DOES MOTOR LEARNING AFFECT THE NEUROMUSCULAR JUNCTION?

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Does Motor Learning Affect The Neuromuscular Junction?

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The activity-dependent regulation and maintenance of the neuromuscular junction, where the chemical synapse between motor neurons and muscle fibers initiating contraction occurs, play a critical role in the formation of memory-like effect with or without the presence of the spinal cord. Prior work has shown that neurons within the spinal cord can learn about environmental relations without input from the brain (Grau et al., 2014, *Neurobiol. Learn Mem, 108,* 155-171). For example, rats that have undergone a spinal transection can learn to maintain a hindlimb in a flexed position to minimize exposure to a noxious shock. Interestingly, after this learning has occurred, cutting communication with the muscle (i.e. sciatic denervation) does not eliminate the flexion response. This implies that a peripheral modification within the muscle helps maintain the flexion response over time. My hypothesis was that this involved an alteration at the NMJ by acetylcholine receptor (AChR)-mediated neural communication. The degree to which the AChR is engaged depends upon both its subunit composition and whether it is positioned in the neural membrane. Past work has shown that inactivity leads to the movement (trafficking) of AChR out of the membrane, which would reduce the strength of the response elicited by the release of acetylcholine (ACh). My premise was that learning leads to a lasting

increase in flexion duration because it has the opposite effect, and traffics AChR into the neural membrane. To explore this possibility, I sought to compare the ratio of AChR protein within the cellular membrane portion to that contained within its interior (cytosol). This required a fractionation procedure to separate the membrane and cytosolic components. Once the tissue was separated, protein antibodies and Western blotting were used to assess AChR protein levels in muscle tissue from rats that have, or have not, undergone training. While fractionation is routinely used to assess receptor trafficking in neural tissue (e.g., Huang et al., 2016, *Exp Neurol, 285*, 82-95), this procedure has not been widely applied to muscle tissue. Thus, the majority of my thesis has focused upon developing an effective fractionation procedure to explore the protein changes within the neuromuscular junction in the skeletal muscle. We specifically developed a protocol for subcellular fractionation of rat skeletal muscle tissue. The purity of the membrane and cytosolic fractions was validated by Western blot analysis and probing with "house-keeper" marker proteins specific for each cellular compartment. The results showed relatively high purities of each fraction and significant difference in the levels of marker proteins among two fractions. This protocol allows the membrane and cytosolic cellular compartments from muscle samples to be rapidly isolated without the use of an ultracentrifuge and thus is time-efficient.

DEDICATION

I would like to dedicate my thesis work to my family and many friends who have supported me throughout the challenges of undergraduate career and life. Without their words of encouragement and endless love I would never have achieved my academic goal.

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NOMENCLATURE

ACh	acetylcholine
AChR	acetylcholine receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BSA	bovine serum albumin
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
QS	quantity sufficient
SCI	spinal cord injury
TA	tibialis anterior
TBST	Tris-buffered saline 0.1% Tween-20

CHAPTER I

INTRODUCTION

The spinal cord is involved in the execution of movements by translating motor commands through the control areas within the brain and transmitting them out to the muscles. Being exceptionally dynamic and complex in its functionality and electrophysiological changes, the spinal cord is of a great interest in studies of learning in the rat. Although learning is typically assumed to depend upon the neural systems in the brain, two simple forms of spinal cord learning, habituation and sensitization, have been observed in the vertebrate model (for review see Groves & Thompson, 1970; Mcmahon & Malcangio, 2009; Thompson & Spencer, 1966; Thompson 2009). Furthermore, using traditional learning paradigms, studies have demonstrated that the spinal cord neurons can also support more complex forms of learning, such as Pavlovian (Alkon, 1984; Bitterman, 1988; Cook & Carew, 1986; Grau, Salinas, Illich, & Meagher, 1990; Hoyle, 1980; Kandel & Schwartz, 1982; Patterson, Cegavske, & Thompson, 1973) and instrumental learning (Grau et al. 1998). As a result, these studies suggest that a learned response can occur through the induction of neural plasticity within the spinal cord.

In agreement with prior studies, recent work suggests that some forms of learning may not require brain input and may even have a peripheral effect at the neuromuscular junction (NMJ). In these studies, rats received a spinal cord transection at the second thoracic vertebra, completely disconnecting the spinal cord from the brain (Grau et al., 2012). The next day, animals were placed in tubes and had a stimulating electrode placed in the tibialis anterior (TA) muscle and a contact electrode taped to the plantar surface of their paw. A salt solution was placed under the animal so that the tip of contact electrode was at a depth of 4mm. Animal then

received leg shock whenever the contact electrode touched the salt solution below. Animals that were trained with response-contingent leg shock learned to maintain the shocked leg in a flexed position that minimized net shock exposure (Grau et al., 1998). To verify that the learning depended upon communication with the spinal cord, Crown et al. (2002) cut the sciatic nerve, which carries signals to/from the TA muscle. As expected, cutting the sciatic nerve blocked learning, implying that spinal neurons play an essential role in the acquisition of the learned response (Crown et al., 2002).

More recently, Hoy (2011) assessed the effect of cutting the sciatic nerve after the behavioral response was acquired. Surprisingly, this manipulation had no effect on the maintenance of the behavioral response or on the facilitation (higher response criterion) of learning. This finding implies that the process that maintains a flexion response (the behavioral memory) does not depend upon continued motor output from the spinal cord. Rather, it appears that spinal learning brings about a peripheral modification, presumably at the NMJ that helps maintain the behavioral response. Immunohistochemical analyses confirmed that training induces an alteration in receptor function at the NMJ (Hoy, 2011). In particular, learning increased the density of the acetylcholine receptors (AChR), which are involved in mediating muscle contraction at NMJ, and increased the trafficking of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; responsible for fast synaptic transmission in the central nervous system) and NMDA (N-methyl-D-aspartate; involved in synaptic plasticity and memory function) receptors to the membrane. Further, both an AChR and a NMDA receptor (NMDAR) antagonist microinjected into the muscle disrupted the acquisition and maintenance of the instrumental response (Hoy 2011).

The results from prior work imply that efferent output from the spinal cord can enhance motor output by up-regulating NMJ function. If this is true, electrophysiological stimulation of the efferent nerve might substitute for the spinal input and drive an increase in leg flexion. M. Strain (2016) tested this by pairing electrophysiological stimulation of the common peroneal nerve (a nerve located in the lower leg that provides motor function and sensation) with shock to the TA muscle. Paired stimulation produced a lasting increase in flexion duration and this was true even when communication with the spinal cord was disrupted by means of a sciatic cut. Taken together, the findings suggest that the NMJ supports a kind of activity-dependent plasticity and may have been associated with the neurobiological changes of AChRs that act to regulate the maintenance of a muscle contraction.

The functional properties and maintenance of the NMJ provide a complex signaling pathway that regulates contractile activity in the skeletal muscle. During the development of the neuromuscular junction, the nicotinic AChR channels undergo progressive electrophysiological modifications. In embryonic muscles, AChR channels exhibit long open time and low conductance (slow channels) while in mature muscles they show a short open time and high conductance (fast channels) (Sakmann and Brenner, 1978). This shift is linked to a change in AChR subunits wherein the γ-subunit is replaced by the ε-subunit (Gu and Hall, 1988; Mishina et al., 1986; Witzemann et al., 1989). In addition, Martinou and Merlie (1991) demonstrated that this reciprocal change in γ and ε levels was also observed in mice; the level of ε-mRNA increased about 10-fold between day 2 and day 15 in the postnatal stage and was approximately 25-fold higher than that of γ-mRNA in the mature muscle.

Although little is known regarding the factors that regulate the developmental shift in NMJ function, others have investigated how denervation affects γ and ε expression. Schuetze and

Vicini (1984) assessed the effect of denervation on the postnatal development of fast channels and found that the conversion from slow fetal-type to fast adult-type AChR channels was delayed after the soleus nerve was transected in neonatal rats. As a result, ε-subunit regulation is thought to be dependent on extrinsic factors such as nerve-evoked activity rather than intrinsic factors, as suggested in Brenner and Sakmann (1983; Brenner et al., 1983, 1987). In agreement with this finding, the levels of mRNAs encoding the γ-subunits of AChR were altered in an unusual way and were increased dramatically (about 3-4 fold) 4 and 8 days after the sciatic nerve had been cut in the adult muscle in mice (Martinou and Merlie, 1991). By contrast, the level of ε-mRNA was unaffected after denervation. This reverse transition from ε to γ expression implies that the neonatal stage of NMJ may have been promoted by denervation.

NMDAR and AChR function is regulated by the trafficking of the receptors to/from the neural membrane. This movement of receptors, and their subunits, can bring about a shift in function without altering the total amount of receptor protein. As a consequence, to determine whether learning affects the trafficking of the NMDAR or AChR, a fractionation procedure is needed to separate the membrane and intracellular (cytosol) portions of the cell. While fractionation is regularly performed on neural tissue (e.g., Huang et al., 2016), this procedure has not been commonly used to investigate receptor trafficking within the NMJ. The purpose of the present study was to establish an effective fractionation protocol to separate the membrane fraction from the interior (cytosol) of the cell. To verify the effectiveness of my procedure, I performed Western blotting for the proteins neuronal cadherin (N-cadherin) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). N-cadherin is a transmembrane protein that plays a role in cell adhesion and is associated with the membrane fraction. GAPDH is an

enzyme that is involved in the intracellular breakdown of glucose for energy and, if my fractionation procedure is successful, it will be isolated to the cytosolic fraction.

CHAPTER II

METHODS

Tissue

The muscle tissue used in this experiment was collected from rats that were used in approved protocols and euthanized. These subjects were scheduled to be euthanized independent of the present research. Because the tissue was collected after the subject's heart had stopped (the criterion used to define death by the Institutional Animal Care & Use Committee at Texas A&M University), and because the euthanasia was unrelated to the current project, the use of this tissue is considered "tissue sharing" and does not require an independent animal use protocol or amendment. The plan for collecting the muscle tissue was submitted, and approved, by Melanie Landis, the Animal Welfare Office Laison within the Animal Welfare Office on October 3, 2016, prior to any tissue being collected.

Tissue fractionation

The tibialis anterior muscles (0.8 cm below the knee joint) were collected and snap frozen in liquid nitrogen for total protein extraction and subcellular fractionation. Briefly, 0.8 cm of tibialis anterior muscles was homogenized in buffer (14.2 mM Tris [pH 7.5] and 14.7% sucrose in 14 ml dH_2O) containing two Complete Mini EDTA-free Protease Inhibitor tablets (Roche Diagnostics). The homogenates were then centrifuged at 5,000g for 5 minutes at 4℃. The pellet (P1) was removed. The supernatant (S1) was collected and centrifuged at 17,000 g for 30 minutes at 4℃. The new pellet (P2) was collected and resuspended in 150 μl cold PBS and used as the membrane fraction. The supernatant (S2) was used as the cytosol fraction. All samples were sonicated and stored at -80℃.

Western blot analysis

Protein concentration was determined by Bradford assay (BioRad, Hercules, CA) for membrane and cytosolic fractions. As a two-day process, Western blotting consisted of gel electrophoresis, blot transfer from gel to PVDF membrane, primary antibody incubation overnight, secondary antibody incubation, and chemiluminescent detection. On Day 1, the preparation steps included making 1X transfer buffer containing 20% methanol from 10X stock solution [30.3 g Tris base, 144.1 g Glycine, and quantity sufficient (QS) to make 1 liter], 1X running buffer diluted from 10X Tris/Glycine/SDS buffer stock solution (BioRad, Hercules, CA), and heating protein samples for 10 minutes; both buffer solutions were remained cold before use. Equal amounts (10 μg) of total protein from each of the fractions were subjected to SDS-PAGE with 15% Tris-HCl Criterion™ precast gels (BioRad, Hercules, CA). Subsequently, cold running buffer was added to the tank to the max-filled line indicated on the side of the tank. Gel electrophoresis was set at constant voltage (suggested 180V for Criterion™) for 1 hr and 15 minutes. Following gel electrophoresis, the gel apparatus was disassembled according to the manufacturer's instructions. The gel was carefully taken out from the gel plate and equilibrated in transfer buffer for 10 minutes. Twenty minutes before gel electrophoresis was complete, blot transfer supplies were prepared: two fiber pads and filter papers (Criterion™ blotter filter paper, BioRad), per gel, were soaked in transfer buffer. PVDF membrane (Immun-Blot® PVDF Membranes for protein blotting, BioRad, Hercules, CA) was activated through a series of steps: 30 seconds in methanol, 1 minute in water, subsequently placed in transfer buffer and ready for use. The order of gel sandwich was set up as followed: black side of transfer cassette, fiber pad, filter paper, gel, PVDF membrane, filter paper, fiber pad, and red side (if using Criterion™) of transfer cassette. An ice pack was placed in transfer tank (BioRad Semi-dry transfer apparatus)

to prevent the temperature from getting too high. The tank was filled with cold transfer buffer to max-filled line. The voltage was set at 100V and blot transfer was run for 1 hour. After run is complete, the blot was cut into half at 75 kDa. The upper portion of the blot for N-cadherin detection was incubated in non-fat milk (5%) in Tris-buffered saline 0.1% Tween-20 (TBST) whereas the lower portion for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) analysis was incubated in 5% bovine serum albumin (BSA) for blocking at room temperature for 1 h. The blots were subsequently incubated with N-cadherin (130 kDa) mouse monoclonal antibody (BD Biosciences, U.S., 1:2000) and GAPDH (37 kDa) rabbit monoclonal antibody (Cell Signaling Technology, U.S., 1:2000) for membrane and cytosolic fraction, respectively, at 4℃ overnight with gentle rocking. Primary antibody incubation was followed by TBST washes (3 times x 10 min) before the blots were incubated with secondary antibody at room temperature for 1 h. An hour following secondary antibody incubation, the blots were washed in TBST (3 times x 10 min). Signals were detected by chemiluminescence (ECL Substrate kit from BioRad), and immunoreactive proteins were visualized using Fluorchem HD2 (ProteinSimple, Santa Clara, CA). Band densitometry was next analyzed.

Statistics

The results were analyzed by analysis of variance (ANOVA) performed using the Prism software package. Data are presented as means ± the standard error of the mean (SEM). Difference were considered significant when the obtained p-values were < 0.05.

CHAPTER III

RESULTS AND DISCUSSION

N-cadherin is a transmembrane protein coded by the CDH2 gene and a cell-cell adhesion molecule that is highly expressed by neural tissue and muscle. The critical role of N-cadherin in skeletal myogenesis and promoting differentiation is well established (Redfield et al., 1997; Goichberg et al., 1998). Found across the plasma membrane, N-cadherin plays a role in embryonic development and tissue morphogenesis (Halbleib and Nelson, 2006; Luo, 2003). N-cadherin has been used as a loading control for total proteins in studies of osteoblast proliferation and survival (Haÿ et al., 2009). These observations suggest that N-cadherin would be a suitable protein label for the membrane fraction. GAPDH, on the other hand, is a well-recognized marker for cytosolic fraction and can be used as a loading control for total proteins (Dimauro et al., 2012; Blesson et al., 2014; Rahimi et al., 2014; Badin et al., 2013). As expected, the membrane fraction was characterized by highly abundant N-cadherin along with barely detectable GAPDH. Conversely, the cytosolic fraction was enriched with GAPDH and essentially undetectable N-cadherin (Fig. 1A).

I first analyzed the brightness of the target band by subtracting the background from the band intensity to obtain the background corrected (BC) average; the higher the protein level is, the brighter the band would be. I then inverted the Western blots to better distinguish the darker areas in contrast to the brighter areas (as shown in Fig. 1A); the darker the band is, the higher the protein of interest is detected. N-cadherin levels in membrane fraction (926.84 ± 327.69) were significantly higher than in the cytosolic fraction (513.32 ± 181.49), $F(1, 7) = 257.43$, $p < 0.0001$. Furthermore, GAPDH levels in cytosolic fraction (3315.14 ± 1172.08) were significantly higher

than in the membrane fraction (2344.70 ± 828.98), $F(1, 7) = 138.53$, $p < 0.0001$. These analyses (Fig. 1B and 1C) showed little contamination between membrane and cytosolic fractions, indicating that a successful fractionation procedure was established and the protocol is suitable for skeletal muscle tissue.

A possible explanation for the bright band of GAPDH in the membrane fraction may come from the insufficient centrifugal force to separate membrane-bound proteins from the cytosol. Technically, however, it is not possible to obtain 100% purify of subcellular fraction. Nonetheless, GAPDH perhaps may have translocated to the external surface of the plasma membrane in the cell for metabolic function.

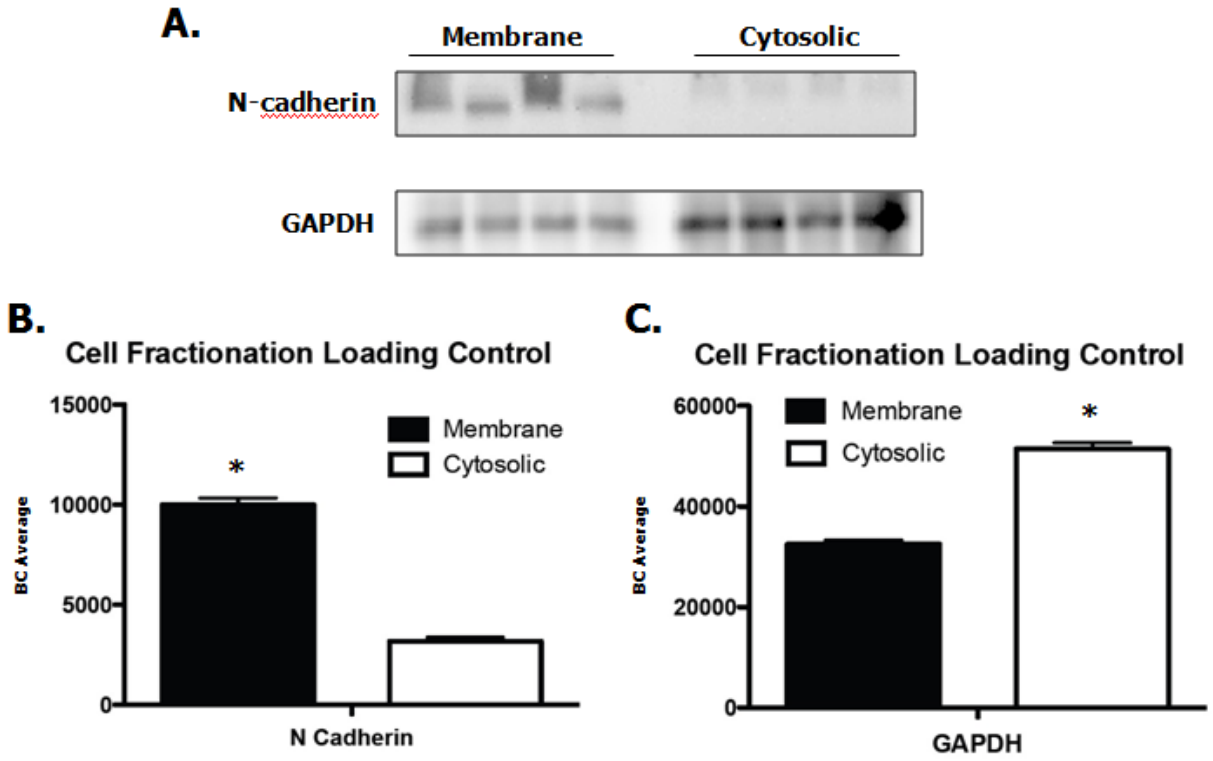

Figure 1: Representative Western blots of N-cadherin and GAPDH in membrane and cytosolic fraction (A). Comparison of measured BC average of N-cadherin in membrane and cytosolic fraction (B). Measured BC average of GAPDH in cytosolic fraction along with average in membrane fraction (C). (n = 8)

CHAPTER IV

CONCLUSION

Prior work using immunohistochemistry suggests that behavioral training can affect the trafficking of the AChR and NMDAR at the NMJ. To study this process, and whether learning affects the subunit composition of the receptors, procedures were needed that would allow the assessment of protein content associated with the plasma membrane versus interior cytosol. The current proposal sought to address this issue by developing a fractionation procedure that could be used to dissociate the membrane and cytosol fractions in muscle tissue.

My results demonstrate how a low cost, and relatively simple, fractionation procedure can be used to separate the membrane and cytosol fractions. Importantly, the procedure does not require the use of an ultracentrifuge. I was also able to show that N-cadherin and GAPDH can be used to evaluate the relative success of the fractionation procedure. Using these markers, I was able to show that the fractionation procedure was quite robust, yielding highly statistically significant differences with a moderate sample. Of course, as with any fractionation procedure, the separation is not perfect and yielded some co-labeling across samples. Nonetheless, relative purity of each sample was comparable to that reported by others (e.g., Huang et al., 2016).

Having established an effective procedure for fractionating muscle tissue, future experiments will examine how behavioral training affects the distribution of the AChR and NMDAR. This will reinforce the results obtained using immunocytochemistry and provide a method to determine whether training affects the subunit distribution of the receptors. My hypothesis is that instrumental training will increase AChR and NMDAR protein levels within the membrane fraction. It is further posited that injury could cause the AChR to revert to a

developmentally earlier state containing a higher proportion of the γ-subunit. Learning could potentially also affect the relative proportion of the γ and ε-subunits.

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