electrophysiological properties are hallmarks of diseases affecting neurons or muscles, aging, cancer, metabolic disorders, and more. As much as it is influenced by

these factors, NMJ health is tremendously important for MNs and muscles as well – changes in NMJ activity can induce changes in muscle fiber type, fiber atrophy, and even motor neuron death (Arnold et al., 2014; Darabid et al., 2013; Gordon et al., 2004; Lin et al., 2001; Tintignac et al., 2015). Given such a complex system and its clinical importance, it is imperative to better understand the molecular mechanisms that establish NMJs and allow them to maintain normal function throughout life.

1d) Arginine methylation and the nervous system

1d.1) Molecular roles of protein arginine methyltransferases: Arginine methylation is among the most common post-translational modifications (PTMs), and is implicated in innumerable molecular processes including regulating transcription, splicing, protein localization, DNA repair, and signalling. Arginine methylation in mammals is entirely performed by the 9-member *Prmt* family, each of which binds a methyl group from S-adenosyl methionine to the arginine's guanidino nitrogens. *Prmts* are divided into 3 types based on how they methylate their substrates: type I (PRMTs 1, 2, 3, 4 [also known as CARM1], 6, and 8) perform asymmetrical dimethylations (aDMA) by dimethylating a single nitrogen, type IIs (PRMTs 5 and 9) symmetrically methylates each of the two nitrogens (sDMA), and the lone type III PRMT7 only monomethylates one nitrogen (MMA). Each type of arginine methylation is thought to have non-overlapping roles; in fact, histone aDMAs are predominately associated with transcriptional activation, while sDMA is most commonly considered to be silencing (Blanc and Richard, 2017). Unlike phosphorylation, arginine methylation does not alter the charge of the protein – instead, it is thought to affect

binding interactions by replacing hydrogen atoms commonly used to interact with other proteins (Fuhrmann et al., 2015). Despite being viewed as a dynamic PTM, no arginine demethylases have been identified *in vivo,* though lysine demethylases KDM3A, -4E, and -5C were found to display some demethylation activity *in vitro* (Walport et al., 2016). Though PRMTs across all classes seem able to "scavenge" each other's substrates (for example, an increase of MMA- and sDMA-proteins are observed when *Prmt1* is deleted), they generally seem to have distinct substrates and roles (Dhar et al., 2013).

PRMTs 1 and 5, the primary type I and II enzymes respectively, are both embryonic lethal when deleted in mice (Tee et al., 2010; Yu et al., 2009). These two preferentially methylate RGG/RG motifs. PRMTs 1 and 5 (and the others, to a lesser extent) have been receiving dramatically increased attention in recent years, partially because a number have been identified as putative targets for cancers, as well as the development of new experimental tools such as conditional knockout mice and specific enzyme inhibitors (Aggarwal et al., 2010; Gu et al., 2012; Wei et al., 2012; Yoshimatsu et al., 2011). For these reasons, and the fact that each are thought to account for the vast majority of aDMA and sDMA marks respectively, PRMTs 1 and 5 were chosen as the main enzymes to study (Blanc and Richard, 2017a; Hadjikyriacou et al., 2015).

1d.2) Prmt1 and -5 in neural development and disease: Several members of the *Prmt* family have been implicated in neuronal development. For type I PRMTs, PRMT1 and -8 were shown to play crucial roles in chromatin remodelling and retinoic acid signalling transduction during neuronal differentiation, respectively (Simandi et al., 2015). PRMT8-deficient mice were also shown to

have clasping phenotypes (in fore- and hindlimbs) and hyperactivity, attributed to defects in Perkinje cell morphogenesis in the cerebellum (Kim et al., 2015a). PRMTs 3 and 6 were implicated in higher-order cognitive processes – the former affects dendritic spine shape in the hippocampus when deleted, suggesting a role in learning and memory, while the latter's expression was markedly reduced in the nucleus accumbens in rodents repeatedly exposed to cocaine (Damez-Werno et al., 2016; Miyata et al., 2010). Both PRMT1 and -5 have been associated with oligodendrocyte differentiation (Hashimoto et al., 2016; Huang et al., 2011). PRMT5 has also been strongly implicated with neural progenitor proliferation, with its deletion leading to p53-dependent cortical NPC apoptosis during late neurogenesis due to dysregulated splicing (Bezzi et al., 2013; Chittka et al., 2012). PRMT7, meanwhile, was shown to prevent MLL4-mediated differentiation of the neuron-like NT2/D1 line, allegedly phenocopying the effects of a Kabuki syndrome-associated mutation preventing MLL4 from interacting with methylated histone residues (Dhar et al., 2012).

Prmt-family genes have also been implicated in motor neuron and neurodegenerative diseases: PRMT1 was identified as associating directly with FUS and TLS, two genes causative for familial amyotrophic lateral sclerosis (ALS) and fronterotemporal lobar degeneration (FTLD; Yamaguchi and Kitajo, 2012), PRMT6 mutations were shown to cause spinobulbuar muscular atrophy in mammalian cells (Scaramuzzino et al., 2015), and PRMT5 and 4 (also called CARM1) have been shown to regulate substrates of SMN1 (the causative gene for spinal muscular atrophy [SMA]; Cuscó et al., 2004). In addition to these presumably-neural roles, PRMTs have been heavily associated with muscle development, providing another avenue by which they may affect motor circuits (Blanc and Richard, 2017b).

1d.3) Prmt7 in vitro *and* in vivo*:* The aforementioned studies have however been limited to type I and II arginine methyltransferases, which catalyze asymmetrical and symmetrical dimethylations respectively – however, save for two recent papers linking rare *Prmt7* mutations in humans to intellectual disabilities, there are no reports of a role in in the nervous system for the poorly-characterized mono-methylating type III enzyme, PRMT7 (Akawi et al., 2015; Kernohan et al., 2016). Prmt7 is structurally unique relative to other PRMTs, and preferentially methylates RxR sites in lysine-rich regions (Feng et al., 2013). This enzyme has been implicated in DNA repair, histone modifications, and pluripotency maintenance (Bedford and Clarke, 2009). Like *Prmt5*, *Prmt7* has been associated with snRNP biogenesis and splicing. It was also shown to regulate epithelial-mesenchymal transition and cell invasion via matrix metalloproteases in breast cancer cells, a processes known to share mechanisms and molecules with axon guidance (Baldwin et al., 2015; Geng et al., 2017; Short et al., 2016).

To our knowledge, only 3 *Prmt7* mice have been generated. Akawi *et al.* and Jeong *et al.* used one knockout; the former reported non-Mendelian birth ratios and features similar to those of human patients with *Prmt7* mutations (which they described as resembling pseudohypoparathyroidism; PPHP), while the latter characterized a muscle metabolism defect resulting in obesity and muscle weakness, potentially through PGC-1α regulation (Akawi et al., 2015; Jeong et al., 2016). Ying *et al.* generated a B-cell specific conditional knockout and described a role in B-cell differentiation via H4R3me1 and H4R3me2, belying a potential interaction with PRMT5 in epigenetic regulation (Ying et al., 2015). Finally, Blanc *et al.* published a defect in SC proliferation which resulted in a "premature aging" phenotype in both full-body

and conditional SC deletions (via the *Pax7* promoter). These mice exhibited obesity and non-Mendelian birth rates, as well as the aforementioned clasping defect when lifted by their tail (unpublished). Interestingly, a case study of a male human child with a homozygotic deletion of *PRMT7*'s start site reported phenotypes similar to those I observe, in addition to severe intellectual retardation: poor coordination and altered reflexes, delayed growth, and abnormal craniofacial and distal limb morphologies. MRI's of his CNS revealed delayed myelination in the corpus callosum and spinal cord before he reached 1 year, but it was described as normal by 5 years. They also reported seizures starting from 1 year and mild epileptiform activity via EEG. They also tested a battery of markers for metabolic dysfunction but found nothing abnormal, contradicting the PPHP hypothesis of the previous analysis of humans with *Prmt7* mutations (Akawi et al., 2015; Kernohan et al., 2016). Together, these observations point to a potential role for *Prmt7* in the mammalian nervous system, though whether this could be developmental in origin or even autonomous to the neuronal lineage is unclear.

2) METHODS

2a) Western blotting and RT Quantitative PCR

Embryonic cortical neurons were harvested from E18.5 Sprague-Dawley rats and dissociated with a neuronal dissociation kit (MACS). They were cultured on plastic at 6% CO_2 at 37^O in a neuronal selection medium to prevent NPC and glial expansion [Neurobasal (Invitrogen), B-27 supplement (1:50, GIBCO), 25 mM L-Glutamine (GIBCO), and Penicillin-Streptomycin (1:100, Wisent), cytosine arabinoside (Ara-C, 2uM)]**.** Cells were lysed in RIPA buffer (50mM HEPES pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 1% Triton X-100,150 mm NaCl, 10 mm EDTA). One plate was lysed each day for 4 days to evaluate expression as the neurons produced axons and matured, and as glia and other cells were eliminated by the media. Protein lysates were resolved by SDS–PAGE and transferred to nitrocellulose membranes, which were visualized by ECL. Rabbit polyclonal PRMT1 and -5 antibodies were generated via affinity purification from peptides KTGEEIFGTIGMRPNAKNNRD and KNRPGPQTRSDLLLSGRDWN respectively, as previously described (Boisvert et al., 2002; Côté et al., 2003). The PRMT7 antibody was purchased from Novus Biologicals (cat no. NBP2-47322).

For the RT-qPCR, 3 wild-type adult (3-6 month) C57BL/6 mice were euthanized in accordance with a protocol approved by the Animal Care Committee at McGill University, and their spinal cords removed. The ventral horns were surgically dissected out via tungsten blades, pooled, and dissociated with a neuronal dissociation kit (MACS). Glia and other contaminants were removed with a neuron isolation kit (MACS) and lysed in TRIzol reagent, according to the manufacturer's instructions (Invitrogen). cDNAs were then amplified by PCR using oligo-dT

primers and MMLV reverse transcriptase, according again to the manufacturer's instructions (Promega). For quantitative PCR, primers were designed and had their efficiency tested according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines. The real-time PCR was performed in triplicates with a 1:4 dilution of cDNA using SyBR Green PCR Mastermix (Qiagen) on a 7500Fast Real-Time PCR System (Applied Biosystems). Values displayed are C_T averages normalized to those of GAPDH.

2b) Ventral embryonic spinal explant cultures

Timed pregnant Sprague-Dawley rats were ordered (Charles River), with embryos aged E14.5-16.5. Rats were euthanized in accordance with a protocol approved by the Animal Care Committee at McGill University. Embryos were harvested and kept in sterile Neurobasal media (Invitrogen) on ice while their spinal cords were removed as open-book preparations. MN-enriched ventral strips of the spinal cord, which corresponded to the LMC in adults, were diced into 0.2-0.4mm diameter explants with tungsten blades. These were plated in motor neuron media [Neurobasal (Invitrogen), B-27 supplement (1:50, GIBCO), 0.5 mM L-Glutamate (Sigma-Aldrich), 25 mM L-Glutamine (GIBCO), and Penicillin-Streptomycin (1:100, Wisent)] and left for 30 mins at room temperature to adhere on laminin-coated glass coverslips (VWR). Equal numbers of explants were used within wells (3 each) and for each treatment, and treatments were mixed up between rows of the culture plate to control for differences in timing during adhesion, fixation, etc. Explants were cultured for approximately 48h at 37^o and 6% CO2. For drug treatment assays, EPZ-015666 was dissolved in DMSO (Sigma) at 1.5uM and applied 4h after

explants were placed in the incubator (Fig. 2a). Cultures were fixed with 15mins in chilled 4% PFA, and then blocked for 15mins (5% NGS, 5% BSA, 0.1% Triton) prior to 1h each with primary and secondary antibodies, in the same blocking solution. The α-β(III) tubulin antibody Tuj1 (Santa Cruz) was used to mark axons, while α-Cleaved Caspase 3 (Novus) was used to assay apoptosis levels. DAPI (Sigma) was included in the secondary solution to visualize nuclei. Coverslips were mounted in Immumount (Thermo Scientific), blinded, and imaged with an LSM780 confocal microscope with Zen Pro software. All explants with at least 3 axons were imaged and included in the analysis.

2c) Analyses of explant survival and outgrowth

Regions of interest encompassing explants and all their axons were manually outlined on blinded images using ImageJ v1.51h with the Fiji platform (Schindelin et al., 2012). Regions for explant bodies (consisting of all cell bodies and processes not projecting outwards) were identified by thresholding the DAPI channel as it has an extremely high signal-to-noise ratio, and performing “dilate” and “close” operations on the binary image; the average intensity of the apoptosis marker antibody α-Cleaved caspase-3 was measured within this region as well as its total area. As equal numbers of explants were used for each treatment, the number which met the condition (being adhered with 3 or more clear axons) for being imaged after 48h in culture was also used as a proxy for survival. Simple metrics for axon growth were obtained from the total pixel value in the axon channel minus the explant body region, as well as the total area covered by the manually-defined region of interest normalized to the body area (Fig. 2b).

A custom ImageJ macro based on the Neurite-J plugin and Conographer macro (Chitsaz et al., 2015; Torres-Espín et al., 2014) was used to preprocess, segment, and more comprehensively analyze explant outgrowth. Compared to Neurite-J, it uses a different strategy for segmentation, takes in more measurements, and forces the user to click through far fewer dialog windows. The algorithm starts by taking three randomly-chosen sample images with their names removed are presented to the user, who is prompted to draw a line representing the maximum width of what they consider an axon – the spatial frequency threshold. Each image is then opened and binarized, using the same thresholds and regions of interest as in the aforementioned analysis. The image is then separated to high and low frequency masks based on the spatial frequency threshold – thinner objects were counted as axons, thicker as cell bodies or noise. Objects with high circularity (>0.8) were removed from the high frequency mask, effectively filtering out small noise particles while leaving long structures corresponding to neuronal projections. The algorithm performs basic measurements of area and circularity on the low frequency mask, and measures intensity and signal distribution in the DAPI channel of a non-thresholded copy. This information about the explants which can be used to normalize the data (such as by explant size) or reject outliers (such as those with low intensities or high kurtosis, both of which are indicative of cells migrating out of explants, and are grounds to discount them as this would affect axon growth measurements). The algorithm then quantifies numbers of axons and their distances from the cell body in the high spatial frequency mask, by drawing concentric rings every 10µm out from the explants body, and counting the number of times it crossed an object. To derive biologically relevant values, numbers of axons, their average distances, and the slope of their number as a function of distance from the cell body (as an

additional descriptor of their distribution) were calculated with the following formulae in Excel 2013 (where *n* = number of counts, *d* = each 10µm distance point, *k* = total distance measured):

$$Number\ Axons = (\sum_{d=2}^{k-1} n_d - n_{d-1}) + n_k$$

$$Average\ Distance = (\sum_{d=0}^{k} n_d * d) \,/\, Number\ Axons$$

$$Slope = \sum (d - \bar{d})(n_d - \overline{n_d}) / \sum (d - \bar{d})^2$$

A rough index of branching/defasciculation was extrapolated from this as well, by counting the number of times counts *increased* with subsequent distance points in Excel (with non-branching axons, the values can only decrease). For statistics, each experiment was independently standardized in R by dividing each value by the mean of the control group, and then pooled. Significance was determined by the non-parametric Mann-Whitney test, using the *wilcox()* function in R (Fig. 3a).

2d) Mouse genetics and behaviour

For the conditional motor neuron deletions, Mnx1$^{tm4(cre)Tmj}$ mice (also known as *Hb9-Cre*; Jackson Labs) were crossed with Prmt1$^{2L/2L}$ or Prmt5$^{2L/2L}$ mice), and their progeny crossed to the

same latter genotype as previously reported (Bezzi et al., 2013; Yu et al., 2009). Females were weighed to track pregnancies and ensure that pups were counted the day they were born to ensure any that died after birth (as can be the case with defective motor neurons) were found. *Hb9:Prmt5* mice were weighed weekly from 4-12 weeks to probe for weight loss as they matured, a common feature of models of motor neuron disease resulting from muscle atrophy. PRMT7 whole body KO were generated as described previously (Blanc et al., 2016). To score the *Prmt*$^{-/-}$ clasping phenotype, mice were lifted by the tail and scored by a blinded observer as absent (1), partial (2; if only one paw is clasped or the limb extension is incomplete for both), or full (3; if both paws are clasped to the chest). The same test was performed on the *Hb9* knockouts, as well as general observations of their locomotion in their cage and on a narrow ledge, with no sign of an effect.

2e) *Prmt7*$^{-/-}$ muscle immunofluorescence

Tibialis anterior (TA) muscles were taken to visualize 3D NMJs from the same mice. These were incubated with Bungarotoxin conjugated to Alexa Fluor 647 (Thermo Fisher) for 30min to label postsynaptic AChRs of neuromuscular junctions prior to 20min fixation in -20^{O} methanol. Frozen muscles were mounted in OCT and cryosectioned into 50µm slices, and stained with the same protocol as spinal cords with SV2. These were imaged with an LSM880 confocal and ZenPro software (Molecular Imaging Center, RI-MUHC). Images were compared qualitatively.

3) RESULTS

3a) Prmt1 and -5 are Dispensable in Motor Circuit Development, but PRMT5 May Negatively Regulate Outgrowth

3a.3) Prmt1, -5, and -7 are expressed by cultured embryonic cortical neurons: Using cultured E18.5 rat cortical neurons cultured in a neuronal selection media that inhibits glial and progenitor proliferation, PRMTs 1, 5, and 7 were found to be expressed for at least 4 days *in vitro* (Fig. 1a; Fig. S1). While I was unable to isolate enough ventral spinal tissue from embryos to assay PRMT expression in developing MNs, RT-qPCR was performed on adult mouse ventral spinals neurons in which *Prmt*1 and -5 mRNA were detected as well (Fig. 1b). PRMT1 expression through the key axon outgrowth timeframe was confirmed throughout the cytosol of cultured MNs of E13.5 and E15.5 rats, with elevated levels in their somatic compartments (Fig. 1c).

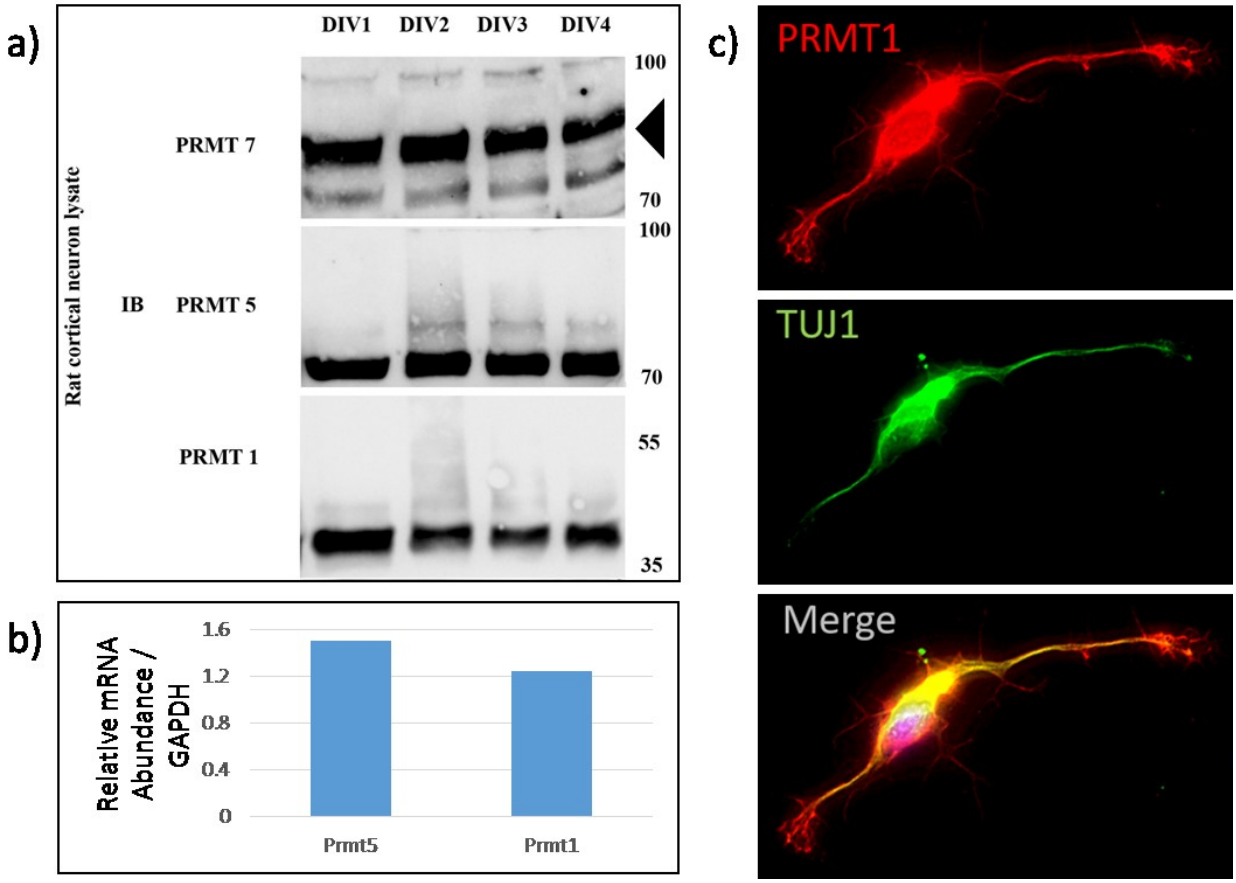

Figure 1: PRMT1, -5, and -7 Expression in neurons. a) Western blot of PRMTs 1, 5, and 7 from E18.5 rat embryonic cortical neurons, cultured in a neuronal selection media. Expression remains constant for at least 4 days *in vitro* (DIV), suggesting each protein is expressed throughout a key window of circuit development. The black arrowhead marks the PRMT7 band at about 79kDa. b) RT-qPCR from adult mouse ventral spinal cords neurons (n=3). Neurons isolated via MACS columns from surgically dissected adult mouse ventral spinal cord confirm *Prmt*1 and -5 mRNA expression (normalized to GAPDH levels). c) Immunofluorescence of E14.5 rat ventral spinal neurons. The protein can be seen throughout the cytoplasm, but is enriched in the somatic compartment.

3a.2) Pharmacological PRMT5 inhibition ex vivo *significantly improves axon outgrowth:* Lacking the ability to satisfactorily transfect primary neurons to knock these genes down, I turned to the recently developed pharmacological PRMT5 inhibitor EPZ-015666 (EPZ) to block its activity in cultured explants. EPZ was reported to significantly reduce the prevalence of SDMA proteins when added to the culture media of several cell lines at 1.5μM, and had a significant effect on tumour proliferation both *in vivo* and *in vitro* (Chan-Penebre et al., 2015). LMC MN-enriched

ventral spinal explants were treated with EPZ dissolved in DMSO at the same concentration or DMSO alone as a control, and cultured for 48h to allow axon outgrowth, in 2 experiments (Fig. 2a). Staining of the apoptosis marker cleaved caspase-3 showed no significant change in intensity, though there was a trend downwards in the EPZ group and roughly 50% more explants survived with enough axon outgrowth to be included in the analysis (Fig. 2b, c, d). Manually measuring the area of axons (stained with the specific neuronal projection marker Tuj1) normalized to their explant's body size (axon density) suggested an increase in axonal content of roughly 50% per explant, inferring a significantly increased level of axon outgrowth (Fig. 2e).

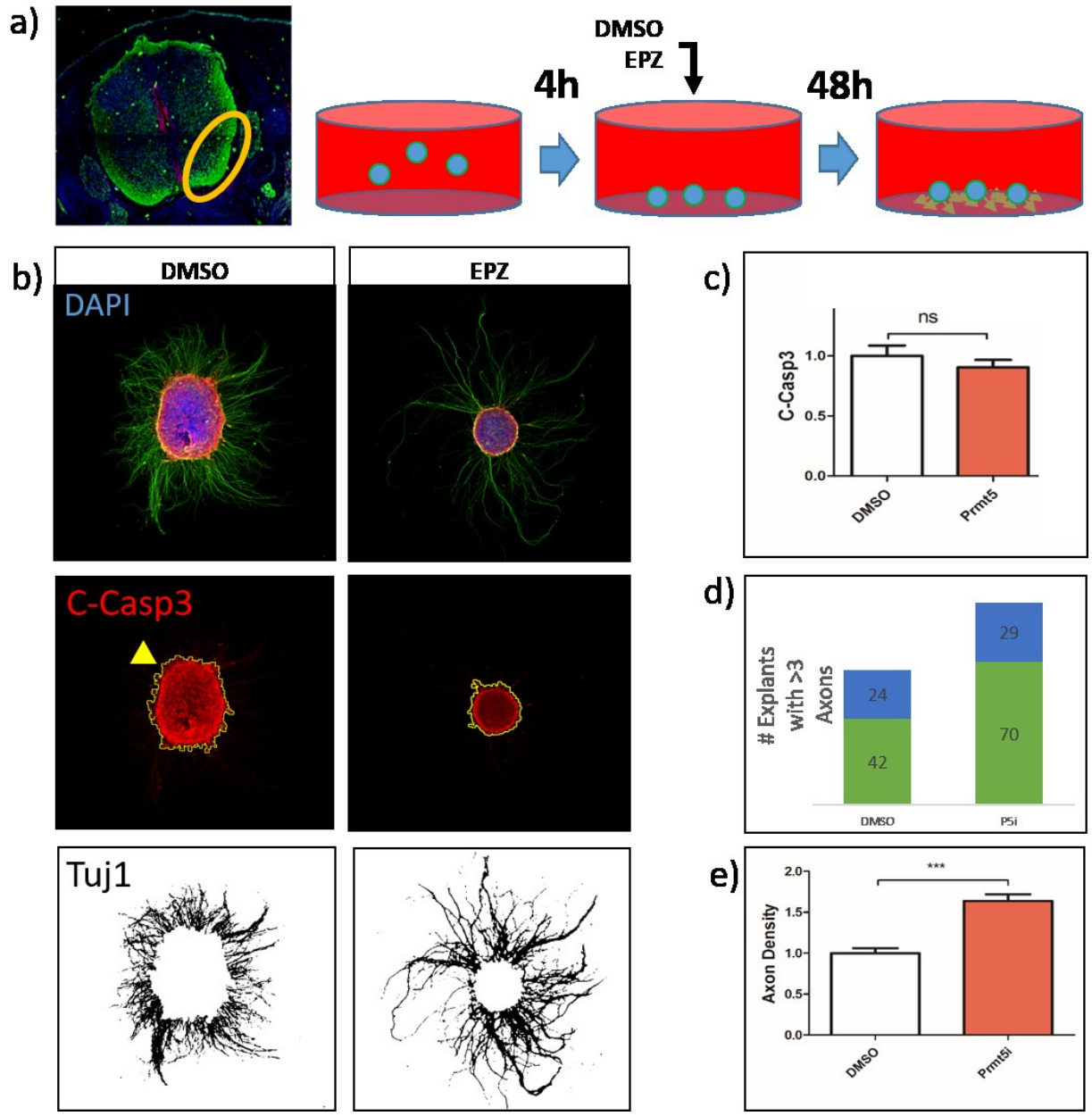

Figure 2: Effects of pharmacological PRMT5 inhibition on explanted LMC tissue. a) Experimental setup: E14.5 rat spinal cords are removed, and their ventral nerve cords are sliced off and diced into 0.2-0.4mm explants. After 4h to adhere in laminin-coated culture wells, either EPZ-015666 or DMSO is added to the media, and the explants are left for 48h to grow before fixation and imaging. b) Sample images from either treatment. Top panels show merged channels, middle show CC3 stains (a marker of apoptosis) with the region of interest used to measure its mean intensity outlined in yellow, and bottom show thresholded masks of axonal marker Tuj1 used to estimate axon outgrowth. c) Quantification of mean CC3 intensity – no difference was found between treatments. d) Quantification of number of explants which were included in the analysis, the criteria of which was having at least 3 discernable axons. As equal amounts of explants were used in each treatment, this was a proxy of overall health. More explants were counted in both experiments (green and blue, respectively), with a total increase of about 50%. e) Quantification of average axon area from binarized Tuj1 channel divided by explant body size, showing a 50% increase in the axonal volume.

Seeking more comprehensive and reliable measurements to determine whether EPZ had an effect on outgrowth, a custom Scholl-like analysis was performed to estimate the number of axons and their distribution relative to the cell body, with a spatial frequency-based segmentation strategy to better resolve axons (Fig. 3a).

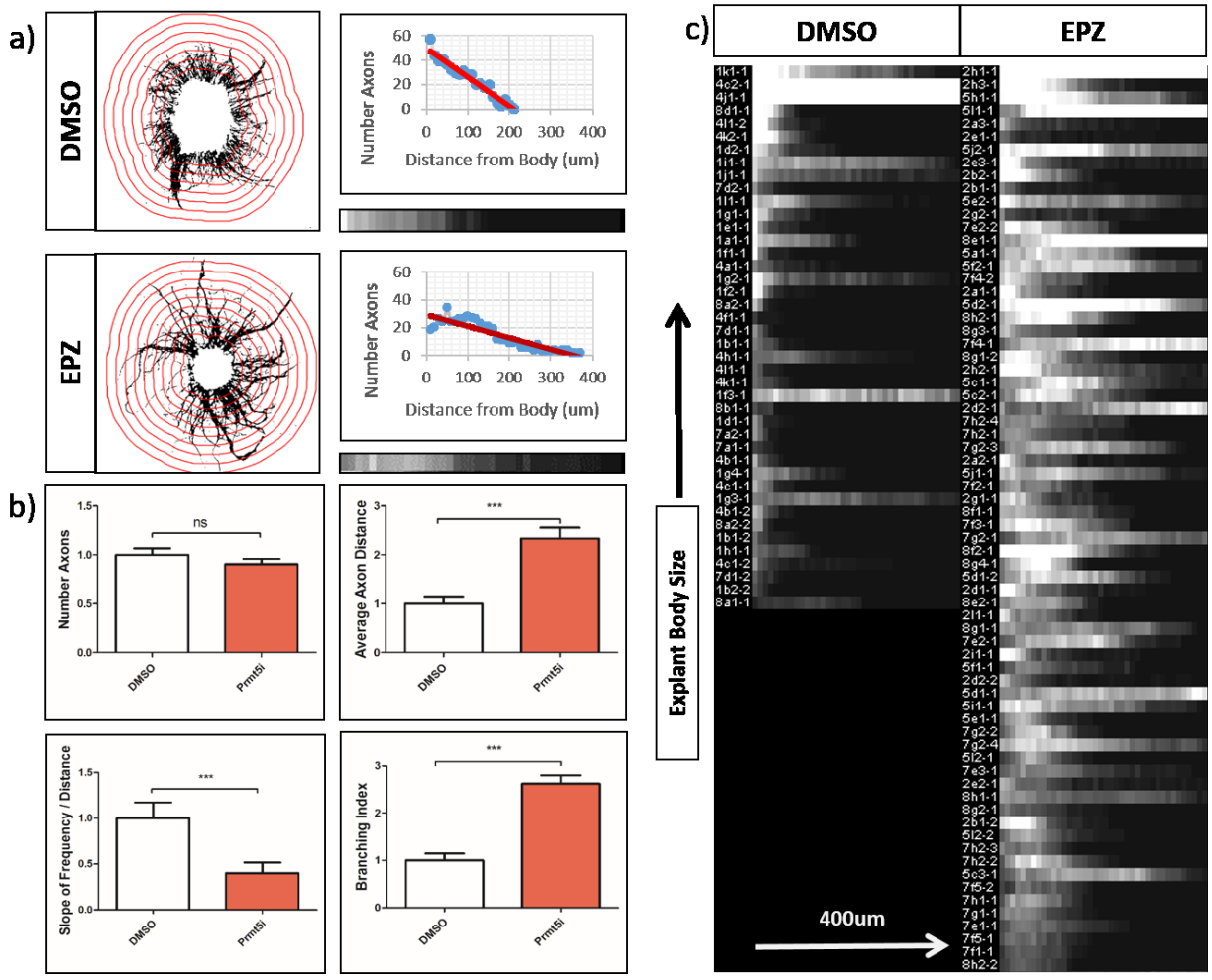

Figure 3: EPZ significantly increases axon outgrowth of embryonic ventral spinal explants. a) Example of the outgrowth analysis from axon masks of the previous explant images. The number of objects corresponding to axons are counted at each 10µm distance step from the explant body. The histograms are shown in the right panel, with the regression line to illustrate the slope of frequency/distance parameter below. Below the histograms are "heat maps" of the frequency as a function of distance, used to make panel c. b) 4 parameters derived from this analysis reflect major increase in axonal outgrowth. Without a significant difference in the total number per explant, there is a >2-fold increase in average length and branching. The slope parameter also reflects this shifted growth rate, as the average slope of the EPZ group is <50% that of the DMSO controls. c) Plots of the outgrowth profiles from all explants measured, sorted from top to bottom in order of explant body size, confirming a striking increase in outgrowth.

These measurements showed that while the total number of axons did not vary, they extended on average more than twice as far with greater levels of branching and/or defasciculating, and (in agreement) a more even distribution as a function of distance from the cell body as determined by the histogram regression slope (Fig. 3b, c).

3a.3) Post-mitotic conditional MN knockouts lack overt phenotypes in vivo*:* In parallel to these *ex vivo* experiments, I generated knockout mice for *Prmt1* and-*5*, using the conditional *Hb9*-Cre driver which is expressed in newly-born MNs. As expected by the absence of adverse phenotypes *ex vivo*, there was no detectable change in survival, basic motility, or weight gain of the progeny as of 10 weeks in the *Prmt5* knockout (Fig. S2). Conditional *Prmt1* mice displayed no noticeable phenotype either (data not shown). Given Prmt1's association with FUS in ALS, and the fact that mice expressing a truncated FUS using the same *Hb9* driver only begin showing symptoms after 3 months, I elected to leave them to age longer in case weight loss or other phenotypes present themselves (McGoldrick et al., 2013; Yamaguchi and Kitajo, 2012).

3b) *Prmt7*$^{-/-}$ mice have abnormal motor activity and NMJ morphologies

3b.1) The Prmt7$^{-/-}$ *clasping defect is reminiscent of many neurodegenerative disease models:* I first sought to characterize the observed "clasping defect" by scoring its severity. Wild-type mice aged at least 4 weeks reflexively extend their hindlimbs outward when lifted by the tail; however when *Prmt7*$^{-/-}$ mice are lifted, they appear to try to do so but are unable (Lalonde and Strazielle, 2011). Instead, they clench their paws and hold them convulsively inwards. Because I was unable to generate more full-body knockouts, these experiments were restricted to a small cohort, which was separated into young (>3 months), medium (3-8 months), and aged (>8 months) groups. I rated their clasping while blinded from 0 (absent) to 3 (severe). There was a robust correlation with the severity of the defect and their age and sex, with old male knockouts displaying it robustly, suggesting it to be progressively worsening (Fig. 5a, b). Though the severity was much more variable, I also found clasping in many heterozygotes. Clasping defects similar to this (and also dependent on age, sex, and genotype) have been described in many rodent models of neurodegenerative diseases, including motor neuron diseases SMA, ALS, and spinal bulbar muscular atrophy (El-Khodor et al., 2008; McManamny et al., 2002; Ricketts et al., 2014). While abnormal reflexes such as this have been observed resulting from defects in higher order motor control areas such as the cerebellum or striatium as well, all descriptions I could find in the literature described additional clear phenotypes which I did not observe such as tremors or major locomotion defects, even in *Prmt7*$^{-/-}$ aged >16 months (Chou et al., 2008; Lalonde and Strazielle, 2011; McKinney et al., 2008; Reddy et al., 1998). Thus, though brain defects cannot be ruled out as a cause of the clasping, I chose to investigate at the level of the PNS.

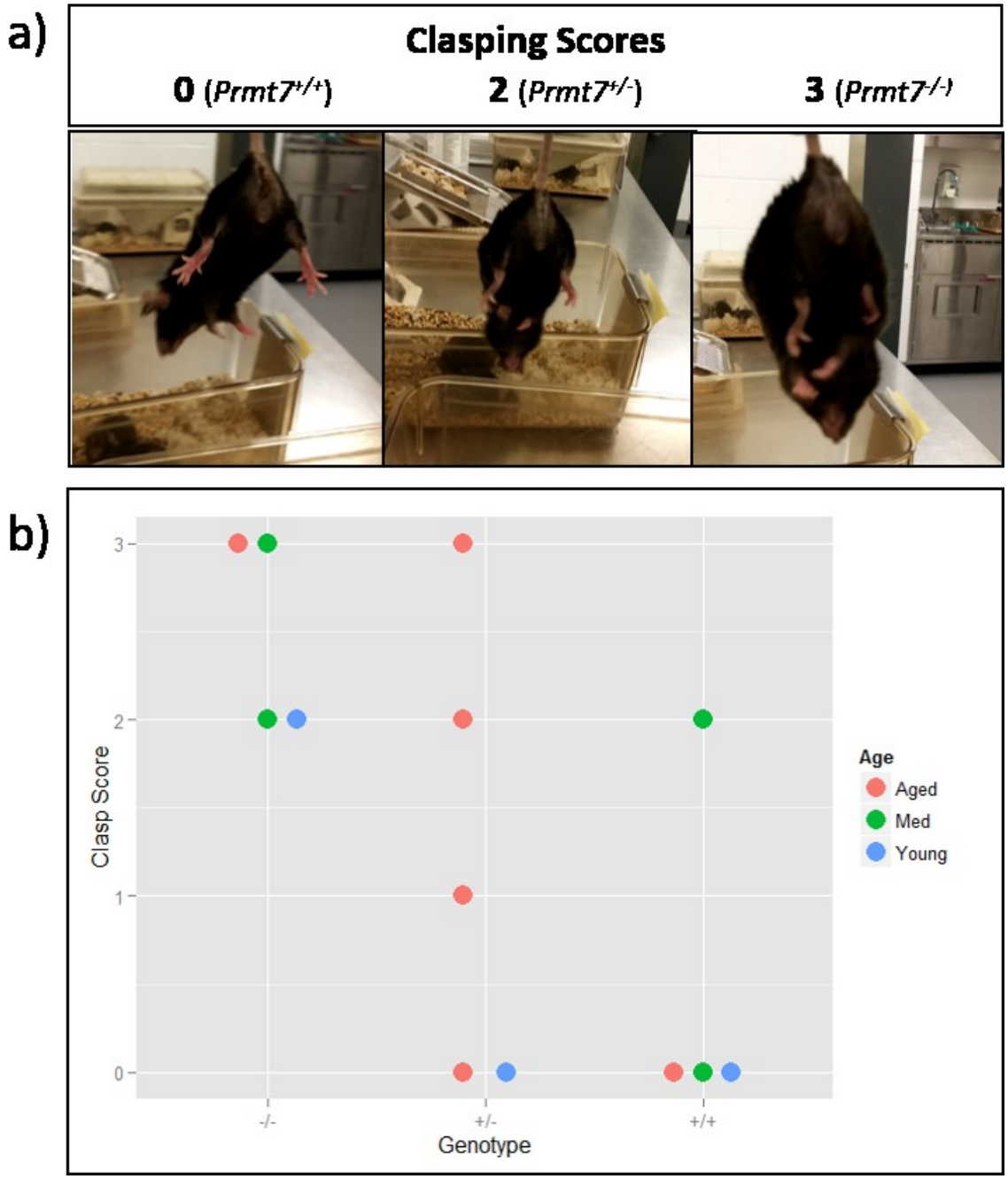

Figure 4: Prmt7 knockout mice display progressively worsening, sex-related clasping defects. a) Examples of age-matched wild-type, heterozygous, and knockout *Prmt7* mice. The first displays no phenotype, the second a moderate clasping, and the third severe clasping (I also scored "minor clasping" when the mice clasped only 1 paw at a time). b) Analysis of clasping phenotype across differently aged mice, divided into young (<3mo), medium (3-8mo), and aged (>8mo) mice. Nearly all knockouts displayed the full phenotype, with only 1 young and 1 medium displaying a moderate phenotype. Most old heterozygous mice showed some degree of the phenotype, while only 1 wild-type mouse showed any.

3b.2) Prmt7$^{-/-}$ *mice exhibit fragmented NMJ morphologies:* Postulating that a defect at the level of the NMJ could explain both the motor and muscle-wasting phenotypes, I stained the presynaptic terminal marker SV2 and postsynaptic marker BTX (which binds to AChRs on the NMJ) in sectioned tibalis anterior (TA) muscles from age-matched *Prmt7*$^{-/-}$ and wild-type mice. This revealed widespread degenerating NMJs with a fragmented morphology, characteristic of numerous neurodegenerative disease models as discussed below (Fig. 5a). Presumably because methanol was used as a fixative, the SV2 stain for many presynaptic NMJ terminals were disrupted, making it impossible to properly quantify the percentage which were innervated. However, of those I could visualize, many more wild-type NMJs were innervated than in *Prmt7*$^{-/-}$ muscle, suggesting that deinnervation could be a cause of the fragmented morphology and perhaps even clasping behaviour (Fig. 5b; Miledi and Slater, 1968).

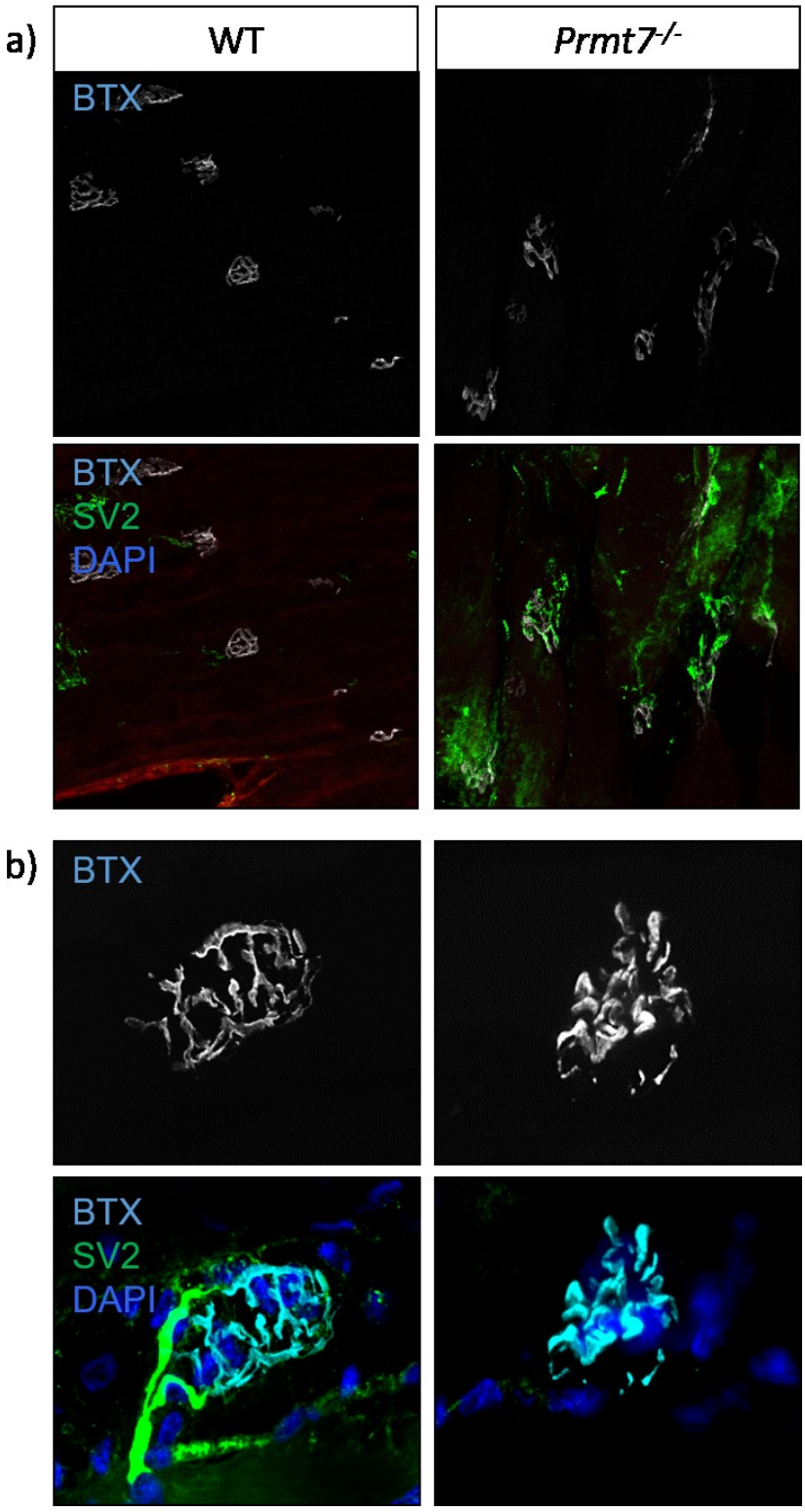

Figure 5: Neuromuscular junctions of *Prmt7*$^{-/-}$ mice have fragmented postsynaptic morphologies. a) *Btx* stains of the muscle endplate show less well-defined AChR clustering, larger areas, and fragmentation in knockout tissue relative to controls. b) Higher resolution NMJ images representative of *Prmt7*$^{-/-}$ and wild-type littermate NMJs. While I could not quantify the percentage where *Btx* costained with presynaptic marker *SV2*, many more could clearly be identified in the control group, pointing towards deinnervation in *Prmt7* knockout mice.

4. DISCUSSION

4a) Major PRMTs 1 and 5 are Dispensable for Early MN Development *In Vivo*

Given the importance of arginine methylation across a huge range of tissues and contexts, it is intriguing that the deletion of the major enzymes in post-mitotic lower MNs have not yet yielded any adverse effects in mice or cultured explants. It is more surprising considering their essential roles in neuronal development: PRMT1 was shown to be required for retinoic acid-induced differentiation in the cortex (retinoic acid also specifies motor neuron progenitors), and PRMT5 was found to be required to maintain the self-renewal potential of brain neural progenitor cells *in vivo* (Bezzi et al., 2013; Novitch et al., 2003; Simandi et al., 2015). If PRMT1 and -5 are indeed dispensable for motor neurons, this information is clinically relevant as well, as PRMTs 1 and -5 are receiving increasing attention as cancer targets; knowing that they can be inhibited safely in MNs inspires confidence that these essential cell types will not suffer side effects (Blanc et al., 2016; Chan-Penebre et al., 2015; Yoshimatsu et al., 2011).

Because the *Hb9* Cre driver used is only expressed right before MN progenitor terminal divisions into non-dividing MNs, it is unlikely that *Prmt1* or *-5* levels are significantly reduced in the progenitors, thereby not contradicting their previously described roles in neuronal specification or differentiation. This suggests that while these enzymes are essential for neuronal generation, they are no longer required in post-mitotic neurons as they form their connections and mature. This being said, there are a number of caveats to consider. Firstly, it is possible that the *Cre*-mediated excision of these floxed alleles failed to result in protein loss, as the *Cre-loxP* system has been known to fail due to poorly placed LoxP sites (Burgess, 2013; Han and Zhang,

2002; Kuhnert et al., 2008; Turlo et al., 2010); however our lab has validated the knockouts using other *Cre* lines and the *Hb9-Cre* is widely used for MN deletions, making this unlikely (Yu et al., 2009; unpublished). Regardless, motor neurons should be isolated from these mice as soon as they can be harvested to validate the absence of PRMT1 and -5. As an additional other caveat, while I showed expression of both in postmitotic embryonic cortical neurons (via Western blot), the expression of *Prmt5* and *-7* in embryonic *MNs* should be confirmed as well, ideally at several points throughout late embryogenesis.

Even if expression of *Prmt1* and *Prmt5* was abolished, the absence of a phenotype in mice is insufficient to conclude that the main arginine methyltransferases are dispensable for motor circuit development. If the enzyme turnover is minimal it is possible that even without transcribing more, embryonic MNs have enough to perform any necessary functions during axon guidance and synaptogenesis. Given the lack of proof of arginine demethylases *in vivo*, it is also possible that they modify the necessary proteins before being degraded, and that their dimethylated substrates remain stable – for example, a classical role of *Prmt5* is depositing repressive H3R4me2s marks, which could depress expression long after PRMT5 is depleted (Fabbrizio et al., 2002). In this way, PRMT1 or -5 could perform a key role regulating key genes with stable SDMAs which remain throughout the MNs' lifespan. Finally, other PRMTs are known to increase their activity when PRMT1 is removed, possibly due to an increased availability of unmethylated substrates – other arginine methyltransferases could thus be compensating for the loss of *Prmt1* and *-5*. This being said, PRMT9 (the only other type II PRMT) was found to be many times less active than Prmt5, as were all type I enzymes relative to PRMT1, so they would

only be expected to mask a phenotype if very low levels of arginine methylation are required (Blanc and Richard, 2017a; Hadjikyriacou et al., 2015).

4b) PRMT5 May Negatively Regulate Axon Outgrowth – A New Therapeutic Tool?

The inhibition of PRMT5 *ex vivo* yielded a profound increase in axonal outgrowth, more than doubling the average axon length after 48h. This outgrowth was consistent across a variety of measurements. While these experiments should be validated with genetic knockdowns or knockouts *ex vivo*, they are promising as a future therapeutic avenue. The peripheral nervous system (including MN axons) has a much better capacity to regenerate than the spinal cord or brain, but it has been shown that prolonged deinnervation (such as in ALS patients where axons continually degenerate, or old patients with slowed nerve regeneration) can lead to the functional loss of NMJs; this is thought to be a major contributor of the progressive neuronal death seen in motor neuron diseases (Sakuma et al., 2016). MNs which cannot connect to muscles eventually die, forcing the remaining pool to innervate their muscles, putting more stress on them and reducing the fidelity of motor commands. By boosting axon regeneration to meet this "critical period" of reinnervation, EPZ-015666 could be a powerful tool to help preserve motor function and ameliorate the progression of certain motor neuron diseases. Before considering use in humans or more human models, EPZ should be administered to mice with peripheral nerve injuries to assay whether it has the same outgrowth-increasing effect *in vivo* and with adult MNs. It was found to be bioavailable and non-toxic when administered twice daily

in two murine models of mantle cell leukemia, making it an excellent candidate for clinical use (Chan-Penebre et al., 2015).

Perhaps the most pressing question is whether EPZ can induce the same effects in the CNS. Spinal cord injuries in particular are notoriously difficult to treat, in part because axon regrowth across injury sites is actively inhibited by glial scarring (He, 2010). A variety of treatments have been proposed, with some having been tested in humans, ranging from local gene therapy to knock down the outgrowth-inhibiting *PTEN*, implantable scaffolds which better encourage axon growth; or "neural bypass" neural prosthetics which record activity directly from electrodes implanted in the motor cortex and relay it to receivers which stimulate MNs or muscles accordingly (Khaing et al., 2014; Ohtake et al., 2015; Sharma et al., 2016). These treatments have unfortunately met very limited success, can be highly invasive, and come with considerable risk. If the seemingly-harmless EPZ's outgrowth-promoting effects can aid CNS injury recovery, whether on its own or in conjunction with other treatments, it could be an invaluable therapeutic agent; currently, no pharmacological agents are approved clinically to aid nerve regeneration. This being said, motor neurons are particularly distinct from most projection neurons of the CNS in terms of their development, physiology, and expression. Their axons face very different environments when growing in embryos, or injured adult tissue, and respond differently as well – for example, while Netrin-1 guides commissural axons attractively in the CNS, it is a repulsive cue for lateral LMC MNs (Moore et al., 2012; Poliak et al., 2015). It is entirely possible that whatever pathway *Prmt5* is potentiating which opposes outgrowth is not present in CNS axons.

As to how PRMT5 activity antagonizes axon outgrowth, the diverse number of functions attributed to the protein lead to a massive number of possibilities. PRMT5 is known to interact with many molecules implicated in the transcriptional control of axon growth and regeneration, including PTEN/mTOR, PKA/CREB, and Jak/STAT3 signalling, suggesting it could be favoring these pro-growth pathways at the level of the nucleus or potentiating these pathways (Banasavadi-Siddegowda et al., 2017; Gao et al., 2012; Ohtake et al., 2015; Shin and Cho, 2017; Tsai et al., 2013; Wei et al., 2014). PRMT5 also has a well-characterized role in inhibiting snRNP biogenesis and therefore SMN-mediated splicing, while alternative splicing of 3' regions is known to be essential for targeting transcripts to the axon; perhaps PRMT5 inhibition increases the localization of growth-associated transcripts to the GC (Andreassi and Riccio, 2009; Fallini et al., 2016; Wei et al., 2014). It could even be acting in GCs themselves by regulating local signalling, such as that of cyclin-dependant kinases, which play a role in GC chemorepulsion and are regulated by PRMT5 (Aggarwal et al., 2010; Kawauchi, 2014). Future experiments could utilize immunofluorescence to identify whether PRMT5 expression or localization changes during axon guidance *in vitro* – by characterizing its spatiotemporal regulation, one could narrow down the candidate pathways.

4c) *Prmt7* and the Neuromuscular Junction

The clasping phenotype in conjunction with the dysmorphic NMJs of the *Prmt7*$^{-/-}$ mice are intriguing, given how frequently similar defects are seen in rodent disease models. On the other hand, of course, the diverse number of potential causes make finding the responsible mechanism

(or even responsible cell type) a daunting task. The NMJ phenotype could have arisen during NMJ assembly, maturation, maintenance in adults, or even as a consequence of impaired regeneration. It could be caused by defects in MNs or their axons, Schwann or endoneural cells of the peripheral nerve, perisynaptic Schwann cells, myofibers, SCs, or some systemic process. In light of previous studies with *Prmt7* mice, however, the muscle does seem to be a likely candidate. Arginine methyltransferases have been strongly associated with skeletal muscle development and regeneration, particularly in SCs; given that SCs also participate in NMJ regeneration, it is plausible that *Prmt7* has an additional as-yet undiscovered role in promoting their maintenance from the muscle side (Blanc et al., 2016; Liu et al., 2015). Jeong *et al.* also postulated that PRMT7 regulates muscle metabolism through PGC-1α, which was reported to be an important retrograde signal by which MNs and myofibers coordinate their type (Arnold et al., 2014; Jeong et al., 2016). Whether this can explain all of the observed degenerating NMJ phenotype is however unclear; conditional knockouts in mouse myofibers will be required to definitively determine their contribution. Ultimately, conditional knockouts and/or *in vitro* models of the neuromuscular system with knockdowns in the different tissues will be required to properly demonstrate where and how PRMT7 is required. Though PRMT7 protein was found in embryonic cortical neurons, I was unable to search in embryonic or adult MNs; before anything else, it should be determined whether they and Schwann cells even express it.

It is also unclear how this and the clasping phenotype are connected, if at all. If the fragmented NMJs significantly alter synaptic transmission, it could certainly lead to abnormal motor activity, however the fact that clasping is restricted only to the hindlimb muscles makes that unlikely (though it would be interesting to check forelimb muscles as well for NMJ

morphology). This also makes a widespread role for PRMT7 in nerves less likely (for example, regulating a process in myelinating glia), as one would expect a more widespread motor phenotype in that case as well. A role for PRMT7 in projections neurons, however, could reconcile both phenotypes. Hindlimbs are thought to be most affected in a range of neurological disorders simply because the upper motor neurons (located in the cortex) must project so much further to reach their MN effectors, presumably putting their axons under greater stress. This could also explain the progressive nature of the phenotype: if PRMT7 knockouts have reduced axon health, these corticospinal projects could deteriorate, resulting in altered MN activity which could in turn produce defective NMJs. By removing *Prmt7* in projection neurons, one could see whether the clasping and/or NMJ phenotype is recapitulated. If it does play a role in axonal health, characterizing its contributions could elucidate new therapeutic pathways for motor neuron diseases, though this could be the case even if it does act indirectly from a different tissue such as the muscle.

4d) Conclusion: New Roles for Arginine Methlytransferases in Neuronal Circuit Development

While small in scope, my experiments served to illuminate new potential roles for arginine methylation in MN circuit development and maintenance. Taken alone, the negative result that deleting the major enzymes *Prmt1* and *-5* in MNs has no deleterious effect is interesting; the fact that the latter may actually antagonize axon outgrowth makes it that much more tantalizing. Though all details of *Prmt7*'s involvement in motor control and NMJs remain unclear, the striking

resemblance between these phenotypes and those of numerous disease models also makes this an attractive avenue to study (Engel et al., 2003; Lee et al., 2011; Moloney et al., 2014; Rocha et al., 2013; Sleigh et al., 2014).

The most pressing future experiment will be to evaluate whether *Prmt5* depletion or inhibition can aid regenerating adult motor axons *in vivo* (after validating the loss of expression in *Hb9-Cre;Prmt5*$^{2L/2L}$). As PRMT1 inhibitors (and more general inhibitors of arginine methylation) have been made, these too could be tested for outgrowth-boosting potential (Blanc and Richard, 2017a). As for PRMT7, it is difficult to say whether the defects described here will be applicable to human disease, however by finding its substrates and mechanism of action, one could hope to identify new pathways which promote neuromuscular health. Much more work is required to validate and further characterize the role of arginine methylation in neuronal circuit development, but these results suggest that it is a worthwhile endeavor. The novel association of an entire class of enzymes with peripheral circuit development and function opens up a whole new avenue of potential therapeutic targets, and will help improve our understanding of how the nervous system self-organizes and maintains its trillions of connections.

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SUPPLEMENTARY FIGURES

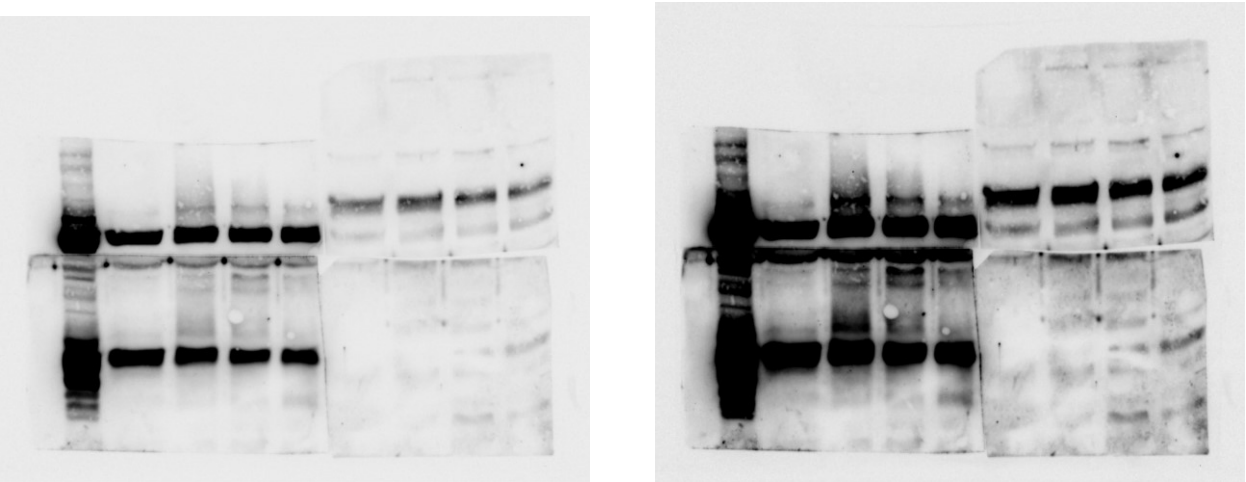

Fig. S1 Raw western blots from Fig. 1. Blots are shown with high (left) and low (right) exposure. The leftmost lane is from lysed HEK293 cells; the other 4 correspond to neurons at days 1-4 *in vitro*. The top blot is Prmt5 (42-44kDa), top right Prmt7 (72kDa), bottom left Prmt1 (78kDa).

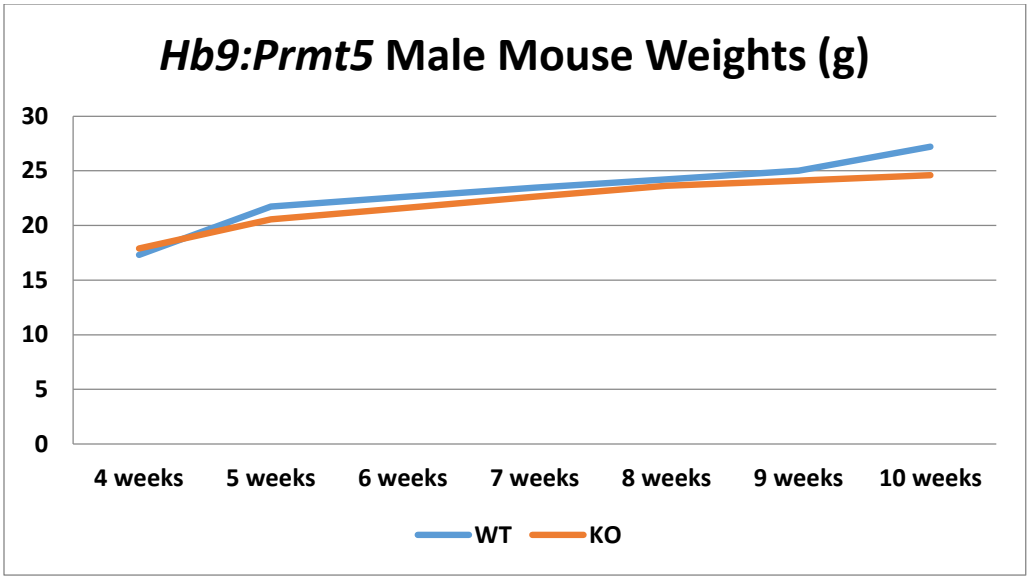

Fig. S2: *Hb9-Cre:Prmt5*$^{2L/2L}$ mice have normal weights for at least 10 weeks postnatally