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UNIVERSITY OF CALIFORNIA, SAN DIEGO

MHC class I is required for activity-dependent structural remodeling at the developing neuromuscular junction

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Neurosciences

by

Marin Alisa McDonald

Committee in charge:

Professor Lisa Boulanger, Chair
Professor Edward Callaway
Professor Lawrence Goldstein
Professor Charles Stevens
Professor Mark Tuszynski

2011

The Dissertation of Marin Alisa McDonald is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011

DEDICATION

This thesis is dedicated to my mother, Carolann McDonald, the source of all my inspiration and fortitude.

If I become just half of the woman she is, I will consider my life a resounding success.

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ACKNOWLEDGEMENTS

My first acknowledgement is extended to my Principal Investigator, Dr. Lisa Boulanger, for immeasurable support and advice throughout my graduate school career. Dr. Boulanger took me on as a very naïve, well intentioned first year and gave me the conceptual and experimental ability to conduct my own independent project. Always striking the correct balance between support and challenge, Dr. Boulanger kept me motivated through many trying circumstances and never failed to applaud my successes, while showing immeasurable patience in the face of my failures. All of this praise and more is well due, but would fall short of the mark if I made no mention of the personal growth I have done under the careful guidance of Dr. Boulanger. Over the last four years, in addition to learning about science, I have also had the privilege of watching Dr. Boulanger grow and expand in her own life, and in so doing, have learned a great deal about the kind of scientist, and the kind of woman, I hope to be one day … and for that I am eternally grateful.

My earnest gratitude is extended to all members of the Boulanger lab, past and present, for experimental, conceptual, emotional and sometimes even physical support. In particular, I would like to thank Timothy Cheng and Michael Spencer for assistance with data analysis, and Dr. Tracy Dixon-Salazar for much experimental advice and assistance. Most special thanks to Dr. Chris Davenport for sharing my rig room, albeit grudgingly at times, and expanding my scientific and musical horizons. No mention of the Boulanger lab would be complete without particular thanks to one Dr. Lawrence

Fourgeaud, postdoc extraordinaire and friend. A consummate professional adhering to the highest rigor of scientific inquiry, Dr. Fourgeaud is a constant inspiration, not only for keen scientific acumen, but for a true generosity of spirit unmatched and unrivaled by any I have met.

My most heartfelt thanks are also extended to the members of my committee, a.k.a. the "Fabulous Four": Ed Callaway, Larry Goldstein, Chuck Stevens and Mark Tuszynski. They were both intellectually and psychologically supportive throughout my graduate school process and never failed to provide excellent experimental advice and invaluable feedback. My work simply would have not progressed to the conceptual and experimental level that it has without their superlative input, and I am truly grateful.

I am also extremely grateful to Dr. Rita Balice-Gordon for protocols, critical discussion, and reagents, particularly at the inception of this project. Dr. Balice-Gordon has been a constant source of inspiration and feedback and is truly a paragon in the science of the NMJ, and in supporting and expanding the place of women in academia. Furthermore, I would like to thank Dr. Ryan Wyatt for technical support provided during a particularly stressful postdoc interview season.

I appreciate the opportunity to pilot these studies using equipment in the UCSD Teaching Labs, with the help of Leonard Lopez. I am also grateful to the Patrick lab for the use of their spinning disk confocal and to Massimo Scanziani for loaned equipment. I would also like to thank D. Raulet and C.J. Shatz for transgenic mice. This work was supported by a Fellowship from the Alfred P. Sloan Foundation,

the Silvio Varon Professorship in Neuroregeneration, and a grant from the Whitehall Foundation (L.M.B.). I have also received additional support by the UCSD School of Medicine general funds.

I would also like to give special mention to the UCSD Neuroscience Program for providing a truly outstanding environment for graduate study. In particular, an almost inexpressible debt of gratitude goes out to Dr. Anirvan Ghosh, as both a program director and mentor this final year. Over the last few months, I have had a chance to witness Dr. Ghosh as a scientific mentor and Principal Investigator and have been thoroughly impressed with both his intellect and ability to manage and motivate a disparate group of truly talented scientists. Seemingly pulled in a million different directions, Dr. Ghosh is somehow able to keep tabs on no less than a dozen different experimental paradigms, to ask salient and provoking questions of his entire lab and to make it his personal responsibility to extend aid and support when needed, whether scientific or personal. I have been truly inspired and revitalized in my pursuit of science and have benefitted immensely, immeasurably from his feedback and support – this is a debt I cannot repay, but will happily spend the remainder of my career trying to be worthy of it.

Lastly, my thanks are turned towards my parents, Michael and Carolann McDonald. Envisioning my future career as an electrician in the family business, or professional Broadway dancer, respectively, my parents were somewhat nonplussed when I announced, at age 16, that I was determined to be a MD PhD candidate. Nevertheless, they have supported, defended, motivated and maintained my position

through all barriers and against all odds. In particular, my mother has been my most stalwart companion and scientific confidante through the last four years. Every failed experiment, every dubious result was laid at her doorstep, and she never failed to provide just the right support to enable me to attack again the next day. From countless hours on the cell phone in front of the confocal, to proofreading abstracts hours before the deadline, my mother has truly been the most valuable reagent in all my work … and for that, she has my thanks, my love and my admiration.

Portions of Chapters 1-4 are currently being prepared for submission. McDonald, Marin A.; Boulanger, Lisa B. The author of the dissertation is the primary author of the manuscript in preparation.

VITA

2003	Bachelor of Sciences, Stanford University
2003-2004	Research Assistant, Level II, Stanford University
2004-present	Trainee, Medical Scientist Training Program, University of California, San Diego Medical Scientist Training Program
2011	Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

MHC Class I Modulates Developmental Synapse Elimination in the Peripheral Nervous System. McDonald, M.A. and Boulanger, L.B. (2009) Soc. Neurosci., 99.

Isoflurane Actions on Noradrenergic Inhibition of CA1 Pyramidal Neurons. McDonald, M.A. and MacIver, M.B. (2003) Anesthesiology, 95.

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Toluene Effects on Glutamate and GABA-Mediated Synaptic Responses. (2003) Choi, D.S., McDonald, M.A., Turnquist, P.A. and MacIver, M.B. (2003) Society for Neuroscience, 93.

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FIELDS OF STUDY

Major Field: Neuroscience (Developmental and Molecular Neurobiology)

ABSTRACT OF THE DISSERTATION

MHC class I is required for activity-dependent structural remodeling at the developing neuromuscular junction

By

Marin Alisa McDonald

Doctor of Philosophy in Neuroscience

University of California, San Diego 2010

Professor Lisa Boulanger, Chair

Mature patterns of connectivity in the mammalian central and peripheral nervous systems require the selective elimination of redundant synaptic connections through activity-dependent mechanisms. At the developing neuromuscular junction (NMJ), individual muscle cells initially receive inputs from multiple motor neurons that are eliminated during the first two postnatal weeks until each motor endplate is monoinnervated. While the requirement for electrical activity in the normal

progression of synapse elimination is well established, the underlying molecular mechanisms remain obscure. Here we identify proteins of the major histocompatibility complex class I (MHCI) as endogenous mediators of activity-dependent synapse elimination at the developing NMJ. MHCI protein is expressed at the NMJ during synapse elimination and genetic reduction of cell surface MHCI significantly increases the number of muscles that remain multiply innervated at the end of the remodeling period. Supernumerary inputs in MHCI-deficient animals are functional and persist into adulthood and the impairment of remodeling in MHCI-deficient animals is associated with a persistent increase in the mean amplitude of the miniature endplate potential (mEPP), suggesting that MHCI is required for the functional weakening of neuromuscular synapses. Additionally, acute blockade of MHCI function by *in vivo* injection of anti-MHCI antibodies is sufficient to impair developmental synapse elimination in wild type mice. These results provide new insights into the molecular basis of activity-dependent synapse elimination at the developing NMJ, and suggest that MHCI is part of a core mechanism of activity-dependent synapse remodeling that is conserved in the peripheral and central nervous systems.

INTRODUCTION

The developing nervous system is presented with the exquisite challenge of generating robust and specific connections between hundreds of thousands of pre- and postsynaptic pairs, often with a paucity of discriminatory information. Consequently, one of the most heavily pursued areas of research in modern neuroscience focuses upon the understanding of the highly dynamic and complex processes that mediate axon guidance, target selection and initial synaptogenesis. However, the development of the nervous system is far from complete after the initial formation of synapses has occurred. It is now understood that early synaptic circuits are extensively refined through a series of functional and structural changes, with the strengthening and stabilization of some synapses occurring preferentially over the weakening and elimination of others. While classic work by Hubel and Weisel first identified the critical role of early activity in this process of circuit refinement (Wiesel and Hubel 1963), the molecular mechanisms that transform neural activity into long term functional and structural change in the developing CNS remain to be fully described.

In the pursuit of this question, the importance of activity-dependent elimination of presynaptic inputs in a variety of functionally diverse areas of the CNS has been uncovered. Beginning with landmark observations in the process of ocular dominance column formation, structures as far ranging as sympathetic ganglia, the cerebellum, and somatosensory cortex have all demonstrated exquisite dependence upon early neural activity, in the form of action potentials, for the spatially precise refinement of

synaptic circuitry (Goda and Davis 2003; Hua and Smith 2004; Holtmaat and Svoboda 2009; Kano and Hashimoto 2009; Luo, Yin et al. 2010). Of particular importance in this refinement process is the general elimination of initially superabundant presynaptic inputs to postsynaptic targets, a phenomenon that is crucially important in the normal development of the mammalian neuromuscular junction (NMJ) (Sanes and Lichtman 1999; Personius and Balice-Gordon 2000; Gillingwater and Ribchester 2003; Lichtman and Sanes 2003; Wyatt and Balice-Gordon 2003; Song, Panzer et al. 2006; Lu and Lichtman 2007).

Immediately postnatal, individual muscle fibers are initially contacted by 5-12 presynaptic motor axons at a specialized postsynaptic concentration of nicotinic acetylcholine receptors, termed the endplate. Over a period of two weeks, this high degree of convergent innervation is reduced in a dynamic competitive process until each muscle fiber is contacted by a single motor. Four decades of work has helped catalog the cascade of functional and structural changes that results in the progressive strengthening of one motor axon over the continued weakening and destabilization of another (for review see (Wyatt and Balice-Gordon 2003). It is now established that progressive functional change, in the form of alterations in probability of release and synaptic efficacy, is coincident with significant structural rearrangement at eliminating terminals, with repeated cycles of weakening and destabilization resulting in the ultimate retraction and resorption of all but one competing axonal input (Balice-Gordon, Chua et al. 1993; Colman, Nabekura et al. 1997; Gan and Lichtman 1998; Kopp, Perkel et al. 2000). Yet, while the NMJ stands as one of the most highly

studied synapses in terms of developmental elimination, the identification of the critical molecular events that are necessary for this refinement have proved elusive.

One potential molecular candidate may yield from an analogous line of research based in the mammalian visual system. Significant insight into the potential mediators of activity-dependent synaptic changes came in the form of an unbiased mRNA screen in the developing lateral geniculate nucleus (LGN) (Corriveau, Huh et al. 1998). This study exploited the well-characterized activity-dependence of eye specific retinal input segregation in the LGN to pull out potential candidate molecules based on differential regulation in response to activity blockade. The result of this blind screen identified a highly surprising candidate: class I major histocompatibility complex (MHCI). Hitherto, class I MHC proteins were best known for their canonical role as immunological signaling molecules, being primary mediators of cytotoxic T cell activity in most nonneural tissues. Given the predominantly immunological context of MHCI function, the identification of these proteins as potential mediators of activity-dependent circuit refinement in the central nervous system presented the field of developmental neuroscience with a triad of provoking implications. Firstly, this result contradicted the prevailing theory that the "immune privileged" status of the CNS reflected a lack of expression of class I MHC molecules. Furthermore, active expression of MHCI in neurons of the LGN under nonpathological conditions suggested a neural, nonimmunological role for this family of immunological proteins. Perhaps most importantly, the timing of MHCI expression coincident with the period of synaptic refinement, coupled with its seemingly robust activity-dependent

regulation, strongly suggested that MHCI may be a mediator of the mechanistic changes that transform neural activity into the structural and functional refinement of synaptic circuits.

Chapter 1: MHCI is expressed at the neuromuscular junction during the period of activity-dependent synapse elimination

1.1 Introduction to MHCI at the neuromuscular junction

Since the initial observation of MHCI in the visual system, it has been shown that class I MHC is expressed by neurons throughout the central nervous system in a spatially and temporally restricted manner (Corriveau, Huh et al. 1998; Huh, Boulanger et al. 2000; Goddard, Butts et al. 2007). MHC I mRNA has been observed in the LGN, hippocampus, somatosensory cortex and retina during the first two postnatal weeks of development, placing these immunological molecules at the right place and time to play a role in activity-dependent structural refinement (Corriveau, Huh et al. 1998; Huh, Boulanger et al. 2000).

Interestingly, both MHCI mRNA and mRNA for β2m, an associated light chain required for proper surface trafficking and expression of many MHCI molecules, have been observed in the cell bodies of motor neurons in the spinal cord of adult mice and rats (Linda, Hammarberg et al. 1998; Oliveira, Thams et al. 2004; Sabha, Emirandetti et al. 2008). Recent studies have expanded upon this work to show MHCI protein is expressed in the presynaptic axons of some motor neurons and at a subset of neuromuscular junctions in a putatively presynaptic distribution (Thams, Brodin et al. 2009). However, virtually nothing is known about the developmental expression patterns of MHCI proteins in the spinal cord and NMJ. Consequently, to determine if MHCI is expressed at a time and place consistent with a role in synapse

elimination, we immunostained whole mounts of mouse diaphragm muscle at several stages of development using a panspecific anti-MHCI antibody (OX18) in combination with antibody against the presynaptic protein synaptophysin (SYPH) and α-bungarotoxin (α-btx) to label postsynaptic nicotinic acetylcholine receptors (nAChRs) at the muscle endplate.

1.2 Experimental methods

All animals were housed and used in accordance with the protocols established by the UCSD Animal Subjects Program. For this study, two genotypes of mice were used: C57B1/6 wild-type (WT, Harlan) and β2m/TAP knock-out ($\beta2m^{-/-}TAP^{-/-}$, courtesy of David Raulet, UC Berkeley, and Carla J. Shatz, Stanford University).

Whole mounts of muscles from mice were immunostained at P7, P15 and P29-50 (adulthood). Animals were deeply anesthetized using inhalational isoflurane and perfused with 4% PFA (Electron Microscopy Systems) in phosphate buffered saline (PBS, Sigma). Diaphragm muscles were dissected, rinsed in PBS, and treated with rhodamine-conjugated α-bungarotoxin (Molecular Probes, 10μg/100μL) to label postsynaptic nicotinic acetylcholine receptors. Muscles were blocked in a solution containing 5% bovine serum albumin, .01% Triton X-100 and .1% sodium azide (Fischer) for one hour. Muscles were treated with primary antibodies against MHCI (OX18, AbdSerotec, 1:100) and synaptophysin (SYPH, Santa Cruz, 1:100) overnight at 4^oC followed by a four hour treatment with Alexa-488-conjugated donkey anti-mouse IgG (Invitrogen, 1:1000) and Cy5-conjugated donkey anti-goat IgG (Jackson,

1:100). Z-serial (.5μm per step, 3μm total per junction) images were collected from entire muscles using a Leica confocal microscope under a 63x oil immersion lens at 1.6x magnification.

1.3 Results

In wild type (WT) animals, MHCI immunoreactivity is detectable at the NMJ on postnatal day 7 (P7), during the period of active remodeling (Figure 1) and overlaps extensively with nAChRs. The majority of junctions imaged at P7 displayed MHCI immunoreactivity (20 out of 23), although intensity varied from site to site in whole mount muscles. Furthermore, MHCI continues to be expressed at the NMJ through the end of the remodeling process (Figure 1, P15, 18 out of 36) and into adulthood (Figure 1, P29-50, 28 out of 39) in a largely postsynaptic distribution. Hence, MHCI proteins are not only expressed at the postsynaptic motor endplate during the establishment of mature patterns of connectivity, but also during the maintenance of those synaptic inputs.

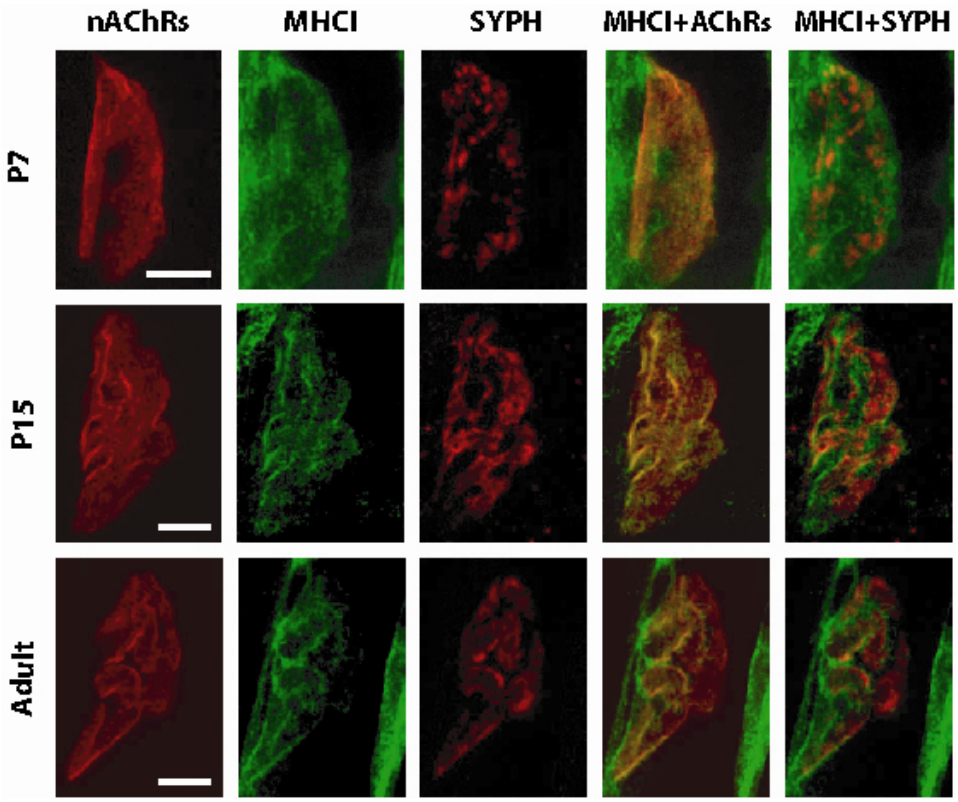

Figure 1. MHCI is expressed postsynaptically at the developing and adult mouse neuromuscular junction. A. Representative images of triple-label immunohistochemistry of the mouse neuromuscular junction in the diaphragm muscle at P7, P15 and adulthood (P29-P35). MHCI immunoreactivity (OX18 antibody; green) colocalizes with postsynaptic nAChRs (α-bungarotoxin; red) but not with the presynaptic marker synaptophysin (SYPH; red). Scale=10μm, n=3 for all ages.

Immunostaining with an isotype-matched IgG1 antibody was also performed on these samples to control for nonspecific binding affinity of the primary antibody. Treating muscles with the IgG1 control primary antibody resulted in nonspecific staining that appears qualitatively different from the pattern revealed by the OX18 antibody (Figure 2). Furthermore, when both OX18 and IgG1 samples were imaged during the same microscopy session and analyzed in ImageJ for relative staining

intensities, a four-fold greater selectivity was observed in the OX18 treatment group compared to control.

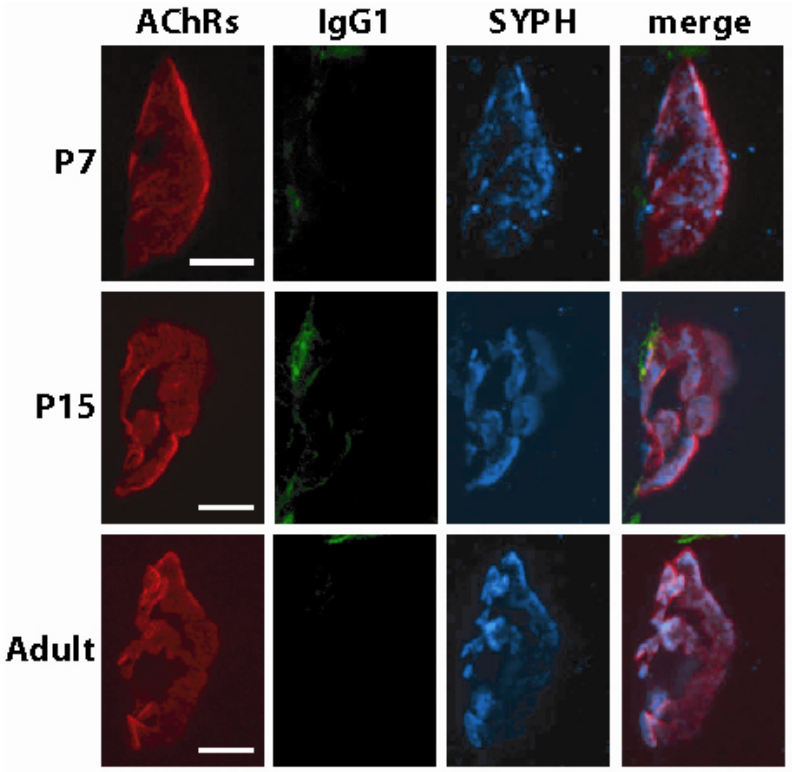

Figure 2. IgG does not label WT NMJs. A. Representative images of triple-labeling of the diaphragm using α-bungarotoxin (nAChRs, red), isotype-specific control antibody (IgG1, green) and anti-synaptophysin antibody (SYPH, blue). Unlike anti-MHCI antibodies, anti-IgG1 does not significantly label the NMJ (compare to MHCI labeling in Figure 1). IgG labeling experiments performed on the same animals as in Figure 1 and imaged on the same day, with identical laser intensity and exposure parameters. Scale, 10μm; n = 3 for all ages.

The present findings are consistent with the observation that both unstimulated myotubes and differentiated myoblasts express low levels of MHCI proteins *in vitro* (Emslie-Smith, Arahata et al. 1989; Nagaraju, Raben et al. 1998). However, recent studies have reported expression of MHCI mRNAs in a fraction of adult mouse motor neurons, as well as the expression of the classical MHCI molecule H2-D^b in a subset of motor neuron terminals at the adult mouse neuromuscular junction (Linda,

Hammarberg et al. 1998; Oliveira, Thams et al. 2004; Thams, Brodin et al. 2009). Our results are not inconsistent with these studies, and the observed distribution of MHCI immunoreactivity may reflect differential localization of MHCI at the NMJ. Specifically, Thams *et al* reported presynaptic localization of MHCI immunoreactivity based on use of the ER HR 52 antibody, shown to be selective for the classical MHCI molecule H2-D^b on nonneural cells (Thams, Brodin et al. 2009). Since the present dissertation utilized the panspecific OX18 antibody, thereby recognizing both classical and nonclassical members of the MHCI family, it is possible that the difference in patterns of immunostaining reflect the detection of nonclassical MHCI at the developing and adult neuromuscular junction.

While MHCI is a gene family with over 60 members in mouse, many MHCI proteins require both the light chain β2m and TAP proteins for stable surface expression. Therefore, in order to study the consequence of loss of function of MHCI on synapse elimination, we used mice with a genetic deletion of both β2m and TAP ($\beta2m^{-/-}TAP^{-/-}$ transgenic mice) that lack stable surface expression of most MHCI proteins (Ljunggren, Van Kaer et al. 1995). Accordingly, when we performed immunostaining on whole mount diaphragms from these mice (Figure 3) we were able to confirm a general decrease in surface protein expression of MHCI.

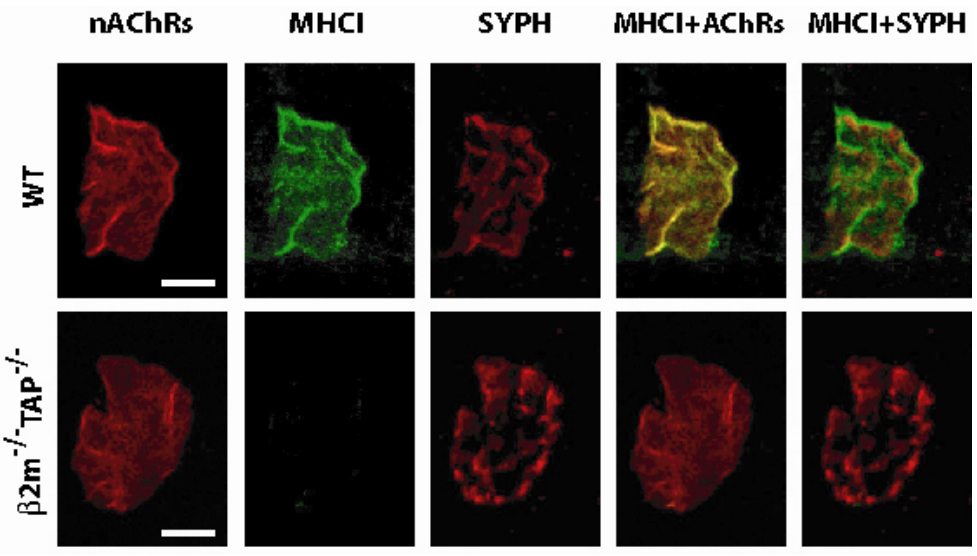

Figure 3. Genetic deletion of β2m and TAP reduces MHCI immunoreactivity at the NMJ. Immunohistochemistry of the NMJ at P15 in WT and $\beta2m^{-/-}TAP^{-/-}$ mice using antibody against MHCI (OX18, green) and synaptophysin (SYPH, red), as well as α-btx (nAChRs, red). Mice lacking β2m and TAP display decreased MHCI protein expression compared to WT controls imaged under identical conditions during the same session. Scale, 10μm; n = 3 for both genotypes.

Chapter 2: Loss of MHCI cell surface expression persistently impairs activity-dependent structural remodeling at the developing NMJ

2.1 Introduction to synapse elimination at the NMJ

Unequivocal monoinnervation of the mature NMJ was first visualized more than 100 years ago using osmium-gold staining (Gillingwater and Ribchester 2003). While this initial observation has withstood the evolution of a legion of experimental techniques, it is now understood that this pattern of mature connectivity, i.e. the singular relationship between a muscle and its sole innervating motor neuron, is neither predetermined nor immutable. Developmentally, all muscles undergo a transient period of polyinnervation, receiving as many as 12 distinct inputs from different motor neurons in the spinal cord at birth (Sanes and Lichtman 1999; Song, Panzer et al. 2006). These initially superabundant inputs are removed in a protracted process called synapse elimination until each muscle fiber, with very few exceptions, receives input from a single motor neuron (Wyatt and Balice-Gordon 2003).

Since the original observation of developmental polyinnervation in rat diaphragm muscle (Redfern 1970), the process of synapse elimination at the NMJ has received nearly four decades of intense scientific study. Occurring well after the period of programmed motor neuron cell death (Clarke and Oppenheim 1995), it is widely held that synapse elimination is governed by local cues at the neuromuscular junction itself that seemingly pit motor neuron terminals in direct competition with

each other for synaptic survival (Brown, Jansen et al. 1976; Thompson, Kuffler et al. 1979; Keller-Peck, Walsh et al. 2001; Buffelli, Burgess et al. 2003). A cascade of structural and functional changes underlies this competitive process in which two initially equivalent synaptic inputs are discriminated into an eventual "winning" and "losing" axon. This manifests as one synaptic input undergoing cycles of physiological strengthening and structural expansion at the functional and structural expense of its competitive partner, until the "losing" axon is eventually eliminated from the NMJ in a retraction bulb (Bishop, Misgeld et al. 2004).

To spite a long history of active inquiry, the molecular mediators of synapse elimination have remained obscure at the NMJ, although it is well appreciated that this structural remodeling process is highly influenced by neural activity (or review (Buffelli, Busetto et al. 2004). Because MHCI has been implicated in activity-dependent remodeling of developing central synapses and axotomized MNs (Huh, Boulanger et al. 2000; Oliveira, Thams et al. 2004), and given that my results suggested this protein family was expressed at a time and place consistent with a role in the development of the NMJ, I investigated whether MHCI is a mediator of structural synapse elimination at the developing NMJ.

2.2 Experimental methods

To determine if MHCI is required for synapse elimination at the neuromuscular junction, fluorescence microscopy was used to assess NMJ innervations in WT and mice lacking cell surface expression of MHCI. Whole mounts of muscles from mice were immunostained at P0, P7, P15 and P29-50 (adult).

Animals were deeply anesthetized using inhalational isoflurane and perfused with 4% PFA (Electron Microscopy Systems) in phosphate buffered saline (PBS, Sigma). Diaphragm muscles were dissected, rinsed in PBS, and treated with rhodamine-conjugated α-bungarotoxin (Molecular Probes, 10μg/100μL) to label postsynaptic nAChRs at the endplate. Muscles were then blocked in a solution containing 2% bovine serum albumin (Jackson), .2% Triton X-100 (Fischer) and .1% sodium azide (Fischer) for one hour. Motor neuron terminals were visualized by treating muscles overnight at room temperature with a mouse primary antibody cocktail consisting of anti-neurofilament (NF; Invitrogen, 1:200) and anti-synaptic vesicle glycoprotein 2 (SV2; Developmental Hybridoma Studies Bank, 1:100). Primary antibodies were incubated for four hours with Alexa-488-conjugated goat anti-mouse IgG (Jackson, 1:100).

Antibody injections. To test the temporal and molecular specificity of MHCI in NMJ development, injections of a function blocking anti-MHC antibody (OX18) were used(Smits, Kuppen et al. 1994; Blom, De Bont et al. 1999). P7 WT mice received daily intraperitoneal injections with 10μg/g body weight of either panspecific anti-MHCI antibody (OX18; AbdSerotec) or isotype-matched mouse IgG1 control (AbdSerotec). Mice were perfused at P15 and diaphragms were either immunostained for neuromuscular morphology as described above, or were treated with Alexa 488-conjugated goat anti-mouse IgG1 (Invitrogen, 1:100) for four hours to visualize OX18 and IgG1 isotype-control immunoreactivity.

Z-serial images were collected from entire muscles using a Leica spinning disk confocal microscope under a 63x oil immersion lens at 1.6x magnification. At least 50 terminals from each diaphragm were examined from individual animals at each age, blind to genotype. Innervation was assessed by counting the number of distinct motor nerve terminals clearly contacting each postsynaptic junction. Postsynaptic nAChR perimeter and area were quantified and measured using ImageJ software (NIH). All data are expressed as mean ± standard error and tested for significance using an unpaired student's t-test.

2.3 Results

Consistent with previous results (Redfern 1970; Kopp, Perkel et al. 2000), synapse elimination is largely complete in WT mice by P15, with nearly all muscle cells receiving input from a single anatomically distinct axon (Figure 4). In contrast, in mice that lack cell-surface MHCI, a significantly larger subset of endplates received visible inputs from more than one MN at P15 (5.5% ± 1.5% in WT vs. 17.9% ± 3.6% in $\beta 2m^{-/-}TAP^{-/-}$ mice, $p = 0.020$, unpaired t-test). The presence of multiple innervation at P15 may reflect a delay of synapse elimination or a persistent failure of activity-dependent remodeling. To distinguish between these two possibilities, innervation was assayed anatomically in adult WT and $\beta 2m^{-/-}TAP^{-/-}$ mice (P29-50). Surprisingly, mice lacking cell surface MHCI retain multiple inputs at a significant number of junctions into adulthood (Figure 5; multiple innervation 2.3% ± 1.7% in WT vs. 18.6% ± 4.3% in $\beta 2m^{-/-}TAP^{-/-}$; $p = 0.012$, unpaired t-test), representing roughly an 8-

fold increase in the level of multiple innervation over WT at this age, indicating that synapse elimination is persistently impaired in the absence of cell surface MHCI.

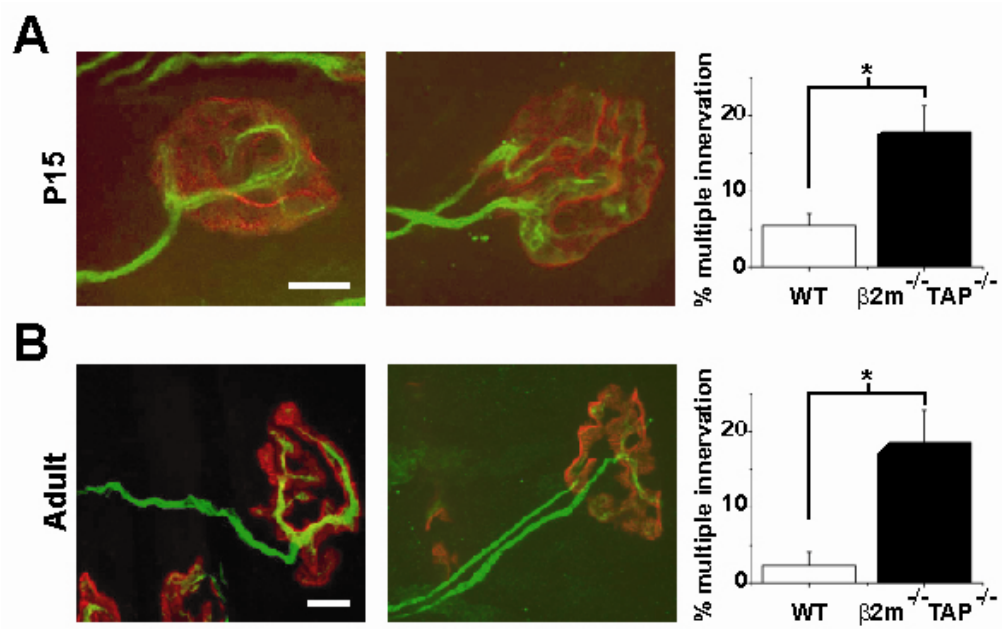

Figure 4. Loss of cell surface MHCI persistently impairs synapse elimination at the NMJ. A. Immunostaining of motor axons and presynaptic nerve terminals (NF and SV2, green) and postsynaptic motor endplates (α-bungarotoxin, red) in mouse diaphragm. *Left,* typical NMJs from WT and β2m$^{-/-}$TAP$^{-/-}$ diaphragm at P15 (A) and adulthood (P29-50, B). *Right,* quantification of anatomically assessed polyneuronal innervation. Statistically significant levels of polyinnervation of individual motor endplates are retained in β2m$^{-/-}$TAP$^{-/-}$ but not WT muscle in P15 and adult animals. P15: n=5 β2m$^{-/-}$TAP$^{-/-}$, n = 4 WT; adult: n = 4 both genotypes. Scale bars, 10μm.

Previous work has demonstrated that excessive motor axon sprouting and outgrowth in response to elevated levels of growth factors, such as GDNF, can delay synapse elimination at the NMJ(Nguyen, Parsadanian et al. 1998; Keller-Peck, Feng et al. 2001). In contrast, gross phrenic nerve morphology and axonal branching were qualitatively normal in MHCI-deficient animals at P0 (data not shown). Furthermore, the percentage of multiply innervated junctions at earlier ages in β2m$^{-/-}$TAP$^{-/-}$ mice were indistinguishable from WT (Figure 5) and muscles were initially contacted by

similar numbers of axons, suggesting that the observed remodeling impairment is not likely to reflect a defect in axon branching or inability to remove an abundance of supernumerary axonal inputs.

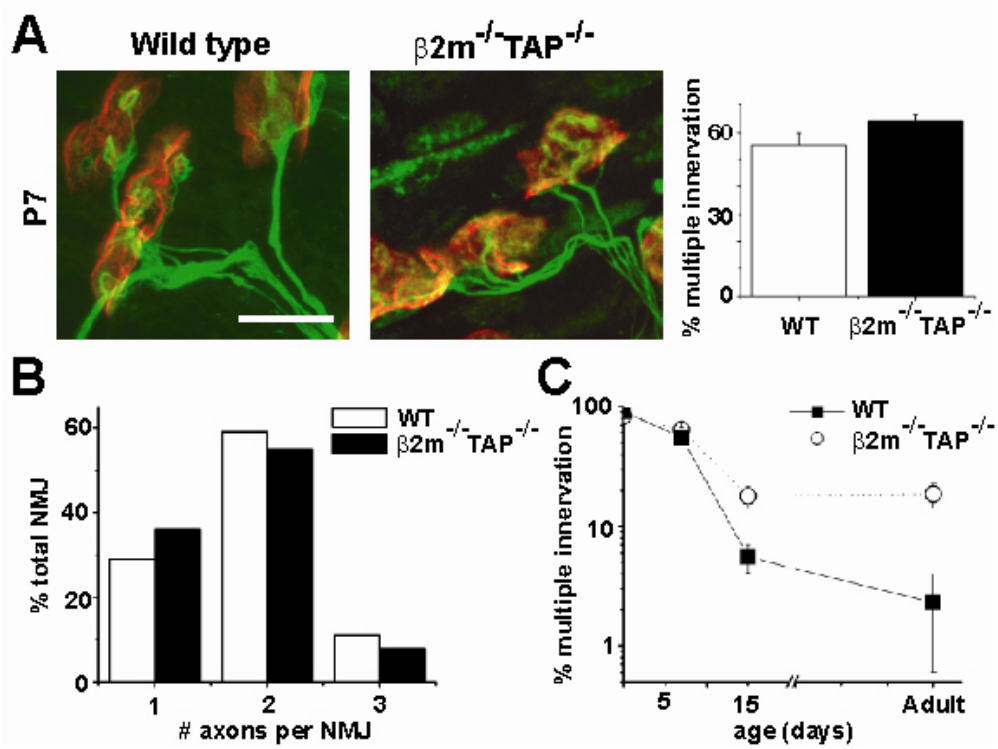

Figure 5. Impaired synapse elimination in BT mice is not due to an overgrowth of motor neuron terminals. A. Immunostaining of motor axons and presynaptic nerve terminals (NF and SV2, green) and postsynaptic motor endplates (α-bungarotoxin, red) in mouse diaphragm. *Left,* typical NMJs from WT and β2m$^{-/-}$TAP$^{-/-}$ diaphragm at P7. *Right,* quantification of anatomically assessed polyneuronal innervation. Levels of polyinnervation are indistinguishable between WT and BT mice at P7. Multiple innervations P7: 55.1 ± 4.8% in WT vs. 64.1 ± 2.2% in β2m$^{-/-}$TAP$^{-/-}$, p = 0.20; unpaired t-test. B. Number of discrete axons contacting each motor endplate at P7 in WT and β2m$^{-/-}$TAP$^{-/-}$ mice. There is no observable difference in the proportion of axons contacting individual muscle fibers in mice lacking cell surface MHCI. C. Logarithmic plot of the time course of synapse elimination in WT and β2m$^{-/-}$TAP$^{-/-}$ mice. Synapse elimination is significantly impaired in β2m$^{-/-}$TAP$^{-/-}$ mice by P15, and aberrant levels of polyneuronal innervation persist into adulthood. Percent polyneuronal innervation P0: 90.2 ± 4.6% in WT vs. 83.3 ± 3.1% in β2m$^{-/-}$TAP$^{-/-}$, p = 0.13. P0: n = 4 both genotypes.

Another potential way in which synapse elimination could be impaired in mice deficient for cell-surface MHCI is if loss of gene function resulted in a general delay

of development. Consequently, we examined the gross development of the diaphragm muscle at P15 and found no difference in the overall distribution of endplates, in both the intact whole mount muscle, and when examined in individually teased muscle fibers (Figure 6). Additionally, postsynaptic endplates appeared to mature normally in mice lacking surface MHCI and mean area (152 ± 5.7μm^2 vs. 157 ± 4.2μm^2, $\beta 2m^{-/-}$ $TAP^{-/-}$ and WT, respectively; $p = 0.50$) and perimeter of nAChRs at endplates (117 ± 11.9μm vs. 101 ± 3.0μm, $p = 0.22$) did not differ between WT and $\beta 2m^{-/-}TAP^{-/-}$ mice at P15 (Figure 7). Consequently, it appears that mice lacking surface MHCI not retain polyinnervation due to a disruption of the development of the NMJ.

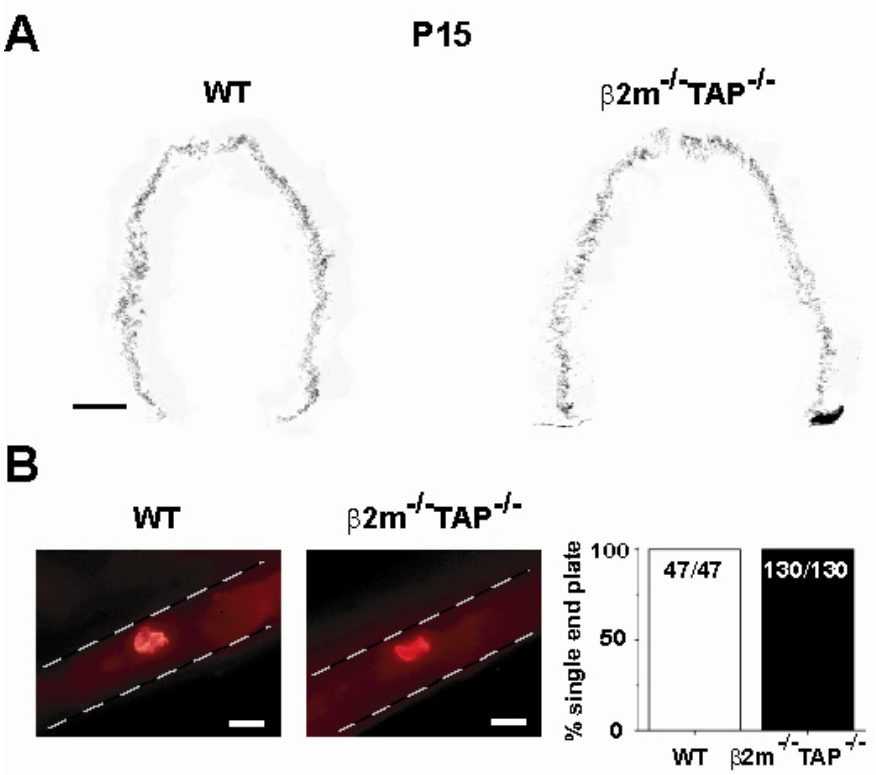

Figure 6. Endplate band morphology and junctions per muscle are normal in mice lacking surface MHCI. A. Montage images of nAChRs labeled using α-btx. Diaphragm muscles develop normally and postsynaptic specializations are confined to the endplate band in the center of the muscle in a distribution comparable to WT. Scale, 2mm. B. *Left,* Representative images of single muscle fibers teased from intact diaphragms from P15 WT and β2m$^{-/-}$TAP$^{-/-}$ mice labeled with α-btx (red). Dashed lines mark the boundary of individual muscle fibers. *Right,* quantification of the number of individual endplates per single muscle fiber in WT and β2m$^{-/-}$TAP$^{-/-}$ mice. Scale, 20μm. n = 3 for both genotypes.

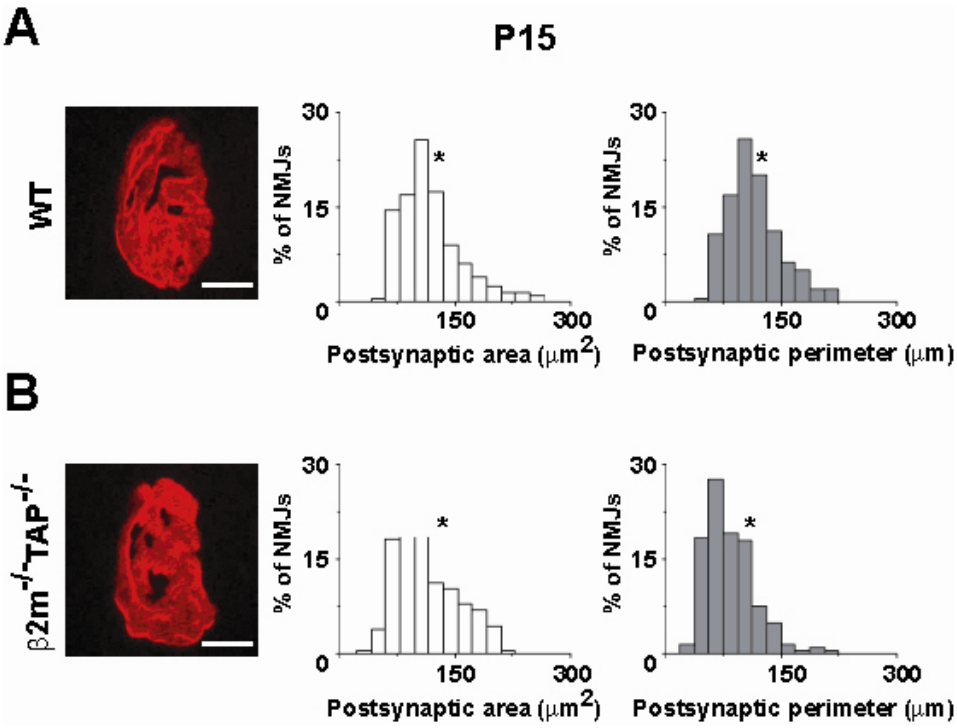

Figure 7. Postsynaptic endplate maturation, area and perimeter are normal in MHCI-deficient animals. A, Representative images showing mature distribution of nAChRs at the endplate at P15 in both WT and β2m$^{-/-}$TAP$^{-/-}$ animals. Scale, 10μm. B. Quantification of the perimeter and area of nAChR labeling in WT and β2m$^{-/-}$TAP$^{-/-}$ mice at P15. There is no difference in the mean area (157 ± 4.2μm^2 in WT vs. 152 ± 5.7μm^2 in β2m$^{-/-}$TAP$^{-/-}$; p = 0.50) or perimeter (101 ± 3.0μm in WT vs. 117 ± 11.9μm in β2m$^{-/-}$TAP$^{-/-}$; p = 0.22) of endplates in mice lacking cell surface MHCI relative to controls. Asterisks mark mean values in each graph. n = 5 animals for each genotype; WT 214 junctions, β2m$^{-/-}$TAP$^{-/-}$ 216 junctions.

However, given that our mutant mice lack the genes encoding the proteins β2m and TAP, it is possible that persistent polyneuronal innervation reflects previously uncharacterized functions for these two proteins, rather than specific involvement of cell surface MHCI. To directly address this question, MHCI function was acutely manipulated during synapse elimination using a function-blocking antibody (OX18) (Smits, Kuppen et al. 1994; Blom, De Bont et al. 1999). Daily intraperitoneal injections of OX18 or an isotype-control antibody (IgG1) were administered to WT mice from P7 to P15 and diaphragm muscles were processed for immunostaining.

Injected OX18 was visible at the NMJ following detection with labeled secondary antibody and lead to a 293% increase in the percentage of polyneuronal innervation compared to IgG1 injected littermate controls (Figure 8A & B; 3.0 ± 0.8% polyneuronal innervation in IgG-injected animals vs. 8.8 ± 1.7% in OX18-injected animals; p = 0.020, unpaired t-test). Thus the retention of multiple motor neuron inputs in $\beta 2m^{-/-}TAP^{-/-}$ mice is due to a loss of MHCI function, and reflects a requirement for MHCI specifically during the period of remodeling.

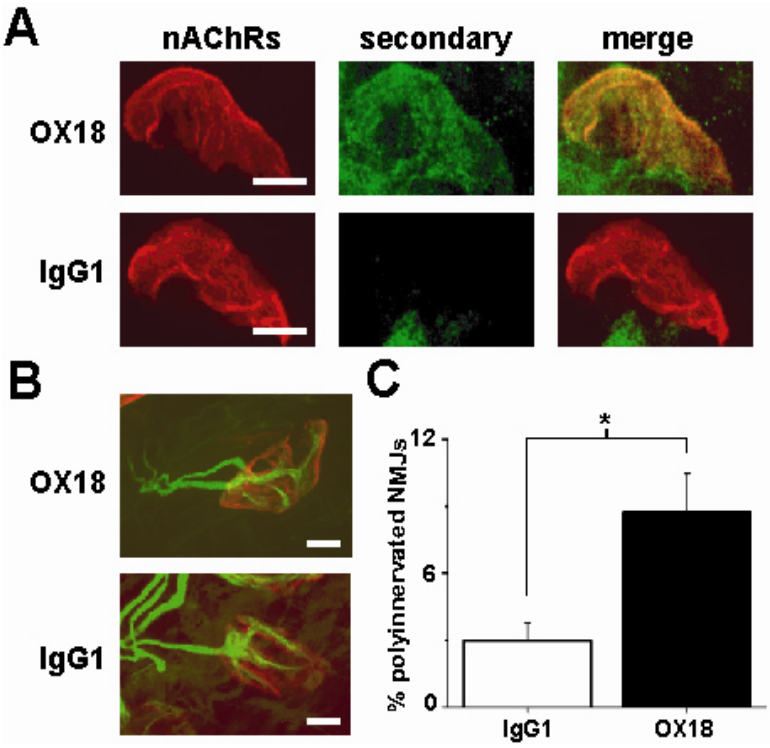

Figure 8. Injection of anti-MHCI antibodies impairs synapse elimination at the developing NMJ. A. Labeling of nAChRs at the postsynaptic endplate (α-btx, red) and detection of injected anti-MHCI or anti-IgG1 primary antibodies with anti-mouse secondary antibody (green; see Methods). Injected OX18 antibody colocalizes with postsynaptic nAChRs at the NMJ. B. Example NMJs from IgG1- and OX18- injected animals. nAChRs, red; presynaptic axons and terminals labeled with anti-SV2 and anti-neurofilament, green. C. Quantification of polyinnervation in IgG1- and OX18-injected animals. Acute treatment with OX18 results in a statistically significant increase in the level of polyinnervation compared to control. All animals imaged at P15. Scale bar, 10μm. OX18, n = 9 animals from 4 litters; IgG1, n = 6 animals from 3 litters.

Chapter 3: Impaired synapse elimination is associated with an increase in quantal size in mice lacking cell surface MHCI

3.1 Introduction to functional plasticity during synapse elimination at the developing NMJ

Synapse elimination at the developing NMJ is characterized by a cascade of structural and functional changes whereby one axon expands and strengthens its input into adulthood at the competitive expense of its synaptic partner, which is weakened and eventually withdrawn. Physiologically, one of the first hallmarks of functional asymmetry between two initially equivalent terminals is a disparity in the probability of release of neurotransmitter, with the eventual winning axon shifted towards a state of higher probability of release than its synaptic counterpart (Kopp, Perkel et al. 2000). Immediately after, differences in quantal size and quantal content, indicators of overall synaptic strength, begin to emerge (Colman, Nabekura et al. 1997). The end result is that one axon develops nearly 4-fold increased synaptic efficacy compared to its competitive partner immediately preceding structural withdrawal.

Therefore, one potential hypothesis in explanation of the observed impairment in synapse elimination in mice lacking cell-surface MHCI is that MHCI proteins normally mediate a portion of the functional changes that are thought to be integral to structural withdrawal. Consequently, in the absence of MHCI, competitive neural activity may not be translated into a reduction in functional synaptic strength that is required for the structural removal of synaptic inputs at the NMJ during synapse

elimination. To investigate this possibility, I used electrophysiological techniques to examine the functional characteristics of monoinnervated muscle in both WT and transgenic animals lacking cell surface MHCI.

3.2 Experimental methods

Hemidiaphragm-phrenic nerve preparations were used from WT and $\beta 2m^{-/-}$ $TAP^{-/-}$ animals at P14-16 and P29-P45. Muscles were dissected from anesthetized animals under cold, oxygenated (95% CO_2, 5% O_2) Rees mammalian ringers (NMR) consisting of (in mM): 135 NaCl, 5 KCl, 15 $NaHCO_3$, 1 Na_2HPO_4, 1 $MgSO_4$, 2.5 Ca gluconate, and 11 glucose, pH 7.4 (Rees, 1970). Contraction blockade was achieved in muscles either via bath perfusion with 3-6μM tubocurarine, 30 minute (adult) or 1 hour (P14-16) pretreatment in oxygenated 2μM μ-conotoxin (Bachem). When tubocurarine was used, the extracellular calcium concentration was raised to 10mM to improve the stability and quality of recordings. Muscles were transferred and pinned to a Sylgard-lined recording chamber and superfused (1mL/min) with oxygenated NMR at 22-24^{o}C. Intracellular recordings of muscles fibers were performed using recording electrodes (30-69 MΩ) filled with 3M KCl. Endplate potentials (EPPs) were evoked by suprathreshold stimulation of the phrenic nerve with a suction electrode (square pulses, .2ms duration, .5Hz). Electrodes were guided to neuromuscular junctions by oblique illumination and only responses with rise times of less than 1.5ms were included for study. Membrane potentials were continuously recorded and only muscles in which potentials were <-55mV and stable within 5mV

were selected for analysis. Membrane responses were amplified by an Axoclamp 2A amplifier (Axon Instruments), low-pass filtered at 1 kHz, digitized and recorded using WinWCP software (John Dempster; WinWCP).

Levels of innervation were assessed by providing graded stimulation to the phrenic nerve and recording the number of elicited inputs of differential amplitude and latency. Miniature end-plate potentials were also recorded using gap free traces and analyzed after collection using MiniAnalysis software (Justin Lee; Synaptosoft). Quantal content was calculated by evoking endplate potentials and recording miniature events in the following 2 seconds. Quantal content was estimated as the ratio of the mean endplate potential amplitude to the mean miniature endplate potential amplitude (Wood and Slater 2001). Paired pulse recordings were also made at 10, 20 and 1000 ms interstimulus intervals to provide an estimation of the facilitation index of each recorded junction by dividing the amplitude of the second EPP by the amplitude of the first EPP.

3.3 Results

Graded stimulation was applied to the phrenic nerve and the number of discrete increases in the amplitude of the endplate potential (EPP) were counted, which reflect the progressive recruitment of multiple synaptic inputs to a single muscle (Redfern 1970). In WT animals at P15, graded stimulation produced endplate potentials of a single amplitude in all muscles measured, indicative of functional monoinnervation (Figure 9A, 16 out of 16). In muscles from P15 MHCI-deficient

animals, however, graded stimulation lead to a stepwise increase in the amplitude of the EPP at 21% of the junctions evaluated (Figure 9A, 4 out of 19), reflecting the presence of at least two functional axonal inputs. Remarkably, multiple functional inputs could still be recruited in 13% of the adult junctions in MHCI-deficient adult animals (Figure 9B, 5 out of 38). At both ages, the percentage of functionally polyinnervated muscle fibers measured electrophysiologically was comparable to that observed anatomically, indicating that many, if not all, of the visible inputs are functional.

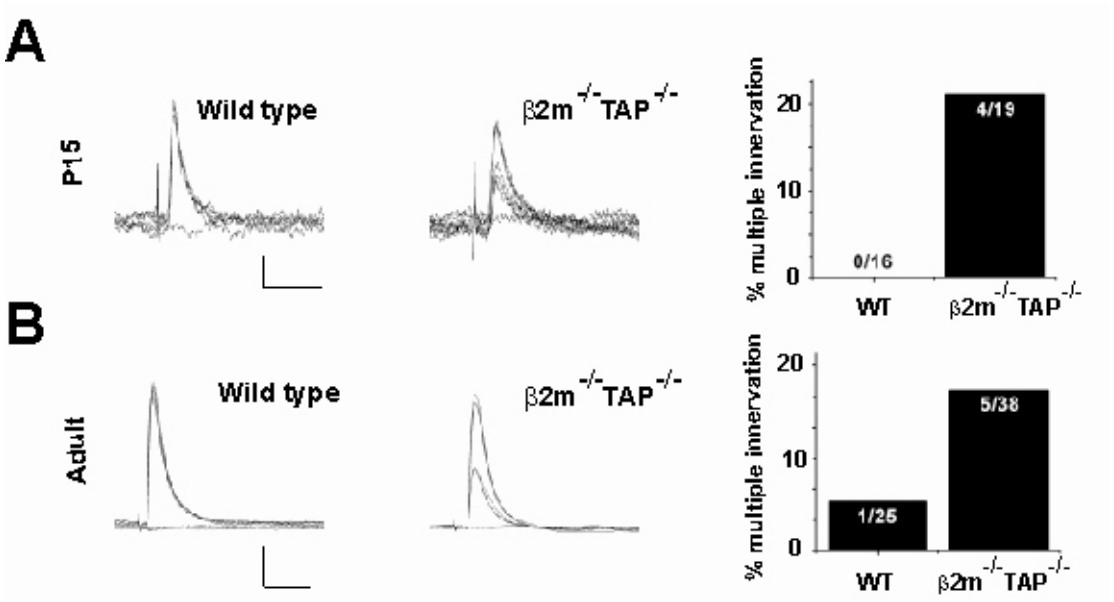

Figure 9. Loss of cell surface MHCI results in the retention of functional polyneuronal inputs. A, Electrophysiological recordings of EPPs in P15 mice using 3-6μM tubocurarine. Graded stimulation evoked multiple EPPs of different amplitudes at a subset of junctions in β2m$^{-/-}$TAP$^{-/-}$ mice (*center*); Scale, 1mV, 15msec. *Right,* quantification of the extent of functional polyinnervation in WT and β2m$^{-/-}$TAP$^{-/-}$ mice (WT, n = 16 muscles from 8 animals; β2m$^{-/-}$TAP$^{-/-}$, n = 19 muscles from 6 animals). B. Electrophysiological recordings of EPPs in adult (P29-45) mice using 2μM μ–conotoxin pretreatment. Functional polyinnervation persists into adulthood in β2m$^{-/-}$TAP$^{-/-}$ mice (WT, n = 25 muscles from 5 animals; β2m$^{-/-}$TAP$^{-/-}$, n = 38 muscles from 8 animals; Scale, 10mV, 10ms).

The persistence of functional polyinnervation in MHCI-deficient animals suggests that MHCI is not required solely for the structural removal of synaptically silent inputs, but rather may be involved in the functional weakening of individual motor axon synapses that is thought to lead to synapse elimination. One of the first functional asymmetries observed between competing motor neuron inputs during synapse elimination is a change in the paired pulse ratio, an indirect measurement of the probability of release at an individual synapse (Kopp, Perkel et al. 2000). To test this hypothesis, I used sharp electrode recordings to record paired evoked EPPs from muscles receiving single inputs in both mutant and wild-type mice at P15 in the presence of tubocurarine. Mice lacking cell-surface MHCI display a systematic shift towards an increased paired pulse ratio at all stimulus frequencies tested, and the paired pulse ratio was significantly larger at both 10msec and 20msec intervals (Figure 10; 10msec: WT 1.015248 ± 0.10 vs. $\beta 2m^{-/-}TAP^{-/-}$ 1.36 ± 0.05, $p = 0.030$; 20msec: WT 0.92 ± 0.09 vs. $\beta 2m^{-/-}TAP^{-/-}$ 1.24 ± 0.09, $p = 0.042$; unpaired t-test), indicating a lowered probability of release under these experimental conditions.

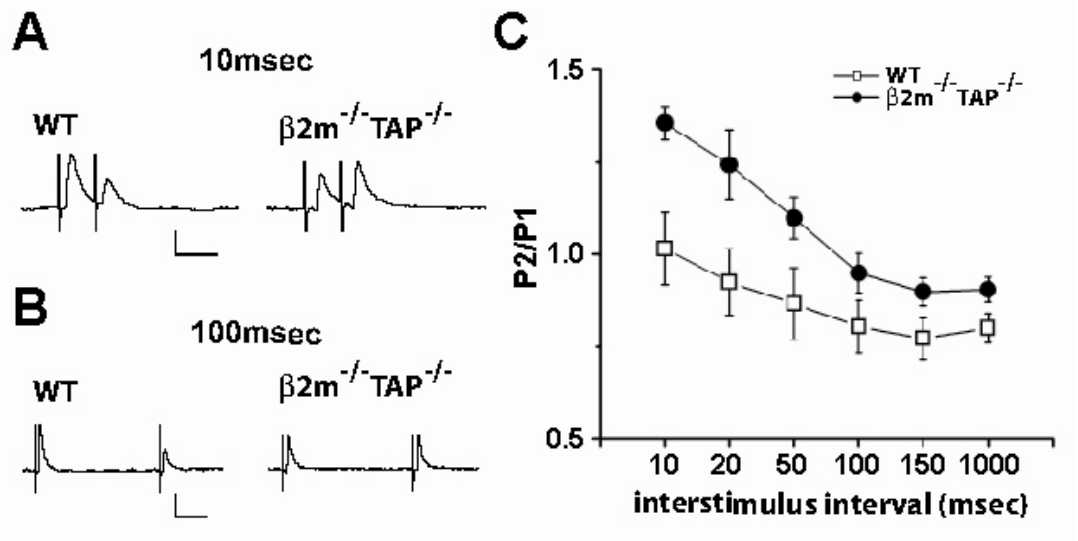

Figure 10. The paired pulse ratio is increase in mice lacking cell surface MHCI in 10mM Ca^{2+}. A. Sample traces of paired EPPs evoked 10msec apart in the presence of 3-6μm tubocurarine in WT (*left*) and $\beta2m^{-/-}TAP^{-/-}$ (*right*) animals at P15. Although high calcium shifts the normally facilitating WT synapse towards depression, $\beta2m^{-/-}TAP^{-/-}$ mice display facilitation of the paired pulse response, indicative of a lower probability of release under these conditions. B. Sample traces of paired evoked EPPs separated by 100msec. C. Plot of the paired pulse ratio obtained at different interstimulus intervals in WT and mutant mice. At 10 and 20msec, $\beta2m^{-/-}TAP^{-/-}$ mice have significantly larger paired pulse ratios when compared to WT controls. (WT, n = 9 muscles from 4 animals, $\beta2m^{-/-}TAP^{-/-}$, n = 5 muscles from 3 animals. *Top*, Scale = 1mV, 10msec. *Bottom*, Scale = 1mV, 25msec.)

It is important to note that this experiment was conducted in the presence of increased external calcium (10mM, see experimental methods 3.3) to improve both stability and signal to noise in neonatal muscles. However, probability of release is highly influenced by both intra- and extracellular calcium levels (Thomson 2000). Consequently, WT paired pulse ratios, which are normally greater than 1, are shifted towards lower values, or towards short-term depression. While this observed shift in probability of release was not displayed in $\beta2m^{-/-}TAP^{-/-}$ mice, it remained possible that the observed phenotype reflected a difference in the amount or sensitivity of calcium channels and/or calcium-sensing release machinery that was unmasked by increased

exogenous calcium, rather than a basal difference in probability of release. To distinguish between these two possibilities, I repeated measurements of the paired pulse ratio in extracellular solution using physiologic levels of extracellular calcium (2mM, see experimental methods 3.3) and blocked muscle contraction using 2μm-conotoxin. Surprisingly, no statistically significant difference in the paired pulse ratio was observed in mice lacking cell-surface MHCI under conditions of normal calcium homeostasis (Figure 11). Taken together, these results indicate that loss of MHCI expression at the NMJ does not change the overall probability of release at the developing NMJ under physiological calcium concentrations. Instead, the observed difference in the paired pulse ratio at P15 in 10mM calcium may reflect decreased entry of, or sensitivity to, increased extracellular calcium in BT mice.

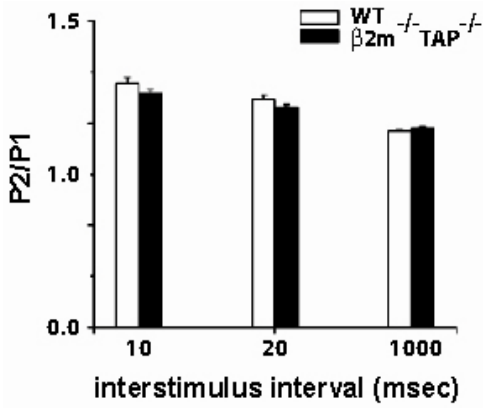

Figure 11. Paired pulse ratios are normal in 2mM Ca^{2+} in mice lacking surface MHCI. Pooled data of paired pulse ratios at 10,20 and 1000msec in WT and $\beta2m^{-/-}TAP^{-/-}$ mice. Mice lacking surface MHCI have statistically indistinguishable paired pulse ratios compared to WT controls. 10ms: WT 1.20 ± 0.03 $\beta2m^{-/-}TAP^{-/-}$ 1.15 ± 0.02, p = 0.18; 20ms: WT 1.12 ± 0.02 $\beta2m^{-/-}TAP^{-/-}$ 1.08 ± 0.01, p = 0.016, 1000msec: WT 0.96 ± 0.01 $\beta2m^{-/-}TAP^{-/-}$ 0.97 ± 0.01, p = 0.20. WT, n = 11 muscles from 6 animals, BT, n = 8 muscles from 4 animals.

Immediately after differences in probability of release emerge between competing inputs, changes in the postsynaptic effect elicited by each individual

quantum, termed quantal size, become apparent (Colman, Nabekura et al. 1997; Kopp, Perkel et al. 2000). In order to establish if loss of cell-surface MHCI alters quantal size, I used sharp electrode recordings from muscles receiving a single input in both mutant and wild-type mice and recorded postsynaptic responses evoked by individual quanta, or miniature endplate potentials (mEPPs). Surprisingly, at P15 mice lacking cell-surface MHCI showed an increase in the amplitude of mEPPs (figure 12A, 1.57 ± .09mV vs. 1.26 ±.12mV, $\beta 2m^{-/-}TAP^{-/-}$ and WT, respectively; $p = 0.043$, unpaired t-test) without a significant change in frequency. Although the average amplitude of the evoked EPP at P15 was larger in $\beta 2m^{-/-}TAP^{-/-}$ mice, this increase was not statistically significant and quantal content estimates were accordingly indistinguishable between transgenic and wild-type mice (Figure 12A). Additionally, the observed increase in mEPP size persisted into adulthood (Figure 11B, 1.0 ± .08mV vs. .66 ± .06mV; $p = 0.004$, unpaired t-test), although at this age the frequency of miniature events had also decreased, potentially as a homeostatic response to the increased mEPP size. Consequently, it appears that MHCI is critically important for the initial establishment of quantal size at the developing NMJ.

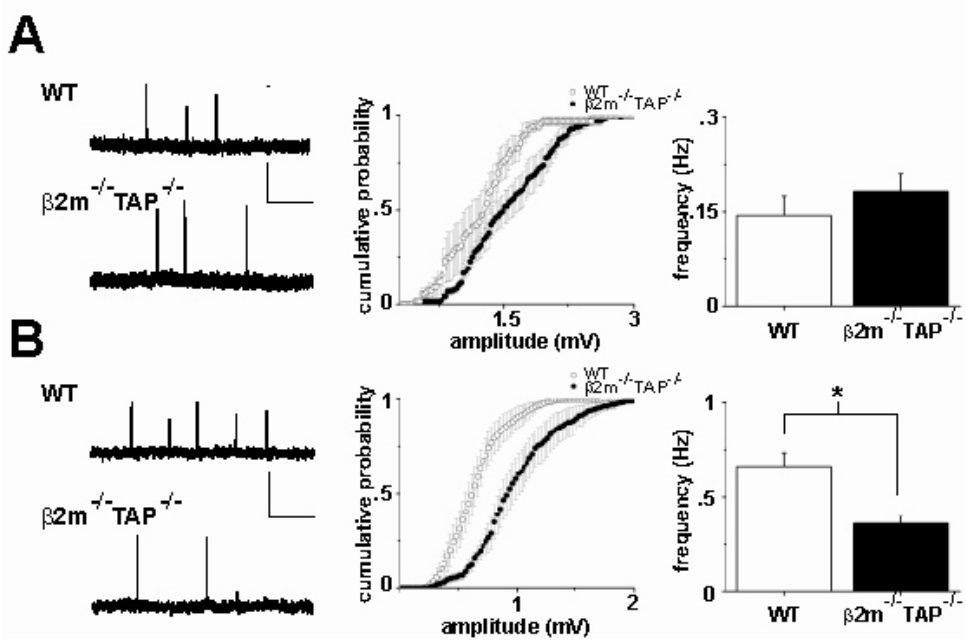

Figure 12. Quantal size is increased in mice lacking surface MHCI. C. *Left*, representative recordings from P15 muscles after 2μM μ–conotoxin treatment. mEPP amplitudes are shifted towards larger values in mice lacking cell surface MHCI (*center*), but mEPP frequency is indistinguishable from WT (*Right*; WT: frequency = 0.12 ± 0.03 Hz, n = 7 muscles from 5 animals; β2m$^{-/-}$TAP$^{-/-}$, frequency = 0.13 ± 0.04 Hz, n = 6 muscles from 5 animals; p = 0.88; unpaired t-test. Scale, 0.5mV, 500ms.) D. *Left*, representative recordings from adult muscles after 2μM μ–conotoxin treatment. mEPP amplitudes are shifted towards larger values (*center*), and mEPP frequency is significantly decreased (*right*) in adult mice lacking cell surface MHCI compared to control. (Amplitude, WT n = 11 muscles from 7 animals, β2m$^{-/-}$TAP$^{-/-}$ n = 10 muscles from 8 animals. Frequency, WT n = 10 muscles from 7 animals, β2m$^{-/-}$TAP$^{-/-}$ n = 9 muscles from 8 animals. Scale, 0.5mV, 500ms.)

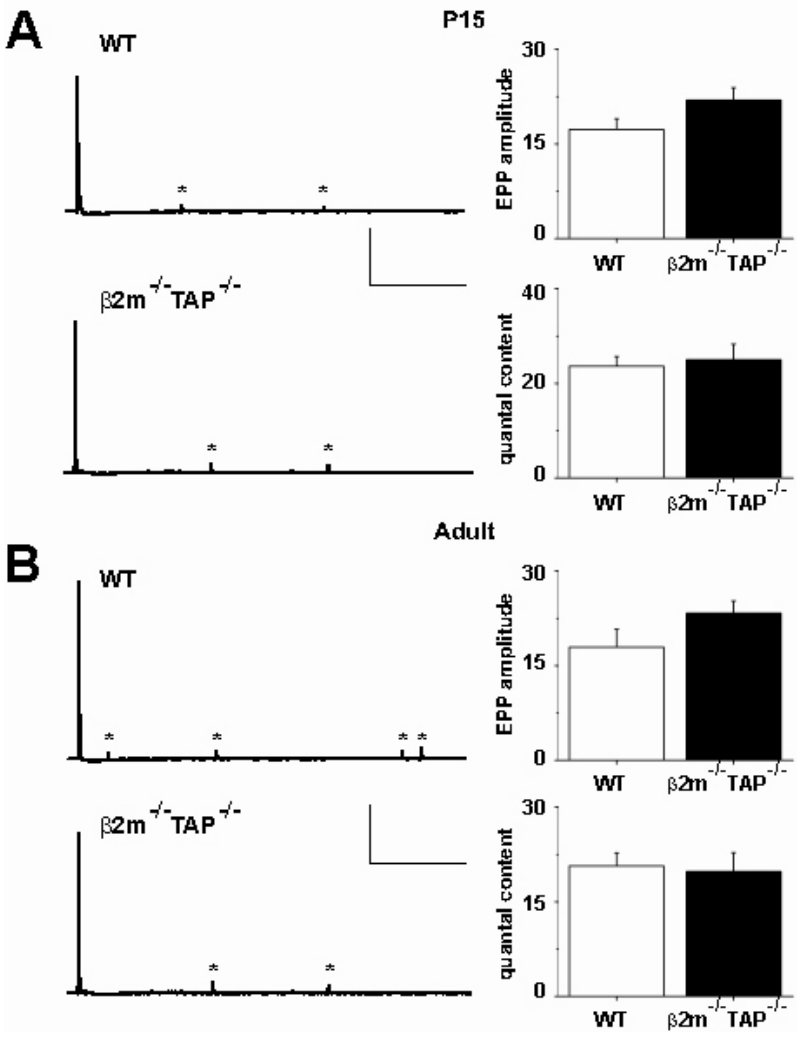

Figure 13. EPP amplitude and quantal content are normal in P15 and adult mice lacking surface MHCI. ***Left,*** **Sample traces showing evoked EPPs and spontaneous mEPPs after 2μM μ-conotoxin pretreatment. A. Recordings performed in P15 mice. Individual mEPPs are marked with asterisks. Scale, 10mV, 500ms.** ***Right,*** **Quantification of EPP amplitude and calculation of quantal content show that EPP amplitude and quantal content are unchanged in P15 MHCI-deficient animals. (WT, n = 7 muscles from 5 animals; β2m$^{-/-}$TAP$^{-/-}$, n = 4 muscles from 4 animals). B.** ***Left,*** **Sample traces showing evoked EPPs and spontaneous mEPPs after 2μM μ–conotoxin pretreatment in adult mice. Scale, 10mV, 500ms.** ***Right.*** **EPP amplitude and quantal content are also unchanged in adult MHCI-deficient animals (WT, n = 6 muscles from 4 animals; β2m$^{-/-}$TAP$^{-/-}$, n = 4 muscles from 3 animals).**

Chapter 4: MHCI is required for activity-dependent synapse elimination at the developing NMJ: implications and future directions

Many molecules have been identified as mediators of pre- and postsynaptic development of the NMJ, yet the key determinants of synapse elimination have remained elusive. Here we report that MHCI is required for normal activity-dependent synapse elimination at the developing NMJ. MHCI is expressed at the postsynaptic motor endplate during remodeling, and loss of cell surface MHCI is sufficient to persistently impair synapse elimination at a subset of junctions in the mouse diaphragm. This impairment of synapse elimination is measurable both anatomically and functionally and is associated with an increase in the quantal size in $\beta 2m^{-/-}TAP^{-/-}$ mice. Thus, endogenous MHCI is required both for the normal elimination of surplus motor axons and the establishment of appropriate quantal size at the NMJ, offering the intriguing possibility that changes in quantal size, and in particular, limiting the amplitude of the mEPP, is a critically important step in the functional cascade leading to structural withdrawal during synapse elimination.

In current models, the functional strengthening of some inputs and the weakening of others at the NMJ are mediated by a combination of "protective" and "punishment" signals. Quantal size, an important indicator of synaptic strength, is increased in MHCI-deficient animals, and at multiply-innervated junctions retained at P15, both inputs still evoke robust postsynaptic responses. Both the increase in q and the presence of multiple strong inputs at a single P15 endplate support a role for MHCI in reducing synaptic strength at the developing NMJ and raise the possibility

that MHCI may normally act as a postsynaptic element of the “punishment” signal that mediates functional weakening of a subset of MN inputs prior to synapse elimination.

It is as yet unknown how endogenous MHCI limits quantal size, although possibilities include postsynaptic (changes in nAChR expression levels, subunit composition or sensitivity, changes in muscle size/permeability), intrasynaptic (decreased acetylcholine esterase (AChE) activity, changes in the size of the synaptic cleft) and presynaptic mechanisms (changes in the neurotransmitter content or size of vesicles). The fact that mEPP frequency is unchanged in MHCI-deficient animals at P15 (Figure 12A and B) is inconsistent with an increase in nAChR levels or increased sensitivity at the synapse. Furthermore, α–btx labeling of nAChR clusters in MHCI-deficient animals does not reveal differences in the area or perimeter of labeling at individual junctions (Figure 7). Additionally, input resistance was indistinguishable between WT and mutant muscles, indicating that increased quantal size is not due to differences in the size or ionic permeability of individual muscles. The decay kinetics of the EPP are normal in P15 MHCI-deficient animals (Figure 14), arguing that changes in nAChR subunits and AChE activity are unlikely to be responsible for the increased quantal size (Wang, Li et al. 2005). Additionally, electron microscopy does not reveal gross differences in the morphology or width of the synaptic cleft in adult mice lacking surface MHCI, although this data is preliminary and needs to be repeated (data not shown). Nevertheless, these results, when taken together, suggest that MHCI may regulate q by modifying the size or neurotransmitter content of ACh-containing synaptic vesicles in presynaptic MN terminals.

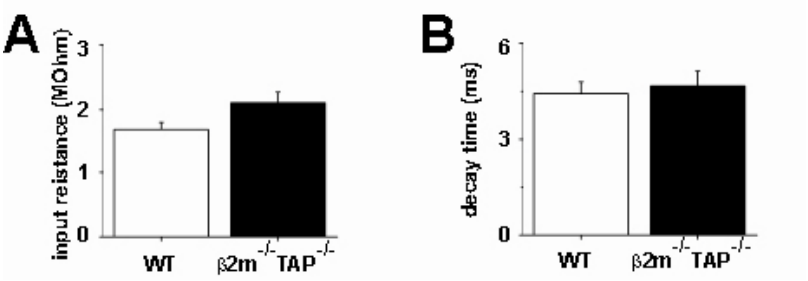

Figure 14. Input resistance and decay kinetics are normal in mice lacking cell surface MHCI. A. Input resistance is indistinguishable between transgenic and WT mice at P15 (WT, n = 6 muscles from 2 animals; $\beta 2m^{-/-}TAP^{-/-}$, n = 10 muscles from 3 animals). B. Decay time of the EPP was comparable in mutant and WT muscles at P15 (WT, n = 8 muscles from 5 animals; $\beta 2m^{-/-}TAP^{-/-}$, n = 5 muscles from 4 animals).

Seminal work by Bernard Katz first highlighted the role of the quantum as a key determinant of synaptic strength (Del Castillo and Katz 1954). While many studies since that initial finding have demonstrated the importance of postsynaptic parameters, in terms of receptor expression and sensitivity, in altering quantal size, presynaptic modulation of individual vesicles has received considerably less investigation (for review (Turrigiano 2007). Nevertheless, changes in the number of transmitter molecules in a vesicle also affect quantal size (reviewed by (Liu 2003)) and work in cultured neurons demonstrates that global alterations in network activity can lead to changes in the presynaptic loading of both glutamatergic and GABAergic synapses (Wilson, Kang et al. 2005; Hartman, Pal et al. 2006). Additionally, i*n vivo* evidence from Steinert *et al* suggests that experience-dependent changes in quantal size in *Drosophila* result from altered vesicle size and filing during a novel behavioral paradigm (Steinert, Kuromi et al. 2006).

It is well established that chronic blockade of presynaptic activity, via TTX, increases quantal size at the murine NMJ (Lomo and Rosenthal 1972; Lomo and

Westgaard 1975). Interestingly, recent experimental evidence points towards a modulation of quantal size via alterations in the amount of ACh released from presynaptic vesicles in response to activity blockade in this system (Wang, Li et al. 2005). Given that loss of cell surface MHCI also increases quantal size in the absence of increased nAChRs or alterations in decay kinetics, it will be interesting to determine if MHCI is involved directly in the modulation of presynaptic activity, or in the ultimate read-out of neural activity into alterations in the ACh content of presynaptic vesicles.

Some experimental evidence exists that suggests MHCI occupies a role downstream of patterned presynaptic activity in other areas of the nervous system. Corriveau *et al* found that MHCI mRNA is highly regulated by neural activity in the developing visual system and adult hippocampus. Specifically, MHCI mRNA in the LGN is downregulated by blockade of activity in retinogeniculate projections, and upregulated by increased activity during kainite treatment in the hippocampus (Corriveau, Huh et al. 1998). While it remains to be determined if similar expression "rules" apply at the NMJ, the possibility may offer tantalizing insight into the molecular link between the neural activity of competing motor neurons at the NMJ, and the functional and structural changes that mediate synapse elimination.

Presynaptic activity is not required for synapse elimination at the NMJ, per say, as chronic activity blockade slows, but does not prevent, the normal developmental loss of axonal convergence (Thompson, Kuffler et al. 1979). Rather, a plethora of studies now suggest that structural remodeling at the NMJ is highly

influenced by presynaptic activity. Developmentally, there is a progression in the synchrony of neural firing patterns between electrically coupled motor neurons, with downregulation of gap junction expression leading to increased asynchrony in the firing patterns of individual motor units (Personius and Balice-Gordon 2001; Buffelli, Busetto et al. 2002). This transition to asynchronous activity is temporally correlated with synapse elimination at the NMJ, and mice lacking connexin 40, which display lowered levels of correlated motor unit firing during development, have an accelerated time course of synapse elimination at the NMJ (Personius, Chang et al. 2007).

Landmark work by Busetto *et al* established the first causal link between asynchronous neural activity and synapse elimination (Busetto, Buffelli et al. 2000). Studying the elimination of multiply innervated ectopic synapses on transplanted muscle, the authors found that imposing synchronous activity across both innervating motor neurons at a single junction was permissive for the retention of multiple inputs at the regenerating NMJ. Interestingly, the presentation of asynchronous activity, via focal stimulation distal to endogenous activity blockade, facilitated synapse elimination. Even more provokingly, when synchronous activity was again imposed, but this time in the absence of TTX, thereby allowing the presence of low levels of endogenous, asynchronous neural activity, synapse elimination then proceeded along a normal time course. These results, coupled with developmental correlations, suggest that it is the presence of asynchronous neural activity, even at very low levels, that is instructive for synapse elimination at the NMJ.

While this line of experimentation clearly supports the theory that differential activity among competing inputs is a critical factor in synaptic competition, previous work suggests that the role of activity is more complex. Specifically, it remains unclear as to whether competitive advantage is conferred to the more or less active motor neuron at the NMJ during synapse elimination. In particular, a series of studies that differentially stimulated competing inputs during synapse elimination found that more active inputs can displace less active counterparts at the NMJ (Ribchester and Taxt 1983; Ridge and Betz 1984). Analysis of mice genetically engineered to harbor a subset of synaptically silent MN terminals support this finding as well (Buffelli, Burgess et al. 2003). However, similar experimental approaches reliant upon differential stimulation suggest that inactive MNs may have a competitive advantage at the NMJ (Callaway, Soha et al. 1987; Ribchester 1993). Although much controversy still remains as to the nature of the role of activity in mediating synapse elimination, the majority of presently available data suggests that differential activity between synaptic inputs is a critical factor in the progression of competition during synapse elimination.

However, it is as yet unknown how asynchronous neural activity translates into the functional and structural asymmetries that result in the process of synapse elimination. Critical insight into potential mechanisms underlying these phenomena comes from classical experiments by Poo and colleagues at the amphibian NMJ. Utilizing neuron-muscle cocultures from *Xenopus,* the authors demonstrated that neural activity from one axon in dually innervated muscle results in the short-term

impairment of neurotransmitter release in its competitive partner (Lo and Poo 1991; Dan and Poo 1992; Lo and Poo 1994; Cash, Dan et al. 1996). This "heterosynaptic" depression does not require the physical presence of a second competitive partner, as it can be mimicked by the application of focal ACh, but is dependent upon the presence of the postsynaptic muscle fiber. Studies that combine heterosynaptic depression of neural activity, via focal blockade of the endplate with α-bungarotoxin, with *in vivo* imaging support a similar mechanism in adult mammals (Balice-Gordon and Lichtman 1994). Focal blockade of nAChRs at part of the endplate results in localized loss of receptor density, and the eventual withdrawal of presynaptic terminals from that area of the endplate. In contrast, blockade of the entire endplate does not result in loss of postsynaptic receptor density, nor does it alter the distribution of presynaptic components, indicating that it is asynchrony between presynaptic and postsynaptic activation that is permissive for the removal of synaptic structures at the NMJ.

The work summarized above leads to the question of what signaling might induce the size and strength of competing inputs to a neuromuscular junction to become progressively different. The bulk of evidence available to date favors a model in which heterosynaptic interactions result in active synapses destabilizing and displacing inactive ones, involving the postsynaptic cell as an intermediary, in a more or less Hebbian fashion. Therefore, when convergent inputs are synchronously active, each nerve terminal produces a combination of "protection" and "punishment" signals that balance, resulting in a net maintenance of polyneuronal innervation. However, when one input is active asynchronously, its synaptically silent counterpart is no

longer subject to the protection conferred by postsynaptic depolarization, and instead is influenced by the "punishment" signal of its more active competitive partner. In theory, this punishment signal would result in decreased synaptic efficacy, in the form of reduced quantal size and content, such that after repeated cycles of weakening, the more inactive MN would be less efficacious at the synapse, resulting in its eventual removal and the establishment of mature mononeuronal innervation.

Therefore, if MHCI is regulated by activity at the NMJ, as has been previously shown in the CNS, it may prove to be the link between asynchronous neural activity and heterosynaptic depression. We can conjecture that during synapse elimination, one nerve terminal increases synaptic activity, which results in an upregulation of MHCI expression. MHCI then functions to limit the quantal size of the synaptically silent competitive partner in a Hebbian fashion, potentially through a transsynaptic signaling event that reduces ACh release. If this brief reduction in quantal size, a potential "punishment signal", temporally summates, then the losing axon, even when synaptically active, will elicit a decreased postsynaptic effect, further weakening at the synapse until it is eventually removed. While this is an intriguing possibility, further work remains to determine if MHCI is indeed regulated by activity at the NMJ in a differential pattern based on competing inputs, and if so, as to the nature of any "protective" signals that would allow the eventual "winning" axon to escape synaptic depression induced by MHCI upregulation in the postsynaptic endplate.

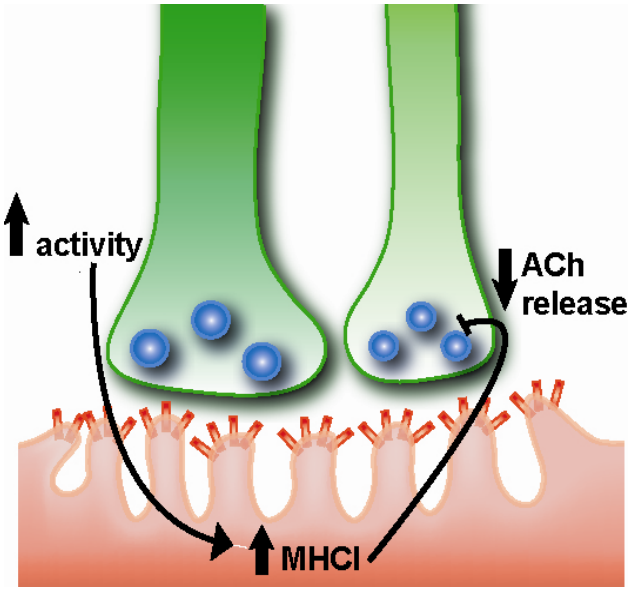

Figure 15. Model of MHCI action at the developing NMJ during synapse elimination. MHCI may function as a putative "punishment" signal at the NMJ by transducing increased neural activity in one competing input (*left)* into a decrease in quantal size, via presynaptic reduction in ACh release in its competitive counterpart (*right*). Continued cycles of Hebbian-like heterosynaptic depression, via MHCI activity, result in the eventual elimination of the synaptically weakened input.

Another outstanding question raised by this work is why loss of MHCI cell-surface expression only impairs remodeling at a subset of junctions. There are several factors that could potentially contribute to this selectivity, including muscle-fiber type and motor unit type. It is also possible that members of the MHCI family are expressed at NMJs in a mosaic fashion and are differentially affected by the loss of β2m and TAP. For example, while most MHCI proteins require β2m for stable surface expression, some members can traffic to the cell surface independently of β2m expression, such as the classical MHCI molecule H2-D^b (Allen, Fraser et al. 1986). Accordingly, recently work by Thams *et al* identified a role for a classical MHCI molecule in the establishment of endplate band architecture and density (Thams,

Brodin et al. 2009). Gross analysis of such parameters in mice lacking β2m and TAP did not reveal similar defects (Figure 6), possibly reflecting the expression of H2-D^b in a β2m-free form. Therefore, the ability of some muscles to remodel in the absence of β2m and TAP might represent a role for non-β2m dependent MHCI members in remodeling, or functional compensation resulting from a developmental knock out.

The present study is the first to suggest a developmental role for MHCI in synapse elimination during the establishment of mature connectivity at the NMJ. Previous work has shown that MHCI is upregulated after peripheral nerve injury or axotomy, and lowered MHCI levels are associated with an impairment in axon regrowth after nerve damage (Linda, Hammarberg et al. 1998; Oliveira, Thams et al. 2004; Thams, Brodin et al. 2009). Although it is well established that motor neuron axotomy results in a transient period of polyinnervation, followed by a loss of axonal convergence that is highly reminiscent of synapse elimination, it remains to be determined if MHCI is required for the normal progression of this form of structural plasticity. Therefore, it will be interesting to investigate if altered regulation of MHCI proteins has consequences unique to neural function at the interface between motor axons and muscles during injury and disease.

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