P2X2 Receptors in Mouse Skeletal Muscle

by

Connie Truong

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of Bachelor of Science

With Honors in Neuroscience

University of Michigan

April 1, 2013

Mentor:

Dr. Hume

ACKNOWLEDGEMENTS

I would like to thank Dr. Richard Hume for his guidance and for being a wonderful mentor. He has always challenged me and allowed me to be independent in research. Through working with him, I have grown a lot as a scientist.

I would like to also thank Dr. Isabel Martinez-Pena y Valenzuela for helping me with the electroporation experiments and for volunteering a significant amount of her time to assist me. Thank you to Dr. Sukanya Punthambaker for your willingness to take the time to answer my questions about molecular biology and other things at lab. To Dr. Marcelo P. de Oliveria and Dr. Mohamed Aittaleb, thanks for teaching me how to image and culture C2C12 cells.

I would like to thank Dr. Mohammed Akaaboune, whose willingness to share the resources that made this project possible.

INTRODUCTION

The first part of this introduction will be focused on synapses and receptors. These general neurobiology topics can be found in a variety of textbooks in this field. For my introduction, I will be referencing *Neuroscience: Exploring the Brain* by Bear, M. F. et al. Next, I will provide background information on the P2X2 receptor and the nicotinic acetylcholine receptors in the neuromuscular junction, which is the focus of my thesis. Lastly, I will go into detail about the goals of the project.

Cells in the nervous system communicate via synapses, where neural information is transferred from one cell to another cell otherwise known as synaptic transmission. Synapses can be found throughout the nervous system. This mechanism of the nervous system is critical since it has an integral role in many different biological functions. These include, but are not limited to, sensory information sent to the brain to perceive pain, taste, hot, or cold. Synaptic transmission also includes signals from the brain sent to muscles. For example, if someone accidentally sets his hand on a hot stove, the sensory information that the brain receives will be perceived as noxious pain. Then signals will be sent from the brain to motor neurons to cause the individual to lift his hand off the hot stove.

In all synapses, the ultimate information that is passed on during synaptic transmission is in an electrical form. This electrical form is known as action potentials if the cell is depolarized past threshold. At threshold, voltage-gated channels open and generate action

potentials that lead to excitation. However, when inhibitory messages are passed on, the signal is hyperpolarizing, which makes the cell more negatively charged. The driving forces behind action potentials are an electrical gradient and a chemical gradient. This electrical form of communication between cells in the nervous system specifically occurs at the synapse.

Defining features of a synapse include the pre-synaptic cell and the post-synaptic cell; information is passed from the pre-synaptic cell to the post-synaptic cell. There are two types of synapses: electrical synapses and chemical synapses. Electrical synapses are more common in the central nervous system (CNS), specifically in the brain. The CNS is composed of the brain and the spinal cord. In electrical synapses, two neural cells are joined together through gap junctions, which are ion channels. This set up for allows positive charge to flow freely through the gap junctions and depolarize the post-synaptic cell directly. Furthermore, the cells that participate in electrical synapses are usually neurons.

On the other hand, chemical synapses are throughout the peripheral nervous system (PNS). In the PNS, a synapse is usually composed of a neuron and a cell. In this type of synapse, neurotransmitters are carried in vesicles to the axon terminal of the pre-synaptic neuron, and then neurotransmitters are then released into the synaptic cleft, which is the area between the pre- and post-synaptic cells. Neurotransmitters act as a ligand, which is a molecule that can bind to their corresponding receptors and activate them. In chemical synapses, the chemical message, which is initiated by neurotransmitters binding to their

respective receptors, is converted into an electrical signal. This conversion from chemical to electrical occurs when the neurotransmitters cause ion channels to open directly or indirectly by downstream signaling of these receptors, which activates ion channels. Some examples that involve chemical synapses are: the neuromuscular junction, the site where effects of psychoactive drugs are carried out, and where the inhibitory effects of alcohol are carried out.

The synapse of interest in my project is the neuromuscular junction (NMJ). At this site, motor nerves from the CNS make a synapse with muscle cells in the PNS. To specify, the pre-synaptic cell is the motor neuron and the post-synaptic cell is the muscle fiber. The motor neuron causes action potentials to occur in the muscle fiber it innervates. Essentially, the neuromuscular junction can be found on each skeletal muscle fiber; this synapse is where the initial stages of muscle contraction occur.

NMJs occur at a specific location on the muscle fiber, and this location is marked by the part of the muscle fiber that has a group of numerous superficial folds. The part of the superficial folds that is closer to the surface of the muscle fiber contains a high density of receptors. Neurotransmitters are contained inside numerous vesicles at the axon terminal of the pre-synaptic neuron. In synaptic transmission that causes excitation, the pre-synaptic neuron becomes depolarized. When this occurs, calcium ions enter the axon terminal of the pre-synaptic neuron, which cause the neurotransmitter vesicles to undergo exocytosis and fuse with the membrane pre-synaptic membrane. The contents in the vesicles are then released into the synaptic cleft. The vesicle-rich areas in the axon

terminal are aligned precisely with the concentrated areas of receptors at the neuromuscular junction to provide the most efficient neuronal transmission.

The most prevalent receptor found at the NMJ is the nicotinic acetylcholine receptor, which uses acetylcholine as its ligand. This type of channel is a ligand-gated ion channel; this means that when its ligand binds, the channel becomes activated and opens to allow ion exchange to occur between the intra- and extracellular regions. Sodium and potassium ions are able to travel through the AChR. Furthermore, the AChR causes synaptic excitation. This occurs because when this ion channel opens, cations tend to flow into the cell down their concentration gradient and also since the cell tends to have a more negative charge relative to the extracellular environment.

Another type of acetylcholine receptors that exist in the body is the muscarinic acetylcholine receptors (mAChR). The difference between this type of receptor and the nAChR is that the mAChR is not an ion channel. Instead the mAChR is a G-protein coupled receptor. Some physiological roles of the mAChR are that it can slow down heart rate and cause smooth muscle contraction. However, for the purposes of this thesis, the nicotinic acetylcholine receptor (nAChR) will be focused on. From here, I will refer to the nicotinic acetylcholine receptor as the AChR for the remainder of this thesis.

There are also many other types of receptors that are ion channel receptors. One example is the P2X2 receptor, which is another receptor examined in this study. This receptor is also a ligand-gated ion channel; it uses ATP as its ligand. Both of the nicotinic

acetylcholine receptor and the P2X2 receptors mediate synaptic transmission that causes excitation. The structure of these two channels is very different as they come from different receptor families.

There are three ligand-gated ion channel families that have been known to date. These are the cys-loop receptor superfamily, the purinergic receptors, and the ionotropic glutamate receptors. The cys-loop receptors are composed of five subunits and they are organized in a pentameric manner to form a central pore. Purinergic receptors are composed of two plasma membrane-spanning domains with a large exracellular domain. These purinergic receptors have three subunits that form a pore, and these subunits can be composed of identical or different subunits. Combinations of different subunits from P2X receptors have different properties. A common characteristic of all purinergic receptors is that ATP is their ligand. An example of a purinergic receptor is the P2X2 receptor. Ionotropic glutamate receptors are composed of four subunits, and each subunit has an extracellular amino terminal; all of these receptors use glutamate as the ligand. Surprisingly, although the P2X2 receptor and the acetylcholine receptor are structurally different and come from different ligand-gated ion channel families, there appears to be an interaction between these two receptors. There have been previous studies done that demonstrated a cross-inhibition interaction that takes place between these two receptors.

Regarding the goals of this thesis, the AChRs (cys-loop) and the P2X2 receptors (purinergic) were studied closely. Both of these receptors are found throughout the CNS and PNS (Albuquerque et al., 2009; Ryten et al., 2007). Biological functions of AChRs in

skeletal muscle contraction are quite well known, however, the physiological functions of the P2X2 receptor have not been as well established. Previous studies have shown that P2X2 receptors have a broad range of physiological mechanisms that involves detecting damaged tissues to sending signals to the intestines (North, 2002).

PROJECT BACKGROUND

In previous research, the nicotinic acetylcholine receptor and the P2X2 receptor have seemed have a slight cross-inhibition interaction when they are expressed in close proximity. This interaction has been observed in HEK-293 cells and in myenteric neurons when the two receptors are expressed in close proximity (Decker and Gallagan, 2009). Although there is an increase in the total current sum in the cell when these two receptor channels are expressed in close proximity, the total current is at a lower value than predicted (Figure 1).

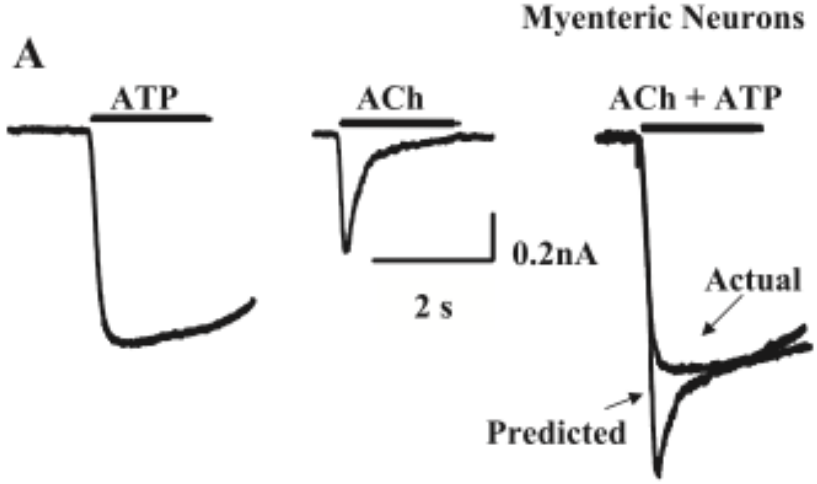

Figure 1: Cross inhibition of the nAChR and the P2X2 receptor in myenteric neurons. When the P2X2 receptor and the nicotinic acetylcholine receptors are activated independently, the sum current of these two receptors is greater than the sum current of these two receptors activated simultaneously in close proximity. This figure is taken from an *American Journal of Physiology-Gastrointestinal and Liver Physiology* article by Decker and Gallagan (2009).

When most of the C-terminal tail in the intracellular region of the P2X2 receptor was truncated (P2X2-TR), the cross-inhibition disappeared and the current sum was equal to the predicted current sum (Decker and Gallagan, 2010). Therefore, that part of the P2X2 receptor has some role in the slight inhibitory effects. The interaction between the P2X2 receptor and the nicotinic acetylcholine receptor has not been extensively studied in skeletal muscle. Skeletal muscle was also chosen at the biological platform to observe the AChR and the P2X2 receptor as the former receptor is prevalent in skeletal muscle. This study investigates the interaction between the AChR and P2X2 receptors in skeletal muscle. This will add to the base of knowledge of the P2X2 receptor in the nervous system, specifically at the neuromuscular junction.

P2X2 receptors appear in rat and mice skeletal muscle during early stages of development. For mice, the P2X2 receptors are endogenously expressed in the early stages of muscle development from days P7 to P15 and have an important role in the formation of the AChRs at the synapse (Ryten et al., 2007; Ryten et al., 2001). These receptors were not maintained in skeletal muscle in adulthood. Based on research done by Santos et al. (2003), ATP appeared to be released by the motor nerve end terminal into the synaptic cleft in rats. However, post-synaptic effects of ATP were not observed in adult rats. This could be due to the lack of endogenous expression of P2X2 in later stages of skeletal muscle development in rats and mice.

While P2X2 receptor disappears before mice reach adulthood, AChRs are usually present in muscle cells throughout the lifespan of a healthy organism. Previous research done by M. Ryten et al. (2004) showed that the P2X2 receptor was associated with regeneration in mice muscle dystrophy mutants, *mtx*. In these mutants, there was a small amount of co-localization between the P2X2 receptor and AChR on myotubes during regeneration of muscle cells (Ryten et al., 2004).

To study how P2X2 receptors would be expressed in developed muscle fibers, the rat-P2X2A-eGFP construct was electroporated into mouse skeletal muscle. The specific muscle electroporated was the sternomastoid muscle. The purpose of electroporation was to transfect the muscle fibers with P2X2 receptors because the age of mice used in this study, which was 4 months, do not endogenously express this receptor. Therefore, the goal of this project was to first observe whether the P2X2 receptors would be expressed in close proximity to the acetylcholine receptors in skeletal muscle and whether the interaction between these two receptors would be consistent to their behavior in myenteric neurons and HEK-293 cells.

The goal of this project was to observe if these two receptors would also be expressed in close proximity in skeletal muscle and whether this interaction between the P2X2 and the acetylcholine receptors would be consistent. As P2X2 receptors and nicotinic acetylcholine receptors have been found to be expressed in close proximity in previous research, it was hypothesized that a sequence exists in the P2X2 receptor that causes it to colocalize with the AChRs (Decker and Gallagan 2009; Decker and Gallagan 2010;

Ryten et al., 2007). Therefore in this project, I expect to see some co-localization between the P2X2 receptors and the AChRs in muscle fiber *in vivo*. Experiments were also done in C2C12 cells as these cells are derived from mouse skeletal muscle cells, and they were found to be a good representation of skeletal muscle *in vitro* (Bruattini et al., 2004). This cell line is ideal *in vitro* experiments, and my prediction is that observations *in vivo* will be similar to experiments done *in vitro*.

Studying how this receptor is expressed in skeletal muscle fibers would also provide insight on muscle development. P2X2 receptors have been known to play a role in the early development of the neuromuscular junction (Ryten et al., 2007). Furthermore, this study can lend insight on future therapeutic applications for muscle diseases that target the neuromuscular junction using the P2X2 receptor. For the purposes of this thesis, I will be investigating how the P2X2 receptors are expressed in skeletal muscle fibers in mice.

MATERIALS AND METHODS

Electroporation

All animal usage followed methods approved by the University of Michigan Committee on the Use and Care of Animals. Skeletal muscle in mice was electroporated with a pcDNA 3 derived plasmid in which the rat P2X2A receptor fused at its C-terminal end to eGFP was driven by the CMV promoter. This fusion allowed for the visualization of the P2X2 receptor in microscopy. In this study, wild type non-Swiss albino mice were used.

The specific skeletal muscle that was examined in this study was the sternomastoid muscle; this muscle is located in the anterior part of the neck. Each mouse has two sternomastoid muscles, one on the left side and one on the right side of the neck. Mice that were electroporated were approximately four months old, and they were in the juvenile stage. The concentration of the P2X2-eGFP plasmid was 500 - 650 ng/μl and 15 μl was electroporated to the left and right sternomastoid muscle in mice.

Details for the preparation for electroporation can be found with specific explanations in previous papers (Martinez-Pena y Valenzuela et al., 2011). Sternomastoid muscles were examined at 4 days, 10 days, and 14 days after electroporation with P2X2-eGFP. In order to observe the muscle post-electroporation, mice were anesthetized by intraperitoneal injection of 72 mg/kg ketamine and 12 mg/kg xylazine. Afterward, the sternomastoid muscles were saturated with Alexa Fluor 488-conguated conjugated α-Bungarotoxin (BTX-Alexa488; Invitrogen) to label AChRs (15 mins, 5 μl/ml). This was done to locate the neuromuscular junction. This application took place ten minutes before viewing the electroporated muscle fibers under the fluorescent microscope. The nonbound fluorescence was washed out with lactated Ringer's superfusion continuously for 10-15 minutes and synapses were imaged.

Techniques for in situ imaging of mature synapses include animal preparation, sternomastoid exposure, and neuromuscular imaging have been described in detail previously (Lichtman et al., 1987; van Mier and Lichtman, 1994; Akaaboune et al., 1999). The mouse that was anesthetized was placed on its back on the stage of an

epifluorescence microscope. Further information has been described about imaging synapses in previous papers (Martinez-Pena y Valenzuela et al., 2011). The synapses were viewed and imaged with a customized epifluorescence microscope, and superficial neuromuscular junctions were viewed (Olympus UAPO 20XW3/340 0.7 NA) and imaged (IPLab Software; BioVision Technologies). At the end of an imaging session, the incision was closed and the mouse was allowed to recover until reanesthetized and prepped for the next imaging session. The images were TIF files and the contrast was adjusted with Adobe Photoshop CS6.

Cell Culture and Differentiation

C2C12 cells were used for cell cultures, and this line of cells was derived from mice (*mus musculus*) skeletal muscle cells. C2C12 cells were cultured in a 35 mm dish with the Dulbecco's Modified Eagle Medium (DMEM), containing 20% FBS (Fetal Bovine Serum) and 5% of penicillin-streptomycin. 35 mm dishes contained a total of 1.5 ml of media for optimal growth of the cells. These smaller dishes were passaged from 10 cm dishes (contained 10 ml of media). The cells were incubated in a 37 °C environment with 5% CO_2 and were split every 48 hours.

To differentiate the myoblasts to myotubes, we used DMEM media with 5% Horse Serum. This differentiation media was changed every other day. It took approximately 4-6 days for robust myotube development. As for the C2C12 cells that were transfected, the

DMEM media provided for the cells after the transfection was changed to DMEM with 5 % Horseserum 8 hours after the transfection.

Isolation of RNA from C2C12 cells for RT-PCR

RNA was collected from 35 mm dishes of C2C12 confluent myoblasts and mature C2C12 myotubes. The mature myotubes were given differentiation medium 4-5 days prior to preparing these cultures for RNA isolation. The RNA was collected using TRIzol and the protocol for the TRIzol Reagent for RNA Isolation provided by Ambion. Subsequently, the RNA was stored at -80 °C. First strand cDNA synthesis was performed by superscript III from Invitrogen. After cDNA synthesis was performed, the PCR (Polymerase Chain Reaction) was used to determine what specific purinergic receptors and myosin proteins were expressed in the two sets of C2C12 cells at different developmental stages.

The set up for the PCR included: forward primer for gene of interest (1.25 µl); reverse primer for gene of interest (1.25 µl); cDNA template of C2C12 myoblast or myotube (1 µl); dTNP mix (0.8 µl); 5X Herculase Buffer (10 µl); Herculase polymerase (1 µl); distilled water (34.7 µl). The PCR cycles were: (1) Held at 94 °C for denaturation; (2) Held at 94 °C for an additional 30 seconds; (3) Annealed the primers onto the two strands of DNA at 65 °C; (4) The temperature was then held constant at 68 °C for elongation; (5) Cycles two through four are then repeated 30 times; (6) The thermocycler is then held at 68 °C for 1 minute. After the PCR cycles, the samples were held at 4 °C.

Analysis of the PCR reaction was carried out with 1 % agarose gels and 1 μg/ml of Ethidium Bromide to identify the DNA bands. Overall, 20 μl of each sample was added to the gel, and 1 μl of loading buffer with blue dye was added for every 5 μl of DNA sample. Therefore, a total of 4 μl of the loading buffer was used for each of the samples. Furthermore, the 100 BP DNA Ladder from Fisher Scientific was used to detect the sizes of the DNA fragments. All the sequences for these receptors to make the primers were obtained from GenBank, and the A Plasmid Editor software was used to generate the primer sequences.

The primers used are listed below:

Primers for RT-PCR

Gene	**Primer Sequence ('5→3')**	**T_m (°C)**	**Size (bp)**
$P2X_2$	F- TACTTCGTGTGGTACGTCTTCATCG R- GGGATAGTGGATGCTGTTCTTGATG	60 59	407
$P2X_4$	F- CATGGGTATCCAGATCAAGTGGGAC R- AGGCTGAGTGGGATTGAAATGTAGG	60 60	396
$P2X_5$	F- TCACTTCAGCTCCACCAATCTCTAC R- CCCTCACCTTCTCAAACTTCTTGTC	60 59	424
$P2Y_1$	F- CAAGAGGTCTAGCAAGTCTCAACAG	59	452

	R- GAGTCAGAGACAGCACTTCGTAAGG	60	
Non-muscle myosin	F -GAGAAGAAACTGCTGGAAGACAGAG R- CCGTTTCTGCTTCTCGGCTTTATTC	59 60	380
Skeletal muscle mysoin	F - CAGAAGAAGATCAAGGAGTTGCAGG R - TCTTCACTTCACTCAGTTGGTCCTC	59 60	420

These sequences were obtained from GenBank (NCBI)

Transfection

Proliferating C2C12 cells at 70% confluency on laminin-coated plates were transfected with rat-$P2X_2$-GFP. Lipofectamine 2000 was used as the transfection reagent, and 10 μl of this reagent and 2 μg of rat-$P2X_2$-GFP (approximately 800 ng of DNA) were sufficient for the cells in the 35 mm dishes. DMEM was added immediately after the $P2X_2$ DNA and the transfection reagent to ensure optimal transfection conditions. The transfection took place during the myoblast developmental stage then the cells were allowed to mature and differentiate into myotubes. The transfected cells were stored in a 37 °C incubator with 5% CO_2.

Preparation of C2C12 cells for microscopy

Proliferating C2C12 cells at 70% confluency grown on a laminin-coated plate were transfected with rat-P2X2-GFP. Laminin was required for this procedure in order for the endogenous acetylcholine receptors to aggregate on the differentiated C2C12 myotubes. To prepare these plates, the 1 ml of 1X ornithine was added to each 35 mm dish, from a stock solution of 200X ornithine stored at -20 °C. These dishes were left in the cell culture hood without the lid on to dry overnight. The following day, the 100X laminin stock was diluted to 1X with L-15 Medium with 0.2 % Sodium Bicarbonate; 1 ml of this solution was therefore administered to each 35 mm dish, these were incubated for 3 hours at 37 °C. Afterward, the remaining solution in these dishes were aspirated and C2C12 myoblasts were split onto dishes with laminin; this substance was used to induce clustering of the acetylcholine receptors. The transfection took place when the cells were on the laminin dishes.

4-5 days after the transfection procedure, the myoblasts were diffentiated into myotubes. The reason that C2C12 microscopy experiments were done in C2C12 myotubes was that differentiated muscle cells display aggregates of acetylcholine receptors when treated with certain chemicals. Myoblasts were not used in this experiment as there is no AChR clustering in this stage of muscle development in mice.

1 μl of Alexa 594 α-Bungarotoxin (α-BGT) was added to 1 ml of media in the transfected, mature myotubes. The Alexa 594 α-BGT was used to label the AChR with red fluorescence. These dishes were incubated at 4 °C for 1 hour, and the α-BGT was washed away with PBS. To fix these cells for imaging, 4 % paraformaldehyde (PFA) was

administered to fix the C2C12 cells on the plate for 5 minutes. To mount the cells, 20 µl of glycerol was added to the cells and a coverslip was placed over the cells. The mounted cells were stored at -20 °C.

The IP Lab software was used for imaging these transfected cells on a fluorescent microscope (Olympus UAPO 20XW3/340 0.7NA). The exposure for the red fluorescence was 2500 or 1800 ms whereas the exposure for the green fluorescence, which labeled the $P2X_2$ receptors, was at 1800 or 1000 ms. The gain and offset were adjusted accordingly to each image taken.

RESULTS

P2X2 receptor clustering in the neuromuscular junction

To observe how P2X2 receptors were expressed in skeletal muscle of mice beyond a point when very young mice express this receptor endogenously, electroporation experiments were carried out. The skeletal muscle that was electroporated in this study was the sternomastoid muscle. Based on previous studies regarding electroporation of genes into *in vivo* skeletal muscle, stable expression was shown to occur one to two weeks after electroporation (Martinez-Pena y Valenzuela et al., 2011). Therefore, muscle fibers were initially observed 14 days after being electroporated with the P2X2-eGFP construct. Approximately ten minutes before viewing the electroporated fibers, the

muscle was saturated with α-Bungarotoxin tagged with red fluorescence (Alexa 594) in order to visualize the AChRs.

The rate of successful electroporation of sternomastoid muscle fibers was low. However, in our initial series, there were several wild type mice that yielded results from the electroporation (n = 3). Results were consistent in all green fluorescent electroporated muscle fibers (n = 9 NMJs). Based on visual observations to the best of my ability, there was green fluorescence throughout the electroporated fiber, with a higher intensity of green fluorescence that overlapped with the synapse. Two examples of electroporated fibers are shown in Figure 2.

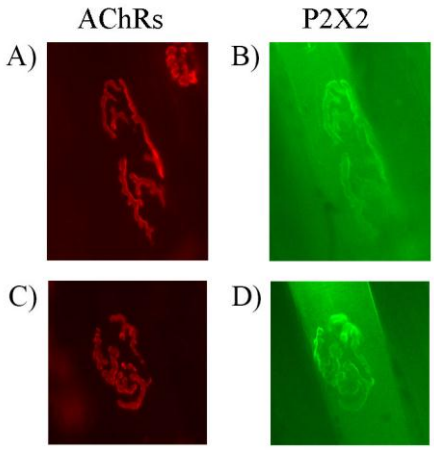

Figure 2: High concentration of P2X2 receptors in the neuromuscular junction. This figure displays two synapses in the mouse sternomastoid muscle that was electroporated with P2X2-eGFP. Figures 2A and 2B represent one synapse, and Figures 2C and 2D are of a second synapse. These two pairs show high clustering of some P2X2 receptors that overlap with the synapse. These images were acquired 14 days after electroporation. In the images of AChRs, there are additional labeled ACh receptors to the right of the synapse in Figure 2A and to the left of the synapse in Figure 2C are AChRs on adjacent muscle fibers.

Looking at the red fluorescent image of figure 2A and 2C, there is a bright red fluorescence in a continuous pretzel-like shaped synapse in the center of the image against a relative dark background. As α-Bungarotoxin tagged with red fluorescence was used and as it is well known that α-Bungarotoxin binds with AChRs at a high affinity, it can be drawn from these results that the red fluorescence represents acetylcholine

receptors (Figures 2A and 2C) (Bruneau and Akaaboune, 2006). These labeled acetylcholine receptors are highly expressed at the neuromuscular junction, and the density of these receptors inside the extrasynaptic space was almost zero.

As for the green fluorescent images, there is a band of green fluorescence that extends above and below the synapse, which indicates the electroporated muscle fiber (Figures 2B and 2D). Within the muscle fiber, it can be observed that there is a higher intensity of green fluorescence that overlaps with the synapse. Based on the results, there was only one band of green fluorescence in each of these images, and it can be inferred that there was only this one muscle fiber that was electroporated in the field of the green fluorescent figures. Furthermore, there was no indication of a green fluorescent muscle fiber at the top right of Figure 2B nor a green fluorescence muscle fiber to the left of the synapse in 2D. Therefore the adjacent muscle fibers where acetylcholine receptors were detected in Figures 2A and 2C were not electroporated with P2X2-eGFP. In the background of the green fluorescent images, there was autofluorescence of unelectroporated fibers.

Through viewing the images of these two synapses to the best of my ability, the main difference between the red and green fluorescent images was that there is green fluorescence present throughout the entire muscle fiber (Figure 2). Whereas the nicotinic acetylcholine receptors are primarily concentrated to the neuromuscular junction, and any receptors that may be expressed outside of the synapse cannot be seen in this image (Figure 2A and 2C). The intensity of the green fluorescence was markedly higher in the

muscle fiber specifically where the synapse is located (Figure 2B and 2D). Therefore, it was reasonable to conclude that P2X2 receptors are highly clustered at the synapse but they are also expressed in other areas of muscle fibers. P2X2 receptors were present on the muscle fiber outside of the synaptic area at a higher density than acetylcholine receptors were outside of the synapse. But overall, P2X2 receptor expression was found throughout the muscle fiber, this receptor was still much more highly clustered at the neuromuscular junction, as indicated by the higher intensity of green fluorescence at the synapse.

To check that eGFP did not inadvertently affect where the P2X2 receptors were expressed in the muscle fiber, a control experiment was performed. This was carried out by electroporating the mouse sternomastoid muscle with GFP-WT, which is a GFP-alone construct. After waiting several days, the electroporated muscle fiber was observed (Figure 3).

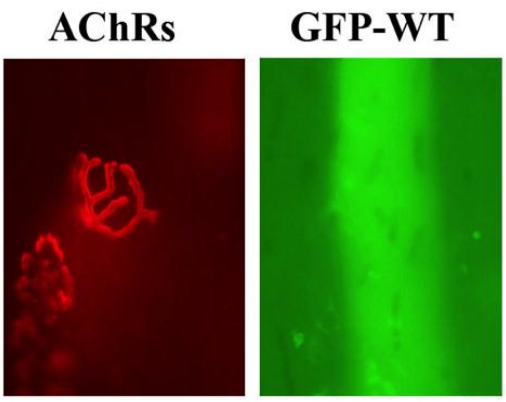

Figure 3: Electroporation of the GFP-only into the sternmastoid muscle. The left image shows AChRs labeled by α-Bungarotoxin tagged with red fluorescence. There are several synapses from adjacent muscle fibers, as indicated by the bright red fluorescence. The right image shows the muscle fiber electroporated with GFP.

Through viewing the muscle fibers that were electroporated with GFP, the green fluorescence was constant and uniform throughout the entire muscle fiber. There were no clusters of higher green fluorescence intensity at the synapse. Based on these observations, it seemed reasonable to infer that GFP alone is not likely to influence the

high intensity of green fluorescence at the synapse. Furthermore, previous experiments have been conducted to show that electroporation of solely GFP into skeletal muscle serves as a viable control experiment; GFP tends to be displayed throughout the entire muscle fiber since it is a small and diffusible protein (Rana et al., 2004).

Increased P2X2 expression in the neuromuscular junction over time

To examine whether the clustering of some of the P2X2 receptors at the synapse was affected by length of time, a time course experiment was conducted on mice electroporated with P2X2-eGFP. Several mice were observed four days after electroporation (n = 3). As indicated in previous study that focused on electroporation by Donà et al. (2003), high levels of gene expression *in vivo* were detected around days 3-5 after electroporation. In the time course experiment, muscle fibers were observed at 4 days after electroporation, and then 10 days after electroporation. The purpose was to observe whether any changes in expression patterns occurred in this six day time period. Interestingly, it appeared that the P2X2 receptor expression overlapped more with the AChR in the synapse 10 days after electroporation rather than four days after electroporation (Figure 4). All synapses in muscle fibers four days after electroporation with P2X2-eGFP provided consistent results (n = 7 NMJs).

When synapses were observed four days post-electroporation, there was some green fluorescence expressed by several muscle fibers. However, the green fluorescence was diffuse in the middle of the muscle fibers during this time point (Figure 4: Day 4). Interestingly, when one of the fibers from day four was imaged at day ten, the green fluorescence in the middle of the muscle fiber that overlapped the red fluorescence labeled AChRs. The specific synapse that was looked at in figure 4 was imaged at different angles as it was difficult to acquire an image from the exact same angle in these two time points as these images were taken in vivo. As the transfection rate for the electroporation was low, further electroporation experiments with the same goal were carried out. However, these mice died after electroporation before expression of the transfected gene occurred.

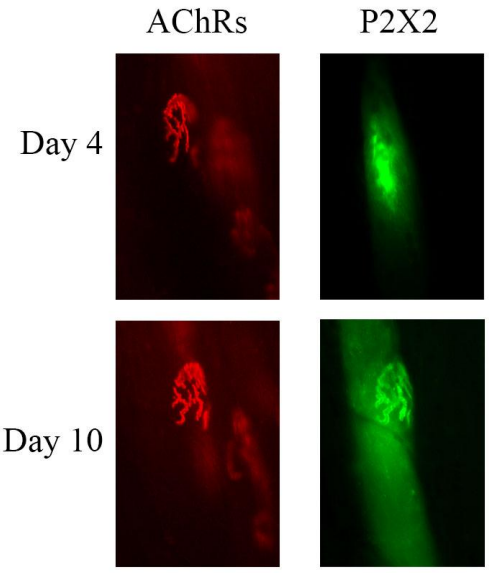

Figure 4: P2X2 receptor expression at the synapse increases over time. The same synapse on a muscle was observed at days 4 and 10 after electroporation of the p2X2-GFP construct. The top row displays the synapse observed at day 4, and the lower row displays the same synapse observed six days later, on day 10. The synapse was imaged from two different angles, which accounts for minor differences in how these brances are oriented in the figure. The red blurs in the AChR image are labeled NMJs on adjacent muscle fibers that were not electroporated with P2X2-eGFP.

P2X2 receptors expressed in C2C12 myoblasts

Since there was co-clustering of P2X2 receptors and acetylcholine receptors at the skeletal muscle synapse *in vivo*, C2C12 cells, which are derived from mouse myoblasts

were used to further experiments regarding this. The advantages of potentially studying the P2X2 and acetylcholine receptors in C2C12 cells are that these cells are easily transfected and can be manipulated to find mechanisms that cause P2X2 clusters at the synapse. Before experiments in C2C12 cells with similar goals to the electroporation experiments were carried out, the endogenous expression patterns of the P2X2 receptor was studied in C2C12 myoblasts and C2C12 myotubes. Based on previous research, it is known that P2X2 receptors are expressed endogenously in mice from P7 to P15, but these receptors disappear before P21 (Ryten et al., 2007). Therefore RT-PCR was performed on RNA extracted from untransfected C2C12 myoblasts and untransfected C2C12 differentiated myotubes to determine the levels of expression of different purinergic receptors between these two cell types. It was found that P2X2 receptors were endogenously expressed in myoblasts, but not in myotubes (Figure 5).

PCR primers were ordered for P2X2, P2X4, P2X5, and P2Y1 receptors, as well as non-muscle myosin and skeletal muscle myosin. The non-muscle myosin and the skeletal muscle myosin were used as controls for the experiment. Non-muscle myosin is expressed in myoblasts and myotubes this substance has a role in skeletal muscle fiber formation (Swailes et al., 2006). Muscle myosin is much more concentrated in myotubes than myoblasts (Burratini et al., 2004). The other P2X receptors and P2Y1 was used to observe how other purinergic receptors were expressed in different stages of C2C12 cell development.

There appeared to be P2X2 receptors expressed in myoblasts, but not in myotubes, as predicted (Figure 5). P2X4 receptors were expressed in both mononucleated cells and in differentiated cells. There was a strong expression of P2X5 receptors in myotubes than in myoblasts. P2Y1 was not detected in either myoblasts or myotubes. There was a stronger band for non-muscle myosin in myotubes than myoblasts. Muscle myosin was expressed much more in myotubes than in myoblasts.

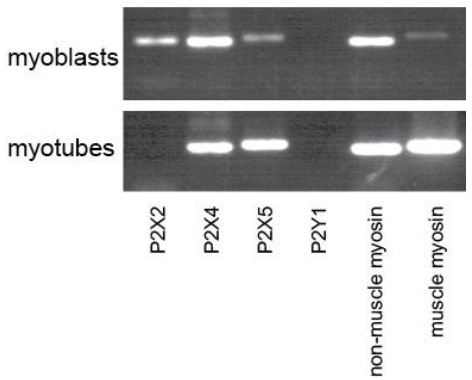

Figure 5: Endogenous P2X2 receptor in C2C12 myoblasts. RT-PCR analysis of C2C12 myoblasts and myotubes. The myoblasts were collected at 85-90% confluency; the myotubes were collected 4-5 days after differentiation.

Results when this experiment was repeated were consistent for P2X2 receptor expression but not for some other purinergic receptors. Repeats of this experiment gave different results for the expression of P2X4 receptors and skeletal muscle myosin in myoblasts. Due to time limitations, the discrepancy in the P2X4 receptor expression was not investigated because this receptor was not the focus of this project.

Absence of P2X2 receptor and AChR clustering in C2C12 cells

Since there was endogenous P2X2 receptor expression in C2C12 myoblasts, similar to how P2X2 receptors are endogenous in skeletal muscle in the early developing stages, experiments were carried out to observe whether P2X2 receptors and acetylcholine

receptors co-localize in C2C12 myotubes. However, the results were unexpected. There was virtually no overlap of these two receptors in C2C12 myotubes that were transfected with P2X2-eGFP.

Results were consistent among the transfected and differentiated myotubes imaged (n = 42 images). There was no obvious co-clustering of P2X2 receptors and acetylcholine receptors in the differentiated muscle cells (Figure 6). In the AChR labeled images, the myotubes showed red fluorescence throughout the entire cell rather than just at the synaptic site. However, as expected, there was much higher intensity of red fluorescence at specific sites on the C2C12 myotubes. Since laminin was used to induce AChR clusters in the myotubes, it was reasonable to say that the high intensity of red fluorescence on the myotubes were clusters with a high density of acetylcholine receptors. Interestingly, this pattern of red fluorescence was similar to how the P2X2-eGFP was expressed in the sternomastoid muscle *in vivo*.

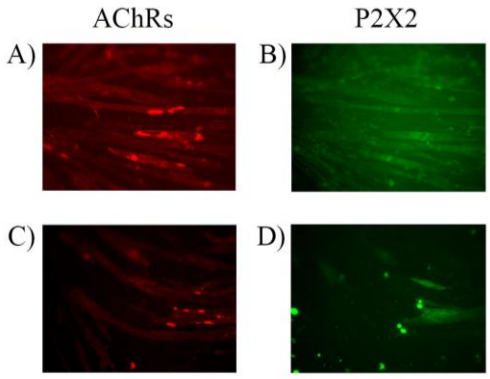

Figure 6: C2C12 cells exhibit no co-localization of P2X2 receptors and nAChR. There are two sets of cells examined in this figure, 6A and 6B represent one group of C2C12 myotubes; 6C and 6D show another group of C2C12 myotubes imaged. Images 6A and 6B were taken at an exposure of 1800 ms for green fluorescence and 2500 ms for red fluorescence. Images 6C and 6D were taken at an exposure of 1800 ms for red fluorescence and 1000 ms for green fluorescence.

The transfections with P2X2-eGFP were not 100%. There were regions of higher green intensity, higher than the average fluorescence in the image. But overall, there appeared to be a relatively equal green fluorescence throughout the myotubes without significant higher intensity of brighter green fluorescence on the myotubes at sites where AChR clusters were expressed. Therefore it was reasonable to conclude that the P2X2 receptors did not cluster where the AChRs were expressed on C2C12 myotubes transfected with P2X2-eGFP.

DISCUSSION

Based on the findings in the experiments done in this thesis, when mouse skeletal muscle express P2X2 receptors, these receptors appeared to highly cluster at the synapse. Using electroporation to cause skeletal muscle to express this exogenous protein, the expression of P2X2 receptors at the synapse seemed to increase over time. Interestingly, when this experiment was done in C2C12 cells, the high levels of clustering between the P2X2 receptors and the acetylcholine receptors were not observed. This result was quite unexpected as C2C12 cells are derived from mouse skeletal muscle. Furthermore, the endogenous expression of P2X2 receptor expression was observed in C2C12 myoblasts, and this expression was similar to the expression of this receptor in mouse skeletal muscle *in vivo*. Overall, the major finding in this project was that the P2X2 receptor is expressed throughout the muscle fiber *in vivo* and clustered at a higher level where the synapse is located. All these experiments should also be repeated many more times with a higher pool of mice and C2C12 cells.

There have been some studies that have shown how electroporation is one of the more effective methods of gene transfer *in vivo* compared to injecting DNA into muscle fibers (Donà et al., 2003). In this paper, the yield for electroporation was higher, however, there are many limitations for effective electroporation. These include the access of the plasmid DNA to the muscle fiber surface and the type of construct the DNA of interest is placed in (Donà et al., 2003). Another limitation was the concentration of DNA construct used in the electroporation, as the optimal electroporation into skeletal muscle *in vivo* occurs with 1 µg/ml. The DNA used in the electroporation experiments had a lower level of concentration.

Future directions with this project should include doing experiments to confirm whether the P2X2 receptors are functional when expressed in muscle. Sharp intensity of fluorescence on the edge of the synapse usually indicate that protein is expressed at the surface, which was observed with the acetylcholine receptors and the P2X2 receptors in the results (Figures 2, 3, 4). A limitation with the electroporation experiments in my project was that it the images only showed presence of P2X2 receptors that overlapped with the synapse. However, future experiments should be aimed to check that the P2X2 receptors are expressed on the surface of the membrane, and not inside the muscle fibers; otherwise this protein would not be physiologically functional.

One method to test if the P2X2 receptors are located at the surface of the muscle fiber is to use an antibody that has an epitope to the extracellular component of the P2X2

receptor. Furthermore, this epitope should be accessible to the antibody after the protein is folded. An antibody that could be used for this experiment is one that is specific to the extracellular epitope of the mouse and rat P2X2 receptor (Alimone Labs); as indicated by the company, this antibody can be used in immunohistochemistry and western blots. Another antibody that targets the extracellular component of the P2X2 receptor can be found in the Hume lab, however, this antibody has only been previously used in western blots. This antibody would be applied directly to the surface of live muscle fibers. Afterward, there would be a secondary antibody tagged with fluorescence that targets the antibody with the epitope of the extracellular component of the P2X2 receptor.

An approach to test the functionality of the P2X2 receptors at the neuromuscular junction can be tested is to perform a functional assay using electrophysiology. There were attempts in carrying out voltage clamp experiments for my thesis, however, due to technical difficulties of the electrophysiology equipment and time constraints, these experiments were not successfully carried out. Rather, I am going to explain the set up that I used for this experiment and these steps should be revisited when carrying out further experiments. The sternomastoid muscle in mice that was electroporated with P2X2-eGFP will be isolated 10-14 days after electroporation for recording. This time point was chosen because based on previous experiments, the expression of the P2X2-eGFP in skeletal muscle was at a high level of intensity then. The logic behind this set of experiments is that the application of ATP should elicit a current response from the muscle fiber electroporated with P2X2-eGFP if these receptors are expressed on the surface of the membrane. From previous solutions used in previous mouse skeletal

muscle recordings, the Tyrode's solution in this experiment was composed of (in mM): 146 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 10 HEPES, at pH of 7.4 (Collet et al., 2002; Jacquemond and Allard, 1998; Solares-Pérez et al., 2010). The calcium free Tyrode's solution is composed of (in mM): 146 NaCl, 5 KCl, 0.5 $CaCl_2$, 2.5 $MgCl_2$, 11 glucose, 10 HEPES, at pH 7.4. The limitation with electrophysiology experiments is that it does not provide information about how many P2X2 receptors are on the surface of the membrane at the synapse relative to the total number of P2X2 receptors the muscle fiber that was electroporated expresses.

Another important aspect to consider in future experiments is signal trafficking. As the P2X2 receptor is electroporated into skeletal muscle, there is continuous transcription and translation for the production of this protein. There is an intracellular pool of this receptor at all times in the muscle fiber because it is transcribed in the nucleus, then translated in the cytoplasm. Furthermore, this protein also undergoes post-translational modifications after it detaches from the ribosome after translation. The protein then goes to the rough endoplasmic reticulum and afterward, it is transported to the golgi apparatus, where the receptor protein buds off in vesicles to be inserted into the membrane. Regarding how proteins are made in the cell, it is reasonable to conclude that at all times, there is a higher number of P2X2 receptors total in the muscle fiber than in the number of this protein on the surface of the cell.

To determine the proportion of P2X2 receptors expressed on the surface of the cell compared to the number of P2X2 receptors inside the cell, a third approach for future

experiments can be to pull down the surface P2X2 receptors by using Biotin. This technique was previous described in detail by Mouslim and Aittaleb et al. (2012). Biotin can be applied to live skeletal muscle that was electroporated with P2X2-eGFP to react with lysine residues on surface receptors on cell. Then, the muscle fibers should be lysed and protein will be extracted from lysate to perform a western blot with an anti-body that recognizes the P2X2 receptor. Incubate this protein with the avidin or streptavidin beads for a while. After a couple washes, elute the bound protein off of beads with selective precipitation to retrieve the P2X2 surface protein. The mass of the elute should be reconcentrated and the volume should then be adjusted. To observe the relative proportion of surface P2X2 receptors to total P2X2 receptors, the intensity of the western blot of surface P2X2 protein should be compared to the total P2X2 protein on the cell using the band on the western blot.

Furthermore, an additional control experiment can be carried out by electroporating the skeletal muscle with a membrane protein, which is known to not co-localize the synapse, fused with GFP in a plasmid with a CMV promoter. This should be done to make sure that not all membrane proteins have a high level of localization to the synapse.

Another experiment to be carried out is a denervation experiment with skeletal muscle electroporated with P2X2-eGFP receptors. As the behavior of acetylcholine receptors change and become more sensitive under denervated conditions, it would be interesting to observe how P2X2 receptors would be expressed under these conditions (Albuquerque and McIsaac, 1969). Furthermore, if the P2X2 receptors appear to have high clusters

where the remaining acetylcholine receptors are after denervation, then it supports the hypothesis that there is a sequence within the P2X2 receptor that causes it to be expressed with acetylcholine receptors.

Although there were time course experiments carried out in skeletal muscles that were electroporated *in vivo*, a time course experiment should also be carried out *in vitro* as well. As for time course experiments *in vivo*, these should be conducted with more and shorter intervals to get a better understanding of P2X2 receptor expression in muscle fibers after electroporation. Since there was a major difference in P2X2 receptor expression in the NMJ between days 4 and 10, it was likely that during this six-day time period, the P2X2-eGFP expression was spreading through the fiber. However, when the expression reached the synapse, there was a much higher intensity of green fluorescence at the NMJ. Future studies should look at more and shorter intervals during this six-day period to further examine how the P2X2-eGFP expression spreads. One limitation of this is that mice need time to recover after the viewing of electroporated fibers, and it would not be ideal for the mouse if muscle fibers were observed in one-day intervals. Mice would not have enough time to recover in such a short time period therefore different mice would need to be used for these time intervals. With this circumstance, the same synapse in a mouse would not be able to be observed for the time course project, given that the same synapse could be located for all of these intervals as it can be difficult to relocate the same synapse in a mouse.

Time course experiments should be revisited with C2C12 cells as well. It is possible that the C2C12 myotubes were still undergoing post-translational modifications within the cells before being inserted into the membrane. Therefore, an experiment with a time course can examine whether a longer time period post-transfection would cause the P2X2 receptors to cluster with the acetylcholine receptors. As the sternomastoid muscle fibers that were electroporated with P2X2-eGFP did not display a high level of intensity of clustering until around 10 days after electroporation, it is possible that the myotubes were examined before optimal P2X2 receptor expression.

As there was green fluorescence expressed from the myotubes it is clear that P2X2 is indeed expressed. The question now is whether the receptors are expressed on the surface and functional. Electrophysiology experiments can also be done with the transfected C2C12 myotubes to check if there is any response from the P2X2 receptors. If there was a response, and the P2X2 receptors were found to not cluster with acetylcholine receptors, then it is a possibility that there is a mechanism present in skeletal muscle cells *in vivo* that acts either directly or indirectly to cause the P2X2 receptors to cluster with acetylcholine receptors. For instance, the differentiated C2C12 myotubes were not innervated; there could be a protein or some type of factor on the motor neuron that could also influence P2X2 receptors clustering with AChRs. However, if there were no current response when recording from myotubes transfected with the P2X2 receptor by applying ATP, it would indicate that this membrane protein was not functional. That could mean that either the protein folded incorrectly, or the P2X2 receptor was undergoing post-

translational modifications and was in the golgi apparatus instead of being transported in to the surface.

The long-term applications of this project can contribute to an understanding of muscle development and therapeutic methods for diseases in the neuromuscular junction. From the findings of this study, P2X2 receptors have been found to highly cluster at the synapse when electroporated into skeletal muscle. These findings provide insight on a potential therapeutic role for P2X2 receptors in the NMJ. With further studies, the P2X2 receptor could rescue synaptic transmission at the neuromuscular junction in neuromuscular diseases. For instance, if the acetylcholine neurotransmission is deficient, the P2X2 receptor could potentially rescue the synaptic signals at the NMJ. An example is myasthenia gravis, a muscle weakness condition. Myasthenia gravis is due to the production of antibodies to nicotinic acetylcholine receptors causes the electrical impulses communicated to the muscle fibers to fail. Experiments in the future would focus on using P2X2 receptors to restore neurotransmission in muscles in those with myasthenia gravis.

REFERENCES

Akaaboune M., Culican S.M., Turney S.G., Lichtman J.W. (1999) Rapid and reversible effects of activity on acetylcholine receptor density at the neuromuscular junction *in vivo.* Science **286**:503–507.

Albuquerque, E. X., Pereira, E. F., Alkondon, M., & Rogers, S. W. (2009) Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function. Physiological Reviews, **89**:73–120.

Albuquerque, E.X., McIsaac, R.J. (1969) Fast and Slow Mammalian Muscles After Denervation. Experimental Neurobiology, **26**:183-202

Burattini, S., Ferri, P., Battistelli, M., Curci, R., Luchetti, F., Falcieri, E. (2004) C2C12 Murine myoblasts as a model of skeletal muscle development: morpho–functional characterization. Eur J Histochem **48**:223–234.

Collet, C., Strube, C., Csernoch, L., Malouk, N., Ojeda, C., Allard, B., Jacquemond, V. (2002) Effects of extracellulr ATP of freshly isolated mouse skeletal muscle cells during pre–natal and post–natal development. Eur J Physiol **443**:771–778.

Decker D.A., and Galagan, J.J. (2009) Cross-inhibiton between nicotinic acetylcholine

receptors and P2X receptors in myenteric neurosn and HEK-293 cells. Am J Physiol Gastrointest Liver Physiol, **296**:G1267-1276.

Decker D.A., and Gallagan, J.J. (2010) Molecular mechanisms of cross–inhibition between nicotinic acetylcholine receptors and P2X receptors in myenteric neurons and HEK–293 cells. Neurogastroenterol Motil, **22**:901–e325.

Donà M., Sandri, M., Rossini, K., Dell'Aica, I., Podhorska-Okolow, M., Carraro, U. (2003) Functional in vivo gene transfer into the myofibers of adult skeletal muscle. Biochemical and Biophysical Research Comunications, **312**:1132–1138.

Jacquemond, V., and Allard, B. (1998) Activation of Ca2+–activated K+ channels by an increase in intracellular Ca2+ induced by depolarization of mouse skeletal muscle fibres. J. Physiol. – London **509**:93–102.

Lichtman, J.W., Magrassi, L., Purves, D. (1987) Visualization of neuromuscular junction over periods of several months in living mice. J Neurosci **7**:1215–1222.

Khakh B.S., Zhou X., Sydes J., Galligan J.J., Lester H.A. (2000) State–dependent cross–inhibition between transmitter–gated cation channels. Nature, **406**:405–410.

Martinello, T., Baldoin, M.C., Morbiato, L., Paganin, M., Taricone, E., Schiavo,

G., Bianchini, E., Sandona, D., Betto, R. (2011) Extracellular ATP signaling during differentiation of C2C12 skeletal muscle cells: role in proliferation. Mol Cell Biochem, **351**:183–196.

Martinez–Pena y Valenzuela, I., Mouslim, C., Pires–Oliveira, M., Adams, M.E., Froehner, S.C., Akaaboune, M. (2011) Nicotinic Acetylcholine Receptor Stability at the NMJ Deficient in α–Syntrophin *In Vivo*. J Neurosci, **43**:15586–15596

Mouslim, C., Aittaleb, M., Hume, R.I., Akaaboune, M. (2012) A Role for the Calmodulin Kinase II–Related Anchoring Protein (alpha kap) in Maintaining the Stability of Nicotinic Acetylcholine Receptors. J Neurosci, **32**:5177–5185.

North, R.A. (2002) Molecular Physiology of P2X Receptors. Physiol Rev, **82**: 1013–1067.

Rana, Z.A., Ekmark, M., Gundersen, K. (2004) Coexpression after electroporation of plasmid mixtures into muscle in vivo. Acta Physiol Scand, **181**:233–238.

Ryten, M., Hoebertz, A., Burnstock, G. (2001) Sequential Expression of Three Receptor Subtypes for Extracellular ATP in Developing Rat Skeletal Muscle. Developmental Dynamics, **221**:331–341.

Ryten, M., Koshi, R., Knight, G.E., Tumaine, M., Dunn, P., Cockayne, D.A.,

Ford, W., and Burnstock, G. (2007) Abnormalities in Neuromuscular Junction Structure and Skeletal Muscle Function in Mice Lacking the P2X2 Nucleotide Receptor. Neuroscience, **148**:700–711.

Ryten, M., Yang, S.Y., Dunn, P.M., Goldspink, G., Burnstock, G. 2004. Purinoceptor expression in regenerating skeletal muscle in the mdx mouse model of muscular dystrophy and in satellite cell cultures. FASEB Journal, **18**:1404–1438.

Sánchez, J.A., Zentella–Dehesa, A., García, M.C., Coral–Vázquez, R.M. (2010) Intracellular Ca2+ transients in delta–sarcoglycan knockout mouse skeletal muscle. Biochimica et Biophysica Acta, **3**:373–397

Santos, D.A., Salgado, A.I., Cunha, R.A. (2003) ATP is released from nerve terminals and from activated muscle fibres on stimulation of the rat phrenic nerve. Neuroscience Letters, **338**: 225-228.

Swailes, N.T., Colegrave, M., Knight, P.J., Peckham, M. (2006) Non-muscle myosins 2A and 2B drive changes in cell morphology that occur as myoblasts align fuse. Journal of Cell Science, **119**: 3561–3570.

van Mier P., Lichtman J.W. (1994) Regenerating muscle fibers induce directional

sprouting from nearby nerve terminals: studies in living mice. J Neurosci **14**:5672–5686.