Title: Constructing a neuromuscular-prosthetic interface and actuator system for limb reconstruction.

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M.B.B.S


Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

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Produced on archival quality paper
Preface

I would like to acknowledge the contributions made by Mr. Cameron Nowell from Walter and Eliza Hall institute for his help with writing the software needed for image analysis of the neuromuscular junctions in chapter 2, Dr. Justin L Bourke for his support in coating the multi-electrode arrays with polypyrrole as detailed in chapter 5 and support with analysis of multi-electrode array recording data and the staff from Intelligent Polymer Research Institute for manufacturing the trilayered polypyrrole actuators used in chapter 5. Parts of the methods and results of chapter 2 and 4 have been adapted from the original article (1) that has resulted from the work detailed in this thesis. These sections have been referenced where appropriate.
Abstract

A neuromuscular prosthesis provides an ideal solution to functional restoration of the limb after amputation. In such a system, the severed nerves at the stump are implanted into denervated muscles and the innervated muscle is coupled to a human-machine interface which detects the body's signals and transmits it to the actuator. This thesis will present studies that address key components of this bio-prosthetic actuator system, namely the neuromuscular junction, the muscle electrode junction and the actuator system. An in vitro nerve muscle co-culture system was established as a model platform for studying the neuromuscular junction. The effect of agrin and laminin on the innervation of muscle cells was studied with immunocytochemistry, real time PCR and liver cell imaging. Agrin and laminin were found to sensitize muscle cells to innervation by PC12 cells forming more functional neuromuscular junctions and promoting muscle maturation.

An in vitro model of the neuromuscular prosthetic interface was created from PC12 neural and primary mice myoblasts grown on multi-electrode arrays. Electrodes of the array were further coated with a conducting polymer polypyrrole to create a low impedance interface between the muscle cells and the electrode. The effect of polypyrrole coating thickness on the quality of the cell recording was assessed. The thickness of polypyrrole coating had no impact on the strength of the cell recording.

Finally biocompatibility studies were performed on trilayer polypyrrole based actuators. Trilayer polypyrrole based actuators are known for their superior work density compared to natural muscle and existing actuators. When implanted into rabbit muscle, actuators that had pores engineered into it to encourage tissue integration showed significant polypyrrole delamination from the actuator. The degradation was slowed by sealing the cut edges of the pores on the actuator with polypyrrole. The biocompatibility results provide valuable insight
into required design upgrades to existing polypyrrole based trilayered actuators.

The work presented in this thesis serve as a basis and platform for further studies in integrating the neuromuscular junction, muscle electrode junction and the actuator into one unit for future translation.
Declaration of Authenticity

The thesis contains only original work towards the degree of Doctor of Philosophy except where indicated otherwise. Due acknowledgements have been made in the text to all other materials used. The thesis contains fewer than 100,000 words exclusive of tables, maps, bibliographies and appendices.

Bill G.X Zhang
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Finally I would like to thank my parents for their encouragement and guidance throughout my PhD journey.
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List of Abbreviations

%- Percentage
µg-Microgram
µl- Microliter
µM- Micromolar
µm- Micron
AC- Alternating current
ACh- Acetyl Choline
AChE- Acetylcholinesterase
AChR- Acetylcholine receptor
AChR β1- Acetylcholine receptor β 1 component
AChR ε- Acetylcholine receptor ε component
AChR γ - Acetylcholine receptor γ component
Ag- Agrin
α BTX- α bungarotoxin
ANOVA- Analysis of variance
APC- Adenomatous polyposis coli
ARIA- Acetylcholine receptor inducing activity
ARP2/3- Actin-Related Proteins
ATP- Adenosine triphosphate
BDNF- Brain-derived neurotrophic factor
BF₄⁻- Tetrafluoroborate
bFGF- basic fibroblast growth factor
BIIIIT- Beta III tubulin
cAMP- Cyclic adenosine monophosphate
Cdc42- Cell division control protein 42 homolog
ChAT- Choline acetyltransferase

Chrb1- gene encoding acetylcholine receptor β 1 component

Chrne- gene encoding acetylcholine receptor ε component

Chrν- gene encoding acetylcholine receptor γ component

Cl- Chloride

CLASP2- CLIP-associating protein 2

CLIP170- Cytoplasmic linker proteins 170

ClO₄⁻- Perchlorate

cm- Centimetre

CM- Conditioned media

CNT- Carbon nanotubes

CNTF- Ciliary neurotrophic factor

Co- Co-culture

CO₂- Carbon dioxide

Col- Collagen

ColQ - Acetylcholinesterase collagenic tail peptide

CP- Conductive polymers

CPK- Creatinine kinase

CRD- Cysteine rich domain

CRK- CT10 regulator of kinase

CRK-L- CT10 regulator of kinase like

DAPI- 4',6-diamidino-2-phenylindole

DC- Direct current

Dex-P' Dexamethasone phosphate

DHPR- Dihydropyridine-sensitive L-type voltage-dependent Ca2+ channels

DM- Differentiation media

DMEM- Dulbecco’s Modified Eagle Medium
DNA- Deoxyribonucleic acid
Dok7- Docking protein 7
Dvl- Dishevelled
EAP- Electric electroactive polymers
E-C coupling- Excitation-contraction coupling
ECM- extracellular matrix
EGF- Epidermal growth factor
EMG- Electromyogram
EPP- End plate potentials
ERK2- Extracellular signal regulated kinase 2
FGF- Fibroblast growth factor
FIJI- version of ImageJ software for image analysis
GAG- Glycosaminoglycan
GalNAc- N-acetylgalactosaminyl
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GDF-8- Myostatin 1b
GDNF- Glial cell line-derived neurotrophic factor
GGT- Geranylgeranyltransferase I
Gl- Glass substrate
GRB2- Growth factor receptor-bound protein 2
GSK3β- Glycogen synthase kinase 3 β
GTPases- Guanosine triphosphatases
H&E- Haematoxylin and eosin
HBGAM- Heparin-binding growth-associated molecule
HBGF- Heparin bound growth factors
HCl- Hydrochloric acid
Hprt- Hypoxanthine phosphoribosyltransferase 1
HSPG- Heparin sulfate proteoglycans

HZ- hertz

JNK- C-Jun N-terminal kinase

kHz- Kilo hertz

LIF- Leukemia inhibitory factor

Ln- Laminin 1

LRP4- Low-density lipoprotein receptor-related protein 4

MAPK- Mitogen-activated protein kinase

MEA- Multi-electrode array

MEJ- Muscle electrode junction

MEK- Mitogen/extracellular signal-regulated kinase

mg- Milligram

MHC- Myosin heavy chain

mL- Millilitre

mm- Millimetre

mM- Millimolar

mRNA- Messenger ribonucleic acid

Mus- Muscle monoculture

MuSK- muscle specific kinase

Myh1- Type II X myosin heavy chain gene

Myh3- Embryonic myosin heavy chain gene

Myh8- Peri-natal myosin heavy chain gene

NaDDS- Dodecylsulfate

NaV 1.4- Sodium voltage gated channel alpha subunit 4

NaV 1.5- Sodium voltage gated channel alpha subunit 5

NGF- Nerve growth factor

nm- Nanometer
NMJ- Neuromuscular junction
NMN- Nerve to muscle neurotization
nNOS- Neuronal nitric oxide synthases
No.- Number of
NO$_3^-$- Nitrate
NPI- Neuromuscular prosthetic interface
NS--1-Naphthalenesulfonate
NT3-Neurotrophin-3
NT4- Neurotrophin-4
°C- Degrees celcius
PAK- p21 activated kinase
PBS- phosphate buffered saline
PC- post coitum
PCR- Polymerase chain reaction
PI3-K- Phosphoinositide 3-kinase
PI- Plastic substrate
PLGA-Poly(lactic-co-glycolic acid)
Ppy- Polypyrrole
PSC-Peri-synaptic Schwann cells
PSS Poly(styrene sulfonate)
pTS Paratoluene sulphonate
RNA- Ribonucleic acid
RT-PCR- Reverse transcription polymerase chain reaction
RyR-Ryanodine receptors
s- seconds
Scn4a- gene encoding sodium voltage gated channel alpha subunit 4 (mouse)
Scn5a- gene encoding sodium voltage gated channel alpha subunit 5 (mouse)
SEM- Standard error of the mean
SHP2- Src homology 2-containing tyrosine phosphatase
SKM1- Sodium voltage gated channel alpha subunit 4
SKM2- Sodium voltage gated channel alpha subunit 5
SNARE- Soluble NSF Attachment Receptor
SO₄²⁻-Sulfate
SR- Sarcoplasmic reticulum
Src- Proto-oncogene tyrosine-protein kinase
Tid1- Mammalian Homologue of Drosophila Tumorous Imaginal Discs
TMR- Targeted muscle re-innervation
Trk- Tyrosine receptor kinase
TTx- Tetrodotoxin
VVA-B4- GalNAc-binding Vicia villosa lectin
Publications arising from this work

Oral Presentations

Australian Orthopaedic Association (AOA) 75th annual scientific meeting, Brisbane, Queensland, 13th October 2015, “Improving innervation of denervated muscle using agrin and laminin”.

Royal Australasian College of Surgeons (RACS), 52nd Annual Scientific Meeting of the Surgical Research Society of Australasia, Sydney, NSW, 13th November 2015. “Building a bio-artificial neuromuscular-electrode interface for tissue reconstruction”.

Publications

Chapter 1

1. Current state of research on neuromuscular prosthetic interface, neuromuscular junction, muscle-electrode junction and polymer actuators.

1.1 Introduction

Loss of limb due to trauma or tumour resection can lead to significant functional deficits for the patient. Up until recently, such injuries are often debilitating forcing the patient to compensate for the lost limb with crutches and to make major adjustments to their life and career. However convergent developments in material science, engineering and biology have led to development of prostheses that can potentially restore function to the amputated limb. Body and externally powered prosthesis have been implemented with varying degrees of success (2). With further advances in technology, the dream of creating prostheses capable of performing complex movements with increasing degrees of freedom may now be realized. However for such machinations to be utilised to its full potential, it must be effectively “connected” to the human body. A human to machine interface is required to convert the neuromuscular activity in the limb stump into electronic signals that are in turn fed into the artificial prosthesis. Such an interface must display high fidelity of signal transduction, reliability as well as biocompatibility. Currently, the development of human-machine interfaces lag far behind that of the prosthesis. Most myoelectric limb prostheses rely on surface electrodes for signal detection which are limited in the quantity, accuracy and reliability of signal transfer (3). Some have attempted to increase quantity of signal transfer by surgically creating neuromuscular units in the limb stump to enhance the number of discrete signalling points with the prosthesis in a process known as targeted muscle re-innervation (TMR) (4, 5). Others have trialled newer electrode materials to
promote greater integration and electrical coupling between the body and the electrodes (6, 7). In this thesis, we propose the construction of a neuromuscular prosthetic interface (NPI) by marrying together surgery, biology and material science. In such a construct, the remaining muscle of the limb stump is first surgically innervated by nerve implantation. The new neuromuscular unit is then interfaced with an array of electrodes that is implanted intramuscularly. Such a system potentially provides high volume and accurate signalling at the body-machine interface that is far more reliable than existing surface electrodes. Similar constructs have been briefly investigated in rats in vivo (8-11). However it is unclear whether the results of the procedure can be improved upon by optimization of the individual components of this construct. This review will first briefly outline the current state of research on body-machine interfaces and its limitations and how NPI can effectively overcome these shortcomings. It will then focus on the two main interfaces of the NPI, namely the neuromuscular junction (NMJ) and the muscle electrode junction (MEJ). The background of NMJ as well as means of enhancing muscle innervation and NMJ formation will be explored. The main determinants of signal transmission at the MEJ will be summarized and the use of polypyrrole- a conducting polymer- as a coating of the electrodes to facilitate signalling at the MEJ will also be covered. Finally the review will delve into the development of novel actuators that may one day replace complex externally powered prosthesis with special emphasis on a polypyrrole based trilayer actuator.

1.2 Body-machine interface and neuroprosthetic interface

The first prosthesis used to restore function to limbs after amputation were body powered. These constructs were controlled by a series of cables and pulleys that were connected to muscles of the limb stump and the torso. However they were limited by the amount of
movements available and the power that can be generated (2). As a result externally powered prosthesis driven by electric motors were created. The electrical limbs were capable of multiple degrees of freedom of movement across multiple joints and capable of performing complex tasks. These machines were connected to the limbs through surface myoelectric sensors that picked up electromyogram (EMG) signals emanating from the stump muscles (3). However the number of surface sensors and therefore signal input into the prosthesis was limited by the low number of remaining muscles in the limb. For an example, after trans-humeral amputation of the upper limb, few flexor and extensor muscles remain in the arm stump. Consequently, only two sensors can be placed on the limp stump, one on the flexor and one on the extensor compartment (12). This essentially limits the number of inputs to the prosthesis to two. The two inputs must then control multiple combinations of movements across the elbows, wrist and fingers on the prosthesis. The patient needs to use an external switch to isolate the field of control to particular movements on particular joints so that the two inputs can suffice after which the control field is then shifted to the next joint by the external switch (4, 5, 12). For an example, to pick up a cup in a trans-humeral amputee, the patient must use the two inputs to first bend or extend the elbow, then sequentially using the external switch to change the locus of control to wrist flexion and finally to finger grasping. It also requires a degree of learning as the patient needs to learn to use muscles that are normally associated with elbow flexion and extension to direct finger grasping. The whole process can be cumbersome and time consuming sometimes forcing people to abandon their use.

More recently, attempts have been made to increase the number of signal inputs into the prosthetic limb through the process of targeted muscle re-innervation (TMR) (4, 5, 12-14). During TMR, the remaining proximal segments of nerves that previously innervated the lost limb are transposed onto discrete regions on an adjacent muscle such as pectoralis muscle
or triceps. Over time the transposed nerves innervate key areas of the muscle as well as its overlying skin (4, 5, 12-14). Surface electrodes placed directly above these entry points can capture electrical signals emanating from the underlying muscle in response to activation of each nerve. As a result, a surface sensor can be allocated to each nerve from the limb stump greatly increasing the number of electrical inputs to the prosthesis and enabling simultaneous control of multiple activities across several joints (4, 5, 12-14). It also makes prosthetic control a more intuitive process as the sensors and inputs can be arranged so that the nerves in the limb stump are matched with prosthetic motions that best approximate the natural function of the nerve. However the main drawback of TMR is the reliability of the sensors. The surface sensors are prone to disruption due to changes in surface condition of the skin such as sweat and oil. Furthermore skin electrodes can detect ‘cross-talk’ activity from surrounding neuromuscular units thus reducing its accuracy (3).

Much research has been focused on the development of implantable electrodes. Compared to surface sensors, implantable electrodes allow for direct apposition of the electrode with the tissue improving the specificity as well as the strength of signal recording. However most of the work in the discipline relate to neural-electrodes (15). Various two dimensional and three dimensional electrode arrays have been developed for in vivo recording of neural activity (5). These electrodes tend to suffer from fibrous encapsulation after implantation due to a modulus mismatch between the soft neural tissue and the hard electrode (16, 17). The fibrous tissue between the electrode and the neural tissue significantly denigrates signal transmission at the interface (16, 17). Furthermore, neural activity tends to be of low amplitude and may be difficult for signals to be recorded. In comparison, implantable electrodes for muscle such as those proposed in a NPI will have better performance in both respects. Muscle is relatively stiffer and can theoretically act as a much needed intermediary between the nerve and the electrode reducing the modulus mismatch with the electrode
and the resultant fibrotic reaction. Furthermore, muscle effectively amplifies the neural activity improving the signal to noise ratio of the electrode recording (3). In essence, the NPI combines the benefits of TMR and implantable muscle electrodes into one construct that is able to deliver simultaneous control of the prosthetic limb with high fidelity and reliability. Others have attempted to create constructs similar to NPI in rats (8-11). However these experiments were done without prior optimization of the individual components of the construction such as the NMJ, MEJ. As a result it is unclear whether the final outcome from the experimental procedures can be further improved upon.

1.3 Neuromuscular Junctions

The neuromuscular junction is a critical component of the neuromuscular prosthetic interface. The injured muscle needs to be first surgically innervated or “neurotized” by a nerve before becoming connected to the electronic array for signal detection. This process involves formation of new NMJ between the implanted nerve and the muscle. The NMJ is the principle site where the complex interaction between nerve and muscle takes place. It is the means by which nerve signals are transduced into muscular ones underpinning the integrity of locomotor system. Yet the topic has received far less attention compared to the extensive body of knowledge on nerves and muscles. Much of the biological processes that are responsible for inducing and maintaining NMJ remain unknown. Traditionally it was thought that the nerve was the primary factor in NMJ formation (18). However recent evidence suggests that muscles also have a role in NMJ regulation (19). More specifically it is now believed that muscles can generate aneural acetylcholine receptor (AChR) clusters on their membrane without nerves and that these aneural AChR clusters serve to prime muscle for synaptogenesis (19, 20). The following sections provide an update on current
understanding of aneural AChR clusters and their role in NMJ formation. Basic structure of NMJ and synaptogenesis will be first reviewed. This will be followed by a discussion on relative importance of nerve and muscle factors on NMJ formation and aneural AChR clustering. The structure and function of AChR clusters will be explored and the range of agents that are responsible for regulating it are discussed with particular attention to agrin and laminin-1; both well-known AChR clustering agents. Other factors that impact on NMJ differentiation will be briefly mentioned. We will also examine some of the surrogate markers that are used to quantify innervation in in vitro nerve-muscle co-cultures. Finally the clinical relevance of NMJ research will be discussed in the setting of constructing a neuromuscular prosthetic interface.

1.3.1 Structure of development NMJ

The neuromuscular junction consists of three core components- nerve terminal, post-synaptic membrane and peri-synaptic Schwann cells (figure 1-1). The three components are specifically aligned to maximize the efficiency of neurotransmission. The nerve terminal contains multiple synaptic vesicles that house neurotransmitters. The principle neurotransmitter deployed at NMJ is acetylcholine (ACh). It is synthesized by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-CoA (21). There are two pools of synaptic vesicles, one that is ‘docked’ at the terminal and ready to be released on depolarization and those that act as ‘storage vesicles’ (22). Upon the arrival of action potential at the nerve terminal, calcium channels at the terminal membrane open allowing influx of calcium into the cytoplasm activating a series of Soluble NSF Attachment Receptor (SNARE) proteins on the docked vesicles and the nerve terminal membrane (23). SNARE proteins are a family of 60 proteins that are responsible for vesicle fusion and exocytosis (24).
After activation by intracellular calcium, the v-SNARE on the synaptic vesicles fuse with the t-SNARE at the subsynaptic membrane of the nerve terminal emptying the ACh into the synaptic cleft (22-25). Exocytosis occurs at key regions of the subsynaptic nerve terminal known as ‘active sites’ (22, 23, 25). The expended pool of docked synaptic vesicles is further replenished and ready for activation next time (22). The released ACh binds to AChR on the post-synaptic membrane. The effect of ACh is temporary and is soon inactivated by the enzyme acetylcholinesterase (AChE) present in the synaptic cleft. AChE cleaves the ACh back into choline which is recycled back into the nerve terminal for further ACh synthesis (22).

The structure of the post-synaptic membrane is ergonomically organized to expedite signal transmission between the nerve terminal and the post-synaptic apparatus. The post synaptic membrane consists of membrane indentations known as primary synaptic clefts (22). The gutter shaped primary synaptic clefts give rise to further smaller membrane invaginations that resemble ‘folds’ on the membrane known as secondary synaptic clefts. The crests of the folds contain AChR while the troughs house voltage gated sodium channels. The primary synaptic cleft is aligned with the nerve terminals while the crest of the synaptic folds are positioned across the active sites on the terminal thus minimizing the distance that ACh must diffuse to reach the ACh receptors (22). Upon activation by ACh from the nerve terminal, AChR activates ligand gated sodium channels producing regional membrane depolarizations known as ‘end plate potentials (EPP) (26). The EPPs travel from the crest of the synaptic fold down to the trough to activate the voltage gated sodium channels and induce an action potential (26). The synaptic folds help to amplify the endplate potential to thresholds required to open voltage gated sodium channels (26).
The primary synaptic cleft is replete with extracellular matrix molecules and further walled off from the surrounding areas by peri-synaptic Schwann cells (28). Unlike Schwann cells that appose axons, peri-synaptic Schwann cells do not produce myelin and their cellular processes do not enter the synaptic cleft (28). The role of peri-synaptic Schwann cells remains unclear and will be further discussed later in the review. In general, these cells aid in synapse maintenance and differentiation. The extracellular matrix in the synaptic cleft consists of various laminins, collagens, perlecans and heparin sulfate proteoglycans (29). Unlike standard muscle basement membrane, the extracellular matrix (ECM) molecules at synaptic sites are specific to NMJs and subserve important functions of which will be further discussed elsewhere in the review. The various features of the NMJ were developed in two
phases- the primary specialization phase (before birth) and the secondary specialization phase (after birth). During primary specialization the nerve terminals develop varicose protrusions that line up with depressions in the muscle membrane to form primary synaptic clefts. ACh clusters form on the post-synaptic membrane and the per-synaptic Schwann cells cap off the synaptic cleft (22). Secondary synaptic specialization is characterized by further differentiation of the rudimentary synapse. The pre-synaptic vesicles become concentrated near the subsynaptic membrane on the nerve terminal and active zones develop on the membrane (22). On the post-synaptic membrane, secondary synaptic folds form and the AChR become concentrated at the crests and the sodium channels at troughs. The pre and post synaptic apparatus become spatially aligned and the synaptic ECM further matures. Initially each post-synaptic membrane and its AChR clusters are innervated by multiple nerves from different motor units and each muscle fibre may contain multiple NMJ (22). However after a process of synaptic remodelling and ‘competition’ the synapses undergo pruning resulting in each muscle fibre being left with one NMJ and that each post-synaptic apparatus is innervated by one nerve. (30, 31)

1.3.2 Neurocentric vs. Myocentric Model of Synaptogenesis

Synaptogenesis between muscle and nerve is a finely orchestrated process that involves a multitude of muscle as well as nerve factors. However the relative importance of nerve and muscle in dictating this complex process has been the subject of much debate. Traditionally, most researchers subscribed to the ‘neurocentric model’ of synaptogenesis which posits that the nerve was principally responsible for inducing synaptogenesis. This was supported by the observation that nerves can induce ectopic NMJs on denervated muscle. When the peroneal nerve of a rat was transected and transposed onto a denervated gastrocnemius muscle at a...
site outside its old endplate, the implanted nerve formed a functional NMJ with the muscle in two weeks (32-34). Clearly the nerve supplied important signals that not only stimulated post-synaptic membrane development but also determined the location of the synapse on the sarcolemma.

However this traditional hypothesis has been challenged in light of recent findings on the ability of muscle to regulate NMJ formation. It has always been known that the state of a muscle is critical to the success of the re-innervation. Chronically denervated muscles become refractory to re-innervation (30). Furthermore muscles that are innervated become resistant to further innervation by another nerve. In one study the peroneal nerves that were transposed onto gastrocnemius muscles with the original nerve supply left intact failed to form ectopic NMJ while synaptogenesis occurred in gastrocnemius muscles with their original nerve supply divided (35). These examples illustrate the importance of the target muscle in the regulation of receptivity to incoming neurites. However the most compelling evidence for the importance of muscle factors in NMJ formation was the observation that muscles are capable of assembling the post-synaptic apparatus independent of neurons. Transgenic mice that lack nerves develop aneural AChR clusters on the muscle membrane suggesting that the muscles are pre-programed to form neuromuscular junctions and the aneural AChRs may provide critical cues to inducing synaptogenesis (20).

1.3.3 Aneural AChR Clusters
Aneural AChR clusters maybe an important means by which muscles regulate NMJ formation. It is believed that AChR clusters convey positional information to the incoming neurites and guide it to the target to synapse. This theory is supported by three pieces of evidence. Firstly during synaptic pruning, as one axon retracts from their post-synaptic site another competing axon will direct its branches to occupy the just vacated area (36). It is likely that the vacated AChR cluster produces geographical cues that guide the competing axon towards itself to form new synapses. Secondly, dennervated muscle grafts with intact end plates are more amenable to forming NMJ after implantation compared to grafts with the endplate zone excised. This also translated into superior functional recovery after implantation, suggesting that the AChR clusters on muscles can potentially prime muscle for innervation by interacting with incoming neurites (30, 37). Thirdly, during development, aneural AChR clusters develop on a restricted central zone on muscles (19, 38) (figure 1-2). This zone also corresponds to the first region on the muscle to be innervated by the incoming neurite (19). Interestingly the same study also found that neurites were initially unable to induce new synapses outside the central zone of the muscle suggesting that aneural AChR clusters are capable of earmarking regions of the muscle membrane for synaptogenesis, sensitizing it to innervation (19). Similar observations have been reported for muscle after denervation. In an innervated muscle, AChRs are only present at the NMJ. After denervation new aneural AChR clusters develop in extra-junctional regions (39, 40). Like during development, it is believed that these aneural receptor clusters ‘flag’ the muscle for innervation sensitizing it to the incoming neurite. However this guidance process is not completely pre-programed and muscle-centric as many aneural AChR do not eventually become innervated (19, 41). The final location of NMJ likely results from a dynamic interplay between the incoming neurite and the muscle membrane. However the gross location of the synapse is likely set by the aneural AChR.
Figure 1-2 NMJ development in utero. Aneural AChR clusters convey positional cues to the arriving neurites and restrict synaptogenesis to a specified area on the sarcolemma. Image created by author. Information adapted from (19, 41).

Unlike mature AChR clusters on NMJ, aneural AChR clusters contain the embryonic $\gamma$ subunit.

Nicotinic AChRs are pentamers consisting of two $\alpha_1$ subunit and one of $\delta_1$, $\beta_1$ and either a $\gamma$
or ε subunit (42). Mature isoforms of nicotinic AChRs contain the ε subunit while embryonic forms contain the θ subunit. Aneural AChR that are present on muscles during development are mainly the embryonic isoform. Transgenic mice without the θ subunit of AChR failed to show spontaneous aneural AChR clustering during early development (43, 44). Furthermore this led to aberrant synaptogenesis with excessive neuronal branching and failure of neurites to stop at AChR clusters (44, 45). This indicates that the θ subunit of the aneural AChRs are important to its ability to stop branching neurites and induce synaptogenesis. Like in in vivo studies, in vitro experiments also show spontaneous clustering of aneural AChR on cultured muscle cells. Amongst all aneural AChRs on cultured myotubes, 95-99% belong to the embryonic subtype, i.e. containing the θ subunit (46, 47). Aneural AChR clusters appear only on myotubes and not on myoblasts (48). These aneural AChRs are functional and linked to the excitation-contraction machinery of the myotube. Bath application of ACh increased the contractility of the myotube while the AChR blocking toxin α bungarotoxin blocked myotube contraction (49, 50). Furthermore aneural AChR clusters are also important in myoblast fusion and in the maturation of the excitation-contraction (E-C) coupling mechanism (51-53). Myotubes with aneural AChR clusters generally display greater spontaneous calcium currents and contractions compared to those without receptor clusters (53).

1.3.4 Factors that affect AChR clustering

Given the importance of aneural AChR clusters to priming muscle for innervation, much study has focused on examining agents with AChR clustering effects. The following paragraphs will discuss the various agents that are known to affect AChR transcription and clustering.
Agrin is one of the earliest biomolecules discovered to have AChR clustering activity and is believed to be the main mediator of post-synaptic organization and differentiation (18, 54). Agrin is a heparin sulfate proteoglycan that is widely present in Schwann cells, muscle and motorneurons (55). There are many distinct spliced variants of agrin with the neural isoform displaying the most potent AChR clustering activity (56). Neural agrin is secreted by motorneurons into the synaptic cleft where it is deposited with the extracellular matrix at the synaptic site. It differs from the muscle isoform of agrin by the insertion of 8, 11, or 19 amino acids at the Z exon (55). Mouse deficient in neural agrin showed aberrant NMJ formation with dispersed AChRs and poor juxtaposition between nerve endings and AChRs (57). Furthermore gene transfection of denervated muscle with neural agrin induced florid AChR clusters at extra-synaptic sites and assembly of key molecules of the post-synaptic apparatus at the receptor cluster site (58). However this effect of agrin depends on the innervation status of the muscle with innervated muscles showing marked attenuation of AChR clustering at ectopic sites in response to agrin (59). This is consistent with the paradigm that innervated muscles are resistant to synaptic stimuli and therefore cannot be further innervated by a new nerve. Agrin promotes redistribution of existing AChRs on the muscle membrane without altering its overall transcription (57, 60). It also stabilizes AChR clusters increasing its half-life (61). The effect of agrin on AChR clusters persists for up to 7 weeks after it has been removed (32, 62). This “footprint” effect of agrin likely explains the resilience of old endplates on denervated muscle against AChR cluster dispersion (63). The original endplates on the denervated muscles were previously exposed to agrin from the nerve and therefore were able to maintain stability for prolonged periods even after the nerve was removed.
On a molecular level, agrin clusters AChR through cytoskeletal remodelling (figure 1-3). The effects of agrin can be broadly classified into two broad categories: cytoskeletal polymerization and the stabilization of AChR clusters. Most of the effect of agrin is mediated by activation of its receptor MuSK (27, 64). Agrin binds to the MuSK receptor in conjunction with Low-density lipoprotein receptor-related protein 4 (LRP4) leading to receptor tyrosine phosphorylation (65). MuSK then activates a multitude of downstream pathways and targets to remodel the cytoskeleton. MuSK activates the Rho family of small guanosine triphosphatases (GTPases) Rac and Cell division control protein 42 homolog (Cdc42) (66, 67). Rac promotes formation of AChR micro-clusters while further stimulation of Rho condenses the micro-clusters into larger complexes (67). The Rho GTPases recruit p21 activated kinase (PAK) in the presence of Dishevelled (Dvl) to affect cytoskeletal polymerization (68). The link between MuSK activation and Rho GTPases is the subject of ongoing research. A variety of possible mediators have been proposed including docking protein 7 (Dok7), geranylgeranyltransferase I (GGT), Phosphoinositide 3-kinase (PI3-K) and Mammalian Homologue of Drosophila Tumorous Imaginal Discs (Tid1). Dok7 is an adaptor protein that binds to MuSK and recruits CT10 regulator of kinase (CRK) and CT10 regulator of kinase like (CRK-L) and possibly activates Rho GTPase. It is indispensable in agrin induced AchR clustering (69, 70). GGT is a zinc metalloenzyme that tethers proteins to plasma membranes. Inhibition of GGT reduces both Rho GTPase activation and AChR clustering in response to agrin (71). PI3-K is widely involved in regulation of cell proliferation, motility and survival. Inhibition of PI3-K also impairs Rho GTPase activity after agrin stimulation (72). Tid1 has been found to act downstream to MuSK and is important in both Dok7 recruitment and Rho GTPase activation (73). Apart from Rho GTPases, MuSK also activates cortactin which is a Src tyrosine kinase substrate that activates Actin-Related Proteins 2/3 (Arp2/3) complex leading to actin polymerization (74, 75).
Agrin stabilizes AChR clusters by capturing cytoskeletal filaments at sub-synaptic sites and linking the filaments to the receptors. Through MuSK activation, agrin inactivates Glycogen synthase kinase 3 β (GSK3β) and recruits CLIP-associating protein 2 (CLASP2) and cytoplasmic linker proteins 170 (CLIP-170) thus capturing microtubules at the NMJ (67, 76). In order for the receptors to be linked to the captured cytoskeletal filaments, the β and δ subunits of the AChR must be phosphorylated (77, 78). This is mediated by activated Src kinases (Src and Fyn) which are also recruited by MuSK (79-81). Src and Fyn are important in stabilizing the
clustered AChR after agrin has been removed. In Src and Fyn knockout myotubes, AChR clusters newly induced by agrin quickly fragment after agrin is removed (79). The stability of the AChR cluster is modulated by a balance between Src kinase activity and Src homology 2-containing tyrosine phosphatase (SHP2) activity (79, 82-84). Agrin stimulation recruits both kinase as well as phosphatases (79-81, 84). Unfettered Src-kinase activity or phosphatase activity both lead to reduced AChR clusters stability after agrin stimulation and a delicate balance is required between the two agents (79, 82). The induced AChR clusters are further linked to the cytoskeleton by molecules such as rapysn and Adenomatous polyposis coli (APC). Rapysn is activated downstream to MuSK and is indispensable in activation of src kinases and in AChR clustering by agrin (80, 85-87). Rapysn knockout mice fail to form AChR clusters (87). Like rapysn, APC is aggregated at AChR clusters by agrin and links the actin and microtubules to the β subunit of the AChR (88).

In addition to affecting post-synaptic development, agrin has also been found to regulate the electrophysiological behaviours of muscle cells and the maturation of the excitation-contraction (E-C) coupling mechanism. Agrin aggregates sodium channels at AChR clusters and upregulates the expression of mature isoforms of voltage gated sodium channels (89-91). Agrin also promotes mechanical linkage between the dihydropyridine-sensitive L-type voltage-dependent Ca\(^{2+}\) channels (DHPR) on the sarcolemma to the ryanodine receptors (RyR) on the sarcoplasmic reticulum (SR) which is characteristic of innervated muscles (92). In immature myotubes, depolarization opens up DHPRs on the muscle membrane allowing for influx of calcium ions. The calcium ions activate the RyR receptors allowing for release of calcium stores from the SR triggering contraction of the myosin and actin (93). However due to the dependence on extracellular calcium influx, such processes are sensitive to extracellular calcium concentrations. In innervated myotubes, a mechanical link develops
between the DHPRs on the muscle membrane and the RyR allowing for depolarization induced calcium release from the SR that is independent of extracellular calcium (93). This maturation of excitation-contraction (E-C) coupling by agrin also leads to added contractility of the muscle. Agrin treated three dimensional muscle constructs display superior contractility and tetanic force generation in response to direct stimulation (94). Interestingly the same study did not find any difference in expression of mature forms of myosin in the treated muscle suggesting that improved efficiency of the E-C coupling rather than myosin maturation is the likely mediator of agrin on muscle contractility (94).

Given the central role of MuSK in mediating the AChR clustering effects of agrin, the receptor has been subject to much research. It is now known that agrin isn’t the only activator of MuSK. In fact, muscles in mice without nerves (Hb9/-/-) show intact aneural AChR clustering despite never being exposed to neural agrin from neurons (20). However prepatternning is abolished in MuSK knockout mice (95). This indicates that MuSK can be activated by agents other than agrin through a mechanism that has yet to be elucidated. Unlike during agrin stimulation, agrin-independent AChR clustering by MuSK does not involve receptor phosphorylation (96). MuSK is important in conveying positional cues to innervating neurites. During embryonic development, MuSK mRNA is concentrated at the central band of the myotube sarcolemma that also corresponds to both aneural AChR clustering and also the first region in the muscle to be innervated (19, 97). Furthermore disruption of the expression pattern by allowing MuSK to be widely expressed across the muscle membrane allowed for aneural AChR clustering and NMJ formation outside the central band at early stages of development (97). MuSK restricts NMJ formation to a portion of the sarcolemma. However MuSK activation is not necessary for formation of pre-synaptic terminals. When muscle grafts from MuSK knockout mice are transplanted into neurovascular tissue beds in wild type mice, neurites were able to form rudimentary
synaptic boutons and arborize at the muscle membrane. However the nerve branches were highly labile with constant remodelling, extending and retracting and NMJ failed to form (98). This indicates that MuSK aids in capturing neurites at synaptic sites secondary to retrograde signals from the post-synaptic apparatus.

1.3.4.2 Electrical Activity

The role of electrical activity on AChR clustering has been long suspected with the apparent discrepancy in phenotype between mice with no nerves and those without agrin expression. Although agrin knockout mice show dispersed AChR clusters on the sarcolemma, mice that are genetically modified to have no nerves and therefore no agrin show normal prepatterning of AChR clusters (20, 57). This suggests that nerves contain other signals that counteract agrin and the removal of both agrin and the counteracting signal restored AChR cluster size. The identity of this signal is likely to be electrical activity as mice lacking the AChT enzyme needed to produce ACh display larger AChR clusters compared to wild type mice (99, 100). Furthermore, inhibition of electrical activity in re-innervated muscle by neuromuscular blockers prevented the elimination of AChR receptor clusters on muscle (33).

Similar observations have been reported in in vitro cell experiments where the number of spontaneous AChR clusters on myotubes decreases after beginning of spontaneous electrical activity and contraction in the cells (40). These results all suggest that electrical activity disperse AChR clusters. However other observations seem to show the contrary with electrical activity increasing the size of AChR clusters. In denervated muscles that are re-innervated by another nerve, AChR clusters that form at nerve-muscle contact sites undergo a process of competitive elimination whereby the stronger AChR clusters survive and enlarge while the weaker ones are eliminated (32, 33). More importantly if the re-innervated muscle
is rendered idle by further denervation, the process of AChR cluster elimination is aborted. However if the denervated muscle is further stimulated with electricity the competitive elimination of AChR clusters resumes (33). This indicates that electrical activity is the key component of innervation that disperses some AChR clusters while bolstering others. The fate of AChR clusters in the event of electrical stimulation depends on its prior exposure and stabilization by nerve derived signals such as agrin (62). Electrical stimulation stabilizes and enlarges the AChR clusters that have been previously induced by neurites or agrin by increasing its half-life while dispersing any other aneural receptor cluster (61). The number of bolstered AChR clusters depends on the delay between denervation and beginning of electrical stimulation. As the vestigial effects of agrin and neurites wane, the AChR clusters become progressively destabilized and less able to survive the electrical stimulation leaving behind fewer AChR clusters (33). By voiding the muscle membrane of aneural AChR clusters, electrical activity renders any site of muscle membrane free from nerve contact refractory to further innervation. In fact, electrical stimulation of denervated muscles inhibited its synaptogenesis with neurites (101). This likely relates to the lack of aneural AChR clusters which is important in conveying positional cues to approaching neurites.

1.3.4.3 Laminin 1

Laminin-1 is an ECM molecule found in developing embryonic tissues and in some epithelial membranes in adults that is found to cluster AChR (102-105). It is capable of both MuSK dependant and independent AChR clustering depending on the manner it is presented to the myotubes. When laminin-1 is added to the cell culture media, it binds to α dystroglycans on the myotube membrane and clusters AChR in a MuSK independent fashion (106-108) (figure 1-4). The receptor clusters are generally larger than those induced by agrin.
and assume a rudimentary oval plaque shape (107). However when laminin-1 is immobilized at high concentrations onto the cell culture substrate, MuSK is required for AChR clustering (48). Myotubes grown on laminin-1 immobilized polyornithine coated wells develop complex pretzel shaped AChR clusters that is reminiscent of mature AChR clusters at NMJs (48, 109). Differences in laminin presentation to myotubes may lead to subtle changes in molecular structure leading to disparate results on AChR cluster morphology. Despite acting through different receptors, both laminin-1 and agrin share many common downstream molecular targets and effectors. Like agrin, rapyn is indispensable for both immobilized and suspended laminin-1 induced AChR clustering (110). Laminin-1 also activates Rho GTPases (Rho, Rac, Cdc42) to cluster AChR as well as rely on Src kinases Src and Fyn to stabilize the receptor clusters once laminin-1 is removed (109-111). Furthermore like agrin, prolonged incubation with soluble laminin-1 leads to tyrosine phosphorylation of the β and δ unit of AChR (110). In addition to sharing common molecular pathways, laminin-1 also interacts synergistically with agrin to increase both AChR cluster number and size (107, 112). Laminin binds to integrin α7 β1 and sensitizes myotubes to agrin by 20 folds (113). Laminin-1 is also capable of binding and immobilizing agrin to the extracellular matrix (112). Agrin contains both a C and N terminal. The C terminal is responsible for binding to MuSK while the N terminal is responsible for binding laminin (60). Given the close proximity of agrin to laminin, it may be difficult to segregate the effects of laminin-1 from agrin in in vivo experiments.
1.3.4.4 Acetylcholine receptor inducing activity (ARIA)/Neuregulin

ARIA is a protein component of the synaptic extracellular matrix that regulates the transcription of AChRs (115, 116). ARIA is produced by the motorneurons and deposited at the synaptic cleft. Unlike agrin which is mainly present at the primary synaptic gutter at the crests of the synaptic folds and close to the AChR clusters, ARIA is positioned at the troughs of the synaptic folds in the secondary synaptic cleft (117). This places the molecule in close proximity with its receptor ErbB on the muscle membrane (117). ARIA belongs to the neuregulin-1 family group of proteins. Neuregulin-1 proteins are structurally similar to the
epidermal growth factor (EGF) with EGF like domains and its corresponding receptor ErbB 2, 3, 4 also resemble EGF receptors (116). There are multiple isoforms of neuregulin-1 due to alternative splicing. Each isoform differs in their EGF like domain (α vs β), their N terminal sequence (type 1, 2, 3) and whether they are synthesized as transmembrane or non-membrane proteins. ARIA is one of many isoforms that are encoded by the neuregulin-1 gene (116). ARIA was first synthesized from brain extracts and found to upregulate α, ε and δ subunits of AChR in C2C12 cells (114, 118, 119). Genetic disruption of ARIA reduced AChR density at synaptic sites in mice (120, 121).

ARIA mainly binds to receptor tyrosine kinases ErbB2, 3 and 4 (122) (figure 4). Upon activation, ErbB undergoes tyrosine phosphorylation, dimerization and binds to adaptor proteins Shc and Grb2 (119, 122). Shc is indispensable in neuregulin induced AChR upregulation (122). The adaptor proteins in turn allow for activation of mitogen activated protein kinase (MAPK), Phosphoinositide 3-kinase (PI3-kinase) pathway and the serine/threonine kinase Cdk5 (123-128). The MAPK pathway involves sequential activation of ras, raf, mitogen/extracellular signal-regulated kinase (MEK), extracellular signal regulated kinase 2 (ERK2) and c-JUN N-terminal kinase (JNK) (123-127). Inhibition of either the MAPK or the PI3-kinase pathway blocks AChR upregulation by ARIA (124). In addition to regulating AChR transcription, ARIA is also capable of interacting with agrin and modulating AChR clustering. When ARIA is added with agrin to cells, the final effect can range from bolstering of AChR clustering to accelerated dispersal of existing clusters. The response of the interaction depends on the length of time that the cells are exposed to ARIA. Short periods (4 hours) of co-incubation with agrin and ARIA bolster the number of large AChR clusters on myotubes (129). However longer treatments with agrin and ARIA for 12 hours lead to accelerated dispersal of large AChR clusters compared to the same cells treated with agrin.
alone (130). The dispersal of AChR clustering by ARIA may be related to its activation of SHP2 phosphatases (131). SHP2 phosphatases can destabilize AChR clusters predisposing it to dispersal (83). The difference in effect between short and longer incubation may be related to the use of recombinant neuregulin instead of naturally occurring neuregulin (130). The recombinant neuregulin only contains the EGF domain of neuregulin and therefore can only partially simulate the action of neuregulin. Despite most of the literature ascertaining the importance of the EGF domain of ARIA to its biological activity, additional functions of its N terminals and other domains cannot be excluded.

WNT

Recent research has shown WNT to play a central role in aneural AChR prepatterning that is independent of agrin. WNTs are lipoglycoproteins involved in neuronal development in both vertebrates and invertebrates. WNT proteins consist of a family of 19 different proteins that are important in regulating cell proliferation, survival and cell fate (132). WNT predominantly act through a G coupled 7 transmembrane receptor known as Frizzled (132). Depending on the WNT family member, different downstream molecules are then recruited by Frizzled (132). WNT 3 and 11R both promote AChR clustering through activating Rho GTPases Rho and rac and JNK in the presence of Dvl thus reorganizing the cell cytoskeleton (132-135). Mice with genetic knockout of WNT 11 display loss of aneural AChR clustering similar to MuSK knockout mice with abnormal nerve branching (133). WNT 3 recruits rac to form microclusters of AChR which are unstable over time and spontaneously disperse without further stabilization (134). However when agrin is further added to WNT3 there is a bolstering of AChR clustering as agrin is able to recruit Rho to stabilize the receptor microclusters formed by WNT3 and condense it into larger AChR clusters (134). Other WNTs are capable of inhibiting agrin induced AChR clustering. WNT3A activates Frizzled in the presence of co-receptor LRP5/6 to reduce the breakdown of β catenin by inhibiting glycogen
The increase in β catenin inhibits agrin induced AChR clustering. The Frizzled receptor is structurally related to the MuSK receptor as both share a cysteine rich domain (CRD) (137). As a result some WNTs such as WNT 4A, 9A and 11 can also activate MuSK in a similar fashion to agrin to induce AChR clustering (135). This ability of WNT to cross react with MuSK may explain the apparent activation of MuSK in agrin deficient mice to form aneural AChR clusters (57).

1.3.4.5 ECM bound factors

The synaptic ECM is an important reservoir for various factors that affect post-synaptic AChR clustering. The ECM contains heparin sulfate proteoglycans (HSPG) such as agrin that immobilize heparin bound growth factors (HBGF) by its glycosaminoglycan (GAG) side chains. HBGFs with AChR clustering properties include basic fibroblast growth factor (bFGF), heparin-binding growth-associated molecule (HBGAM), Midkines and GalNAc-binding Vicia villosa lectin (VVA-B4) (60, 138-140). bFGF is a component of the muscle basement membrane. Its AChR clustering property was first discovered when it was found that blockage of bFGF signalling inhibited AChR clustering secondary to polystyrene beads (138). Polystyrene beads clustered AChR in xenopus myotubes at areas of bead muscle contact (138, 141). This phenomenon was related to the bFGF immobilized by HSPG on the muscle membrane as pre-incubation with heparin or co-incubation of the beads with anti-FGF inhibited AChR clustering by the beads (138). The heparin cleaved the HSPG thus depriving the muscle membrane of all HBGF including bFGF while anti-bFGF prevented the endogenous bFGF binding the bFGF receptor. The beads clustered AChR by capturing the endogenous bFGF on the muscle membrane and presenting it to the bFGF receptors that are also on the sarcolemma. More interestingly bath application of bFGF did not affect AChR clustering while polystyrene beads with surface adsorbed bFGF clustered AChR (138, 141).
This indicates that the bFGF needs to be presented to the muscle cells in specific manner in order for AChR clustering to occur efficiently and explains the reason why florid spontaneous AChR clustering does not occur despite the prevalence of bFGF on the muscle membrane. The nerve terminal acts like the polystyrene bead to bind the endogenous bFGF, likely changing the molecule and allowing it bind to its receptor to cluster AChR.

HBGAM and Midkines are both HBGFs with AChR clustering ability (139, 140). HBGAM is present diffusely across the muscle membrane but more concentrated at areas of AChR clusters in both innervated and aneural myotubes (139). Midkines are produced by neural cells and not present on myotubes (140). Bath application of HBGAM or midkine did not affect AChR clustering while polystyrene beads coated with HBGAM or midkine redistributes AChR to areas of bead-muscle contact (139, 140). Hence, like bFGF both HBGAM and midkines need to be presented to the myotubes in a specific manner to be bioactive. VVA-B4 lectins are synapse specific carbohydrates that bind specifically to saccharides with N-acetylgalactosaminyl (GalNAc) terminals to cluster AChR (60). GAINAc is a component of many biomolecules including HSPG. Agarose beads complexed with VVA-B4 redistribute existing AChRs on the myotube to areas of bead muscle contact without affecting the total number of AChRs on the membrane (60). VVA-B4 potentiates AChR clustering by agrin while disruption of binding of GAINAc inhibited agrin induced AChR clustering (60). This indicates that agrin signalling also requires GAINAc binding by endogenous lectins like VVA-B4. Apart from AChR clustering agents, the ECM also contains AChR dispersing agents such as sialic acid. Sialic acid partially inhibited AChR clustering by agrin and VAA-B4 lectin reducing MuSK and AChR β subunit phosphorylation (142). Cleavage of sialic acid by the enzyme neuraminidase promotes AChR clustering (60, 96). This indicates that sialic acid likely exerts constant inhibition on AChR clustering and is as a means of regulating AChR clustering.
1.3.5 Role of ECM, support cells and Growth Factors in NMJ development

Apart from AChR clustering agents discussed, the NMJ contains many other cellular, humoral and matrix based factors that affect both pre and post-synaptic development. The following paragraphs provides a brief overview of the role of synaptic ECM components; synaptic laminins and collagens; support cells; perisynaptic Schwann cells and sensory neurons; and neurotrophic growth factors in the development, maturation and maintenance of NMJ.

1.3.5.1 Synaptic laminins and collagens

The synaptic ECM consists mainly of laminins, collagens, heparin sulfate glycosaminoglycans (GAGs) such as agrin and other miscellaneous proteins such as perlecan, tenascin, nidogen and fibronectin (29). Amongst these factors synaptic laminin and collagens are the most thoroughly investigated factors. This section will briefly discuss the current understanding of the role of synaptic laminins and collagens in NMJ development. Laminins are heterotrimers of α, β and γ chains (105). There are multiple forms of each of the chains. All muscle laminin contain γ one chain. Muscle laminin can be further classified into synaptic and extrasynaptic depending on its position on the sarcolemma. In adult muscle extrasynaptic laminin consists mainly of laminin 2 or 211 (α2, β1, γ1) (105). Synaptic laminins all share the β2 chain and consist predominantly of laminin 4 or 221 (α2, β2, γ1), laminin 9 or 421 (α4, β2, γ1) and laminin 11 or 521 (α5, β2, γ1) (105) (refer to table 1-1). During embryonic development, laminin 2 is initially expressed in both synaptic and extrasynaptic sites (105). However with time the synaptic laminin 2 disappear restricting it to the extrasynaptic membrane (105). The
Synaptic laminins develop soon after synaptogenesis in utero and are present exclusively at the NMJ.

Much of the information on synaptic laminins has been gleaned from mice with genetic knockout of the laminin β2 chain that is shared by all synaptic laminins. These mice do not express any synaptic laminin show abnormal pre-synaptic development with reduced active zones in the pre-synaptic terminal and loss of the polarized organization of synaptic vesicle at the nerve terminal (143, 144). The post-synaptic membranes on these mice have reduced secondary synaptic folds. There is also disruption to the peri-synaptic Schwann cells with Schwann cell processes protruding into the synaptic cleft and blocking NMJ transmission (143, 144). More importantly there is misalignment of the pre and post-synaptic apparatus with the active zones no longer lining up with the AChRs on the crest of the secondary synaptic folds (143, 144). These abnormal ultrastructural features are reflected in the electrophysiological behaviour of these NMJs with increased incidences of failed conductance of action potentials, reduced frequency of spontaneous endplate potential, reduced amplitude of evoked endplate currents and increased synaptic depression with high frequency stimulation (145). These observations all point to synaptic laminin playing a critical role in the maturation of NMJ rather than on the induction of synaptogenesis. β2 containing synaptic laminins are important in ‘fine-tuning’ the alignment of the pre and post-synaptic apparatus and conveying ‘stop’ signals to Schwann cell process protecting the synaptic space from process intrusions. In fact the synaptic laminin 11 has been shown to inhibit both Schwann cell and neurite extension in vitro (105, 146). Much of the effect of the synaptic laminin on neurite extension stems from the Leu-Arg-Glu (LRE) situated at the triple-stranded coiled-coil region of the assembled, trimeric laminin molecule (147).
β2 laminins act through both pre and post-synaptic receptors. On the pre-synaptic terminal, the β2 laminins bind to Q type voltage gated calcium channels. Mice without q type voltage gated calcium channels show reduced active zones on the pre-synaptic terminal that is similar to β2 knockout mice(29). Binding of the channels occurs between the LRE domain of laminin β2 and the 11th extracellular loop of the calcium channel (29). Little is known about the receptors for laminin β2 on the post-synaptic membrane. However mice with knockout of α4 chains; a critical component of synaptic laminin 9 or 421; show delayed maturation of the ovoid AChR clusters at the NMJ into pretzel shaped ones and simultaneous knockout of both α4 and 5 chains; important component of laminin 9 and 11; abolished the maturation of AChR clusters(148). Whether this effect on post-synaptic membrane is mediated by a specific laminin β2 receptor or by an indirect pathway through the pre-synaptic terminal remains to be elucidated.
<table>
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<th>Site</th>
<th>α2</th>
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<tr>
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<tr>
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<td>x</td>
<td>X</td>
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<table>
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</tr>
<tr>
<td>laminin 2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
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<tr>
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<td>2</td>
<td>1</td>
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<td>2</td>
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<td>laminin 10</td>
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<tr>
<td>laminin 11 (synaptic)</td>
<td>5</td>
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</tr>
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Table 1-1 Laminins and their tissue distributions (105).

Synaptic collagens comprise predominantly of collagen IV, XIII and Acetylcholinesterase collagenic tail peptide (ColQ). Collagen IV consists of three subunits known as α (IV) chains.
The three α (IV) chains form a triple helical structure. There are 6 isoforms of α (IV) chains encoded separately by 6 genes; α1-6 (IV) chains (29). Although according to permutations there are >50 different combinations of trimers possible. However in reality only three trimers are found (α1) (α1)( α2), (α3)(α4)(α5), and (α5)(α5)(α6). Both collagen IV α 345 and collagen IV α 556 are restricted to the synaptic region (29). The synaptic collagen IVs emerge at around three weeks postpartum and are responsible for NMJ maturation. Synaptic collagen IV is important in neurite branching, alignment of pre and post-synaptic apparatus and post-synaptic maturation (29, 143). Mice with genetic knockout of the collagen IV α5 chains do not express any synaptic collagen IV as the α5 chain is a common component of both collagen IV α 556 and 345. These mice show retraction of axons from ACR cluster, reduced AChR coverage by nerve terminals and fragmented ACR clusters (143). The NC1 domain of collagen IV is also important in pre-synaptic development as antibodies against the NC1 domain of collagen IV inhibited neurite extension of sympathetic neurons grown on collagen IV substrate (143, 149). Furthermore recombinant NC1 domains of collagen IV α2, 3 and 6 chains; all components of synaptic collagen IV; clustered synaptic vesicles at the nerve terminal (143, 149).

Like collagen IV, collagen XIII is only upregulated one month after birth and is important in maturation of the pre-synaptic terminal, post synaptic apparatus and the peri-synaptic Schwann cell. Collagen XIII is a transmembrane molecule exclusively present at the NMJ. Collagen XIII knockout mice show immature post-synaptic specialization with fragmented, plaque like ACR clusters (150). There was reduced accuracy of alignment between pre and post synaptic apparatus with poor clustering and polarization of the synaptic vesicles in the nerve terminal (150). Schwann cell processes were seen invaginating into the synaptic cleft and reducing the area of apposition between the pre and post- synaptic apparatus. There was decreased frequency of mini-endplate potential and reduced response after higher
frequency stimulation. This all indicates a smaller quantal content and storage of available neurotransmitter with reduced probability of spontaneous release of neurotransmitters. The ectodomain of collagen XIII can also be shed and the domain is capable of promoting AChR cluster maturation in vitro (150).

ColQ is a non-fibrillar collagen that is localized to the NMJ (29). It binds AChE and anchors it to the synaptic basement membrane. ColQ is important in the regulation of AChR transcription at the post-synaptic site and maturation of NMJ (151). Mice with ColQ knockout display increasing percentage of NMJ with fragmented AChR clusters and degeneration of the subsynaptic cytoplasm (152). AChE is absent in the synaptic sites and the Schwann cells intrude into the synaptic cleft and enwrap the nerve terminals blocking nerve terminal –AChR cluster apposition (151, 152). ColQ is also important in downregulating AChR subunits through a MuSK based mechanism (151).

1.3.6 Schwann cells and sensory neurons

Both Schwann cells and sensory neurons provide support for neuromuscular transmission at the NMJ. Unlike Schwann cells that reside on axons, peri-synaptic Schwann cells (PSC) do not produce myelin. PSCs sit at the NMJ walling it off from the surrounding environment (28). PSCs are activated during neurotransmission as evident by the increasing calcium flux in the cells during NMJ activity (153). Furthermore this calcium flux is inhibited with cessation of neurotransmitter release (153). PSC guide re-innervation of endplates in denervated muscle. After denervation the PSC extend processes to the adjacent NMJ thus forming a cellular bridge that guides adjacent axons to innervate the vacated endplate (154). PSCs are also important in maintaining synapses (155). Selective ablation of PSC leads to retraction of
neurites from the NMJ and reduced nerve evoked muscle twitch (155). Much of the effect of PSC is mediated by the various growth factors it secretes (156). Schwann cell conditioned media increases frequency of spontaneous synaptic activity, AChR cluster formation and alignment between nerve terminal and post-synaptic apparatus in neural-muscle co-cultures (157). Some researchers have identified TGF-β1 as the active component in the conditioned media as antibodies against the factor inhibited some of the effects of the conditioned media (158). However others have shown TGF-β1 independent effectors in the conditioned media (157). The Schwann cells are also important in functional innervation of muscle. Schwann cells inhibit neuritogenesis in motorneurons and neural muscle co-cultures without Schwann cells fail to show signs of active neuromuscular transmission (128). This indicates that Schwann cells may switch neural cell from a ‘growth’ mode to a ‘maturation’ mode that is needed for effective synaptogenesis. Sensory neurons are also important in effective innervation of muscle cells. Nerve muscle co-cultures containing Schwann cells but without sensory neurons fail to show functional synaptogenesis (128). Therefore both Schwann cells and sensory neurons play important roles in functional innervation of muscle.

1.3.7 Neurotrophic Factors and Cytokines

Most of the neurotrophic factors and cytokines promote retention of NMJ leading to polyinnervated muscle fibres. bFGF, Ciliary neurotrophic factor (CNTF), Brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF), Neurotrophin 3/4/5 (NT3, NT4/5) and Leukaemia inhibitory factor (LIF) all inhibit synapse elimination in developing NMJs to varying degrees (159-162). Some growth factors must be delivered during the synapse elimination phase in order to retain synapses such as CNTF (163). However many of the extra synapses that form maybe silent and redundant showing no signs of active neuromuscular
transmission (159). Neurotrophic factors also affect pre and post-synaptic development. FGF 7, 10 and 22 promote development of vesicle rich and varicose nerve terminals (143). Glial cell line-derived neurotrophic factor (GDNF) increases neurotransmitter release by the nerve terminal (164). On the post-synaptic membrane, BDNF and NT4 inhibit agrin induced AChR clustering on muscle cells (165, 166). Inhibition of the BDNF receptor Tyrosine receptor kinase B (TrkB) bolstered AChR clustering on muscle indicating a baseline level of TrKB activity is present in myotubes (165). These observation all support the notion that neurotrophins are important in growing neurites and not differentiating them. Therefore it is understandable that neurotrophins may inhibit differentiation and maintain the neural cells on the path of growth. Some neurotrophic factors such as GDNF also are capable of increasing AChR insertion into the muscle membrane (167). This is mediated by a Src, Cyclic adenosine monophosphate (cAMP) and MAPK based pathway (167).

1.3.8 Measures of innervation

One of the problems that beset researchers when conducting in vitro experiments on NMJ using nerve-muscle co-cultures is how to accurately gauge innervation of muscle cells. Both the micro and ultrastructural features of NMJs can be difficult to appreciate under the microscope. As a result many have resorted to using surrogate markers of innervation as an objective measure to compare the level of synaptogenesis. Innervation leads to a series of downstream phenotypical changes in both the nerve and the muscle. These changes can potentially be used to study the NMJ formation in in vitro nerve muscle co-cultures. The following paragraphs will discuss some of these surrogate innervation markers such as AChR subtype, voltage gated sodium channel subtype, myotube electrical and contractile activity and myosin heavy chain expression.
1.3.8.1 AChR Subtype

Nicotinic AChRs are receptor gated sodium channels that are important in producing endplate potentials needed to stimulate an action potential in response to ACh. As discussed earlier, nicotinic AChR on muscle can be broadly classified into the embryonic isotype containing the \( \gamma \) subunit and the mature isotype containing the \( \varepsilon \) subunit. Compared to \( \gamma \) containing AChR, \( \varepsilon \) containing AChRs have a shorter open time as well as refractory period making it more suitable for high frequency stimulation (168). At birth the \( \gamma \) AChR subunit is present at very high levels while \( \varepsilon \) AChR subunit is relatively low (46). However over the course of two to three weeks, the \( \gamma \) subunit decreases to undetectable levels while the \( \varepsilon \) subunit rises to its peak (46, 169). By 6-7 weeks after birth the \( \varepsilon \) AChR subunit decreases and plateaus to a steady level (169). After denervation, the \( \gamma \) subunit increases dramatically while restoration of nerve supply again reduces \( \gamma \) subunit expression (168, 170, 171). However the link between innervation and \( \varepsilon \) is not well defined. Some studies show that denervation of neonatal rodent muscle did not affect the rise in \( \varepsilon \) AChR subunits (172). It is likely that brief periods of contact with nerves is all that is required to trigger the rise in \( \varepsilon \) AChR subunit (172). Loss of nerve supply after initial priming of the muscle likely does not impact on \( \varepsilon \) AChR subunit expression.

Innervation regulates \( \gamma/\varepsilon \) subunit expression through two main mechanisms: nerve contact and muscle activity (173, 174). When denervated rodent muscles were re-innervated with an ectopic nerve, pharmacologic blockage of neurotransmission did not prevent the shortening of AChR open time suggestive of a reduction in \( \gamma \) AChR subunit (175). Furthermore, if the re-
innervated muscle was again denervated but stimulated by electrical activity the AChR open
time also continued to shorten like in normal re-innervated muscle (175). In the first
scenario nerve contact without neuromuscular transmission was sufficient to drive the θ/ε
switch. Some evidence suggests that part of this contact-driven mechanism is due to neural
agrin (176). One study showed that approximately 30% of agrin induced AChR clusters
contained a combination of ε and γ subunits while 70% contained only γ subunits (177). In
the second scenario electrical activity was sufficient to promote AChR subtype change
provided the muscle had previously been primed by the nerve. Despite the overwhelming
evidence for the link between innervation and AChR subtype switching, use of AChR subtype
expression has rarely been used as a surrogate marker for innervation in nerve muscle co-
cultures. One study showed that both three dimensional myotube cultures and neural-
muscle co-cultures expressed ε AChR subunit mRNA while two dimensional myotube
cultures failed to show any ε AChR mRNA (178). The presence of ε subunits in three
dimensional muscle monocultures suggests that expression of ε subunit is not restricted to
innervated muscle cells in vitro. The absence of ε subunits in two dimensional cultures
suggests that ε mRNA expression is also sensitive to the scaffold that the muscles are
immobilized in.

1.3.8.2 Voltage gated sodium channel subtype

voltage gated sodium channels are responsible for converting small depolarisations
secondary to opening of AChR gated sodium channels into action potentials at the NMJ.
These channels are strategically positioned at the trough of the secondary synaptic folds (22).
As depolarization spreads from the AChR gated sodium channels at the peak of the synaptic
fold to the trough and becomes subsequently amplified, it exceeds the threshold potential for channel activation triggering the channels to open (22). Like AChRs, voltage gated sodium channels are predominantly clustered at NMJs with low levels present at extra junctional areas (179). The channels develop embryonically and are present diffusely across the sarcolemma at birth (180, 181). However by two to three weeks after birth the voltage gated sodium channels become concentrated at the endplates (181). This clustering mechanism is nerve induced as denervation increases extra junctional channel density (179, 181). The channels are anchored to the muscle membrane by cytoskeletal proteins such as ankyrin and can bind to dystroglycans through syntrophin (182-184).

There are two isoforms of voltage gated sodium channel in mammalian muscle- mature and immature. The mature channels, also known as sodium channel protein type 4 subunit alpha (NaV1.4 or Skm-1), have higher sodium conductivity, shorter activation and inactivation times and are activated at a more positive voltage compared to the immature channels, also known by sodium channel protein type 5 subunit alpha (NaV1.5 or Skm-2) (185). Many older studies on voltage gated sodium channels distinguished the two isoforms by their sensitivity to neurotoxins Tetrodotoxin (TTx), Geographutoxin and Saxitoxin with NaV1.4 sensitive and NaV1.5 resistant to the toxins (186-188). NaV1.4 is concentrated at the NMJ in adult muscle with low levels detected in extra-junctional regions (189). At birth NaV1.4 levels is relatively low. However this increases by 4 fold in the first month after birth with general increase in the NaV1.4 expression in both junctional as well as extra-junctional regions (90, 180, 190). However the increase at junctional regions was more prominent with 8 fold difference in expression between endplates and non-endplate regions (180, 189). The underlying mechanism for the enhanced NaV1.4 expression is likely nerve derived. Neural muscle co-cultures display higher levels of NaV1.4 activity compared to muscle-only cultures which predominantly express NaV1.5 (191-194). Furthermore this phenomenon on NaV1.4
activity can also be reproduced by incubating the myotubes with extracts from spinal cords (195). Key components of neural tissues that mediate the upregulation of NaV1.4 include neural agrin and neuregulin (90, 196). When ectopic regions of innervated muscles were transfected with neural agrin, there was local upregulation of NaV1.4 expression (90). Neuregulin purified from chick brains increased NaV1.4 activity in chick myotubes (196).

The effect of denervation on NaV1.4 depends on the stage in development that denervation is introduced (197). In adult muscle, denervation does not change the distribution or the expression level of NaV1.4 (189, 198). Like with AChR clusters, the nerve likely exerts a ‘footprint’ effect on NaV1.4s. The clustering effect of the nerve terminal on NaV1.4 lingers even after denervation allowing for maintenance of the distribution and expression level of the channel. If denervation occurs early at 5 days after birth, NaV1.4 expression undergoes two phases of changes. In the first phase NaV1.4 expression continues to rise like in innervated muscle until it is 47-57% of the value of that in adult muscle (90, 197). This indicates that ongoing nerve supply is not required during this phase of NaV1.4. It is possible that brief neural contact with the muscle is all that is needed to ‘prime’ the muscle to upregulate the channels at previous areas of nerve-muscle contact. After day 11 post-partum, the muscle enters stage two and NaV1.4 decreases and plateaus to a level that is still well above that at birth (197). This suggests that ongoing neural contact is required for NaV1.4 to reach mature levels.

The immature NaV1.5 is present in relative abundance at birth and diffusely distributed at both junctional and extra-junctional regions (180). By two to three weeks after birth NaV1.5 decreases to very low levels with most of the reduction occurring in extra-junctional regions (180, 187, 189, 190). There is some discrepancy between studies that examined the expression of NaV1.5 in adult muscles. Studies that measured NaV1.5 mRNA show undetectable levels of NaV1.5 in adult innervated muscle while those that used NaV1.5
function as a gauge of NaV1.5 level by measuring the level of TTx resistant Na current show persisting low levels of the channel at NMJs (90, 180, 189, 190). This may be related differences in experimental setup, sensitivity of the detectors and unknown effects of post-translational modifications on the function of both NaV1.4 and NaV1.5. In adult muscle, denervation leads to a general increase in NaV1.5 expression in both extra-junctional as well as junctional regions (189). However NaV1.5 is predominantly clustered at the endplates after denervation (189). In the first three to five days after denervation, NaV1.5 rises by 100 fold, peaks and levels off before plateauing at 30% of the peak value but still well above that before denervation (187, 190, 198). The rise in NaV 1.5 is likely muscle activity related as blockage of neuromuscular transmission by botulinum toxin upregulates NaV1.5 in innervated muscles in a similar manner to denervated muscle (198). Furthermore electrical stimulation of denervated muscle prevents the rise in NaV1.5 expression (189).

1.3.8.3 Spontaneous electrical activity and muscle contraction

Spontaneous myotube depolarisations and contractions are often observed in muscle-only as well as neural-muscle co-cultures. Muscle cells are highly excitable and possess sodium, calcium dependant potassium channels and calcium channels that are important in spontaneous electrical activity and mechanical contractions (199, 200). Sodium channels first develop in the myoblast stage of development and persist on the cell membrane throughout its lifespan (201). Both NaV1.4 and NaV1.5 channels are present with a predominance of NaV1.5. Both have been implicated in spontaneous myotube contraction in muscle cultures (202, 203). Upon fusion of myoblast to myotubes, spontaneous calcium transients develop with the appearance of calcium channels. Initially the transients are not linked to any contraction (204). However with further maturation each calcium flux becomes linked to
visible myotube contractions (204). Up to 75% of rodent myotubes eventually display calcium linked contractions (205). There are two types of calcium channels present on myotubes- L-type dihydropyridine and T-type voltage dependant calcium channels (201). The L-type channels precede the appearance of T-type channels. As the myotubes mature the proportion of T-type channels increase (201). It is believed that the T-type channels are important in inducing pacemaker like electrical activity and contractions in the myotubes while the L-type channels are mainly responsible for replenishing the intracellular calcium stores (201).

The presence of spontaneous myotube contraction also varies with the species of myotubes. Unlike rodent muscle, human muscle cells do not display spontaneous contractions unless they are innervated (128). In such cases, myotube contraction can be a direct marker of innervation. However use of human cells is limited by availability and the need for invasive procedures to obtain adequate muscle samples. In contrast, rodent muscle cells are widely available but limited by non-discrete development of myotube contractions in both muscle-only and nerve-muscle co-cultures (202, 206). As a result it is difficult to use contraction or electrical activity by itself as a marker of innervation. Many studies have attempted to overcome the problem by using the AChR antagonist tubocurarine to differentiate between spontaneous and nerve induced muscle activity (206-209). The tubocurarine will block any activity that is a result of ACh action at the NMJ. However this approach is also flawed as muscle-only cultures also display tubocurarine responsive contractions (210). Myotubes are capable of releasing ACh like compounds that act on its aneural AChR in an autocrine fashion to induce spontaneous contractions (211, 212). In fact ACh like compounds have been isolated from myotube conditioned media (211, 212). In order to deduce the level of NMJ induced muscle activity in rodent cultures, it is critical to establish multiple controls with muscle-only cultures treated with tubocurarine and conditioned media from co-cultures.
This series of controls and interventions will effectively rule out any spontaneous muscle activity as well as any paracrine effect neural cells may have on muscle, thus allowing the exact level of NMJ induced activity to be found.

1.3.8.4 Myosin heavy chain

Myosin is the principle component in muscle that is responsible for contraction. It actively hydrolyses adenosine triphosphate (ATP) effectively transducing the chemical energy released in the process into physical filament displacement. Myosin is a hexameric molecule consisting of two myosin heavy chains (MHC) and two pairs of non-identical light chains (213, 214). The MHCs provide the motor function of the myosin molecule. MHCs are expressed in skeletal muscle, cardiac muscle, smooth muscle and non-muscle tissues (214). Skeletal muscle MHCs exist as multiple isoforms: embryonic, neonatal, α, I/β, Iia, Iib, IIX/d, extraocular and mandibular (213, 214). Each MHC isoform is encoded by a different MYH gene as detailed in table 1-2. The isoforms differ in the rate of ATP hydrolysis and shortening thus impacting on the mechanical properties of the muscles that house the MHC. Type I/β MHCs have slower rates of ATP hydrolysis and are present mainly in type 1 slow muscle fibres that are generally small and rely on oxidative phosphorylation for energy supply (213, 214). Type II MHCs are mainly present in fast fibres and the isoforms have relative rates of shortening so that type Ila <X<b (215). Type Ila MHCs are present in red fast fibres that are medium in size and also rely on oxidative phosphorylation for energy (213). Both type Iib and X MHCs are present in fast white muscle fibres that derive energy principally from anaerobic glycolysis (213). The type of MHC expressed also depends on the species. Unlike in rodents, human muscles do not express type Iib MHC (213).
Both developing and adult muscles are hybrids of multiple MHC isoforms (214). Developing muscles mainly express embryonic and neonatal MHCs that are gradually replaced by either type I or 2 MHCs overtime. The switch from embryonic/neonatal towards more mature MHCs is a complex process regulated by multiple factors including innervation. In rodents innervation of muscles occurs during the embryonic stage at approximately 14 days post-coitum (pc) (214). This time point coincides with the switch from embryonic to the foetal stage of development in rodents. During the embryonic stage myoblasts fuse to form primary myotubes that express predominantly embryonic MHC with lower levels of neonatal as well and type I MHC (214). After innervation at day 15 secondary myotubes form as new myoblast continue to fuse with the primary myotubes. This corresponds to dramatically increased neonatal MHC and raised neonatal-to-embryonic MHC ratios (214). In fast fibres type II MHCs appear in the secondary myotubes after innervation at day 18 pc and progressively replaces the immature MHCs (214). In slow fibres type I/β MHCs gradually increase to become the predominant MHC. Most of the increase in adult MHCs takes place after birth with 50% and 90% of MHCs in slow and fast fibres in neonates consisting of immature MHCs respectively (216, 217). However by three to four weeks after birth both neonatal and embryonic MHCs disappear as most of the immature MHCs are replaced by adult forms (217). Denervation soon after birth inhibited the rise in type I MHC in slow fibres and IIb MHC in fast fibres resulting in relatively increased levels of type IIa and X MHC in both fibre types (216, 218). In essence denervation makes slow fibres faster and fast fibres slower (218) (216). This observation suggests that innervation is important for functional maturation of both fast and slow muscle fibres. The presence of structured organization of myosin and actin in myotubes in the form of striations has also been observed in various neural muscle co-cultures (207, 219-221). The striated myotubes indicate increased maturity of the contractile machinery secondary to innervation. Various neural muscle co-cultures
have reported striated myotubes as evidence for innervation of the muscle cells (207, 219-221).

<table>
<thead>
<tr>
<th>Gene</th>
<th>MHC subtype</th>
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<tbody>
<tr>
<td>MYH 1</td>
<td>MHC 2X</td>
</tr>
<tr>
<td>MYH 2</td>
<td>MHC 2A</td>
</tr>
<tr>
<td>MYH 3</td>
<td>MHC embryonic</td>
</tr>
<tr>
<td>MYH 4</td>
<td>MHC 2B</td>
</tr>
<tr>
<td>MYH 7</td>
<td>MHC 1</td>
</tr>
<tr>
<td>MYH 8</td>
<td>MHC neonatal</td>
</tr>
</tbody>
</table>

Table 1-2 Genes encoding for isoforms of myosin heavy chain.

1.3.9 Nerve to Muscle Neurotisation

Research on aneural AChR clusters and muscle priming for innervation can find clinical relevance in the construction of the neuromuscular prosthetic interface. In a NPI, the muscle needs to be innervated by process known as direct nerve to muscle neurotisation (NMN). NMN refers to direct implantation of severed nerve endings into the substance of the muscle after injury to the NMJ (222, 223) (figure 1-5). The procedure is suitable for scenarios where the muscle and its innervating neurites and endplate have been damaged by trauma or surgery necessitating innervation of the remnants of the damaged muscle. NMN has been seldom investigated in human studies and there are no randomised controlled trials that have examined the procedure’s efficacy. Most of the evidence comes from small case series which show good functional outcome after surgery (222, 223).
However it is difficult to ascertain from the results whether the recovery is due to collateral innervation by intact surrounding motor units or from innervation by the reimplanted nerve. A study that accounts for collateral innervation would be required to divide all intact nerves in the vicinity of the neurotised muscle which is ethically unacceptable. As a result, most NMN studies have been performed on rat models (224-227). Classically, in such a model, segments of the tibial and sensory branch of rat sciatic nerves are excised and tied off. The common peritoneal nerve is transected and the proximal nerve stump is transposed onto the gastrocnemius muscle at a site away from the old endplate and attached in place (224). The functional recovery of the rat hind limb is then monitored. A major strength of the animal model is that it minimizes the effect of collateral nerve sprouting on the recovery of the re-innervated muscle. By excising both the tibial and sensory branch of the sciatic nerve the model ensures that the recovery of the denervated muscle comes solely from innervation by the transplanted common peroneal nerve.
Figure 1-5 Nerve repair and nerve to muscle neurotisation. (A) Neural damage at the level of the axon is amenable to nerve conduit repair. (B) Damage at the level of neuromuscular junction such as nerve avulsion requires implantation of nerve ending into muscle belly (nerve to muscle neurotisation). (C) Neurotisation also required when transplanting tissue engineered muscle grafts. Image created by author. Information adapted from (30, 224).

Recovery after NMN in animal models have been inferior compared to nerve grafting or normal muscle (224, 226-228). Some researchers have attempted to improve the result by regular injection of muscle with neurotrophins such as NGF with some improvement in function. But the final recovery was still incomplete. A possible reason for the poor recovery
in NMN may be related to the loss of the original endplate. It is known that transplanting the common peroneal nerve with its previous endplate attached as a neuro-muscular pedicle onto the gastrocnemius enhances the functional recovery compared to plain NMN (226). Furthermore muscle grafts with the original endplate excised show poor functional outcomes when neurotised compared to those with the endplate intact (37, 225). Clearly the old endplate on the muscle is capable of conveying biological cues that promote re-innervation of the muscle. The old endplates consists of aneural AChR clusters that have been previously primed by a nerve. It is of interest in future studies to investigate the effect of artificially increasing the number of aneural AChR clusters on denervated muscle with nerve derived signals such as agrin and further neurotising the denervated muscle with a transplanted nerve. The agrin serves to ‘prime’ the muscle and induce endplate like structures on the sarcolemma thus expediting re-innervation.

1.3.10 Future Directions in NMJ research

Research on aneural AChRs has come a long way since its discovery on developing muscle in embryos. It is clear that aneural AChR clusters play an important role in the re-innervation of muscle both during development and after denervation. However the mechanism underlying aneural AChR clustering is still unclear. A variety of biological agents have shown to cluster AChR both in vitro and in vivo. However it remains unclear as to whether the AChR clusters that form in response to different agents are qualitatively and functionally equivalent. Are the aneural AChR clusters that are artificially induced the same as those that spontaneously form during development? Do the induced receptor clusters also convey positional information to neurites and prime muscle for re-innervation? These questions have direct implications on the prospect of using AChR clustering agents for treatments of
neuromuscular injuries. Furthermore the experimentation methods employed to investigate aneural AChR clustering and re-innervation must also be improved upon. Firstly the methods of neural-muscle co-cultures must be standardized to reduce variability between studies. More importantly more attention needs to be devoted into developing surrogate markers of innervation in in vitro experiments to better objectify and measure innervation in neural-muscle co-cultures. This helps to both compare results between studies and summarize and synthesize new ideas from existing experimental results. Finally, it will be of great interest to apply the theory of AChR clustering and muscle sensitization to clinical procedures such as nerve to muscle neurotisation. Re-innervation of denervated muscle is a major challenge for surgeons of all specialties ranging from orthopaedics to neurosurgery. The prospect of using AChR clustering agents to supplement existing re-innervation procedures can potentially transform treatment of denervation injuries.

1.4 Muscle electrode junction

The muscle electrode junction (MEJ) is a critical component of the NPI, transforming biological impulses into electronic signals that drive the prosthetic limb. Most of the research on cell-electrode electrical coupling relate to neural-machine interfaces. Due to the lack of research on MEJ, the findings and concepts from work on neural-machine interfaces will be generalized to MEJs during discussion. The following sections will briefly explain the chief determinants of signal transmission at the cell-electrode interface. This will be followed by an outline of the current state of research on using electrode coatings to improve the signalling at the cell-electrode interface with special emphasis on polypyrrole- a conducting polymer- as coating material for cell recording.
1.4.1 Determinants of cell-electrode electrical coupling

For activity to travel across the cell-electrode interface, the electrical signal must transverse multiple structures. The signal begins as an ionic current inside the cell cytoplasm before crossing the cell junctional membrane - the part of the membrane facing the electrode- to enter the cleft of extracellular fluid interposing between the cell and the electrode. From there, the ionic current is finally transduced into an electrical current at the fluid-electrode interface before travelling through the substance of the electrode. Several key structures can impact on the signalling transfer during this process, namely the cell junctional membrane, the distance between the cell and electrode, the seal between the cell and the electrode and the electrode surface.

The distance between the cell and the electrode is a critical factor in determining signal strength. Electrical signals decay with distance according to the formula $\Delta E \propto \frac{1}{r^2}$ where $E$ stands for fluctuations in electric field due to changes in membrane potential while $r$ is the distance (229). The closer the cell is to the electrode the stronger the recording. However, close apposition of the cell against the electrode is not always possible as the cell is separated from the underlying substrate by layers of glycocalyx, cell debris and the biological coatings that are often applied to the substrates at the time of culture (229, 230). The glycocalyx refers to the multitude of adhesion and signalling molecules that are scattered across the cell lipid membrane. The glycocalyx and substrate coating together act as a cushion diluting the strength of the extracellular field potential (230). To further complicate the picture, the distance between the cell and the substrate is also not uniform with areas of focal adhesion closer to the substrate than others, leaving behind a thin cleft of extracellular fluid between the cell and the electrode (231-233). This distance varies with the cell type and the substrate coating used. It is unclear whether greater adhesion conferred by surface
coating necessarily reduces cell-electrode distance as the surface adhesive coatings also occupy volume, potentially pushing the cell away from the electrode. In addition, cell debris and protein deposition may collect at the cell-electrode junction over time leading to unpredictable effects on cell-electrode signalling.

Figure 1-6 Schematic diagram illustrating the components at the muscle-electrode junction and the factors affecting signal transmission. Image created by author. Information adapted from (229, 230).

The cell junctional membrane is defined as the portion of the cell membrane that is directly opposite the electrode. It can be represented as a resistor and capacitor in parallel as it can act both as a resistor and a capacitor (Figure 1-6). The cell phospholipid membrane behaves like a dielectric in a capacitor dividing the cytoplasmic charge from the extracellular environment (234). The channels in the membrane allow a limited level of
electrical conductivity through the membrane at a constant resistance. Fluctuations in cytoplasmic potential can be conveyed extracellularly by either capacitive coupling at the lipid membrane or through the ion channels that traverse the membrane (234). Like capacitors, the capacitance of the junctional membrane can be enhanced by increasing the junctional membrane surface area thus improving the strength as well as the speed of signal transfer (235). The area of the junctional membrane is dependent on the morphology of the cell. Cells that assume a spread and flat morphology will have a greater junctional membrane area allowing for better capacitive coupling and signal transmission (229, 230, 235). Therefore, it is critical to incorporate biological cues into electrode surfaces to promote cellular spreading and augment cell-electrode signalling.

Like the junctional membrane, the fluid-electrode interface can also be modelled by a resistor and capacitor in parallel. The electrode surface has the capacity to act as a barrier dielectric blocking the entry of ions from the extracellular fluid while sequestering charge within the substance of the electrode. Electrical current from the extracellular fluid can traverse the interface either by inducing capacitive currents in the electrode near the interface or by crossing the barrier through redox reactions (234, 236). Redox reactions require voltages that are many folds higher than those seen in biological tissues. Therefore, signal transfer at the fluid electrode interface is predominantly through capacitive coupling. Like in junctional membranes, electrical coupling with the electrode can be improved by expanding the electrode surface area and increasing its capacitance. However greater surface area can also lead to larger electrodes limiting the number of electrodes that can be fitted into each array and compromising its spatial resolution (235). More recently with advancements in material science electrode coatings with micro and nano-scale surface texture have been developed that increase surface area without enlarging the gross dimensions of the electrode (237, 238).
As the electrical current leaves the cell and travels across the cleft of extracellular fluid between the cell and the electrode, much of it is lost through current leak along the cleft. The “leakage” current is determined by the level of “seal” between the cell and the electrode. Cells that extend processes to cover the entire surface of the electrode have a high ‘seal resistance’ that minimize current leakage thus preserving the integrity of the signal (230, 239-243). In one study the electrodes were designed with a ‘mushroom’ shape to encourage endocytosis by the neural cells. Once the cells had ‘engulfed’ the electrodes, strong signal recordings were obtained (244). The authors credited the enhanced recording to the high seal resistance developed by the engulfing neural cells.

1.4.2 Conducting polymers and Polypyrrole electrode coatings

Conducting polymers (CP) are a new class of electroactive materials that have been studied as a coating for biological electrodes. CPs draw from the superior electroactive properties of metals and marries it with the ease of processing and chemical modification that is characteristic of polymers (245). CPs have of a conjugated polymer backbone consisting of alternating single and double bonds and electrons that are readily delocalized and can flow through the polymer. The backbone is stabilized and made conductive by the addition of a dopant molecule which is generally a negatively charged anion. Upon application of a potential voltage, the dopants move in and out of the molecule disrupting the polymer backbone and allowing for free passage of the electrons (245). Compared with standard metals, CPs have a lower modulus that better approximates that of soft tissue thus dampening the modulus mismatch and reducing fibrous reaction *in vivo* (245). CPs also contain surface microstructures that increase surface area thus reducing the electrical impedance and increasing both the charge injection capacity as well as charge storage in
comparison with metal electrodes (6, 7, 237, 238, 246, 247). More importantly CPs can be readily modified to contain biological molecules that promote cell adhesion and differentiation which contrasts with the inert surface of metal electrodes (246-249).

The most thoroughly investigated CP is polypyrrole (PPy). PPy has been used as an electrode coating to improve the electrical as well as biological performance of electrode arrays. PPy coatings reduced the impedance and increased the charge injection capacity of metal electrodes (6, 7, 246, 247). On a microscopic level, the PPy is dotted with globules of various sizes (237, 238). This microtextured surface significantly increases PPy roughness and surface area thus increasing its capacitance and reducing electrical impedance. The surface area and roughness of polypyrrole varies positively with thickness (237, 250-253). As more PPy is deposited, the microscopic surface globules coalesce to form larger “cauliflower” structures further expanding its surface area. However this positive correlation changes once the PPy thickness reaches a critical level after which both impedance and surface area plateaus (253, 254). It is believed that after successive layers of deposition of polymer, further additions of PPy become more condensed in structure thus not further adding to the total surface area. In fact in some CPs, further increases in thickness may paradoxically increase impedance likely due to the changed conformation of newly deposited polymers (253, 254).

The type of dopant used to dope PPy can also impact on its electrochemical as well as physical properties. Common dopants include paratoluene sulphonate (pTS), perchlorate \( (\text{ClO}_4^-) \), poly(styrene sulphonate) (PSS) and dexamethasone phosphate (Dex-P), chloride (Cl\(^-\)), sulfate \( (\text{SO}_4^{2-}) \), dodecylsulfate (NaDDS), nitrate \( (\text{NO}_3^-) \), tetrafluoroborate \( (\text{BF}_4^-) \), or 1-naphthalenesulfonate \( (\text{NS}^-) \) anions (17). Larger dopants tend to smoothen the complexion of Ppy while smaller one roughens the film. Ppy doped with with Cl\(^-\) and ClO\(_4^-\) was rougher than those doped with the larger dopants sulfate and dodecylsulfate (237, 238). Similarly, PPy doped with larger anions pTS and NS were smoother than those doped with the smaller
ClO$_4^-$, NO$_3^-$, BF$_4^-$ (237, 238). Interestingly the same study also reported that larger dopants produced more conductive Ppy compared to small dopants despite having smoother surface. The study posited that the difference in conductivity was due to the ease at which charge moved through the substance of the PPy rather than due to effect at the fluid/PPy interface.

More recently attempts have been made to modify Ppy with dopants that are components of natural biological molecules (246-249). The biological dopants impart a bioactive surface to the Ppy enhancing its interaction with cells. PPy doped with fibronectin fragments and various biological peptides have been studied as substrates for neurons, glial cells and fibroblasts and neural cell lines and have enhanced cell adhesion, survival and growth (246-249). Furthermore, PPy films have also been used as sustained delivery devices for drugs and growth factors in response to electrical stimulation (6, 255). Apart from in vitro studies, various authors have examined the tissue response to Ppy in vivo (6, 248, 256-259). PPy implants have shown fibrotic reactions that are at least comparable and sometimes better than materials that are currently approved to be used clinically as scaffolds such as Teflon and Poly(lactic-co-glycolic acid) (PLGA) (256, 257). One of the main drawbacks of Ppy is its lack of physical stability. PPy films are known to delaminate from its substrate upon repeated electrical stimulation by an outside source (260-262). It is theorized that repeated stimulation leads to redox reactions in the PPy resulting in volume changes to the material (260-262). The cyclical volume change predisposes it to delaminate. This inherent instability of the PPy coating is related to the $\alpha$-$\beta'$ couplings in its structure which lead to defects sites along its polymer chain that serve as main sites of breakdown when over oxidized (263, 264). Some attempts have been made to rectify the situation such as roughening the substrate before PPy coating to strength the interlock between the Ppy and the substrate (265). Others embedded the conducting polymer in a semi interpenetrating polymer network to
overcome the delamination problem (266). However from the perspective of recording electrode coating, this should not be a major issue as the PPy coatings will be used to detect low level activity from cells rather than being repeatedly electrically stimulated by an external high voltage source.

1.5 Conducting polymer based trilayer actuators

In order to restore function to the amputated limb, the human machine interface must be connected to an external actuator to fully transduce the biological signals from the stump to a mechanical one. Much research has been conducted in developing new forms of actuators for artificial limbs. Some of the important parameter used to assess actuators includes stress, strain, strain rate, work density, power density and electro-mechanical coupling (267). Stress refers to force generated by unit cross sectional area of the actuators, strain refers to the percentage deformation achievable by the actuators. Strain rate is the rate that the deformation occurs. Work and power density is the amount of work or power output by generated by the actuator per unit mass. The amount of electromechanical coupling refers to the percentage of input energy that is converted to mechanical energy by the actuator. Traditional actuators include internal combustion engines, electric motors and piezoelectric motors. Internal combustion engines and high speed electric motors have higher power density and stress compared to muscle (268, 269). However, they are principally designed for continuous activity whereas actuators for artificial limbs should be optimized for intermittent and aperiodic motions. The inertia, friction, backlash and gear compliance associated with internal combustion engines and geared electric motors precludes rapid control and response of actuator output that is often required by artificial limbs (268, 269). As a result, direct-drive electric motors have been considered as an alternative due to its fast
response rate and low inertia. However direct-drive motors require substantially larger volumes to produce same amount of power as compared to muscle tissue (269). Piezoelectric motors enjoy high stress as well as power density. But they have low strain of approximately 0.1% and are not ideal in situations where significant displacement of the actuator is required (268).

Recently with advances in material science, a new class of polymer actuators have been developed with high stress, work density and are ideal for intermittent actuation with minimal volume requirements. Polymer actuators can be broadly classified into two categories; electric electroactive polymers (EAP) and ionic EAP (267, 268, 270). Electric EAPs require an electric field to drive the deformation of the actuator. Ionic EAPs operate in an electrolyte environment and actuate in response to a flux of ions through the polymer (267, 268, 270). Various electric EAPs have been produced including dielectric elastomers, electrostrictive polymers. Dielectric elastomers are capacitors with a compliant dielectric (271-273). As the capacitor charges up, the electric field across the capacitor also known as Maxwell stress compresses the compliant dielectric deforming it and causing it to expand laterally (271-273). Electrostrictive polymers include relaxor ferroelectric polymers, graft elastomers and liquid crystal elastomers (274-280). These polymers contain dipoles that realign in response to an electric field leading the polymer to deform. All electric EAPs produce high stress, high strain and also high power density (271-280). However they require high voltages to sustain the electric field (268, 281). Unfortunately actuators for limb prosthesis will likely be powered by low voltage batteries. As a result extra AC-to-DC converters will need to be engineered into the system increasing the mass and volume of the prosthesis.

Ionic EAPs include carbon nanotubes (CNT), conductive polymer actuators and ion-metal composites (281). The main benefit of ionic EAPs is its low operating voltage which is ideal
for portable batteries fitted on artificial limbs (281). All ionic EAPs require an accompanying electrolyte to actuate. Carbon nanotubes deform when immersed in an electrolyte. When a voltage is applied across the carbon nanotube and a counter electrode, opposite charges in the electrolyte adsorb onto the CNT and the counter electrode. The repulsive interactions between like charges on the surface of the CNT counteract the carbon to carbon bond in the CNT causing it to deform (282). CNT actuators generate high stress and have very high power density (282). However they have very low strain of <2% mainly owing to their high modulus (268). Conducting polymer actuators are mainly made from polypyrrole. Like CNT, PPy needs to be immersed in electrolyte for it actuate. Upon application of an electric potential, the Ppy undergoes redox reactions and ions in the electrolyte flow in and out of the polymer chains to balance the charge leading to volume change of the film (283). PPy actuators have high stress, high work density, low working voltage and are silent and light (270). The main drawback of the material is its low electromechanical coupling of <1% meaning that fewer than 1% of the energy input is transformed into actuation and low strain of approximately 1-3% (270, 284). More recently some studies have managed to improve the strain to 12.4% (285). The rate of strain (rate of deformation) of Ppy actuators is also low and it becomes even slower as the thickness of the polypyrrole actuator increases (270). This is due to increased resistance to diffusion of ions through the substance of the polypyrrole as it thickens. Recently there have been improvements in the manufacturing of the Ppy actuators improving the strain rate to 3%/s (284). In order to engineer Ppy actuators that match the strain rate of muscle, the thickness of the actuators must be significantly reduced which raises a new challenge for manufacturing. The electrolyte used for the actuator must also be properly encapsulated so it is sealed within the substance of the polymer and not allowed to be dry up.
The configuration of the polypyrrole actuator also impacts on its physical and mechanical properties. Broadly speaking PPy actuators can either be linear or bending. Linear PPy actuators move in a linear fashion while bending actuators deflect upon oxidation/reduction. Linear actuators generate more stress while bending actuators sacrifice stress generation for greater displacement (283). Bending actuators are either bilayered or trilayered (283). Bilayered actuators consist of an active layer of PPy and a passive layer (286, 287). When a voltage is applied, the PPy layer contracts or expand bending the actuator in one direction (286, 287). In a trilayered actuator, a layer of electrolyte is sandwiched between two active layers of polypyrrole (288-290). Upon application of a voltage across the two PPy layers, one layer under goes oxidation and the other reduction leading to reciprocal changes in volume with one side expanding and the other contracting further increasing displacement of the actuator. If the voltage difference is reverse, the actuator will deflect in the opposite direction (288-290). Ion-metal composite actuators function in a similar fashion to PPy trilayered actuators whereby a polyelectrolyte consisting of polymer chains and ions is sandwiched between two layers of compliant metal electrodes (291). Under an electrical potential, cations in the polyelectrolyte move towards negative electrodes leading expansion of the electrolyte near the negative electrodes and shrinkage near the positive electrode thus bending of the actuator trilayer (291). Ion-metal composite actuators are capable of achieving high stress but also suffer from low strain of 3% (291).

Despite attempts at replicating the function of muscle, none of the polymer actuators developed thus far satisfy all the criteria for an artificial limb. In fact there are certain features of normal muscle that cannot be recapitulated in artificial actuators. Firstly, muscle is able to alter the modulus of the fibres. Contracting muscle fibres are stiffer than those that are relaxed. This is critical for completion of certain tasks such as catching a speeding object whereby a compliant surface is required to minimize the damage to the muscle (267). It is
also critical in the control of muscle power. Muscle alters its power output by gradated recruitment of muscle fibres. Fibres that are not recruited become compliant to avoid impeding the contraction of activated muscle fibres (267). This allows for better regulation of muscle force output. However none of the currently available actuators are capable of such a feat. Secondly, muscle fibres have a very high efficiency for energy conversion that is unmatched by any extant polymer actuator (267). Thirdly, muscle fibres are capable of regeneration allowing it to remain intact after millions of cycles of activity over decades. None of the current actuators can claim such a durable and reliable track record (267). However many of the current polymer actuators already have power densities and stress that are at least as good as or better than muscle (268). Future developments of polymer actuators should aim to improve the strain and strain rate of the actuators. More studies are required to assess the durability of the actuation by the new polymers and means of sealing the electrolyte within the polymer actuator.

1.6 Future Directions

Construction of a NPI is a multidisciplinary undertaking requiring input from fields as diverse as physics, material science, engineering and biology. This review has summarized the state of research in three key components of the NPI; neuromuscular junction, muscle-electrode junction and new generation of polymer actuators that can be connected to the NPI. The neuromuscular junction research is critical for effective neurotisation of muscle for interfacing with machines. The possibility of enhancing NMJ formation and neurotisation by increasing aneural AChR has been reviewed. Research on aneural AChRs has come a long way since its discovery on developing muscle in embryos. It is clear that aneural AChR clusters play an important role in the re-innervation of muscle both during development and
after denervation. However the mechanism underlying aneural AChR clustering is still unclear. A variety of biological agents have shown to cluster AChR both in vitro and in vivo. However it remains unclear as to whether the AChR clusters that form in response to different agents are qualitatively and functionally equivalent. Are the aneural AChR clusters that are artificially induced the same as those that spontaneously form during development? Do the induced receptor clusters also convey positional information to neurites and prime muscle for re-innervation? These questions have direct implications on the prospect of using AChR clustering agents for constructing neuromuscular units for interfacing with electrode arrays in NPI. Furthermore the experimentation methods employed to investigate aneural AChR clustering and re-innervation must also be improved upon. Firstly the methods of neural-muscle co-cultures must be standardized to reduce variability between studies. More importantly more attention needs to be devoted into developing surrogate markers of innervation in in vitro experiments to better objectify and measure innervation in neural-muscle co-cultures. This helps to both compare results between studies and summarize and synthesize new ideas from existing experimental results. Finally, it will be of great interest to apply the theory of AChR clustering and muscle sensitization to clinical procedures such as nerve to muscle neurotisation which can then be connected to an electrode array for human-machine interfacing.

Muscle electrode junction is critical for transduction of muscle signal into electronic ones in a NPI. However little research has been focused on establishing a reliable and high fidelity recording interface for muscle. Most studies on cell-machine interfacing have mainly concentrated on neural tissues and cells. The conditions that are required to optimize cell-electrode interfacing also remain to be elucidated. It is unclear whether the presence of various biological cues on electrode surfaces enhances signal transduction at the cell-machine interface. The biomolecules may promote cell spreading and focal adhesion
formation, however they also form a space occupying barrier between the electrode and the cell potentially diluting the cell-electrode electrical coupling. It would be of interest to investigate the effect of different ECM molecules that are commonly used to coat substrates for cell culture on cell-electrode coupling. Such information will greatly inform the design of biological active electrodes for NPI. With recent advances in material science, conducting polymer electrode coatings have become the focus of much research. They possess superior electrical properties as well as being readily amenable to biological modification. However most of the studies on these electrode coatings have been conducted in acellular environments. As a result the conclusions drawn from such studies may not account for other cellular and in vivo environmental factors that may impact on the performance of the electrode. Parameters of electrode coating such as roughness, thickness and the types of micro/nanostructured topography need to be studied within an cell based environment to better assess its impact on cell-electrode electrical coupling.

The ultimate aim of constructing a NPI is to restore function of the lost limb. The activity from the NPI will be used to drive an actuator. Existing actuators based on internal combustion, electric and piezoelectric motors are unsuitable for use as artificial muscles owing to high operating voltages and difficulty with intermittent actuation. New classes of polymer actuators have shown promise with high power to weight ratios and low operating voltages. More importantly they do not have some of the drawbacks of conventional actuators such as high inertia, backlash and friction making them suitable for aperiodic activity. However these actuators still suffer from low strain, slow strain rate as well as low electromechanical coupling. More research is required to improve the electromechanical properties of these novel actuators. Furthermore, little has been done on examining the biocompatibility of polymer actuators and their interactions with cells. Most of the studies on polymer actuators are conducted in bench side experiments without cells. Given the
intended use of the polymer actuator is to replace muscle, more research is required to
gauge the response of cells and tissue to the actuators and to evaluate the impact of
biological tissues on the performance of these actuators.

As our knowledge in NMJ, MEJ and actuators mature, effort must be eventually channelled
towards integrating the exciting accomplishments in these fields into constructing the NPI.
Some preliminary studies involving similar constructs to the NPI have been conducted by
others (8-11). However these tend to lack fine tuning and optimization as the parameters for
both the neurotisation as well as the setup of the muscle electrode junction lacked scientific
underpinning and more studies are needed to investigate the parameters that impact on
different components of the NPI. Such a task will require collaboration between experts of
each individual field. More importantly the design of the NPI and its actuator system must
involve clinicians and surgeons who are involved in the day-to-day care and treatment of
amputees. This ensures that the design of NPI remains clinically relevant and plausible.
Another area that will need to be developed for the NPI actuator system is a viable sensory
feedback loop. In normal muscle, muscle spindles and joint proprioceptors constantly relay
signals back to the CNS leading to changes in muscle tone and power. Similarly, the NPI –
actuator system will also require a viable sensory feedback mechanism that is able to ensure
the activity of the actuator is appropriate and regulated.
1.7 Structure of the thesis

Construction of the NPI involves establishing a NMJ between the muscle and the nerve and also creating a MEJ by implantation of the electrodes. The NPI will also be connected to an actuator to restore limb function. Chapter two to 4 will be concentrated on the NMJ. Chapter two will explore the effect of AChR clustering agents agrin and laminin on the number of NMJ formed between muscle and neural cells. Chapter three examines a range of molecular markers that may be used as an indication of innervation and whether agrin and laminin treatment increases the expression of the markers. Chapter 4 seeks to answer the question of whether the increased NMJ show in chapter two in cells treated with agrin and laminin are also functional. It explores means of gauging functional NMJ activity in cultures treated with agrin and laminin. Chapter 5 will demonstrate some of the preliminary work conducted on optimizing the muscle electrode junction (MEJ). It will display some work on developing an in vitro model of NPI as a platform for MEJ optimization. It will also showcase results that show the effect of electrode coating with the conductive polymer polypyrrole on the signal recording. Chapter 5 will also show results from biocompatibility studies on novel polymer actuators that may one day become an alternative to electric powered prosthetic limbs. Finally the future directions for development of NPI will be discussed.
Chapter 2

2. The effect of agrin and laminin pre-treatment on the number of neuromuscular junctions formed in nerve muscle co-cultures

2.1 Introduction

The first step in establishing a NPI is to neurotise the remaining muscles on the limb stump that are de-functioned by the limb amputation in the process known as targeted muscle re-innervation. This increases the number of neuromuscular units leading to more signal inputs for guiding the prosthetic limb. Neuromuscular junction formation (NMJ) is the key step in establishing new nerve-muscle junctions. An *in vitro* neural-muscle co-culture system is an important experimental tool that allows NMJ formation to be studied in a controlled and reproducible environment. Other authors have used neural-muscle co-culture systems have been developed to explore a range of NMJ topics (206, 207, 219-221). Most of these models consist of dissociated motorneurons; stem cell derived neural cells or spinal cord explants co-cultured with muscle cells. The source of the neural cells is invariably from embryonic rodents while the muscle cells originate from both embryonic as well as neonatal sources (206, 219, 220). However the main downside of using primary neural cells is the lack of control over the cell populations. Spinal cord explants and dissociated neural cells are heterogeneous populations of glial, fibroblasts, sensory and motor neurons (as found by colleagues at our laboratory). The proportion of each cell type varies with the batch harvest and is difficult to control for with standard cell culture techniques. This precludes scientific study of NMJs introducing unwanted variability to the final experimental result. These issues can resolved by the use of neural cell lines which show reproducible behaviour with passaging and are able to exist as a homogenous cell population. Other authors have generated cell-line based neural –muscle co-cultures consisting of PC12 neural
cells and L6 myoblast cell lines (292). However the synaptogenesis in these models have not been thoroughly characterized underscoring the need to further optimization.

Aneural AChR clusters are present during development on the muscle membrane before the neurites arrive at the muscle (20). They are believed to convey positional cues to the neural processes priming the muscle for innervation. However it is unclear whether increasing the number of AChR cluster number and size above a level that is physiologically present is able to further enhance synaptogenesis (19, 30, 36, 37, 293). This topic has major implications in use as a priming agent to expedite neurotising muscle during NPI construction. However in order to study the hypothesis a reliable means of manipulating both AChR cluster size and number must first be explored. Innervation is a well-known factor that affects AChR cluster size and number. *In vivo* studies show innervation increasing the size of AChR cluster with neural contact while diminishing those at extra-junctional site (33). However this phenomenon has been seldom demonstrated in *in vitro* nerve muscle co-cultures. Furthermore the exact mechanism underlying the AChR cluster consolidation needs to be further clarified. It is unclear whether there are also paracrine factors released by neural cells that cluster AChR and can subsequently be isolated and used for manipulating AChR cluster morphology in future studies.

Agrin and laminin are two agents that are known to cluster AChRs. Agrin is a nerve derived proteoglycan that clusters AChR through a MuSK receptor based mechanism (18). Laminin-1 is an ECM that is present mainly on developing muscle and on some adult epithelial membranes (102-104). It also clusters AChR through an agrin and MuSK independent fashion (107, 108). It unclear whether pre-treating muscle cells with a combination of agrin and
laminin to boost the number of aneural AChR clusters can further sensitize muscle to innervation.

This chapter will first outline the steps taken to develop and optimize the neural PC12-muscle co-culture system. It will then detail the steps taken to characterize the effect of innervation on AChR cluster morphology in the neural muscle co-culture with specific attention focused at identifying possible agents underlying the AChR clustering. Finally, the effect of increasing both the number and size of aneural AChR clusters using agrin and laminin on muscle innervation will be presented.

2.2 Aims

1. To develop a PC12 neural–primary myotube co-culture system for NMJ research.

2. To define the effect of innervation on AChR cluster morphology in an in vitro neural-muscle co-culture system.

3. To investigate the effect of increasing both aneural AChR cluster size and number on the level of NMJ formation in vitro.
2.3 Methods

2.3.1 Primary myoblast culture

Myoblast cultures were prepared from skeletal muscle removed from the hindlimbs of 3-4 week old C57BL/6 mice as described by (Todaro et al., 2007). Myoblasts were maintained in growth media containing Hams F10 (Gibco), 20% Fetal Bovine Serum (Gibco), 2.5ng/mL, recombinant human bFGF (Peprotech), 2mM L-glutamine (Gibco), 100U/mL penicillin and 100μg/mL of streptomycin (Gibco) at 37°C under 5% CO2 and passaged at 80% confluence using TrypLE (Gibco). Myoblasts from passage 6 to 7 were used for all experiments described in this study.

2.3.2 Myotube differentiation

(As adapted from (1)) Clear 96 well flat bottom polystyrene plates (Nunclon delta microplates) were used for initial characterization of morphology of PC12-muscle co-cultures. 12 mm diameter 1.5 circular cover glass (Menzel Glasser) were used in experiments that required measuring of the dimensions of AChR cluster morphology in PC12-neural co-cultures, muscle-only cultures and muscle cultures treated with conditioned media. Glass bottomed, 96 well plates (In vitro Scientific) were used for optimization of PC12-neural co-cultures and for all experiments that involve use of agrin and laminin. The tissue culture surfaces were coated with type 1 rat tail collagen (BD Bioscience), laminin-1 (L2020 Sigma) or collagen 1 followed by laminin-1. For type 1 collagen coating, wells were incubated with collagen type 1 at 2μg/cm² in 2mM HCl for two hours at room temperature followed by two
5 minute washes with phosphate buffered saline (PBS, Life Technologies) before use. For laminin coating, the wells were incubated with laminin-1 at 2µg/cm² in sterile PBS for two hours at 37°C followed by two brief washed with PBS before use. For initial characterization of PC12-muscle co-cultures and for measurement of AChR clusters in PC12-muscle co-culture experiments, myoblasts were seeded at a density of 7.4 X 10⁴ cells/cm². For experiments using agrin and laminin, myoblasts were seeded at a density of 2.5 X 10⁴ cells/cm². The seeded myoblasts were suspended in myoblast growth media and left to adhere at 37°C under 5% CO₂ for 24 hours after which the media was changed to myoblast differentiation media (DM) (containing Dulbecco’s Modified Eagle Medium (DMEM, Lonza), 2% horse serum (Gibco), 100U/mL penicillin and 100µg/mL streptomycin). A full media change was conducted every 24 hours and the cells were exposed to myoblast differentiation media for a total of three days. For experiments using agrin and laminin, rat agrin (R&D Systems) was included in the media at 10ng/mL. In laminin treated groups, a dose of laminin-1 (Sigma) at 100nM was added to the myoblast differentiation media after 48 hours of myotube differentiation. For agrin and laminin co-treated groups, laminin was added to the agrin containing differentiation media after 48 hours of treatment with agrin containing myoblast differentiation media. The differentiating cultures were then left for a further 24 hours. For experiments that evaluated the effect of agrin and laminin on AChR clustering, the treated myotubes were fixed after three days of myotube differentiation.

2.3.3 Myotube and PC12 Co-cultures

(As adapted from (1)) PC12 cells were passaged in proliferation media consisting of DMEM (Lonza), 10% horse serum, 5% FBS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml of
streptomycin. Cultures were maintained for three days at 37°C and 5% CO₂ until 70% confluence was reached at which point the cells were harvested for co-culture. PC12 cultures were pelleted by centrifugation and resuspended in PC12 differentiation media (DM) (DMEM, 1% horse serum, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 50ng/ml mouse NGF-7S (Sigma)). The media from the adherent myotube cultures after three days of differentiation (described above) was discarded and PC12 cells in PC12 differentiation media were seeded onto differentiated myotubes with or without agrin and laminin pre-treatments, as described above. A PC12 seeding density of 8600 cells/cm² was used for experiments that involve initial characterization of morphology of PC12-muscle co-cultures and measuring of the dimensions of AChR cluster morphology in PC12-neural co-cultures, muscle-only cultures and muscle cultures treated with conditioned media. A PC12 density of 12,500 cells/cm² was used for optimization of PC12-muscle co-cultures and all subsequent experiments that used agrin and laminin. The co-cultures were then incubated at 37°C and 5% CO₂ for three, four or five days with daily half media changes. At day three, four or five all cultures were fixed and stained.

2.3.4 Conditioned media (CM) experiment

Co-cultures and PC12 cultures were prepared on collagen coated flasks. The cultures were incubated in PC12 DM at 400μl/cm² of flask area. The media was not changed after beginning of culture and collected on day three, centrifuged at 3000rpm for 10 minutes
and sterile filtered through a 0.22µm syringe driven sterile filter (Millipore) to remove dead cells and debris. The conditioned media was stored in aliquots at -80 degrees. 96 well glass bottom plates were coated with collagen as above. Myoblasts were seeded into the wells and differentiated for three days as described above. Upon differentiation the myotubes were changed to either co-culture CM, PC12 CM or control media PC12 DM. All conditioned media were diluted 1:1 with base media (PC12 DM). The media was completely changed every day for three days.

2.3.5 Immunocytochemical analysis

(As adapted from (1)) For visualization of AChR, cells were incubated with Alexa Fluor 488 conjugated bungarotoxin (Molecular Probes) dissolved in cell culture media (1:1000) for one hour at 37°C and 5% CO₂ before fixation. All cells were fixed with ice cold methanol/acetone (50:50) for 10 minutes and washed with PBS. Cells were then incubated with 0.5% Triton X in PBS for 5 minutes and blocked with 10% donkey serum (Millipore) in PBS. The cultures were then incubated in 10% donkey serum containing the appropriate primary antibody combinations (mouse anti-Desmin IgG1 (Novocastra Laboratories) diluted 1:200, mouse anti-synaptophysin SP17 (Covance) diluted 1:1000 and rabbit anti β III tubulin (Covance) diluted 1:1000. The cells were then briefly washed in PBS before incubation with the appropriate secondary antibodies diluted in 10% donkey serum (Alexa Fluor 594 conjugated donkey anti-mouse IgG (Molecular Probes) diluted 1:200, Alexa Fluor 488 conjugated donkey anti rabbit IgG (Molecular Probes) diluted 1:2000 and Alexa Fluor 594 conjugated goat anti-mouse IgM diluted 1:2000). For negative controls, the primary antibodies were omitted from the secondary incubation step and a negative control for α bungarotoxin staining was also included by the omission of labelled α bungarotoxin from the primary incubation step. For visualization of cell nuclei, all cultures were incubated with DAPI (Sigma)
(2µg/mL in PBS) for 5 minutes. The immuno-labelled cells were then stored in PBS in the dark for subsequent analysis.

2.3.6 Scanning Electron Microscopy

Myotube cultures grown on collagen coated 15mm diameter Nunc™ Thermanox™ Coverslips were pretreated with and without agrin and laminin as described above and co-cultured with PC12 cells for 5 days. The coverslips were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 48 hours. The samples were further washed in PBS followed by distilled water and dehydrated in increasing concentrations of ethanol consisting of 50, 70, 90 and 100% ethanol for 60 minutes each step. The samples were then dried in a Balzers CPD030 critical point dryer (Balzers, Liechtenstein, Germany) and mounted onto 25mm aluminium stubs with double-sided carbon tabs. The coverslips were coated with gold using a Xenosput sputter coater (Dynavac, Wantirna South, Australia). The cells on coverslips were imaged with a Philips XL30 field-emission scanning electron microscope (Philips, Eindhoven, Netherlands) at a voltage of 2.0 kV and spot size of 2.

2.3.7 Microscopy and Image Analysis

(As adapted from (1)) Immunostained cultures were imaged with a Nikon Eclipse Ti confocal microscope for quantification and co-localization analyses. 5 random fields were selected from each well and the AChRs visualized by Alexa Fluor 488 labelled α bungarotoxin staining at 400x with an oil immersion lens (Plan Fluor, NA1.3). Z stacks of 3.88µm (12 bit) were generated for each view. For initial experiments that measured AChR cluster
dimensions in co-cultures without any agrin and laminin treatment, the gain, offset and laser power of the confocal experiment was individually adjusted for each set of experiment. Fiji software was used to count and assess area of ACh receptor clusters after manual thresholding. Given the high offset of the image during acquisition there was no background staining as confirmed by negative control. The post acquisitional threshold was set so the any pixel with one unit intensity was picked up. The particle analysis function on Fiji was used to calculate the number and area of clusters and any cluster with area <4squm was excluded. The areas of individual clusters on all five fields from each well were pooled together and the percentage of clusters greater than 50µm² was calculated as was the average number of clusters per field. For co-localization analysis of synaptophysin and αbungarotoxin staining the Co-localization Threshold tool in Fiji was used. The program determines the co-localization threshold on the stack according to the Coste’s method in which any pixel pair with intensities below the set threshold showed no correlation (Pearson coefficient <0) (49). The area of the clusters with synaptophysin co-localization was calculated with particle analysis. For experiments that involved agrin and laminin, the confocal images were automatically batch analysed using a custom script written in the Fiji distribution of ImageJ (Schindelin et al., 2012). The analysis performed a threshold (Renyi entropy) to extract the stains of interest. The total number of αbungarotoxin stained AChR clusters and the number of large clusters in the culture were counted. A standard cluster was classified as a staining area above three µm² while a large cluster was any staining above an area of 15µm². The results were expressed as the average number of discrete clusters found per microscopic field. For co-localization between synaptophysin and αbungarotoxin, areas of overlapping stains with area above one µm² were extracted using a binary reconstruction. The results were again expressed as the number of discrete co-localized clusters found for each microscopic field. For qualitative assessments, cultures were imaged with an Olympus IX70 wide field microscope using a LCPlanFI 20X lens with
numerical aperture (NA) of 0.4. The images were acquired with a gain of one and exposure of 5000ms using a Spot RT Slider digital camera and Spot Advanced software, version 4.8 (Diagnostic Instruments).
2.3.8 Data and Statistical Analysis

Three experiments, with six to eight replicates in each treatment group were performed for all assays in this study. Each replicate value in each treatment group was normalized to the mean of the control muscle treatment group within the same experiment. The normalized values for all three sets of experiments were combined within a single data set and represented in one graph. Each well was treated as an independent and separate result (n=1). Graphpad Prism (Version 5.0) was used to perform the Student’s paired and unpaired t-test and a one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test, to assess statistical significance between treatments. Statistical significance was set at $p < 0.05$. 
2.4 Results

2.4.1 Characterization of PC12-muscle co-culture
Figure 2-1 Immunocytochemical staining of (A, B) control neural PC12-myotube co-cultures, (C,D) control muscle-only cultures, (E,F) agrin and laminin treated co-cultures and (G,H) agrin and laminin treated muscle-only cultures at three days. PC12 cells stained for βIII tubulin (green, Alexa Fluor 488nm), myotubes stained for desmin (red, Alexa Fluor 568nm) and nuclei stained with DAPI (blue, 358nm). Note the PC12 neural processes that anchor onto myotubes in both control and agrin and laminin treated cultures at this time point.
Figure 2-2 Immunocytochemical staining of (A, B) control neural PC12-myotube co-cultures, (C,D) control muscle-only cultures, (E,F) agrin and laminin treated co-cultures and (G,H) agrin and laminin treated muscle-only cultures at 6 days. PC12 cells stained for βIII tubulin (green, Alexa Fluor 488nm), myotubes stained for desmin (red, Alexa Fluor 568nm) and nuclei stained with DAPI (blue, 358nm). Note the relative proliferation of PC12 cells at this time point. PC12 cells form columns lining and adhering to the side of myotubes.

PC12 neural cell lines were co-cultured with neonatal black 6 mice myotubes. By three days after co-culture PC12 cells extended process that anchored onto myotubes (Figure 2-1). The PC12 cells lined up next to the myotubes and adhered onto the side of the myotubes rather than on top of it (Figure 2-1). Spontaneous contractions were noted in the co-cultures at an earlier time point in co-cultures compared to muscle-only cultures. Groups of myotubes and attached PC12 cells contracted in singular neuromuscular units at the same frequency. By day 5 and 6, there were proportionately more PC12 cells compared to myotubes reflecting an uneven proliferation between the two cell types (Figure 2-2). The PC12 cells bunched together and formed rows of cells interspersed amongst columns of myotubes (Figure 2-2). The neural processes from PC12 formed varicose protrusions as they anchored onto myotubes (Figure 2-3). There was also marked increase in contraction in the co-cultures at this time point. In co-cultures with myotubes pre-treated with agrin and laminin, a similar sequence of events was observed. Like in control co-cultures, agrin and laminin treated co-cultures also showed PC12 process extension onto myotubes and progressive PC12 proliferation by day 6 after co-culture and heightened contractile activity.
Figure 2-3  Scanning electron micrographs of 5 day (A,C) control neural PC12-myotube co-cultures and (E, G) co-cultures pretreated with agrin and laminin and (B, D and F, H) magnified regions of neuromuscular contact. Note the varicose protrusions from the neurites of PC12 cells anchoring onto myotubes (arrow). (As adapted from (1)) Asterix indicates PC12 neural cells, arrow head indicates myotubes.

2.4.2 Optimization of PC12-myotube co-cultures

When PC12 and mice primary myoblasts were seeded at high densities, the resultant culture detached readily from the culture plate after 4-5 days of co-culture owing to the force of myotube contraction. As a result, cultures were produced with different cell densities on surfaces coated with different biological agents in order to find the conditions that allow for a durable culture that resists delamination while maintaining sufficient number of nerve-muscle contact for analysis. Myoblasts were seeded at either 1X10^4, 2X10^4, 3X10^4 or 5X10^4 cells/cm² on collagen, laminin or collagen and laminin coated surfaces and differentiated for three days. PC12 cells were seeded onto the myotubes at a ratio to myoblast of 1:2 and serial images of the cultures were taken at up to 5 days after beginning of co-culture. At low seeding densities such as 10^4 cells/ cm², laminin coated surfaces enhanced cell spreading and myotube differentiation with increasing number of myotubes compared to collagen coated surfaces (Figure 2-4). However at higher seeding densities of 5X10^4 cells/cm², myotubes differentiated on collagen based surfaces display increased apoptosis and more pronounced differentiation with increased myotubes arranged in a haphazard manner compared to laminin surfaces (Figure 2-4). Myotubes grown on collagen and laminin coated surfaces have phenotypes between those grown on collagen or laminin.
surface alone. They tend to have more myotubes compared to laminin coated surfaces but fewer apoptosis compared to the collagen surfaces (Figure 2-4). Similar trends were also noted after co-culture of myotubes with PC12 cells (Figure 2-5 to 2-9). Collagen surfaces promoted more PC12 cell proliferation compared to laminin surfaces. By day 4 and 5 after co-cultures (Figure 2-8 and 2-9) cultures on collagen containing surfaces showed significantly more PC12 and myotubes compared to laminin only surfaces. The differences in cell densities between groups with different initial seeding densities became less conspicuous at 5 days after co-culture compared to one day after co-culture (Figure 2-5). None of the cultures showed any sign of detachment at 5 days. There was progressive increase in PC12 cell proportions in the culture over time in all treatment groups. The results suggests that the range of cell seeding densities tested were adequate for culturing without detachment for up to 5 days after co-culture. Collagen containing surfaces seemed to promote increased myotube differentiation and proliferation of PC12 cells compared to laminin surfaces.
Figure 2-4 Bright field images of myotube cultures at day 0 just before PC12 seeding. The myotubes were differentiated on collagen, laminin or collagen and laminin coated surfaces at different cell densities.
Figure 2-5 Bright field images of 1 day PC12-myotube co-cultures with different myoblast and PC12 seeding densities and grown on collagen, laminin or collagen and laminin coated surfaces.
Figure 2-6 Bright field images of 2 day PC12-myotube co-cultures with different myoblast and PC12 seeding densities and grown on collagen, laminin or collagen and laminin coated surfaces.
Figure 2-7  Bright field images of three day PC12-myotube co-cultures with different myoblast and PC12 seeding densities and grown on collagen, laminin or collagen and laminin coated surfaces.
Figure 2-8 Bright field images of 4 day PC12-myotube co-cultures with different myoblast and PC12 seeding densities and grown on collagen, laminin or collagen and laminin coated surface.
Day 5 post co-culture

![Figure 2-9 Bright field images of 5 day PC12-myotube co-cultures with different myoblast and PC12 seeding densities and grown on collagen, laminin or collagen and laminin coated surfaces.](image)

<table>
<thead>
<tr>
<th>Myoblast Density</th>
<th>PC12 Density</th>
<th>Surface Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000/cm²</td>
<td>5000/cm²</td>
<td>Collagen</td>
</tr>
<tr>
<td>20000/cm²</td>
<td>10000/cm²</td>
<td>Laminin</td>
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<tr>
<td>30000/cm²</td>
<td>15000/cm²</td>
<td>Collagen + Laminin</td>
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<td>50000/cm²</td>
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Figure 2-10 Comparison of AChR clusters in innervated and myotube only cultures. A-
Column graph showing the number of large (>50µm²) per field in three, 4 and 5 day co-
cultures and myotube cultures of a similar age relative to the 6 day myotube culture. Note
that the 6 day myotube cultures were used to compare with three day co-cultures as the
myotubes in the 3 day co-culture had three days of differentiation prior to beginning of co-culture with PC12 cells. B- Number of clusters (>4µm²) per microscopic field relative to 6 day myotube culture. C- Percentage of AChR clusters in culture that are greater than 50µm² relative to 6 day myotube culture. Column and error bars represent mean +/- SEM. *- p<0.05, **- p<0.01, ***- p<0.001, unpaired student t-test.

2.4.3 Innervation and AChR morphology

Given that, innervation is known to increase the size of innervated AChR clusters and disperse aneural clusters, we investigated whether the same phenomenon also occurred in our PC12-mytube co-culture (33)(34). The AChR clusters in three, 4 and 5 day co-cultures and myotube only cultures were identified by fluorescent α bungarotoxin and their sizes were analysed by post-acquisition image analysis. The size of AChR clusters in muscle-only cultures and PC12-muscle co-cultures were compared (Figure 2-10). There was twice as many large AChR clusters (>50µm²) per microscopic field in 5 day co-cultures compared to myotube cultures (p<0.01) (Figure 2-10A). There was a trend towards increased large AChR clusters in three and 4 day co-cultures compared to their respective myotube controls. However these were not statistically significant. Note that 6, 7 and 8 day myotube cultures were used to compare with three, 4 and 5 day co-cultures respectively. This is due the fact that the myotubes in co-cultures had undergone three days of differentiation prior to co-culture with PC12 cells. Therefore the age of myotubes in the three day co-cultures was equivalent to the age of myotubes in 6 day muscle mono-cultures. There was an increase in the number of large AChR clusters in both co-cultures and myotube cultures from day three to day 4. The number of large clusters increased by approximately 70% (p<0.05) and 50% (p<0.05) from day three to day 4 in co-cultures and myotube cultures.
respectively. However the number of large clusters decreased by approximately 40% in myotube only cultures from day 4 to day 5 ($p<0.05$). This contrasts with plateauing of large cluster numbers from day 4 to day 5 in co-cultures. In order to further investigate whether the rise in large AChR clusters is due to a general rise in AChR clusters of all sizes or a specific increase in larger sized clusters, the total number of AChR clusters (>4 $\mu$m$^2$) was measured for both co-cultures and myotube cultures (Figure 2-10B). Co-cultures show a general increase in AChR cluster number per microscopic field compared to myotube only cultures across all time points ($p<0.05$ 3 days, $p<0.05$ 4 days, $p<0.001$ 5 days). Like in figure 2-10A, there was also an increase in total cluster number in co-cultures from day three to day 4 by approximately 40% ($p<0.05$). A similar but non-significant trend was also observed in myotube cultures during the same period. The number of AChR clusters decreased from day 4 to day 5 in myotube cultures by 30% ($p<0.05$). The number total number of clusters remained the same in co-cultures between day 4 and 5. These results suggest that the increase in large AChR clusters in co-cultures is due to a general increase AChR clusters. This was further supported by figure 2-10C which showed that there was no difference in the proportion of all clusters that was large (>50 $\mu$m$^2$) between co-cultures and myotube only cultures. However it is unclear from the data whether the increase in AChR cluster number is truly general across clusters of all sizes or is there a re- distribution of existing AChR by innervation such that innervated clusters are consolidated and enlarged at the expense of aneural clusters dispersing and shrinking. In both scenarios there may be an overall increase in AChR cluster number with the former resulting from raised levels of clusters of all sizes and the later due to increases in large as well as small clusters. These questions can be clarified by comparing the innervated as well as the non-innervated AChR clusters in the same co-culture.
2.4.4 Identification of NMJ with immunocytochemistry and image analysis

In order to compare innervated as well as aneural AChR clusters in the same co-culture, a means of identifying innervated AChR clusters must first be developed. PC12 myotube co-cultures were stained for pre-synaptic marker synaptophysin and post-synaptic AChR clusters. NMJs were identified by discrete areas of co-localization between the two stains using the “Co-localization threshold” plugin in the image analysis software FIJI (figure 2-11). The “Co-localization threshold” plugin calculates areas of co-localization based on Coste’s method. However as the co-cultures consist of multiple layers of cells, co-localization may be detected between cells that are not in direct contact but separated by other cell layers. As a result confocal microscopy was employed to take z slices of the cultures. Each z-slice would only contain cells in direct proximity to each other, thus minimizing any unintended overlap in stains between vertically distant cells. The analysis results show that not all AChR clusters on the myotubes have neural contact. The non-innervated AChR clusters may belong to non-innervated myotubes or aneural clusters on recently innervated myotubes in the process of dispersal due to innervation.
Figure 2-11 Identification of NMJ by immunocytochemistry. Confocal Z slices of 5 day myotube-PC12 co-culture are acquired by confocal microscopy. (A) Synaptophysin staining of presynaptic terminals. (B) Alpha bungarotoxin staining to identify AChR clusters on myotubes. (C) Merged image of A and B. synaptophysin (red) co-localize with AChR (green) to form discrete white areas of co-localization. (D) Image processing of colocalized image. The colocalized pixels are identified in black.
3day innervated vs aneural clusters

A

4day innervated vs aneural clusters

B

5day innervated vs aneural clusters

C
Figure 2-12: Frequency distribution of innervated and non-innervated AChR clusters in (A) 3 day, (B) 4 day and (C) 5 day co-cultures. Each graph represents pooled populations of AChR clusters from 21 wells across three sets of experiments. The size of each AChR cluster in each set of experiment was expressed relative to the average cluster size of aneural clusters in the same experimental set.

Figure 2-13 Innervation and AChR cluster redistribution. A- Column graph showing the percentage of innervated and non-innervated AChR clusters in 3, 4 and 5 day co-cultures that are above 50µm² relative to 3 day aneural clusters. Columns and error bars represent mean +/- SEM. B- Column graph comparing the percentage of aneural AChR clusters in co-cultures greater than 50µm² to the whole population of AChR clusters in muscle-only cultures. Values were normalized to three day co-culture group. All data from three sets of
experiments with 7 replicates for co-cultures and 8 replicates for myotube cultures in each set. Column and error bars represent mean +/- SEM. *- p<0.05, **- p<0.01, ***- p<0.001, unpaired student t-test.

### 2.4.5 Innervation and AChR redistribution

Having developed a method for identifying innervated clusters in co-cultures, the size of both innervated and non-innervated clusters in three, 4 and 5 day co-cultures were compared. Figure 2-12 shows the frequency distribution of innervated and aneural AChR cluster sizes in the same co-culture. Both innervated and aneural clusters display a skewed distribution with the great majority of clusters occupying the lower range of sizes. Compared to aneural AChR clusters, innervated clusters show greater distribution across large size ranges. This seems to be consistent in three, 4 and 5 day co-cultures. A logarithmic scale is used for the y-axis for ease of illustration. However this may underrepresent the true magnitude of frequency differences between the neural and aneural clusters at lower size ranges. The difference in size between innervated and aneural AChR clusters is further emphasized by the finding that a larger proportion of innervated clusters were large (above 50 µm²) compared to aneural clusters (Figure 2-13 A). There were 6 to 7 times as many large (>50 µm²) AChR clusters in innervated clusters compared to aneural ones (p<0.001). The proportion of large clusters increased from day three to day 5 in both innervated (p<0.05) and aneural clusters (p<0.01). The proportion of large clusters approximately doubled from day 3 to day 5 in aneural clusters while the same parameter in innervated clusters only recorded a 50% increase during the same period. In order to assess whether innervated clusters enlarge at the expense of aneural AChR clusters, the size of aneural AChR cluster in co-culture was compared to those in myotube only cultures. It was found that there was
approximately 50% fewer large clusters in the aneural clusters from co-cultures compared to the muscle-only cultures at day three (p<0.001) and day 4 (p<0.01) (Figure 2-13B). This indicates that aneural AChR clusters are dispersed by innervation. There was no statistical difference in the proportion of large AChR clusters between three, 4 and 5 day myotube cultures. However a clear trend can be observed whereby the portion of large clusters increased from day three, peaking at day 4 before decreasing at day 5.

2.4.6 Conditioned media and AChR cluster size

Innervation may affect AChR clustering by both physical contact or by secreting paracrine factors. In order to elucidate the nature of the AChR clustering mechanism, myotubes were incubated with conditioned media (CM) from either PC12-neural co-cultures or PC12 cultures. The morphology of the AChR clusters in the culture was compared to control myotubes grown in base media (PC12 differentiation media). Figure 2-14 shows a frequency distribution histogram of the relative AChR sizes in myotubes grown in control media as well as PC12CM and co-culture CM. The AChR clusters sizes in all three treatment groups displayed a skewed distribution with concentration at the lower cluster sizes. However myotubes grown in co-culture conditioned media show greater proportions of smaller AChR clusters compared to both the control as well as myotubes grown in PC12CM. Myotube grown in co-culture CM have approximately 55-60% fewer large AChR clusters (>50 µm²) (p<0.001) and AChR clusters in general (>4 µm²) (p<0.001) compared to the control group and myotubes grown in PC12CM (Figure 2-15A and B). The lower number of large AChR clusters was not due to a general decrease in AChR clusters of all sizes in myotubes grown in co-culture CM. In fact myotubes grown in co-culture CM had 20% and 30% lower proportion of large AChR clusters compared to control group (p<0.05) and
myotubes in PC12 CM group (p<0.001) respectively (Figure 15C). This indicates that the co-culture CM had an AChR dispersing effect on the myotubes.
Figure 2-14  Frequency distribution of AChR cluster sizes in myotubes differentiated for three
days in either control media which is PC12 DM, PC12 CM and co-culture CM. All data pooled
from three sets of experiments with 6 replicates for each treatment group per set. All cluster
sizes were expressed relative to the average AChR cluster size of the control group in the
same experimental set.
Figure 2-15 Conditioned media and AChR size. A- Number of large AChR clusters (>50µm²) per microscopic field in myotubes cultured in control media (PC12 DM), PC12 CM and coculture CM for three days. B- Number of AChR clusters (>4 µm²) per microscopic field in myotubes grown in PC12DM, PC12CM and co-culture CM. C- Percentage of all AChR clusters
in myotubes grown in control media, PC12CM and co-culture CM that is greater than 50 µm². Bars represent mean +/-SEM. *-p<0.05, **-p<0.01, ***- p<0.001, unpaired t-test.

1.4.7 Agrin and Laminin and AChR clustering

Given that CM of either PC12 or co-cultures failed to cluster AChR, the main mechanism underlying the receptor clustering effect of innervation is likely contact related. The NMJ is rich in various bioactive ECM molecules. Agrin and laminin are two ECM molecules that may mediate the contact induced AChR clustering by neurites. The effect of agrin and laminin on AChR clustering was assessed in combination with various bioactive coatings- collagen, laminin and combinations of collagen and laminin. The addition of agrin to the media significantly increased the number of receptor clusters (>3µm²) in all groups studied (p<0.001) (figure 2-16A), regardless of which cell adhesion molecule, or combination thereof, was used as a coating. A similar significant increase was seen in the number of large clusters (>15µm²) in the groups treated with soluble laminin (p<0.001), however in groups without soluble laminin treatment there was no significant increase in the number of large (>15µm²) clusters (figure 2-16B).

Most strikingly, the addition of soluble laminin to the cultures resulted in over 7 times the number of clusters (>3µm²) present per field (p<0.001) and the addition of agrin with soluble laminin at least doubled that effect to 18 times the number of clusters (p<0.001), compared to the average number of clusters per field on myotubes grown on collagen coated surfaces alone (figure 2-16A). This effect was even greater in the number of large clusters present per field with 16 fold increase in large clusters with the addition of soluble
laminin (p<0.001) and a tripling of this effect (55 fold increase over collagen coat control) (p<0.001) with the addition of agrin (figure 2-16B).

This demonstrates that both soluble laminin and agrin have significant effects on the frequency of cluster formation, particularly on the frequency of large (>15µm²) clusters. The magnitude of this effect is greatest when agrin and soluble laminin are both present in myotube cultures, in vitro. Unlike its effect on receptor cluster numbers, agrin alone did not significantly increase the number of large clusters (>15µm²) (figure 2-16B), however soluble laminin alone increased the number of large sized receptor clusters (p<0.001). Again, agrin acted synergistically with laminin to further increase the number of large sized AChR clusters (p<0.001) (figure 2-16B). The addition of laminin coating to standard collagen-coated wells reduced the number of AChR clusters. Myotubes on mixed collagen and laminin coated wells had 20% fewer AChR clusters compared to those on collagen coated wells (p<0.05) (Figure 2-16A). Myotubes on laminin coated wells and treated with agrin had 25% fewer AChR clusters compared to myotubes on collagen coated wells and treated with agrin (p<0.001). However laminin coating had no effect on the number of large AChR clusters on myotubes (Figure 2-16B).
Figure 2-16  Agrin and Laminin treatment and AChR cluster size and number. A- Total number of AChR clusters >3µm². B- large, clusters >15 µm². (As adapted from (1)) Culture conditions included combinations of: col- collagen coating, Ln coat- laminin coating, Ln- laminin in media, and Ag- agrin in media. Data represents mean ±SEM. For unpaired t-tests, *** p<0.001, ** p<0.01, * p<0.05, for one-way ANOVA with Bonferroni correction, ^ indicates p<0.001.
1.4.8 Agrin and Laminin and NMJ formation

Having established the AChR clustering effect of agrin and laminin, the implications of this AChR clustering on NMJ was investigated. Myotubes were treated with combinations of agrin and laminin and on various biological coatings and further co-cultured with PC12 cells. As described earlier, the co-cultures were stained with pre-synaptic (synaptophysin) and post-synaptic (α bungarotoxin) markers and analysed for NMJ formation by confocal microscopy and image analysis. A more efficient means of analysing the areas of co-localization between synaptophysin and α bungarotoxin was developed. The level of manual processing associated with these experiments precluded large scale analysis thus limiting the number of experimental groups that can be included in the experiment. As a result an automated AChR cluster analysis software was developed with collaboration from colleagues at Walter Eliza Hall institute that was able to analyse and identify co-localization of greater than one µm² efficiently (Figure 2-17A and B). From the schematic diagrams produced by the computer analysis, it is clear that not all AChR clusters are innervated in the culture and there remained many aneural AChR clusters in the co-culture.

Co-cultures on collagen coated surfaces, pre-treated with either soluble laminin or a combination of agrin and laminin, showed 88% (p<0.001) and 138% (p<0.001) more NMJ compared to untreated myotubes grown on collagen surfaces respectively (figure 2-17C ), however agrin pre-treatment alone did not have any effect on NMJ formation. Interestingly, co-cultures on laminin-coated wells showed significantly reduced NMJ formation. Myotubes grown on laminin coated wells developed 40% fewer NMJ compared to those grown on collagen or mixed collagen and laminin coated wells (p < 0.01). Despite reducing
the number of AChR clusters, the addition of laminin coating to collagen-coated wells did not reduce NMJ formation, suggesting that number of AChR clusters at the time of PC12 seeding is not the absolute determinant of NMJ formation and that other factors are also at play.
Figure 2-17 Agrin and laminin treatment and NMJ formation. (A) Confocal Z slice of co-stained of PC12-myotube co-culture pretreated with agrin and laminin at three days in culture. Stains are α bungarotoxin (green, Alexa Fluor 488nm) for post-synaptic AChR clusters, synaptophysin (red), Alexa Fluor 568nm) for pre-synaptic nerve terminal, and DAPI (blue, 358nm) for nuclei. (B) Same region as (A) analysed by FIJI software for co-localization of α bungarotoxin and synaptophysin, yellow indicates discrete areas of co-localization of α bungarotoxin and synaptophysin (arrow). Some AChR clusters were not innervated (arrow
head). C- Number of discrete, non-contiguous co-localizations between synaptophysin and α-bungarotoxin that was greater than one μm². (As adapted from (1)) Culture conditions included combinations of: col- collagen coating, Ln coat- laminin coating, Ln- laminin in media, and Ag- agrin in media. Data represents mean ±SEM. For unpaired t-tests, *** p<0.001, ** p<0.01, * p<0.05, for 1-way ANOVA with Bonferroni correction, ^ indicates p<0.001.

2.5 Discussion

2.5.1 Characterization and Optimization of PC12-myotube co-cultures

In the past, many authors have developed various in vitro neural muscle co-cultures that consisted of primary neural and muscle cells with varying levels of success (206, 207, 219-221, 294). However these models suffered from several major drawbacks. Firstly the primary neural cells used were heterogeneous populations consisting of neural as well as glial cells and the ratio of the two cell types varied from batch to batch. As a result, data generated by such models were confounded by differences in cell proportions. Secondly these primary co-cultures often required a cocktail of multiple neurotrophic and myogenic factors that have not been thoroughly studied in isolation (206, 221). Their use is partly underpinned by empirical evidence rather than a systematic understanding of their mechanism of action thus potentially and unwittingly affecting the final result. In the current study, a PC12-primary mice myotube co-culture was developed. PC12 cells were capable of extending processes to the myotubes and there was subjectively more contraction in the co-cultures compared to muscle-only cultures (observation only). PC12 cells have been widely used as a model for the study of neural cells (295). The use of PC12 cell lines obviates the problems
with cell type heterogeneity associated with primary neural cultures. Cell lines allow for study of experimental parameters under a controlled and reproducible environment that is more amenable to analysis. Compared to primary neural cultures, primary myoblast cultures from black 6 mice produced at our lab show high myogenic purity with minimal fibroblast content and reliable cellular behaviour. The presence of contractile activity in myotube containing cultures is not necessarily a sign of synaptic activity owing to the ability of myotubes to spontaneously contract in culture. However there was more contractile activity at an earlier time point in co-cultures compared to myotube only cultures that could only be attributed to the additional presence of PC12 neural cells in the culture. Similar co-cultures between PC12 and L6 myotubes have been attempted by other authors and have shown to develop cholinergic synapses (292). The heightened contractile activity observed in our current study likely stems from the cholinergic synaptic transmission between the PC12 and the muscle cells. A more detailed quantitation and evaluation of the contractile process will be presented in another chapter of this thesis.

The results from the PC12-myotube optimization experiment have identified cell seeding density and the type of culture substrate as two factors that impact on cellular behaviour. At lower seeding densities before introduction of PC12 cells, there were more myotubes on laminin coated surfaces. The myotubes grown on laminin surfaces also displayed different morphologies compared to those on collagen with spread out, stretched and aligned orientation compared to those grown on collagen surfaces. This is consistent with studies that show laminin promotes greater myoblast proliferation and differentiation than collagen (296-298). This is mediated by activation of integrin α 7 on myoblast by the E8 fragment of laminin (299, 300). However at later time points after co-culture with PC12 cells there seemed to be fewer myotubes in laminin coated surfaces compared to collagen surfaces.
This was likely related the progressive detachment of myotubes from the cell surfaces. As myoblast fuse to form myotubes, there is concomitant loss of stress fibres and reduced cellular adhesion (301). Myotubes grown on laminin coated surfaces have been shown to detach as they become progressively more contractile (296). When the cell seeding density was increased, collagen was found to promote greater myotube formation compared to laminin. This likely relates the effect of cell density of myotube on differentiation. As myoblast are more closely spaced, they contact each other more frequently and more readily signal through cell adhesion molecules such as cadherins (302-308). In fact inhibition of cadherin blocks myoblast fusion (306, 307). As a result, cultures with higher myoblast seeding densities are more “advanced” compared to those seeded with lower densities with more myotubes at a later stage of development. As a result there would more contractile myotubes detaching from laminin coated surfaces in high density groups compared to the lower density group at the same time point. Consequently the advantage of collagen over laminin coating became obvious at an earlier time point in high density cultures compared to the same cultures seeded at lower densities. Another interesting observation was that there seemed to be more apoptotic cells on collagen surfaces compared to laminin coated surfaces. Muscle regeneration is associated with cell apoptosis (309, 310). In cultures, approximately 30% of myoblast endure apoptosis with differentiation (304). The apoptotic cascade proceeds in tandem with myoblast differentiation and fusion and key apoptotic proteins such as caspase 3 and 9 have been found to play major roles in myoblast differentiation (310-313). The heightened apoptosis in the collagen group at high seeding densities likely results from the increased differentiation. However interestingly there was less apoptosis of myoblast cultured on mixed collagen and laminin coated surfaces despite no clear difference in the level of myotube differentiation. This supports recent evidence that postulates that myoblast apoptosis and differentiation are not necessarily linked and
are regulated independently (304). The laminin may have an anti-apoptotic effect that does not detract from the differentiating effects of collagen.

After co-culture with PC12 cells, the co-cultures became progressively ‘taken over’ by the PC12 cells. The PC12 cells showed disproportionately higher proliferation compared to the myotubes. Like in myotube cultures, there were more PC12 cells on collagen surfaces compared to laminin surfaces. The faster growth of PC12 cells compared to myotubes was likely due to the fact that the PC12 cells were seeded at a later time. Both cells were at different stages of development leading to differences in their growth potentials. Upon PC12 seeding, the myotubes were near confluence and consequently displayed low growth potential. Myoblast cultures consist of three main populations of cells - differentiating cells, proliferating cells and reserve cells (302, 314). During myoblast differentiation, some cells become committed down the myogenic lineage expressing myogenin, p21 and myosin and are irreversibly removed from the cell cycle (315). Other cells continue to proliferate undifferentiated. A small proportion of cells retain a “stem cell” phenotype continuing as undifferentiated cells that are quiescent but still retain the ability to proliferate and give birth to myoblast populations when placed in growth media (314, 316). As the confluence of the differentiating myoblast culture increases with time, the growth potential of the culture decreases while the level of differentiation increases (302-305). However when confluence is reached, both proliferation and differentiation is suppressed resulting in no further population growth (302). Compared to myotubes, PC12 cells were still in an earlier phase of development with greater growth capacity. Like in myoblasts, some PC12 cells continued to proliferate despite differentiation (295, 317). Therefore PC12 cells grew faster than the myotubes at the same time point owing to different seeding times. The presence of more PC12 cells on collagen coated surfaces was likely due to increased amount of extracellular
matrix produced by the more numerous myotubes on collagen coated wells. The confluent myotubes have effectively ‘coated’ the culture surface with cells. More importantly myotubes and myoblasts are known to secrete their own ECM consisting of collagen IV and laminin (318-321). Laminin promotes PC12 differentiation and growth through an integrin based mechanism (322-324). Upon seeding, the PC12 cells were essentially grown on a biologically active layer of myoblasts and laminin. The increased proliferation of PC12 cells observed on collagen surfaces was likely due to the higher number of myotubes present at the time of PC12 seeding. Overall the results of the optimization experiment suggest that a collagen containing coating should be used for PC12-myotube co-culture given the superior proliferation and differentiation. No detachment was noted in the cells seeded at various densities studied on collagen coated wells. Therefore, cells can be grown at a range of cellular densities with reasonable levels of neural muscular contact. Incorporation of laminin into the collagen coating further reduces apoptosis. However unlike collagen, laminin-1 is not a regular component of muscle basal lamina. Any myoblast culture on laminin-1 containing surfaces may not reliably simulate the physiological condition of muscle regeneration as muscles do not regularly contact laminin-1. Most of the studies in the remainder of this thesis will be conducted on collagen coated wells unless specified otherwise.

2.5.2 Characterization of AChR clustering in co-cultures and myotube only cultures

AChR clustering and redistribution by neurites is well defined in various in vivo animal models of synaptogenesis. However the phenomenon has seldom been demonstrated in in vitro nerve muscle co-cultures. Recapitulation of the physiological process in a nerve-muscle
The co-culture model is critical for ascertaining the authenticity and relevance of experimental data generated from such *in vitro* models. To assess the physiological relevance of the PC12-myotube co-culture system, the development of AChR cluster morphology over time was characterized. There was a greater number of large AChR clusters (>50μm²) in co-cultures compared to myotubes cultures of the same age. This was against the backdrop of an overall increase in total AChR clusters (>4μm²) in co-cultures. After factoring the overall increase in AChR clusters, the proportion of AChR clusters that was large was not significantly different between co-cultures and myotube cultures. The increase in large AChR clusters in co-culture was likely due to innervation of the myotubes by PC12 cells. Neurites secrete clustering agents such as the proteoglycan agrin clustering AChR at areas of nerve-muscle contact (18). Innervation also destabilizes and disperses AChR clusters without neural contact in the direct vicinity of the neurite (32-34). The dispersed AChR redistribute to the innervated AChR cluster, thus bolstering its size (32-34). This explains the rise in the number of large AChR clusters in co-cultures. The overall increase in AChR clusters in co-culture was likely due to an increase in both small and large AChR clusters. The large clusters are newly induced by neurites while the extra small clusters are remnants of larger aneural clusters in the process of dispersal by innervation. The number of large AChR clusters and overall number of clusters increased in both co-cultures and myotubes from day three to day 4. The number of large and overall AChR clusters remained the same in the co-cultures from day 4 to day 5 and decreased in myotube cultures over the same period. The decrease in AChR clusters in the myotubes only cultures coincided with the start of spontaneous contractions. Spontaneous contractions signify electrical activity in the myotube. Electrical activity is known to disperse AChR clusters that are not stabilized by innervation (33, 61). This explains the fall in AChR clusters in myotube only cultures. In contrast the large AChR clusters induced by PC12 cells are sufficiently stabilized by the neurites to withstand the dispersing
forces of electrical activity. This is consistent with the observation that the number of AChR clusters plateaued instead of decreasing form day 4 to 5 in co-cultures.

2.5.3 Innervation and AChR redistribution

The discussion thus far has focused predominantly on differences in the overall number and large AChR clusters between co-cultures and myotube cultures. However the results discussed fail to reveal the nature of the AChR redistribution on the innervated myotubes. Was the increase in AChR cluster uniform across all size ranges or was there a polarization of AChR sizes with both small and large AChR clusters increasing in number? In both scenarios, little change would be noted in the proportion of large AChR clusters as found in our previous experiment. To answer this question the innervated clusters in co-cultures must first be identified. The computer analysis of NMJs in PC12-myotube co-cultures demonstrated the presence of many non-innervated AChR clusters. These clusters may belong to uninnervated myotubes or extra-junctional AChRs on innervated myotubes. It can be concluded that not all myotubes in the PC12 myotube co-culture were innervated. The size distribution aneural AChR clusters assumed a skewed pattern with high numbers of small clusters. A similar but less skewed distribution was found in innervated AChR clusters. The innervated clusters had lower proportions of smaller clusters and increased ratios of larger ones. Innervation likely consolidates the innervated AChR clusters by contact factors such as agrin and by inducing myotube electrical activity (32, 33, 54). As shown in figure 2-18, it is postulated that given more time in culture the innervated cluster population will continue to enlarge as it matures under the effect of innervation assuming a more balanced distribution with the median that is larger than that of the aneural cluster population.
Figure 2-18 Schematic diagram of the development of frequency distribution of AChR clusters in co-cultures over time.

The aneural AChR clusters are also a heterogeneous population consisting of both AChR clusters on uninnervated myotubes and extra-junctional AChR clusters on innervated ones that are in the process of dispersal. It is impossible to segregate the two groups from the distribution.

When the innervated and aneural AChR clusters in the co-cultures were compared, it was noted that the innervated clusters generally had a higher proportion of large clusters. More importantly, the aneural AChR clusters from the co-cultures were also smaller than their counterparts in myotube only cultures. This indicates that the increase in AChR cluster noted was not uniform across all clusters sizes. The AChRs were redistributed by the innervation with innervated clusters enlarging and the aneural clusters diminishing in size to the point that they became smaller than standard AChR clusters in myotube only cultures. This phenomenon faithfully reproduces the changes in clusters sizes during re-innervation. When a denervated muscle is re-innervated, the nerve terminal increases both AChR redistribution and transcription (32-34). The redistribution is accomplished by neural derived agrin while the transcription upregulation is mediated by Neuregulin (57, 114, 118, 119, 121, 130, 325). The clustering effect is combined with the AChR dispersal effects of neuromuscular
transmission on uninnervated AChRs to increase innervated cluster size while shrinking aneural ones. Interestingly, the proportion of large clusters in the aneural AChR population in co-culture actually increased from day three to day 5 despite innervation induced receptor dispersal. This is likely a result of presence of many uninnervated myotubes with aneural AChR clusters that continue to mature and enlarge with myotube maturation and are unaffected by the innervation. This population of receptor clusters potentially diluted the effects of innervation of aneural AChR cluster size.

2.5.4 Paracrine factors and AChR redistribution

The results discussed thus far are still insufficient to elucidate the mechanism by which innervation redistributes AChR clusters. Neural cells are known to secrete a range of neurotrophic factors that affect AChR clustering (156, 165). It is possible that the observed AChR cluster changes are a result of paracrine factors secreted by the PC12 cells instead of contact signalling via neuromuscular junctions. To investigate this further, the myotubes were cultured in conditioned media for PC12 cells or co-cultures and the AChR cluster morphology was studied. AChR clusters in standard as well as PC12 and co-culture conditioned media all showed skewed distribution concentrated principally at smaller clusters sizes. More importantly myotubes in co-culture conditioned media had a higher proportion of smaller clusters compared the other groups. The co-culture conditioned media group also reported lower numbers of both large, small clusters and proportion of large clusters. It is interesting that myotubes grown in PC12 conditioned media behaved differently from those grown in co-culture conditioned media. It is likely that muscle contact transforms the PC12 cells prompting it to secrete factors that actively disperse AChR clusters. In fact neurotrophins such as BDNF and NT4 are known to disperse AChR clusters on muscle
through activation of TrKB receptors (165). Neurotrophins act principally to drive neurite extension and proliferation. However proliferation is often associated with de-differentiation in many cell types. It is therefore intuitive that neurotrophins would impede AChR clustering and NMJ formation. Neurotrophins likely play a yet under-researched role of dispersing aneural AChRs together with electrical activity during innervation. The result suggests that much of the AChR clustering effect of innervation stems from its contact effect on the muscle.

2.5.5 Agrin and laminin and AChR clustering

Given that most of the AChR clustering effect of innervation was mediated by contact, factors associated with neural contact were selected as a means to artificially enhance receptor clustering on myotubes. Both agrin and laminin were selected as the biological agents used to cluster AChR and prepattern muscle for innervation. Agrin is a proteoglycan secreted by neural cells that acts to cluster AChR (18). Laminin -1 is an ECM molecule that is present in the muscle basal laminin during embryonic development and also associated with AChR clustering (102-104, 106-108). Myotubes treated with agrin showed increased number of AChR clusters regardless of the type of cell culture substrate. Agrin clusters AChR through co-activation of MuSK receptor with LRP4 leading to actin polymerization and AChR redistribution without changing the total number of receptors on the cell membrane (27, 31, 57, 65, 68, 132). It also stabilizes existing AChR clusters by tethering it to the cellular cytoskeleton (61, 76, 88, 326). Interestingly, we showed that agrin failed to affect the number of large AChR clusters. This conflicts with reports by others that agrin increases large AChR clusters in myotubes (129). The reason likely relates to the time of incubation with agrin as the effect varies with time. Short periods of incubation with neural agrin can
increase the number of large AChR clusters while prolonged incubation of over 16 hours disperse the receptors (129). It is possible that during short term incubation agrin may have a polarizing effect on the distribution of AChR clusters. It may increase the size of large clusters and disperse small clusters. This increases both the total number of clusters and the number of large clusters without impacting total number of receptors on the muscle membrane. In our experiment the myotubes were treated with agrin for 72 hours and its effect on AChR size is likely to be different to previous published data on agrin. The reduction in large AChR clusters with prolonged agrin incubation also raises the possibility that other factors that are present in vivo are required to stabilize the clusters induced by agrin. In addition to agrin, the nerve terminals and the synaptic clefts are known to contain many other matrix bound factors that can stabilize AChR clusters (60, 96, 139-142).

In addition to agrin, our results also showed laminin suspended in cell culture media increased both the number and size of AChR clusters. Furthermore, laminin-1 acted synergistically with agrin to increase both number and size of AChR clusters. These findings are consistent with the background literature on laminin-1 and AChR clustering. Laminin-1 clusters AChR through an agrin and MuSK independent fashion that involves activation of α dystroglycans (107, 108). Laminin complements agrin by binding to integrin α7 β1 thus sensitizing myotubes to agrin by 20 folds (112). Interestingly, unlike suspended laminin-1, laminin-1 coating failed to affect AChR clustering. This may be due to differences in laminin-1 dose. The amount of laminin present on the coating is significantly fewer than the amount added to the media. In fact it has been shown that using special methods of coating that allow for immobilization of high levels of laminin to surfaces, myotubes develop large pretzel shaped AChR clusters (48). However this form of laminin induced AChR clustering is mediated by a MuSK based pathway instead of dystroglycan indicating that the pathway underpinning receptor clustering is sensitive to the manner that laminin is presented to the
myotubes (48). The amount of laminin in the coating required to cluster AChR may be different to the amount needed in the cell culture media due to intrinsic differences in signalling pathway. However the coating method used in this study limits the amount of laminin immobilized to levels that are significantly less that those needed to induce pretzel shaped clusters as reported elsewhere.

Collagen was found to be a better substrate than laminin in promoting AChR clustering. In fact myotubes grown on coating that contained laminin showed reduced AChR cluster number compared to those on plain collagen coating. One possible explanation is that myotubes on laminin coated surfaces readily detach as they spontaneously contract thus reducing the total number of myotubes and the number of AChR clusters present (296). However this argument fails to account for the inferior receptor clustering observed in mixed collagen and laminin coated wells. The collagen in the mixed coating should make up for the inferior adherent properties of laminin. The result indicates that the specific form of laminin coating used in this study impedes AChR clustering in myotubes.

2.5.6 Agrin and laminin and NMJ formation

The role of aneural AChR clusters in sensitizing muscle to innervation and guiding synaptogenesis is supported by several lines of evidence. Firstly during in utero-development in mice the earliest NMJ form within the central band on the muscle that houses previous aneural AChR clusters (19). In non-mammals such as the zebra fish nerves preferentially re-innervate aneural clusters during development (41, 293). Secondly, during synaptic pruning, as one axon retracts from their AChR cluster another competing axon will direct its branches to occupy the just vacated endplate (36). The vacated AChR cluster likely
conveys topographical cues to the competing axon, guiding it towards itself to form new synapses. Thirdly, denervated muscle grafts with intact end plates are more amenable to forming NMJ after implantation compared to grafts with the endplate zone excised (30, 37). This also translated into superior functional recovery after implantation, suggesting that the AChR clusters on endplate can potentially prime muscle for innervation by interacting with incoming neurites. These findings suggest that aneural AChR clusters prime muscle for innervation and guide the incoming neurite to the site of NMJ formation. A corollary of this theory is that increasing the number of aneural AChR clusters beyond which is physiologically present using agents such as agrin and laminin can further make the muscle more “attractive” to the neurite for synaptogenesis. The results of this study showed that myotubes pre-treated with laminin or a combination of agrin and laminin formed more synapses with PC12 cells compared to all other treatment groups. More importantly, agrin treatment alone failed to increase NMJ formation. This indicates that muscle sensitization may be related to both the size of the aneural AChR clusters and the mechanism by which it is formed. Laminin clusters AChR through an agrin independent process mediated by dystroglycans while agrin principally acts through MuSK receptors. Furthermore laminin and agrin/laminin combination were able to increase AChR cluster size while agrin was unable to change cluster size. It is likely that AChR clusters induced by dystroglycan are intrinsically different from MuSK derived clusters and that this is correlated with the priming of muscle for innervation. Interestingly agrin and laminin combination was able to enhance synaptogenesis while agrin alone had no effect. The laminin may have stabilized the aneural AChR clustered by agrin allowing the myotube to be more readily innervated by the incoming neurite. To date, there has been one study that investigated the use of agrin to prepattern muscle and improves NMJ formation. The study showed agrin treated myotubes suspended in fibrin gels developed more NMJ when peripheral the nerve was implanted next to the muscle (327). The difference in result likely stems from the experimental setup.
The muscle basement membrane is known to contain a multitude of biomolecules with AChR clustering potential (96, 139-142, 328). Therefore agrin treated myotubes in the fibrin gel may have been exposed to various AChR clustering agents after *in vivo* implantation with unpredictable effects on muscle innervation. Secondly, unlike the current study which used primary mice myoblasts, the previous study was performed using C2C12 cell lines which show key differences in biological activity compared to primary cells (205).

Due to limitations in experimental design, it was not possible to completely remove the agrin and laminin from the treated myotubes before introduction of PC12 cells to the culture. As a result there is the distinct possibility that the residual agrin and laminin may act on PC12 cells through unknown mechanisms to boost synaptogenesis instead of by increasing AChR clustering. Both agrin and laminin will likely bind to the integrin, MuSK and dystroglycan receptors present on the myotube surface after addition into the culture, precluding removal by simple washing with buffers (27, 31, 107, 108, 329, 330). Any chemical methods to removing the agents risk introducing unpredictable changes to cellular biology thus impacting on the final result. It is possible that AChR clustering is not the main mechanism by which agrin and laminin promotes synaptogenesis. One recent pertinent study by the Granato lab showed aneural AChR being dispensable to NMJ formation (133). In the study, the authors were able to selectively express one of the MuSK isoforms in a MuSK knockout zebrafish thereby induce NMJ formation without any aneural AChR prepatterning (133). However the study was performed on zebrafish which are known for key differences in developmental synaptogenesis compared to mammals (19). More importantly despite being a dispensable factor in NMJ formation, aneural AChR clusters can still be an accelerant of synaptogenesis. Aneural AChR clustering may well be an optional condition for NMJ formation; however increasing aneural AChR can still act to enhance the process as suggested in this study. Furthermore the AChR clusters induced in our study result from
different molecular pathways compared to the ones investigated in the studies conducted by the Granato lab. Theirs was focused on the WNT-MuSK pathway while the current study utilised a laminin/agrin pathway that is underpinned by an interaction between dystroglycan, integrin and MuSK (112, 113, 133, 329, 330). Therefore the aneural AChR receptors in the current study may be intrinsically different to their counterparts in the study by Jing et al. Despite background literature showing agrin enhancing neuron-neuron synaptogenesis, there is little evidence to suggest agrin and laminin improving NMJ formation through a primarily neural mechanism (331-335). In contrast, the link between aneural AChR and NMJ formation has been supported by many authors and it is likely to explain the elevated synaptogenesis observed in the agrin and laminin treated muscle cells in the current study (19, 36, 95, 97).

Results from this study may have clinical relevance, particularly in the field of tissue engineering and reconstruction involving the re-innervation of denervated muscle and in enhancement of muscle regeneration. Re-establishment of neuromuscular supply to denervated muscles is critical for restoring function after injury. Muscle sensitization with AChR clustering agents may provide a means for improving treatment outcome after denervation and ablative muscle trauma. However before the results can be translated in a meaningful way, the function of the synapses need to be corroborated and other evidence of synaptogenesis are needed such as maturity of ion channels and myotubes that are indicative of innervation. Functional studies of the NMJs will be explored in another chapter of this thesis.
2.6 Conclusion

PC12-myotube co-culture reliably recreates the physiological process of synaptogenesis.

Pre-treatment of myotubes with agrin and laminin promotes NMJ formation in vitro.
Chapter 3

3. The expression of synaptogenesis related transcripts in agrin and laminin treated muscle nerve-muscle co-cultures and muscle monocultures.

3.1 Introduction

Reliable molecular markers of innervation are important for the study of NMJ in vitro. There is a large degree of overlap in signalling molecules between aneural AChR clusters and innervated clusters precluding the use of a single molecular marker as a discrete measure of NMJ formation. This presents a challenge for an objective study of synaptogenesis in in vitro neural-muscle co-cultures. Most studies have resorted to demonstrating a co-existence of multiple maturation related markers within the same muscle cell as evidence of innervation (207, 221). However due to lack of standardization, each study uses a different range of factors making inter-study comparison difficult. In this chapter, the messenger ribonucleic acid (mRNA) expression level of several innervation related proteins were analysed in co-cultures and muscle-only cultures including AChR γ subunit, AChR ε subunit, voltage gated sodium channel NaV1.4 and NaV1.5 and myosin heavy chain 2X, myosin heavy chain embryonic and myosin heavy chain perinatal. The levels of these markers were also assessed in cultures treated with agrin and laminin.

Nicotinic AChR are present at the post-synaptic apparatus at the NMJ and act as receptor gated sodium channels. They are pentamers consisting of two α, 1 β, 1 δ and either one ε or one γ subunit (336, 337). Gamma containing AChRs are immature and generally present at high levels in muscle before innervation or after denervation (46, 169-172). ε containing
AChRs are mature and present at high levels in innervated muscles (46, 169-172). AChR ε and η subunits are encoded by the genes *Chrne* and *Chrng* respectively. Like nicotinic AChRs, voltage gated sodium channels also exist in a mature (NaV1.4) and an immature (NaV1.5) isoform (180, 338, 339). Both isoforms are present at the post-synaptic terminal of NMJ, converting endplate potential from the AChR into action potentials. NaV 1.5 is present at very low levels in innervated muscle and increases post denervation (90, 183, 187, 188, 190, 195, 198, 338) while NaV 1.4 is the main form of voltage gated sodium channel in innervated muscle (90, 183, 187, 188, 190, 195, 198, 338). NaV1.4 and NaV1.5 are encoded by genes *Scn4a* and *Scn5a* respectively. The relationship between innervation and AChR and voltage gated sodium channel levels have been elucidated mostly from in vivo studies in rats. However, the expression of these markers in an in vitro setting in neural-muscle co-cultures has not been thoroughly studied.

*Myh* 1, 3 and 8 are genes that encode mature (type 2X), embryonic and perinatal myosin heavy chain respectively (340). Myosins are the main active component of muscle that provides actuation to the contractile filaments. Multiple forms of myosins exist in muscle depending on the type and developmental stage of the muscle (214, 216, 217). Embryonic myosin is the predominant myosin in embryonic muscle before innervation. After arrival of neurites, the level of perinatal myosin increases (214, 216, 217) while the level of mature type 2X myosin increases after birth (214, 216, 217). Guo et al. have shown immunocytochemical staining of neural-muscle co-cultures with myosin specific antibodies (207). However this cannot be easily quantified. Performing western blot to assess protein expression will require a large amount of culture and reagents. As a result, ribonucleic acid (RNA) analysis was used as a surrogate marker of protein expression.
3.2 Aims

1. To identify a list of innervation specific markers that can be readily and reliably used as measuring tools in in vitro neural/muscle co-cultures for NMJ formation.

2. To assess the effect of agrin and laminin pre-treatment on the expression level of these innervation markers.

3.3 Method

3.3.1 Primary myoblast culture

Myoblast cultures were prepared from skeletal muscle removed from the hind-limbs of 3-4 week old wild type C57BL/6 mice as described by Todaro et al (341). Myoblasts were maintained in growth media containing Hams F10 (Gibco), 20% Fetal Bovine Serum (Gibco), 2.5ng/mL recombinant human bFGF (Peprotech), 2mM L-glutamine (Gibco), 100U/mL penicillin and 100μg/mL of streptomycin (Gibco) at 37°C under 5% CO₂ and passaged at 80% confluence using TrypLE (Gibco). Myoblasts from passage 6 were used for all experiments described in this study.
3.3.2 Myotube differentiation and Prepatterning

Glass bottom 6 well plates (In vitro Scientific) were coated with type 1 rat tail collagen (BD Bioscience), as follows. Wells were incubated with type 1 collagen at 2µg/cm² in 2mM HCl for two hours at room temperature followed by two 5 minute washes with phosphate buffered saline (PBS, Life Technologies) before use. Myoblasts were seeded into coated 6 well plates at a density of 2.5 X 10⁴ cells/cm² in myoblast growth media and left to adhere at 37°C under 5% CO₂. After 24 hours the media was changed to myoblast differentiation media (containing DMEM (Lonza), 2% horse serum (Gibco), 100U/mL penicillin and 100µg/mL streptomycin). Rat agrin (R&D Systems) was included in the media at 10ng/mL in the agrin treatment groups. A full media change was conducted every 24 hours for all groups in the study. After 48 hours of differentiation, a dose of laminin-1 (Sigma) at 100nM was added to the myoblast differentiation media in the laminin treatment groups. The differentiating cultures were then left for a further 24 hours. At this point, PC12 cells were added to the co-culture group (as described in section 3.3.3) while the control muscle monocultures were changed into PC12 differentiation media (DMEM, 1% horse serum, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 50ng/ml mouse NGF-7S (Sigma)).

3.3.3 Myotube and PC12 Co-cultures

PC12 cells were passaged in proliferation media consisting of DMEM (Lonza), 10% horse serum, 5% FBS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml of streptomycin. Cultures were maintained for three days at 37°C and 5% CO₂ until 70% confluence was
reached at which point the cells were harvested for co-culture. PC12 cultures were pelleted by centrifugation and resuspended in PC12 differentiation media. The media from the adherent myotube cultures (described above) was discarded and PC12 cells in PC12 differentiation media were seeded onto differentiated myotubes with or without agrin and laminin pre-treatments, as described above, at a PC12 density of 12,500 cells/cm². The co-culture was then incubated at 37°C and 5% CO₂ for three days with daily half media changes.

3.3.4 RNA extraction and preparation

At day three after growth into PC12 differentiation media, both the myotube cultures and the PC12 myotube co-cultures were harvested for RNA. mRNA extraction was performed using the Qiagen RNeasy plus mini kit (Qiagen, Germany) as per manufacturer’s instructions. A volume of 30ul of eluate containing RNA was produced at the end of the extraction process. The purity of the RNA was measured using the Ultraspec 2200 spectrophotometer (GE Healthcare Life sciences). The ratio of RNA to contaminant was estimated using the ratio of absorbance of the sample at 260nm and 280n (A260/ A280). Pure RNA was defined as having an A260/A280 ratio of 1.8-2.3 in 10nM Tris.CL, pH 7.5. The concentration of RNA was estimated by A260. The quality of the RNA was assessed using 1% agarose TAE gel electrophoresis with ethidium bromide staining. If the RNA was deemed adequate, it was further used to generate cDNA with Omniscript (Qiagen) as per manufacturer’s instructions. A negative control was prepared for each sample whereby the Omniscript enzyme was omitted from the reaction mixture. Any unused RNA was stored at -80 degrees centigrade. The presence of contaminating cDNA was assessed by performing a reverse transcriptase polymerase chain reaction (RT-PCR) of the positive and negative cDNA samples for expression of GAPDH and GDF-8. Taq polymerase (New England Biolabs) was
used to amplify the gene products, according to manufacturer instructions using GAPDH (fwd 5’ CCACCATGGAGAAGGCTGG 3’, rev 5’ CTCAGTGTAGCCGCCAGGATGC 3’) and GDF-8 (fwd 5’ GAGCGCCTCCACTCGGGAACTGAT 3’, rev 5’ CCTGGTCCGGAAAGGTACAGCAAGAT 3’) primers (Integrated DNA Technologies). The samples were deemed free of contaminating cDNA if bands were absent in the negative controls and bands present in the positive cDNA reactions. If contaminating cDNA was detected, the samples were further treated with DNase using Turbo DNA free (Ambion) and retested by PCR.

3.3.5 Duplex Real time PCR

Duplex real time PCR was performed on the samples using Taqman assays (Life Technologies) and Taqman gene expression master mix (Life Technologies) as per manufacturer’s instructions in a Light Cycler 480 (Roche). Taqman assays (6FAM™ and VIC®): Hprt (Mm01545399_m1), Chrne (Mm00437411_m1), Chrng (Mm00437419_m1), Chrnb1 (Mm00680412_m1), Scn4a (Mm00500103_m1), Scn5a (Mm01342518_m1), Myh1 (Mm01332489_m1), Myh3 (Mm01332463_m1) and Myh8 (Mm01329494_m1). 50°C for two minutes and 95°C at 10 minutes, the samples underwent 40 cycles of 95°C at 15 seconds and 60°C at one minute. Relative expression was determined using the ΔΔCt method with Hprt as the reference gene.

3.3.6 Statistical Analysis

One set of experiments with three replicate cultures for each treatment group was performed for Chrne, Chrng, Chrnb1, Myh1, Myh3 and Myh8 PCR experiments. Two
independent sets of experiments with three replicate cultures were performed for each treatment group for Scn4a and Scn5a PCR experiments. Each culture was treated as an independent and separate result (n=1). Where more than one set of experiments was performed, the value for each culture was normalized to the average of the control muscle group and pooled together with the results of duplicate experiments. Graphpad Prism (Version 5.0) was used to perform the Student’s paired and unpaired t-test to assess statistical significance between treatments. Statistical significance was set at $p < 0.05$.

3.4 Results

3.4.1 Chrne and Chrng expression

In muscle only cultures, laminin treated myotubes showed higher expression of Chrne compared to control myotubes ($p<0.01$) and agrin treated myotubes ($p<0.001$) (Figure 3-1A). This same phenomenon was not seen in the co-culture treatment groups (Figure 3-1A and B). Laminin treated myotubes also show significantly higher expression of Chrng compared to control myotubes ($p<0.01$) and agrin treated myotubes ($p<0.01$) (Figure 3-2A). Again, there was no difference in expression of Chrng between the co-culture groups (Figure 3-2A and B).
Figure 3-1 *Chrne* mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean±SEM. **p<0.01, ***p<0.001, student t-test.
Figure 3-2 *Chrng* mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean±SEM. ** p<0.01, student t-test.

### 3.4.2 Chrne and Chrng expression relative to Chrnb1

The comparison of *Chrne* and *Chrng* expression between co-cultures and muscle monocultures was confounded by the different makeup of their respective cell populations. Muscle monocultures are homogenous populations of myotubes while co-cultures contain PC12 cells in addition to the muscle cells. Both *Chrne* and *Chrng* are present exclusively in muscle cells while the housekeeping gene *Hprt* used in the experiment was present in both muscle cells and PC12 cells. As a result, the relative gene expression values in cocultures become diluted by the *Hprt* from PC12 cells, artificially lowering the final result. Unfortunately, a housekeeping gene that is specific to muscle cells could not be found, at the time of this work. This problem was resolved by expressing *Chrne* and *Chrng* relative to the total levels of AChR. AChR are pentamers that contain either ε or γ subunit, however every AChR contains one β1 subunit (336). Therefore, the level of mRNA encoding for the β1 subunit (*Chrnb1*) was used as a surrogate marker of total AChR levels and both *Chrne* and *Chrng* were expressed relative to *Chrnb1* expression. The level of *Chrnb1* expression in the different treatment groups was first established (Figure 3-3A and B). There was no statistically significant difference in *Chrnb1* expression amongst co-cultures and within muscle-only cultures (Figure 3-3A and B). Expression of *Chrne* relative to *Chrnb1* did not change the
general trend of *Chrne* expression. As when *Chrne* was expressed relative to *Hprt*, laminin treated muscles had a higher ratio of *Chrne* to *Chrnb1* compared to control muscle (p<0.01) and muscle treated with agrin (p<0.01) (Figure 3-4A). Co-cultures treated with agrin and laminin showed a marginal but significant increase in *Chrne* / *Chrnb1* ratio compared to agrin treated co-cultures (p<0.05) (Figure 3-4B). When comparing muscle monocultures to co-cultures, agrin treated co-cultures had a higher *Chrne* / *Chrnb1* ratios compared to agrin treated muscle-only cultures (p<0.05) (Figure 3-4C). There was otherwise no difference in the ratio of *Chrne* to *Chrnb1* between other co-cultures and their respective muscle counterparts (Figure 3-4C).

Expression of *Chrng* relative to *Chrnb1* was higher in laminin treated muscle when compared to control muscle (p<0.05) (Figure 3-5A). The *Chrng* / *Chrnb1* ratio was higher in laminin treated co-cultures compared to control co-cultures (p<0.05) (Figure 3-5B). There was otherwise no significant difference in *Chrng* to *Chrnb1* ratios between co-cultures and muscle and between different muscle groups or co-culture groups (Figure 3-5A-C).

Figure 3-3 *Chrnb1* mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of:
muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag) and laminin in media (Ln). Data represents mean ±SEM.

Figure 3-4 *Chrne* mRNA expression relative to *Chrnb1* in muscle (A), PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin and a comparison of muscle-only and co-culture expression levels (C). Culture conditions included combinations of muscle-only
cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no
additions (Ctrl), agrin in media (Ag), and laminin in media (Ln) Data represents mean±SEM. *
p<0.05, ** p<0.01, student t-test.

![Figure 3-5](image)

Figure 3-5 *Chrng* mRNA expression relative to *Chrn1* in muscle (A), PC12-muscle co-cultures
(B) and comparison of muscle-only and co-culture expression levels (C) in the presence or
absence of agrin and laminin. Culture conditions included combinations of: muscle-only

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cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM. * p<0.05, ** p<0.01, *** p<0.001, student t-test.

3.4.3 Scn4a and Scn5a expression

The expression level of voltage gated sodium channel Scn4a (mature form) and Scn5a (immature form) was analysed in the co-cultures and muscle cultures. However, the final data for Scn4a (Figure 3-6) and Scn5a (Figure 3-7) did not show any significant differences between treatment groups in the muscle cultures or the co-cultures. As with assessment of AChR subunit expression, the comparison of Scn4a and Scn5a is confounded by differences in composition of the cell populations (muscle only vs. PC12-muscle cocultures) and the non-specificity of the Hprt housekeeping gene. Furthermore, differences in expression level may be due variations in the overall channel levels without any meaningful change in preference towards mature or immature sodium channels. Given that Scn4a and Scn5a are unique to muscle cells and are the only two forms of voltage gated sodium channel on the sarcolemma, a ratio of the two subunits may better gauge the maturity of the channels in the culture (Figure 8A-C). Some difference was noted between laminin treated co-cultures and co-cultures treated with agrin and laminin (p<0.05) (Figure 3-8B). There was no difference in the ratio of Scn4a to Scn5a between co-cultures and muscle only cultures (Figure 3-8C).
Figure 3-6 Expression of *Scn4a* mRNA relative to control muscle group in (A) muscle cultures and (B) co-cultures. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM.

Figure 3-7 Expression of *Scn5a* mRNA relative to control muscle group in (A) muscle cultures and (B) co-cultures. Culture conditions included combinations of: muscle-only cultures
(Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM.

Figure 3-8 Ratio of SCN4A to SCN5A expression relative to the control muscle group in (A) muscle cultures, (B) co-cultures and (C) – comparison of Scn4a to Scn5a ratios between muscle and co-cultures. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM. ^ p<0.05 2-way ANOVA with paired comparisons.
3.4.4 Embryonic, Perinatal and Mature Myosin Heavy Chain expression

The expression of embryonic (*Myh3*), perinatal (*Myh8*) and mature 2X (*Myh1*) myosin heavy chain (MHC) gene transcript was analysed in muscle and co-cultures treated with and without agrin and laminin. *Myh3* expression was lower in agrin and laminin treated muscle cultures compared to control (p<0.001), agrin treated (p<0.001) and laminin treated muscles (p<0.001) (Figure 3-9A). Similarly, co-cultures treated with agrin and laminin show reduced *Myh3* compared to control co-cultures (p<0.001) and laminin treated co-cultures (p<0.05) (Figure 3-9B).

Agrin and laminin treatment also reduced *Myh8* expression in muscle cultures compared to control muscle cultures (p< 0.001) and agrin treated muscle cultures (p<0.01) (Figure 3-10A). Agrin treatment also reduced *Myh8* expression in muscle cells compared to untreated control muscle cells (p<0.01) (Figure 3-10A). In co-cultures, agrin and laminin treatment also reduced MYH8 expression compared to control co-cultures (p<0.001) (Figure 3-10B).

*Myh1* expression was lower in agrin and laminin treated muscle cultures compared to control (p<0.001), agrin treated (p<0.001) and laminin treated muscle cultures (p<0.001). Laminin treated muscle cultures also showed higher *Myh1* expression compared to control (p<0.05) and agrin treated muscle cultures (p<0.01) (Figure 3-11A). Co-cultures treated with agrin and laminin also showed lower *Myh1* expression compared to control (p<0.001), agrin treated (p<0.05) and laminin treated co-cultures (p<0.05) (Figure 3-11B).
Figure 3-9 Embryonic MHC (Myh3) mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM. * p<0.05, *** p<0.001, student t-test.
Figure 3-10 Perinatal MHC (Myh8) mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM. * p<0.05, ** p<0.01, *** p<0.001, student t-test.

Figure 3-11 Mature 2X MHC (Myh1) mRNA expression in muscle (A) and PC12-muscle co-cultures (B) in the presence or absence of agrin and laminin. Culture conditions included combinations of: muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean ±SEM. * p<0.05, ** p<0.01, *** p<0.001, student t-test.
As with the analysis of AChR and voltage gated sodium channel subunit expression, direct comparison of MHC between co-cultures and muscle monocultures is precluded by cell population heterogeneity as discussed previously. Again an attempt was made to normalize the expression of the Myh genes to genes that are only present in muscle. To achieve this, the adult MHC gene Myh1 was expressed relative to the total immature MHC genes (Myh3+8) (Figure 3-12). Agrin and laminin treatment increased the mature to immature MHC ratio in muscle monocultures relative to all other groups (control, p<0.05, agrin, p<0.05, laminin, p<0.05) (Figure 3-12A). Agrin also increased relative adult MHC expression in co-cultures relative to control (p<0.001), agrin (p<0.01) and laminin (p<0.001) groups (Figure 3-12B). Control and laminin treated co-cultures showed higher relative adult MHC expression compared to their respective muscle monoculture counterparts (control, p<0.001, laminin, p<0.05) (Figure 3-12C). There was a trend towards higher relative adult MHC in the co-cultures compared to muscle mono-cultures in agrin treated and agrin and laminin treated groups (Figure 3-12 C). However these differences were not statistically significant.
Figure 3-12 The ratio of $Myh1$ (adult MHC) to $Myh3$ and $Myh8$ (immature MHC) expression in (A) muscle cultures and (B) co-cultures. (C) Comparison of the $Myh1/(Myh3+Myh8)$ ratio between co-cultures and muscle monocultures. Culture conditions included combinations of:
muscle-only cultures (Mus), PC12 muscle co-cultures (Co), standard PC12 differentiation media with no additions (Ctrl), agrin in media (Ag), and laminin in media (Ln). Data represents mean±SEM. *- p<0.05, student t test.

3.5 Discussion

3.5.1 AChR $\varepsilon$ and $\gamma$

Laminin treated muscle only cultures showed higher levels of both Chrne and Chrng expression compared to agrin and control muscles respectively. Otherwise, addition of agrin or laminin did not significantly change Chrne or Chrng transcription in in co-cultures. Chrne and Chrng encode for $\varepsilon$ (mature) and $\gamma$ (immature) subunits of AChRs respectively (336, 337). During development, AChR $\gamma$ subunit is progressively replaced by $\varepsilon$ (42, 46, 169). AChR $\gamma$ subunit is also upregulated after denervation and reduced once the muscle is re-innervated (46, 169-172). However, no study to date has assessed the effect of laminin-1 in media on the switch from AChR $\gamma$ subunit to $\varepsilon$. Our study suggests that laminin increases both Chrne and Chrng transcription in muscle cells, of which the mechanism remains to be elucidated. Interestingly agrin alone did not increase Chrne transcription in muscle cultures. Others have shown that agrin promotes AChR $\varepsilon$ subunit transcription in muscle (176, 342). The difference likely stems from differences in agrin delivery and possible differences in experimental setting. Some authors have transfected muscle cells with agrin to provide a constant mode of delivery of agrin to muscle in vivo (176). The agrin release profile and bioavailability may differ to the current study whereby agrin was suspended in media. It is also possible that differences in agrin dosage may affect AChR $\varepsilon$ and AChR $\gamma$ transcription. Other authors have shown upregulation of AChR $\varepsilon$ transcription in muscle cells grown on
agrin coated surfaces (342). However it is known that like laminin, agrin immobilized to surfaces acts differently to when are suspended in media (48, 107, 108, 343). One such study showed that agrin coating increased AChR ε transcription, while unbound agrin in media had no effect on AChR ε transcription (344). Substrate binding of agrin may introduce conformational changes to the molecule thus altering the signalling pathway, similar to laminin(48). We also found that a combination of agrin and laminin treatment also did not change AChR γ or AChR ε expression in muscle cells. To the best of our knowledge, no study has examined the effect of agrin and laminin on AChR γ or AChR ε transcription or expression in vitro, in muscle only and co-cultures. Our results suggest that a combination of agrin and laminin does not increase either total AChR transcription or the transcription of individual AChR γ and AChR ε subunits.

3.5.2 AChR β1 subunit

Accurate comparison of Chrne and Chrng expression levels was confounded by the non-specific nature of the housekeeping gene Hprt. Hprt is present in both PC12 neural cells and muscles while the genes of interest (Chrne and Chrng) are restricted to muscle cells. Consequently, every unit of mRNA extracted from the co-cultures contain neural mRNA which does not contain any gene of interest compared to the same unit of mRNA from muscle-only cultures. This artificially lowers the expression of both Chrne and Chrng in co-cultures, per unit of mRNA or Hprt transcript. Furthermore, only a proportion of muscle cells are innervated by co-cultures as there are still muscle cells with no neural contact. In fact in our previous studies as detailed in chapter two, there were many acetylcholine clusters in
nerve-muscle cocultures without any neural contact or co-localization with pre-synaptic markers suggesting a population of non-innervated muscle cells in the culture. These muscle cells are incorporated into the mRNA measurements of co-cultures, and may dilute any real difference between co-cultures and muscle-only cultures. These factors combine to confound an accurate comparison of muscle monocultures and co-cultures. In order to overcome the problem, 

\textit{Chrne} and \textit{Chrng} were expressed relative to \textit{Chrnb1} gene. \textit{Chrnb1} encode the β1 subunit of AChR. It is one of five component of the AChR and is an ubiquitous subunit of all muscle nicotinic AChRs and present exclusively on muscle (336, 337). Assuming a positive relationship between protein expression and mRNA, \textit{Chrnb1} can be viewed as a surrogate marker for total AChR levels in muscle. Expressing \textit{Chrne} and \textit{Chrng} relative to \textit{Chrnb1} normalizes the gene against a surrogate marker of total AChR in the culture, thus overcoming the problems associated with differences in cell population composition between co-cultures and muscle monocultures. Furthermore, this also facilitates comparison between muscle only cultures or co-cultures. Observed differences in \textit{Chrne} or \textit{Chrng} transcripts relative to \textit{Hprt} may be due to differences in overall AChR number rather than a difference in \textit{Chrng} or \textit{Chrne} content. All \textit{Chrng} and \textit{Chrne} expression relative to AChRB1 will be referred to as relative \textit{Chrng} and \textit{Chrne} values respectively for the remainder of this section.

In order to normalize \textit{Chrne} and \textit{Chrng} against \textit{Chrnb1}, the expression profile of \textit{Chrnb1}mRNA was first elucidated. No statistically significant difference was found in the expression of \textit{Chrnb1} between cells treated with agrin or laminin and those without treatment. At first glance, these results seem to conflict with our previous data showing AChR clustering induced by agrin, laminin and combinations of agrin and laminin as detailed in chapter two. However, these discrepancies may be reconciled by two possible explanations. Firstly, agrin and laminin may cluster AChR by re-distributing existing AChRs on
the muscle membrane without changing the general expression level of AChR in the muscle. Therefore, the expression level of AChR subunits may not reflect observed changes in AChR clustering. Secondly, the PCR results allude to a mRNA level which is not necessarily proportional to protein translation and expression on the muscle membrane. We will explore these concepts further in the following paragraphs.

The level of AChR clustering on the cell is in a state of dynamic equilibrium between AChR insertion, lateral migration and removal (343, 345). AChR insertions further depend on the interplay between membranous and intracellular pools of AChRs (343, 345). The effect of agrin on AChR depends on its dose (61). At the doses used in the current study, agrin likely clusters and redistributes AChR predominately through lateral migration of AChR without increasing total AChR (57, 60). Studies that have assessed the effect agrin on AChR dynamics have produced disparate results owing to differences in experimental setup and agrin dose.

Muscles transfected with agrin at high efficiencies show elongation of AChR half-life implying that the rate of AChR removal is slowed by agrin (61). When the transfection efficiency was lowered, agrin failed to change AChR half-life (61). Similarly, incubation of muscle cells with agrin at doses that were 10 fold higher than the level used in this study enhanced AChR insertion (343). When the dose of soluble agrin was reduced to 10ng/ml, as in our study, there was no effect on total AChR levels (60). Furthermore, the relationship between AChR on the cell membrane and AChR transcription is confounded by post-translational regulation and AChR trafficking. The level of AChR transcription may not correspond to the level of protein translation (346). In addition, the process of AChR trafficking between intracellular and membranous pools is another level of regulation that may introduce variability between membrane localized AChR and transcription (345).

The effect of laminin on AChR transcription is not well defined with a paucity of work evaluating its effects on AChR dynamics. One study found that immobilized laminin-1
promoted AChR redistribution and insertion without affecting receptor removal (343). However, laminin immobilization changes its mechanism of effect (48). When suspended in the media, laminin clusters AChR through a MuSK independent pathway that involves dystroglycans (107, 108). However, when laminin is immobilized it clusters receptors by activating the agrin receptor MuSK (48). It is unclear what implications these mechanistic subtleties have on AChR insertion and removal. The current study suggests that suspended laminin, like suspended agrin, clusters AChR principally through receptor redistribution without modifying overall AChR levels.

3.5.3 Relative Chrne and Chrng

Our results show that there was no consistent statistically significant difference in relative Chrne or Chrng expression between co-cultures and muscle cultures in all treatment groups as evidenced by the Chrne and Chrng to Chrnb1 ratios. This indicates that the relative expression of Chrne and Chrng are not reliable indicators of innervation within our in vitro nerve-muscle coculture system. This may be due to the variable relationship between gene transcription and translation. Assessment of gene expression at the transcriptional level whilst informative, does not always reflect changes seen at the protein level. In terms of AChR dynamics there is little is known of the relationship between transcription and protein translation. Differences at the protein level may not necessarily be reflected on the transcript level. An interesting finding to note was that laminin increased both mature and immature AChR subunit relative expression. If the relationship between transcription and translation for AChR was constant, there should be a reciprocal relationship between relative Chrne and Chrng levels. Each AChR must contain either a $\varepsilon$ or $\gamma$ subunit and never
both, while the β1 subunit is a part of every receptor. The lack of a reciprocal relationship between relative \(Chrne\) and \(Chrng\) transcript indicates a discrepancy between transcription and translation. This has direct implications in the interpretation of real time PCR results in this scenario as changes in transcript levels may not necessarily reflect differences in protein expression which suggests that RNA analysis, as carried out in the current study, may be a poor predictor of AChR maturity. This is further supported by the findings by Bach et al. where myotubes grown in three dimensional fibrin gels expressed \(Chrne\) mRNA without showing any protein expression suggesting some level of post transcriptional regulation (178).

3.5.4 \textit{Scn4a} and \textit{Scn5a}

\textit{Scn4a} and \textit{Scn5a} encode for NaV1.4 and NaV1.5 voltage gated sodium channels respectively. Both are sodium channels present only on skeletal muscle. NaV1.4 is the mature form that is present in innervated muscle at peri-junctional regions while NaV1.5 is its immature counterpart that is expressed at low levels in innervated muscle and is upregulated in response to denervation (90, 179, 189, 198). The current study investigated the relative levels of \textit{Scn4a} and \textit{Scn5a} transcripts in co-cultures as well as in cultures treated with agrin and laminin. Our results show that there was no consistent difference in \textit{Scn4a} and \textit{Scn5a} transcript levels within co-cultures and muscle monocultures. Comparison between co-cultures and muscle monocultures suffer from the same problems of lack of cell specificity of housekeeping gene as discussed previously. This was resolved by expressing \textit{Scn4a} as a ratio of \textit{Scn5a}. Both genes are exclusively present on muscle and not on neural cells. Contrary to existing literature (174, 338), we found no significant difference in \textit{Scn4a} /\textit{Scn5a} ratio between co-cultures and muscle monocultures. The discrepancy between our results and the
literature may be explained by the fact that most studies demonstrate the causal relationship between innervation and sodium channel subtype in *in vivo* animal models (187, 188, 197, 339, 347, 348). *In vivo* animal models differ from *in vitro* cell culture in that the *in vivo* environment encompasses a broader range of biomolecules with disparate and ill-defined effects on the sodium channel expression. Secondly, many of the earlier studies on sodium channels measured electrical activity of the muscle in response to toxins such as tetrodotoxin that specifically block *Scn4a* (187, 188, 197, 347). Electrical activity of the muscle is a surrogate marker of protein expression at the cell membrane. Furthermore like in acetylcholine gated sodium channels, there is also discord between channel transcription and translation. In rat muscle, denervation decreased the total protein expression of *Scn4a* without changing its mRNA expression (188, 189, 198). This indicates that regulation of final protein expression likely rests at the translational and post-translational level. However little is known of the post-translational modification of muscle voltage gated sodium channels. Most of the research on post-translational regulation of these channels are mainly concentrated on brain voltage gated sodium channels which involve ubiquitination, phosphorylation and arginine methylation (349). A similar mechanism may be presumed to occur in muscle voltage gated sodium channels.

Thirdly as discussed previously, co-cultures are a heterogeneous population consisting of both innervated and non-innervated muscle. Differences between co-cultures and muscle-only cultures may be diluted by the presence of large numbers of uninnervated muscle cells in the co-cultures. Interestingly, neither agrin nor laminin or a combination of agrin and laminin showed any effect on *Scn4a* or *5a* expression. Agrin is known to cluster sodium channels *in vitro* (89). Some studies show agrin upregulating *Scn4a* mRNA (90). However, these studies involved transfection of muscle fibres with agrin plasmids thus allowing for a sustained release of agrin from the fibre (90). The concentration of agrin in such paradigms
will differ from the agrin concentrations present in the culture media in our study which may explain for differences in its effect on Scn4a transcripts. Our results suggest that assessment of Scn4a and 5a transcripts in nerve-muscle co-cultures may not be a viable means of measuring innervation in vitro.

3.5.5 Myh 1, 3 and 8

Innervation likely leads to maturation of the muscle contractile machinery which is evident by altered mature myosin heavy chain expression(350). The aim of the current analysis was to determine whether expression of transcripts for MHC 2x (Myh1), MHC embryonic (Myh3), MHC perinatal (Myh8) can be used as a reliable markers of innervation and to analyse the effects of agrin and laminin on the relative expression of embryonic, perinatal and adult isoforms of myosin heavy chain subunits (i.e. maturation). As with analysis of AChR components and voltage gated sodium channels, comparisons between co-cultures and muscle monocultures encountered the same issue with non-specificity of the Hprt housekeeping gene. To circumvent this issue, the level of adult Myh1 was expressed relative to the total level of immature MHC transcript which is taken as the sum of Myh3 and Myh8. Control and laminin treated co-cultures showed higher ratio of Myh1/(Myh3 + Myh8) than their respective muscle monocultures. There was a trend towards higher Myh1/(Myh3 + Myh8) ratios in co-cultures compared to muscle monocultures in the other treatment groups, in some cases this reached a significant level. The differences in Myh1/(Myh3 + Myh8) ratio between co-cultures and muscle monocultures are consistent with the traditional view that innervation promotes progressive maturation of muscle with replacement of immature embryonic MHC with more mature type II fast MHCs over time (350). Embryonic MHCs are first to develop in utero (351). The embryonic isoforms are then replaced successively by
neonatal isoforms and mature type II MHCs towards the end of in utero development and
during early post-partum period (340, 351). The time frame that these events take place
corresponds with the arrival of neurites and subsequent NMJ formation on the muscles
(351). Denervation similarly leads to loss of specialization of muscle MHC expression with
fast muscle expressing slower MHC while slow muscle upregulates faster MHCs (216, 218).
The results from the current study suggest cells in the process of transition from less mature
embryonic MHCs towards more mature type II MHC as a result of innervation by PC12 cells.
However, the exact mechanism by which innervation induces MHC maturation is unclear.
Some studies claim that muscles have an intrinsic capacity to develop various MHCs
independent of nerves and nerves preferentially innervate muscle fibres with more mature
forms of MHC (352). In fact, muscle from mice rendered aneural in utero still display the
same distribution and MHC expression profile as innervated muscle from age matched
controls (352). Rodent muscle cells are capable of expressing embryonic as well as more
mature Type II MHCs when cultured in the absence of neurons (353-357). Innervation likely
further fine tunes the muscle, upregulating MHCs and adjusting muscle function to match its
location on the body (357-359). This fine tuning process is mediated by induced muscle
activity as adjusting the duration and frequency of electrical stimulation of muscle was
sufficient to alter the expression profile of MHC in the muscle (357-359) . An interesting
finding in the current study was that agrin and laminin also enhanced the ratio of *Myh1 /
(Myh3 +8)* in muscle monocultures. This suggests that a combination of agrin and laminin
exert a direct maturing effect on muscle cells in addition to its effects on innervation. Agrin
is known to promote maturation of excitation/contraction coupling process in myotubes (92,
93). However, it is not known to directly upregulate mature isoforms of MHC (94, 97, 360).
Laminin 1 is known to accelerate myotube differentiation (296-300). However no study has
analysed its effects on MHC expression profile. Laminin 1 is present temporarily in the
basement membrane of muscle during in utero development and soon disappears after
birth (102-104). The expression time frame coincides with the changes in MHC subtype during pre-natal development suggestive of a possible role in muscle maturation. Whether there is synergy between agrin and laminin in promoting muscle differentiation is not known. Our results suggest that agrin and laminin are capable of interacting synergistically to expedite mature MHC expression. The mechanism may be similar to the synergistic effects of agrin and laminin on AChR clustering (107, 109, 343).

There are several limitations to our study. Firstly, only Myh1 was analysed when measuring mature myosin heavy chain expression (type II MHC). Myh1 encodes for type IIX MHC (213, 214). There are three different types of mature MHC (type II MHCs), type IIa, IIb, X/d. It is possible that innervation upregulates other forms of type II MHC and measuring the ratio of Myh1/(Myh3 + Myh8) ratio may not be sensitive enough to the maturing effects of innervation on muscle development. This may explain the lack of consistent statistical difference between the relative expression levels of mature MHC in co-cultures and their respective muscle monocultures in all the treatment groups.

Secondly, the sample size of the experiments limited the statistical significance of the results obtained through real time PCR analysis. A larger sample size is required in order to improve the statistical power of the results. However, due to limitations in time and resources, it was decided that the focus of the studies should be on functional endpoints of innervation rather than analysis of static markers of innervation such as mRNA transcripts. For a full discussion of functional studies of NMJ, please refer to the next chapter. Finally, the mRNA level was measured in this study. Due to the variable relationship between protein transcription and translation, the changes we have found on the mRNA level may not translate into differences on the protein level.
3.6 Conclusion

In this study, the use of muscle specific mRNA transcripts as a measure of innervation in cell cultures was confounded by differences in cell populations between co-cultures and muscle cultures and the lack of a housekeeping gene specific only to muscle. This issue was partially overcome by normalizing the expression of the gene of interest to other related molecules that are limited to muscle cells. Using this approach, it was determined that both AChR receptor subunits and voltage gated sodium channels were unreliable indicators of innervation in muscle cells.

Cocultures generally showed a higher relative Myh1 content compared to their muscle monoculture counterparts. This suggests that the presence of PC12 cells, or innervation by PC12 cells, may enhance relative Myh1 expression. This effect reached significance in control and laminin treated cultures. In addition, a heightened ratio of adult to immature MHC expression was observed in agrin and laminin treated muscle monocultures and cocultures, suggestive of enhanced maturity. This suggests that, agrin and laminin, acting in synergy or in parallel may exert a direct maturing effect on muscle differentiation, reflected in increased Myh1 expression, independent of innervation.

In summary the findings of this chapter indicate that myosin heavy chain subunit expression may be a reliable indicator of innervation in culture studies. In addition, agrin and laminin was found to increase expression of Myh1 relative to immature subunits to a significant level, corroborating the findings in chapter two that suggest synergistic treatment with both of these factors enhance NMJ formation.
Chapter 4

4. The effect of agrin and laminin treatment on functional innervation of myotubes in nerve-muscle co-cultures

4.1 Introduction

Agrin and laminin are components of the extracellular matrix that are known to cluster AChR on the muscle membrane (18, 54, 107). AChR clusters develop spontaneously on muscle membrane at extra-junctional regions after denervation and during development before innervation (19, 32, 33). It is believed that these receptors sensitize muscle to innervation. We have shown in our previous results that agrin and laminin increases the number and size of these aneural AChR clusters and increases the number of NMJ formed. However these conclusions were predicated on static measures of innervation that do not impart any functional information on neuromuscular transmission.

Functional assessment of NMJ formation is an essential component of NMJ research as it demonstrates the dynamic dimensions of synaptogenesis and is more directly linked to the clinical problem of restoring neuromuscular function to muscle after injury. Traditionally the gold standard measure of NMJ function is patch clamping (206). However the laborious nature of patch clamping only allows for the procedure to be performed on one cell at a time thus severely limiting the sample size and the power of the experimental findings. In our study we aim to use myotube contraction in neural-muscle co-cultures as a measure of innervation. Muscle contractility is a sign of maturity of the contractile filaments (205). Innervation of muscle likely leads to maturation of the contractile machinery of myotubes changing myotube contraction (361-363) However use of myotube contractility as innervation marker is confounded by the presence of spontaneous contraction in muscle-
only cultures (201, 204). In this study we aim to develop and refine the use of myotube contractility as an innervation marker by setting up the relevant muscle-only controls in the experimental design and by the use of acetylcholine antagonists to measure neuromuscular junction mediated activity in the culture. Having optimized myotube contractility as an innervation marker, we then seek to use it as a functional endpoint in assessing the effect of agrin and laminin pre-treatment on neuromuscular transmission in neural-muscle co-cultures.

4.2 Aims

1. To develop myotube contractility as a measure of innervation in neural-muscle co-cultures
2. To assess the effect of agrin and laminin pre-treatment on neuromuscular transmission in neural-muscle co-cultures.

4.3 Method

4.3.1 Primary myoblast cultures and PC12 myotube co-cultures

4.3.1.1 Obtaining myoblast cultures

Myoblast cultures were prepared from skeletal muscle removed from the hind-limbs of 3-4 week old C57BL/6 mice as described Todaro et al. (341). Myoblasts were maintained in growth media containing Hams F10 (Gibco), 20% Fetal Bovine Serum (Gibco), 2.5ng/mL recombinant human bFGF (Peprotech), 2mM L-glutamine (Gibco), 100U/mL penicillin and
100μg/mL of streptomycin (Gibco) at 37°C under 5% CO₂ and passaged at 80% confluence using TrypLE (Gibco). Myoblasts from passage 6 were used for all experiments described in this study.

4.3.1.2 Myotube differentiation and Prepatterning

Polystyrene 96 well plates (Nunclon Delta Surface) were coated with type 1 rat tail collagen (BD Bioscience). Wells were incubated with collagen type 1 at 2μg/cm² in 2mM HCl for two hours at room temperature followed by two 5 minute washes with phosphate buffered saline (PBS, Life Technologies) before use. Myoblasts were seeded into coated 96 well plates at a density of 2.5 X 10⁴ cells/cm² in myoblast growth media and left to adhere at 37°C under 5% CO₂. After 24 hours the media was changed to myoblast differentiation media (containing DMEM (Lonza), 2% horse serum (Gibco), 100U/mL penicillin and 100μg/mL streptomycin). Rat agrin (R&D Systems) was included in the media at 10ng/mL in the agrin treatment groups. A full media change was conducted every 24 hours for all groups in the study. After 48 hours of differentiation, a dose of laminin-1 (Sigma) at 100nM was added to the myoblast differentiation media in the laminin treatment. The differentiating cultures were then left for a further 24 hours. At this point, PC12 cells were added to the co-culture group while the control muscle monocultures were changed into PC12 differentiation media (DMEM, 1% horse serum, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 50ng/ml mouse NGF-7S (Sigma)).

4.3.1.3 Myotube and PC12 Co-cultures
PC12 cells were passaged in proliferation media consisting of DMEM (Lonza), 10% horse serum, 5% FBS, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml of streptomycin. Cultures were maintained for three days at 37°C and 5% CO₂ until 70% confluence was reached at which point the cells were harvested for co-culture. PC12 cultures were pelleted by centrifugation and resuspended in PC12 differentiation media. The media from the adherent myotube cultures (described above) was discarded and PC12 cells in PC12 differentiation media were seeded onto differentiated myotubes with or without agrin and laminin pre-treatments, as described above, at a PC12 density of 12,500 cells/cm². The co-culture was then incubated at 37°C and 5% CO₂ for three days with daily half media changes. At day one, three and 5 days after PC12 seeding, the cultures were analysed by live imaging. At day 5 the cells were fixed and stained for Immunocytochemical analysis.

4.3.2 Live imaging assessment of contractile activity

(As adapted from (1)) PC12-myotube co-cultures on collagen-coated 96 well plates were pre-treated with agrin and laminin as described above and co-cultured with PC12 neural cells for 5 days. On the first, third and fifth day of co-culture, the wells were examined live under an inverted microscope (Olympus X70) in an incubation chamber at 37°C and 5% CO₂. The wells were examined with a LCPlanFI 20X lens with NA of 0.4. Five fields from five non-overlapping regions of the well were selected. The position of each field was approximately the same in each well. Each field was observed for 30 seconds and noted for any contractile activity. The percentage of the 5 fields in each well that showed contractile activity was recorded and used as a gauge of total contractile activity of the well. For the remainder of this thesis, the level of contractile activity between treatment groups will be compared by measuring their respective percentage of microscopic fields with contraction after observing each field for 30s. Higher contractile activity is defined as having a higher proportion of contractile fields.
The contractile activity of each well was recorded before and after incubation with (+)-Tubocurarine chloride pentahydrate (at 12.5µM) (93750 Sigma). The wells were incubated with tubocurarine for a total of 15 minutes at 37°C and 5% CO₂. For experiments relating to tubocurarine optimization and investigation of the effect of drug vehicle injection on contraction, three replicate cultures were performed for each treatment group. All other experiments were performed three times with 6 replicates within each experimental group. Each well was treated individually as a separate n. For derivation of tubocurarine responsive contractions, the contractile activity after tubocurarine treatment for each well was subtracted from the level before tubocurarine treatment. For calculation of the level of NMJ mediated contraction, the average tubocurarine responsive contractile activity of control muscle group was subtracted from the activity level of each replicate in the control co-culture group while the average value for the agrin and laminin muscle group was subtracted from each replicate in the agrin and laminin co-culture group. All resulting values were expressed relative to the control co-culture group and values from three sets of experiments were combined.

4.3.3 Immunocytochemical analysis of muscle striation

(As adapted from (1)) All cells were fixed with ice cold methanol/acetone (50:50) for 10 minutes and washed with PBS. Cells were then incubated with 0.5% Triton X in PBS for 5 minutes and blocked with 10% donkey serum (Millipore) in PBS. The cultures were then incubated in 10% donkey serum containing the mouse anti-fast (type 2) skeletal myosin MY-32 (Sigma) antibody diluted 1:400, for two hours at 37°C. The cells were then briefly washed in PBS before incubation with the secondary antibody diluted in 10% donkey serum (Alexa Fluor 488 conjugated donkey anti rabbit IgG (Molecular Probes) diluted 1: 2000). For
negative controls, the primary antibodies were omitted from the secondary incubation step. For visualization of cell nuclei, all cultures were incubated with DAPI (Sigma) (2µg/mL in PBS) for 5 minutes. The immuno-labelled cells were then stored in PBS in the dark. For analysis, five wells were selected from five regions of each well in the same manner as when examining myotube contraction. Micrographs of the fluorescent stains were taken with an Olympus X70 microscope using a 20X LCPlanFl lens with NA of 0.4, 2000ms exposure and a gain of two. The number of striated myotubes and the total number of myotubes were counted using the Cell Counter plugin in Fiji software (364).

4.3.4 Data and Statistical Analysis

Three experiments, with 6 replicates in each treatment group were performed for all assays in this study. Each replicate value in each treatment group was normalized to the mean of the control muscle treatment group within the same experiment. The normalized values for all three sets of experiments were combined within a single data set and represented in one graph. Each well was treated as an independent and separate result (n=1). Graphpad Prism (Version 5.0) was used to perform the Student’s paired and unpaired t-test and a one-way ANOVA with Bonferroni’s post-hoc test, to assess statistical significance between treatments. Statistical significance was set at $p < 0.05$.

4.4 Results
4.4.1 Spontaneous muscle contraction as a measure of innervation

In order for muscle contraction to be an indicator of innervation there must be notable differences in muscle contraction between innervated and non-innervated muscle cells. To this end, muscle cells were differentiated for three days in myoblast differentiation media and then further cultured in PC12 differentiation media in the presence or absence of PC12 neural cells. The level of spontaneous muscle contraction was monitored over time. Spontaneous muscle contraction was noted in both muscle monocultures as well as PC12-muscle co-cultures. However marked differences in contractility developed between innervated and non-innervated muscles after 5 days in PC12 differentiation media (Figure 4-1). There was 1470% more contraction in innervated muscle cells compared to their non-innervated controls (p<0.001). A raised level of muscle contraction is indicative of maturation of the cellular contractile machinery. To explore this further, the same cultures were also analysed by immunocytochemistry for expression of type II fast myosin. Type II fast myosin is a mature form of myosin. It was found that co-cultures contained stained myotubes with distinctive striated appearances while minimal such cells were noted in muscle mono-cultures (Figure 4-2). The striation likely represents a developed form of myosin and actin that underlie the heightened contractility in the co-cultures. The number of striated muscle fibres was further quantified by image analysis. (Figure 4-3A). Co-cultures contained 1410% more striated myotubes compared to muscle mono cultures (p<0.001). Interestingly the proportional difference in contraction between the two treatment arms was very similar to the difference in striated myotube suggestive of a linear relationship between number of striated myotubes and observed contraction. The difference in both muscle striation and contraction was not due to differences in cell number between the two groups. In fact there were 30% more myotubes in muscle monocultures compared to neural-
muscle co-cultures (p<0.001) (Figure 4-3B). The coincidental increase in striation in co-cultures which also show greater spontaneous contractility further corroborates the use of myotube contraction as a measure of innervation.

Figure 4-1 Spontaneous muscle contraction in muscle and co-cultures. 5 day neural muscle co-cultures and their respective muscle monoculture controls were examined for spontaneous myotube contractions using a light microscope. Spontaneous contractile activity was assessed by selecting 5 non-contiguous microscopic fields and observing each field for 30s for presence or absence of contractile activity. The percentage of the 5 fields in each well that showed contractile activity was recorded and used to compare the total level of “contractile activity” amongst groups. Innervated muscle cultures show significantly more spontaneous contractions relative to muscle monocultures. Data represents mean ±SEM Legend- Muscle- muscle monoculture, Co-culture- PC12 neural-muscle co-cultures. *** p<0.001 for unpaired t-test.
Figure 4-2 Innervation and myotube striation. Neural muscle co-cultures (A-B) and muscle monocultures (C-D) were stained with antibody against type II fast myosin. Note the striated myotubes present in innervated muscle cultures (arrow head). Minimal striated myotubes were present in the muscle monocultures.
Figure 4-3 Striated myotube proportions in co-cultures and muscle cultures. The number of striated myotubes was quantitated in co-cultures and also in muscle cultures using immunocytochemistry and image analysis and expressed relative to muscle control. (A) There were significantly more striated myotubes in neural muscle co-cultures compared to muscle monocultures. (B) There were fewer myotubes in co-cultures compared to muscle-only cultures. Data represents mean ±SEM. Legend- Muscle- muscle monoculture, Co-culture- PC12 neural-muscle co-cultures. *** p< 0.001 for unpaired t-test.

4.4.2 Agrin and laminin pre-treatment and myotube contraction

Having established myotube contractility as a potential indicator of innervation, this measure was also used to examine the effect of agrin and laminin treatment on muscle and neural-muscle co-cultures. The level of spontaneous myotube contraction was monitored overtime at days one, three and 5 for muscle and co-cultures in the presence or absence of agrin and laminin (Figure 4-4). Spontaneous contractions in muscle-only cultures generally increased from day one to day three before peaking at day three and then subsiding by day 5 (Figure 4-4A). In comparison, activity in co-cultures continued to increase from day one to day 5 (Figure 4-4A). Activity in control muscle cultures increased by 86% from day one to day three before dropping to 15% of day one level by day 5 (p<0.001) (Figure 4-4B). In control co-cultures, muscle contraction increased from day one to day three increasing by 145% (p<0.01) before plateauing off at 254% of day one level. In agrin and laminin treated muscle cultures, contraction increased by 700% from day one to day three (p<0.01) (Figure 4-4B). Contraction then settled to 488% of day one level at day 5 (Figure 4-4B). In agrin and laminin co-cultures, contraction increased by 558% from day one to day three (p<0.001) and further
to 1563% of day one level by day 5 (p<0.001) (Figure 4-4B). The relative contractile activity of the different treatment groups also changed over time. At day one, no difference in contraction was noted between co-cultures and their respective muscle-only monoculture controls (Figure 4-5A). Agrin and laminin treated muscle monocultures and co-cultures had contractile activities that were 90% (p<0.001) and 78% (p<0.001) fewer than control muscle cultures and co-cultures respectively (Figure 4-5A). By day three differences began to develop between co-cultures and muscle cultures with control co-cultures contracting 91% more than control muscle monocultures (p<0.05) and agrin and laminin co-cultures contracting 165% more than agrin and laminin treated muscles (p<0.01) (Figure 4-5B). Agrin and laminin treated cells continued to contract less than their control counterparts with treated muscle monocultures and co-cultures contracting 60% (p<0.05) and 41% (p<0.05) less than control muscle and co-cultures respectively (Figure 4-5B). By day 5 the difference in contraction between muscle monocultures and co-cultures had further widened with control and agrin and laminin treated co-cultures contracting 2325% (p<0.001) and 987% (p<0.001) more than control muscle and agrin and laminin treated muscle monocultures respectively (Figure 4-5C). There were 34% more contraction in agrin and laminin treated co-cultures compared to control co-cultures (p<0.05) (Figure 4-5C). Unlike at day one and three, there was no difference in contraction between control and agrin and laminin treated muscle monocultures (Figure 4-5C).
Figure 4-4 Contractile activity of muscle-only and PC12-muscle co-cultures over time. A- The level of contractile activity of muscle and co-cultures in the presence of absence of agrin and laminin treatment were recorded at day one, three and 5. Contractile activity was assessed by selecting 5 non-contiguous microscopic fields for each well and observing each field for 30s for presence or absence of contractile activity. The percentage of the 5 fields in each well that showed contractile activity was recorded and used to compare the total level of
“contractile activity” amongst groups. A- Activity in muscle-only cultures peaks at day three before decreasing at day 5. Activity of co-cultures tends to increase over time without decreasing from day three to day 5. B- Comparison of contractile activity between different time points. Data represents mean ± SEM. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, Ctrl- control cultures without agrin and laminin. # p<0.01, ^ p<0.001 for paired t-test.
Figure 4-5 Relative contractile activity of muscle monocultures and PC12 neural-muscle co-cultures with or without agrin and laminin treatment at A- day one, B- day three and C- day five. Data represents mean ±SEM. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, Ctrl- control cultures without agrin and laminin. * p<0.05, ** p<0.01, *** p< 0.001 for unpaired t-test.

4.4.3 Contractility and acetylcholine

Given the presence of spontaneous contractile activity in muscle-only cultures, it is clear that myotube contraction is not specific to innervated rodent muscle cells. Therefore it is important to separate the non-innervated muscle related activity from the neuromuscular activity in neural muscle co-cultures. This problem was further explored with the use of acetylcholine receptor antagonist tubocurarine. Acetylcholine is a neurotransmitter restricted to neuromuscular synapses. The minimal dosage of tubocurarine that was sufficient to saturate the cell receptors in culture and deliver the maximal response was first ascertained. Different doses of tubocurarine were suspended in a small volume of vehicle culture media and added to agrin and laminin treated co-cultures and the level of contractile activity was recorded after incubation (Figure 4-6). Two doses of tubocurarine were tested, 12.5µM and 25 µM. Agrin and laminin co-cultures were selected as they were postulated to have the maximum level of contraction owing to increased NMJ formation as found by our previous work. Addition of 12.5µM and 25µM tubocurarine into the culture media reduced contractions of co-cultures by 95.7% (p<0.001) and 95.8% (p<0.001) respectively (Figure 4-6A). There was no significant difference between the co-cultures before and after treatment with either 12.5µM of tubocurarine or 25µM (Figure 4-6A). In order to rule out the
possibility of the vehicle media itself changing co-cultures contractions, the effect of injection of vehicle media alone on contractions was also tested (Figure 4-6B). Vehicle media did not change the level of contractile activity in co-cultures (Figure 4-6B). These results suggest that 12.5µM of tubocurarine was sufficient to saturate all the AChR in the culture and was equally efficacious as higher doses.

Figure 4-6 Tubocurarine and co-culture contraction. A) Tubocurarine titration. Agrin and laminin treated co-cultures were incubated either with 12.5 or 25 µM tubocurarine suspended in vehicle media and the percentage of microscopic fields in culture with contraction before and after incubation was recorded. B) Vehicle media and contraction.
The contraction of co-cultures before and after injection of vehicle media was recorded. Data represents mean ± SEM. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, ^ p< 0.001 for paired t-test.

4.4.4 Tubocurarine responsive activity

Having established the optimal dose of tubocurarine for contraction suppression, the level of contraction before and after tubocurarine was measured in muscle and co-cultures in the presence or absence of agrin and laminin pre-treatment. Before tubocurarine addition, co-cultures displayed more contractions than their corresponding muscle monoculture controls. Contractile activity of control co-cultures was 355% (p<0.001) greater than control muscles while activity in agrin and laminin treated co-cultures was 1194% (p<0.001) higher than agrin and laminin treated muscle monocultures (Figure 4-7A). Agrin and laminin treatment increased contractile activity in co-cultures by 50.77% compared to control co-cultures (p<0.01) (Figure 4-7A). No difference was noted between control and agrin and laminin treated muscle cultures (Figure 4-7A). Tubocurarine addition significantly reduced the level of contraction in each treatment group. Tubocurarine (12.5µM ) reduced contractile activity in control muscle by 78% (p<0.01), control co-cultures by 84.8% (p<0.001), agrin and laminin treated muscle by 100% (p<0.05) and agrin and laminin treated co-cultures by 93.5% (p<0.001) (Figure 4-7A). There was no significant difference between the treatment groups after tubocurarine addition (Figure 4-7B). The level of tubocurarine responsive contraction for each well was derived by subtracting the contractile activity after tubocurarine incubation from that before tubocurarine. There was again more tubocurarine responsive contraction in co-cultures than their muscle counterparts with control co-cultures showing
419% more activity than control muscle (p<0.001) and agrin and laminin treated co-cultures showing 1234% more activity than agrin and laminin treated muscle cultures (p<0.001) (Figure 4-7C). There was 96% more tubocurarine responsive activity in agrin and laminin treated co-cultures than control co-cultures (p<0.01) (Figure 4-7C). Agrin and laminin treatment did not change tubocurarine responsive activity in muscle monocultures. Interestingly a low level of tubocurarine activity was detected in muscle-only monocultures (Figure 4-7C). This was further accounted for by subtracting the mean tubocurarine activity in muscle-only cultures from their respective co-cultures (Figure 4-7D). The final result represents contractile activity that is both acetylcholine mediated and restricted to innervated muscle and is coined as “NMJ activity”. Agrin and laminin treated co-cultures displayed 113% more NMJ activity compared to control co-cultures (p<0.001) (Figure 4-7D).
Figure 4-7 Tubocurarine responsive myotube contractions. A) Contractile activity before and after incubation with 12.5 µM tubocurarine. B) Contractile activity after incubation with 12.5 µM tubocurarine. C) Tubocurarine responsive activity. D) NMJ activity. (As adapted from (1))

Data represents mean ±SEM. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, Ctrl- control cultures without agrin and laminin, post-tub- post incubation with 12.5 µM tubocurarine. * p<0.05, ** p<0.01, *** p<0.001 for unpaired t-test. & p<0.05, # p<0.01, ^ p<0.001 for paired t-tests.
4.4.5 Conditioned media and contractility

The heightened contractility observed in co-cultures relative to muscle culture may be due to the effect of paracrine factors released by neural cells instead of NMJ formation. To investigate this further, control and agrin and laminin treated muscle cells were grown in either control co-cultures conditioned media or again and laminin co-cultures conditioned media respectively (Figure 4-8). The level of contractile activity of the treated muscle was compared to those grown in standard media. There was no significant difference in contractility between control muscles grown in standard media and those in control co-cultures conditioned media (Figure 4-8A). Likewise, no difference in activity was noted between agrin and laminin treated muscle cells grown in standard media and those in agrin and laminin co-cultures conditioned media (Figure 4-8B).
Figure 4-8 Conditioned media and muscle contractility. A) Contractility of control muscle cells grown in either standard media or control co-cultures conditioned media. B) Contractility of agrin and laminin treated muscle cells grown in standard media or agrin and laminin treated co-culture conditioned media. (As adapted from (1)). Data represents mean ±SEM. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, Ctrl- control cultures without agrin and laminin, standard- standard media, CM-conditioned media.

4.4.6 Muscle striations in agrin and laminin treated cultures
The increased contraction noted in agrin and laminin treated co-cultures suggests increased NMJ formation which likely leads to muscle maturation. The level of muscle striation was used as a surrogate marker of muscle maturity and quantified. Muscle and co-cultures treated with or without agrin and laminin were fixed and stained with type 2 fast MHC fluorescent antibody. The number of striated myotubes in the cultures was quantified by microscopy and post-acquisitional image analysis. Similar to our previous experimental finding, control co-cultures had 1743% more striated myotubes than control muscle monocultures (p<0.001) (Figure 4-9A). Agrin and laminin treated co-cultures had 3256% more striated myotubes than agrin and laminin treated muscle (p<0.001) (Figure 4-9A). Agrin and laminin treatment did not change the number of striated myotubes in muscle monocultures. However, agrin and laminin treatment did increase the striated myotubes in co-cultures by 49.7% (p<0.05) (Figure 4-9A). This difference in striated myotube could not be accounted for by any difference in myotube numbers (Figure 4-9B). There were 29.72% and 30% fewer myotubes in control co-cultures and agrin and laminin treated co-cultures compared to their respective muscle controls (p<0.001) (Figure 4-9B).
Figure 4-9 Agrin and laminin treatment and myotube striation and number. A) Number of striated myotubes per microscopic field in agrin and laminin pre-treated cultures. B) Number of myotubes per microscopic field. (As adapted from (1)). Data represents mean ±SEM.

Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Ag + Ln- agrin and laminin pre-treated, Ctrl- control cultures without agrin and laminin. *p<0.05, *** p< 0.001 for unpaired t-test.
4.5 Discussion

4.5.1 Myotube contractility as a measure of innervation

In this study we attempted to optimize and develop myotube contraction as a reliable measure of \textit{in vitro} innervation. We showed that PC12 neural muscle co-cultures showed significantly more spontaneous contraction than muscle monocultures. The presence of PC12 neural cells also corresponded to greater number of striated myotubes and fewer number of myotubes overall. Myotube contraction is the end result of an electrophysiological phenomenon that has been coupled to a mechanical process. It begins with depolarization of the muscle cells secondary to activation of sodium channels which leads to activation of dihydropyridine calcium channels and subsequent RyR receptors in the sarcoplasmic reticulum (365, 366). The resulting efflux of calcium from the SR activates the myosin and actin filaments in the myotubes to contract (366). Greater contraction in innervated muscle cultures is indicative of a mature excitation-contraction coupling process and contractile machinery. Innervation is associated with phenotypical changes to both AChR and sodium channel subtypes as well as MHC expression (175, 192, 195, 216, 218, 350). It is therefore expected that such changes in molecular expression will translate into heightened contractile activity. Others have shown that aneural human myotube cultures fail to develop acetylcholine receptors, basal lamina and spontaneous contractions (361-363). Addition of neural cells induced sustained myotube contractions as well as ultrastructural changes such as emergence of T-tubules (363). However unlike human cells, our studies were performed using mice muscle cells which are known to spontaneously contract without neural supply (199). Spontaneous muscle contractions in rodent cells are
mediated by a combination of calcium, sodium and small conductance calcium activated potassium channels (199-203), C45. However no study has directly compared myotube contraction in aneural and innervated rodent myotubes. Our results suggest that spontaneous muscle contractions subside in aneural rodent myotubes overtime while large contractions develop in co-cultures likely due to NMJ formation. This maturity of the innervated muscle is further corroborated by the increasing number of striated myotubes in co-cultures. Striated myotubes rarely spontaneously develop in muscle mono-cultures. Their presence in co-cultures suggests that they are likely a result of NMJ formation and increased neuromuscular transmission. Innervation is known to promote transition from immature embryonic MHCs to adult type II fast MHCs (350, 357-359). Our results further support the mainstream view that innervation is a critical component of muscle maturation. Interesting innervation led to a reduction in total myotube number. This may be related to the size of the myotubes in co-cultures. The innervated muscles may be more mature and fuse more readily than their aneural counterparts, thus forming larger syncytium from more cells than aneural myotubes. As a result the overall number of myotubes may be lower despite the total nuclei number being similar between the cultures.

There are few limitations to this result. Firstly the contraction level was assessed by observing 5 microscopic fields from 5 regions of the culture well and therefore only a fraction of the well is observed. There is a possibility that some wells may be misrepresented as the cells may be heterogeneously distributed across the well surface. Secondly, the percentage of contractile area use in our study only measures one facet of contractility. Other contraction parameters such as frequency and strength of myotube contraction may not be reflected in the endpoints used in the current study. Thirdly, the heightened contraction in neural muscle co-cultures may be due to a paracrine effect rather than NMJ formation. This possibility will be explored later in this chapter and was found to be absent.
The quantification of striated myotubes is limited by the resolution of the microscope and what can be physically perceived by the researcher on a computer screen. Some myotubes may overlap others on a conventional microscope image and therefore difficult to distinguish one from the other when counting. Finally our results were performed using primary rodent mice cells co-cultured with PC12 neural cell lines. Rodent cells and cell lines may behave differently from human cells. Therefore any result may be specific to the culture model and may not necessarily be generalized to other species.

4.5.2 Contractile activity over time

Our results show that contractile activity of muscle mono-cultures assumes a biphasic trend. It increased from day one, peaked at day three and decreased again by day 5. In contrast, activity in co-cultures continued to increase from day one to day 5. Spontaneous muscle contraction is a well-known phenomenon in rodent myotube cultures. Others have reported that contraction peaks in rat myotubes at day 7-12 after differentiation (202, 367, 368). Other studies have shown increases in the frequency of spontaneous depolarization and shortening of the time to peak of the action potentials in rat myotubes over time. These studies unanimously support a biphasic trend in spontaneous cellular activity. Initially electrical activity increases and peaks at day 7-14 before subsiding. This is also mirrored by similar trends in the density of sodium channels over the same time frame. The difference in the time of peak activity between our study and those discussed above likely stem from differences in the species of rodent cells as well as the density that the cells are seeded. Myoblast density is known to affect both differentiations of the cells as well as their resting membrane potential (302-305, 369).
Unlike muscle-only cultures, little work has been done on characterizing the temporal profile of spontaneous contractile activity of neural-muscle co-cultures. The bulk of studies on neural-muscle co-cultures have been limited to demonstration of spontaneous contraction at a single time point (361-363). Our results show that myotubes in co-cultures contract progressively more over time. Comparison of trends in muscle and co-cultures indicates that muscle cells have an intrinsic capacity to develop independent of nerve supply to a certain extent after which innervation is required in order to progress further developmentally. Myotubes are initially capable to developing the capacity to contract in a nerve independent fashion. But a neural supply is likely required for the contraction to be maintained in the long run. It is well established that muscles are programmed to mature to a limited extent in the absence of innervation (20, 38, 90, 187, 197, 339, 352, 362). Aneural muscles can develop acetylcholine receptor clusters like those at the neuromuscular junction (20, 38). However these receptor clusters consist of immature receptor components and are ultrastructurally different from those at NMJ lacking the ‘pretzel’ morphology of mature AChR clusters at the NMJ (19, 20, 38, 46, 47). Mature sodium channels in denervated neonatal muscles still increase over time like their innervated controls (90, 187, 197, 339). However the level of immature sodium channels in these muscles is also higher (90, 187, 197, 339). Aneural muscles express the same mature isoforms of myosin heavy chains as innervated muscle (352, 355, 356). However aneural muscles contain significantly fewer slow type myosin (352). An interesting thing to note about the result is that the temporal profile of contraction of muscle monocultures and nerve muscle co-cultures bears striking resemblance to the trend in the number of large AChR clusters on similar cultures over time (Figure 4-10). Our previous work has shown that the number of large AChR clusters in muscle-only cultures also follow a biphasic trajectory, increasing at first, peaking and decreasing afterwards while the number of large clusters in co-cultures increased and plateaued at a high level (Figure 4-10). This similarity provides a glimpse into the interaction
between muscle activity and AChR dynamics and the mechanism underlying the decreasing trend in contraction in muscle monocultures at later times. Muscle contraction results from spontaneous depolarization of myotubes which is known to disperse AChR clusters (22, 27, 31, 100, 370, 371). Initially in muscle monocultures, as myotubes mature the number of AChR clusters increases sensitizing it to spontaneous activation. However this soon becomes a self-limiting cycle without a nerve supply. As the spontaneous activation becomes frequent, it further disperses AChR clusters reducing the cell sensitivity to further stimulation thus reducing its contraction. This self-perpetuating cycle leads to a drop in both AChR clustering and spontaneous contractions in muscle monocultures. In neural muscle co-cultures, as more muscles become innervated, the number of innervated large AChR clusters increases leading to more contraction overtime. However unlike muscle monocultures, these neural induced AChR clusters are resistant to dispersal by muscle depolarization and therefore the innervated muscles maintain their level of contractility (32, 33, 63). Others have shown that the number of AChR clusters begin to decrease with the beginning of spontaneous contractions in muscle-only cultures (40). In our studies, comparison of time points between our AChR clustering results and the current contraction result is hampered by differences in the density of the myoblast culture used to conduct the two individual experiments. Therefore the peak time of AChR clustering may not align as expected with our contraction data. The relative timing of the peaks in AChR clustering and muscle contraction will depend on the balance between the AChR clustering forces that is inherent of myotube development and the receptor dispersing forces of cellular depolarization. Both are subject to various culture conditions such as cell density and substrate (296-298, 302-305).
Figure 4-10 Comparison of AChR clusters in innervated and myotube only cultures. Column graph showing the number of large (>50µm²) per field in 3, 4 and 5 day co-cultures and myotube cultures of a similar age relative to the 6 day myotube culture. Note that the 6 day myotube cultures were used to compare with three day co-cultures as the myotubes in the three day co-culture had three days of differentiation prior to beginning of co-culture with PC12 cells. Data from three sets of experiments with 8 replicates in each treatment group in each set. Column and error bars represent mean +/- SEM. * p<0.05, ** p<0.01, *** p<0.001, unpaired student t-test.

4.5.3 Agrin and laminin’s effect on myotube contraction

Agrin and laminin pre-treatment initially reduced spontaneous contractions in both muscle and neural-muscle co-cultures. However over the next few days activity in the treated cultures increased progressively. By day 5 there was no difference in activity between treated and control muscle monocultures, while the activity of treated co-cultures exceeded
those of control co-cultures. Agrin and laminin clusters acetylcholine receptors on the
myotube membrane. The two agents interact synergistically to increase both the number
and size of AChR clusters (107, 109, 113, 343). The relationship between spontaneous AChR
custers and contraction in myotubes is not well defined. Bernareggi et al. showed that
aneural AChR clusters are correlated with spontaneous myotube twitching in muscle
monocultures (53). Furthermore increasing the number of AChR cluster by the use of agrin
increased the proportion of twitching myotubes (53). By increasing the number of AChR
custers on the myotube, agrin likely induced a hypersensitivity state in muscle cells allowing
it to be depolarized more readily. The aneural receptors are supposedly activated by
acetylcholine like substances released by the myotubes (211, 212). However these studies
were all performed using chick agrin and not the recombinant rat agrin used in this study.
Laminin was not used in conjunction with agrin in the mentioned studies. Despite its effects
on the number and size of AChR clustering, the quietening effect of agrin and laminin on
contraction in muscle-only cultures at day one and three suggests that the receptor clusters
and their downstream excitation-contraction coupling mechanisms in treated cultures may
be intrinsically different to those on control cells. Acetylcholine receptors are pentamers
that can exist as either mature or immature isoforms (42, 46, 169, 336, 337). Studies
performed by Rimer et al. suggest that the new AChR clusters induced by agrin contain both
mature and immature AChR isoforms (177). However it is unclear whether combination of
agrin and laminin treatment may impact on the maturity of the AChR. The effect of agrin and
laminin on the maturity of downstream voltage gated sodium channels and RyR receptors
also remain poorly defined.

Our previous results showed that agrin and laminin sensitized muscle to form more NMJ
with PC12 neural cells. The current result that agrin and laminin increased the level of
contractile activity in co-cultures is therefore a natural corollary of our previous finding. The
increased NMJ likely led to increased neuromuscular transmission and activation of muscle cells promoting muscle maturation. Muscle contraction is an end result of this process. As mentioned previously, the most characterized action of the agrin and laminin combination treatment is aneural AChR clustering (107, 109). Aneural AChR clusters are postulated to convey topographical signals that attract and guide neuritogenesis and NMJ formation (41). This is supported by two lines of evidence. Firstly, during in utero development aneural AChR clusters develop spontaneously at the central regions of muscle fibres. These also correspond to the first areas to be innervated by the incoming neurite (19). Secondly, transfecting regions of the muscle outside this central zone with AChR receptor clusters caused synaptogenesis to occur in the vicinity of the clusters (97). A logical inference from this observation is that by further enhancing the number and size of AChR clusters on muscle before innervation with the agents agrin and laminin, the muscle can be innervated at supraphysiological levels. An interesting point to note is that agrin and laminin treated co-cultures were more quiet than their control counterparts at both day one and day three. However the gap between the two groups narrowed overtime. As shown by our results previously, the myotubes in co-cultures consist of two populations- innervated and non-innervated. The contractile activity in the co-cultures represents a summation of activity from the two populations. The non-innervated myotubes follow the temporal profile of the muscle mono-cultures, peaking at day three before subsiding at day 5. The innervated myotubes contract ever more forcibly with time. Given that our results showed agrin and laminin quieting contraction in muscle monocultures at day one and three, the aneural myotube component of the agrin and laminin treated co-cultures will also show fewer contraction at those time points. Summation of the innervated and aneural myotubes in the treated co-cultures will lead to reduced total contraction levels compared to control co-cultures at day one and day three. However by day 5 the contribution of aneural myotubes to the overall contractile activity becomes minimal as the number of innervated myotube
increases with synaptogenesis. By this time, innervated myotubes played a major role in determining contractions. Owing to increasing number of NMJs, agrin and laminin treated co-cultures will contain more innervated myotubes and therefore greater overall all contractile activity. From an innervation point of view, quietening of contractions of aneural myotubes by agrin and laminin may aid NMJ formation. In order to establish a neuromuscular connection, there must be minimal movement of the target muscle to allow for firm attachment of the neurite. A part of the mechanism underlying enhanced innervation in agrin and laminin treated co-cultures may rest in its ability to immobilize the muscle and preparing it for innervation.

4.5.4 Acetylcholine and myotube contraction

The mechanism underlying the heightened contraction in PC12-muscle co-cultures compared to muscle monocultures remains unclear. Theoretically, the contractions are mediated by acetylcholine released from the PC12 cells activating the myotubes at areas of NMJ formation. In order to ascertain the aetiology of myotube contractions in our neural – muscle co-cultures, we added tubocurarine to agrin and laminin treated neural-muscle co-cultures and noted the resultant contraction. Tubocurarine is an ACh receptor antagonist and should block NMJ transmission. Agrin and laminin treated co-cultures were chosen as they showed the most NMJ formation and contractile activity as demonstrated by our previous results. The result was not due to volume change of the media associated with the addition of the drug as addition of the plain vehicle used to carry the drug did not change myotube contraction in the co-cultures. As expected, addition of the drug to the co-culture silenced most of the contraction in the co-culture. There was some residual contraction that was resistant to tubocurarine and therefore non-ACh mediated. This raised the possibility
that the remaining contractile activity was attributed to non-innervated myotubes. To explore this issue further, we added tubocurarine to both muscle mono cultures and PC12-neural co-cultures. Interestingly tubocurarine also blocked most of the contractile activity in muscle-only cultures treated with or without agrin and laminin. This indicates that acetylcholine receptor activation is responsible for contraction in both muscle-only and neural-muscle co-cultures. Like in co-cultures, there was also residual activity resistant to tubocurarine in all muscle monocultures suggesting that non-acetylcholine related mechanisms play a role in muscle contraction in both innervated and non-innervated muscle. Given the presence of acetylcholine induced contractions in muscle monocultures, tubocurarine responsive activity alone is insufficient to differentiate between innervated and non-innervated muscle cells. To further filter out the non-innervated muscle activity in co-cultures, we analysed the data while accounting for spontaneous activity in muscle monoculture controls. The end result was contractile activity in co-cultures that was both neural induced and mediated by acetylcholine. From the analysis, we deduced that agrin and laminin treated co-cultures showed greater such activity than control co-cultures. This is further supported by the presence of more striated myotubes in the agrin and laminin treated co-cultures which is suggestive of greater muscle maturity secondary to more NMJ formation. These results all support our hypothesis that agrin and laminin enhances innervation of muscle by neurons.

Bandi et al. reported that spontaneous AChR activation is necessary for myotube contraction (212). Blockage of AChR using α BTX inhibited spontaneous depolarisation and contraction of myotubes (53, 212). This concurs with our finding that tubocurarine suppressed contraction in myotube only cultures. The AChR is likely activated by an ACh like substance produced by the myotubes themselves. Conditioned media from the myotubes promoted depolarization and contraction of myotubes (211). An ACh like substance has been isolated from the
conditioned media (212). Furthermore, the enzyme choline acetyltransferase which is crucial to ACh synthesis is also expressed by myotubes in muscle monocultures (51, 212). These results all suggest that myotubes are capable of producing ACh that acts in an autocrine fashion activating the AChR present on its membrane leading to depolarization and contraction. Some have even posited that the spontaneous activation of the AChR serves a developmental role. Stimulation of AChR promoted myoblast fusion while inhibition prevented the process (51, 52, 211). Like in our study, other authors have also noticed a low level of tubocurarine resistant myotube contractions in muscle monocultures (212). It is believed that the mechanism underlying spontaneous contraction may not be uniform within the same population of myotubes (199). In one study, addition of α-BTX to block AChR only abolished 70% of spontaneous electrical activity (212). The suppressed activity was characterized by a slow frequency (0.45Hz) while the tubocurarine resistant activity had a higher frequency (212). The study concluded that the high frequency activity that was tubocurarine resistant was a result of interplay between Ca channels, sodium channels and small conductance calcium activated potassium channels (199). This likely explains the tubocurarine resistant activity present in both muscle monocultures and co-cultures in our study. However, it is unclear whether the different mechanisms of depolarization and contraction co-exist on the same muscle cell or are present on different cells.

Our finding that tubocurarine is capable of suppressing spontaneous muscle contraction in muscle-only cultures raises questions regarding its use as a means of demonstrating NMJ formation. Others have used the suppression of muscle contraction by tubocurarine in neural-muscle co-cultures as evidence of neuromuscular transmission without comparing it to the relevant muscle-only controls (207-209). Most studies that have treated muscle controls with tubocurarine have shown similar results to our study with suppression of myotube twitching (53, 208, 210). In one study tubocurarine did not change the
spontaneous contraction of rat myotubes (206). However the study was conducted using embryonic rat muscle cells which may behave differently to post-natal mice cells that are used in the current study. In light of these findings and our results, future studies that involve demonstration of NMJ activity in neural muscle co-cultures by using tubocurarine should also have relevant muscle-only controls to account for spontaneous muscle activity. Despite showing the elevated activity in the co-cultures to be neurally induced and acetylcholine mediated, it is unclear whether mode of action is contact mediated or paracrine. In our study, conditioned media from control and agrin and laminin treated co-cultures did not change the activity of the myotube monocultures. This would suggest that the activity of the myotubes is contract driven further supporting the hypothesis that the enhanced contractile activity in the co-cultures is due to NMJ function.

4.6 Conclusion

Myotube contraction in neural-muscle co-cultures can be used as a functional measure of innervation provided that the proper muscle-only controls are also provided. Myotube contraction is acetylcholine induced and is present in both innervated and non-innervated muscle cells. However innervated muscle cultures contract more than muscle mono-cultures. By controlling for spontaneous contractile activity in muscle-only controls, the level of NMJ-induced activity can be readily deduced. Agrin and laminin quietens contraction of non-innervated cells. This likely facilitates NMJ formation by immobilizing the muscle for innervation resulting increased synaptogenesis and neuro-muscular transmission. In this chapter we have provided functional evidence that agrin and laminin enhances muscle innervation.
Chapter 5

5. Neuromuscular prosthetic interface and trilayered conductive polymer actuators

5.1 Introduction

Limb amputation due to trauma or tumour ablative surgery can lead to significant functional deficits for patients. Prosthetic limbs provide a viable alternative for functional restoration in amputees. However performance of prosthetic limbs are limited by the fidelity of the body machine interface. Ideally the interface must be durable and be capable of expediting signal exchange between the limb stump and the prosthesis with accuracy and reliability (8-11). Current body-prosthetic interfaces employ surface electrodes placed over the remaining muscles of the limb stump to record muscle electrical activity as a means for driving the prosthesis (2). Such approach limits the number of active inputs feeding into the prosthesis as there is often deficiency of available muscles on the stump for electrode interfacing (4, 5, 12). Others have sought to surgically innervate muscle that were de-functioned by the limb amputation with nerves that originally supplied the lost limb in a process known as targeted muscle re-innervation (4, 5, 13, 14). This approach produces multiple discrete neuromuscular synapses between nerve endings severed by amputation and a stump muscle. Electrodes are placed strategically on skin overlying these synapses thus increasing the number of active inputs into the prosthesis (4, 5, 13, 14). However the skin electrodes used in TMR are prone to interruption by sweat and oil (3). A neuromuscular prosthetic interface (NPI) essentially combines TMR with intra-muscular electrodes (Figure 5-1). In such a system, the de-functioned muscle is surgically innervated by nerves severed by the amputation. The muscle is further interfaced with an array of intramuscular electrodes. Activity from the nerve-muscle pedicles are detected by the electrodes and utilised to
control motion of the prosthetic limb. As compared to existing body-machine interfaces, the NPI offers the advantages of simultaneous control of multiple movements over multiple joints of the prosthesis with high fidelity and reliability.

Figure 5-1 Schematic diagram of a neuromuscular prosthetic interface and actuator system. It consists of a muscle that is surgically innervated by nerves severed by the amputation. The innervated muscle is then apposed to an array of electrodes that are directly linked to a downstream actuator. Image created by author.

An NPI consists of two key interfaces, 1) the neuromuscular interface between the nerves severed by the amputation and the de-functioned muscle and 2) the muscle electrode interface. In the previous chapters of this thesis we have already explored means of enhancing the nerve-muscle interface by priming muscle to develop more neuromuscular junctions with nerves. In the current chapter we will explore other modelling and material aspects of designing the NPI as well as the muscle-electrode interface and its downstream actuator system. Studies on developing an in vitro model of a NPI by marrying a cell based
innervated muscle system with a multi-electrode array (MEA) will be presented. The potential for improving the muscle-electrode interface in a NPI using the electroconductive polymer polypyrrole will also be explored. Finally the biocompatibility of a trilayer polypyrrole actuator that may one day be coupled to the NPI will be elucidated.

Multi-electrode arrays (MEA) are devices that contain an array of electrodes that act as interfaces between biological tissues and electronic circuitry. MEAs have been implanted into animal cortices to provide real-time, high resolution recording of neural activity (372-375). An MEA will also be used in the setting of building a NPI. However in order to expedite experimental work on NPI, an in vitro, MEA based model of the interface needs to be developed. MEAs have been used to detect activity from neural as well as muscle cells (235, 376, 377). In this chapter we constructed an MEA based in vitro model of innervated muscle cultures to simulate the functioning of a NPI. This will serve as a platform for future in vitro experimental work in this discipline.

The choice of material for coating the MEAs used to detect neuromuscular activity is an important design consideration when building a NPI. The base component of recording electrodes in implantable MEA’s has traditionally been metal, which has a propensity to induce an inflammatory response leading to fibrotic encapsulation and a resultant decreased potential for signal recording (16, 17). Polypyrrole coatings may ameliorate some of the shortcomings of metal electrodes. Polypyrrole is a biocompatible conducting polymer that has stiffness closer to biological tissue than traditional metal electrodes, thus reducing scar formation in vivo (6, 245, 248, 256-259). Furthermore it is more versatile and amenable to modification to improve its surface bioactivity. Polypyrrole have been combined with various extracellular matrix molecules to impart bioactivity to its surface (246-249). The performance of different thickness polypyrrole coatings in detecting muscle cellular signal will be gauged in this chapter.
Currently prosthetic limbs are either body powered or externally powered (5). Externally powered prostheses consists of multiple moving parts and are prone to breakdown and require regular maintenance. In the current chapter, a trilayered PPy based actuator was examined for potential use in prosthesis (Figure 5-2). The actuator consists of a platinum coated PVDF membrane sandwiched between two layers of PPy doped with para-toluene sulfonic acid sodium salt (pTS, Sigma Aldrich). Upon passage of electrical current, redox reactions occur on the PPy layers leading to volume change resulting in actuation (270, 283-285). Unlike traditional actuators, such a system is simple in design and offers large...
actuation per unit mass of the actuator. This polypyrrole-based design raises the potential of condensing the NPI and the actuator into one single construct, further lessening the complexity of the system. However the biocompatibility of such an actuator is unknown. In this chapter the tissue response to these actuators will be explored. The results will also add to the existing limited literature about the in vivo biocompatibility of polypyrrole.

5.2 Aim

1. To develop an in vitro model of a neuromuscular prosthetic interface using multi-electrode arrays.

2. To assess the capacity of polypyrrole as an electrode coating to detect muscle cellular activity and to evaluate the effect of polypyrrole thickness on recording cellular signals.

3. To evaluate the biocompatibility of a polypyrrole based trilayer actuator in vivo.
5.2 Methods

5.2.1 Myotube cultures and PC12-myotube co-cultures

5.2.1.1 Obtaining myoblast

Myoblast cultures were prepared from skeletal muscle removed from the hindlimbs of 3-4 week old C57BL/6 mice as described by Todaro et al. Myoblasts were maintained in growth media containing Hams F10 (Gibco), 20% Fetal Bovine Serum (Gibco), 2.5ng/mL recombinant human bFGF (Peprotech), 2mM L-glutamine (Gibco), 100U/mL penicillin and 100μg/mL streptomycin (Gibco) at 37°C under 5% CO₂. Cells were passaged at 80% confluence using TrypLE (Gibco). Myoblasts from passage 6 and 7 were used for all experiments described in this study.

5.2.1.2 Myotube seeding and differentiation

All cell culture substrates were incubated with type 1 rat tail collagen (BD Bioscience) in 2mM HCl at room temperature. The concentration of collagen and the length of incubation varied with the individual experiments. The coated substrates were then washed twice with phosphate buffered saline (PBS, Life Technologies) before use. Myoblasts were seeded onto coated surfaces at various densities in myoblast growth media and left to adhere at 37°C under 5% CO₂. After 24 hours the media was changed to myoblast differentiation media, containing DMEM (Lonza), 2% horse serum (Gibco), 100U/mL penicillin and 100μg/mL streptomycin. A full media change was conducted every 24 hours for all groups in the study.
After 72 hours of differentiation, the myotube cultures were either co-cultured with PC12 cells or simply changed to PC12 differentiation media, containing DMEM, 1% horse serum, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 50ng/ml mouse NGF-7S (Sigma). Daily half media changes were performed from then onwards.

5.2.1.3 PC12-myotube cocultures

PC12 cells were passaged in proliferation media consisting of DMEM (Lonza), 10% horse serum, 5% FBS, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. Cultures were maintained for three days at 37°C and 5% CO₂ until 70% confluence was reached at which point the cells were harvested for co-culture. PC12 cultures were pelleted by centrifugation and resuspended in PC12 differentiation media consisting of DMEM with 1% horse serum, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 50ng/ml mouse NGF-7S (Sigma). The media from the adherent myotube cultures (described above) was discarded and PC12 cells in PC12 differentiation media were seeded onto differentiated myotubes at a PC12 density that was half that of the initial myoblast seeding density. The co-culture was then incubated at 37°C and 5% CO₂ with daily half media changes.

5.2.2 Maximizing myotube contractility experiment

Clear 96 well flat bottom polystyrene plates (Nunclon delta microplates) and 96 well glass bottom plates (In Vitro Scientific) were used. Plates were incubated with type 1 rat tail collagen (BD Bioscience) at 2μg/cm² in 2mM HCl for two hours or at 10μg/cm² in 2mM HCl for 18 hours at room temperature for low and high collagen coatings respectively. Myoblasts were seeded at a density of 2.5 X 10⁴ cells/cm² and at 1 X 10⁵ cells/cm² for low and high density.
seeding density groups respectively. For cocultures, PC12 seeding density was half that of the initial myoblast seeding densities; $1.25 \times 10^4$ cells/cm² for low seeding density and $5 \times 10^4$ cells/cm² for high seeding density. All cell cultures were conducted as per protocol described above.

5.2.2.1 Live imaging assessment of contractile activity

At one, three and 5 days following incubation in PC12 differentiation media, cultures were transferred to an incubation chamber at 37°C and 5% CO₂ and imaged (Olympus X70, LCPlanFl 20X objective with NA of 0.4). For each well, 5 fields from non-overlapping regions were selected, with the position of each field approximately the same in each well. Each field was observed for 10 seconds and noted for any contractile activity, with the percentage of fields in each well with contractile activity recorded and used as a gauge of total contractile activity. Each treatment group consisted of 6 wells, with each well treated individually as a separate n.

5.2.3 MEA electrodes

5.2.3.1 Polypyrrole electrochemical deposition on MEA electrodes

Pyrrole monomer (Fluka, 98%) was distilled to purity. A coating solution was prepared containing 0.2M pyrrole and 0.25M pTS, in MilliQ H₂O. Room temperature coating solution was placed into the gold MEA plates (60EcoMEA, Multi Channel Systems) and the MEAs
placed into the recording system (USB-MEA system, Multi Channel Systems). After isolating the MEA internal reference electrode, a platinum mesh and Ag-AgCl reference electrode were placed into the MEA bath, with the platinum mesh electrode parallel to the recording electrode surfaces. The reference electrode and platinum mesh were connected to the electrochemistry equipment (eCorder 401, eDAQ), which was controlled by proprietary software (eChem version 2.1.16, eDAQ). Four MEA recording electrodes at a time were selected for stimulation through software (MEA-Select, Multi Channel Systems), with the working electrode of the electrochemistry unit routed to these 4 MEA electrodes. Voltage was cycled from -0.2 to +0.85 V and then reversed at 100mV/s, either two, 4, or 8 times to polymerise the pyrrole and electrochemically attached it to the gold electrode surfaces. The number of cycles of cyclic voltammetry electrochemical deposition has been designated ‘CV’. Groups of 4 electrodes were coated in each batch, with three batches for each of the three coating thicknesses, resulting in 12 electrodes at each of 2, 4, and 8CV on each MEA. The remaining 23 electrodes of each MEA uncoated (0 CV). For each MEA, electrodes were assigned to each group randomly.

5.2.3.2 Cell seeding on MEA

MEA (Multi Channel Systems) were sterilized by immersion in 80% ethanol at room temperature for one hour, rinsed three times in MilliQ H₂O for 30 minutes, followed by incubation with type 1 rat tail collagen (BD Bioscience) at 10µg/cm² in 2mM HCl for 18 hours at room temperature. Myoblasts were seeded at a density of 2.5 X 10⁴ cells/cm², 5 X 10⁴ cells/cm² or 1 X 10⁵ cells/cm². All muscle monocultures and PC12-muscle co-cultures were performed as per protocol described above.
5.2.3.3 MEA recording

At various times in culture, MEA with cells attached were transferred to a recording system (USB-MEA system, MEA1060 amplifier, Multi Channel Systems). Spontaneous electrophysiological activity was recorded at 25kHz (MC Rack software, Multi Channel Systems) with recordings completed in differentiation media at 37°C. The recordings were generally conducted over 60 seconds and the number of active electrodes was also noted. Upon completion of each recording, media was replaced and MEA returned to the incubator at 37°C and 5% CO2. Repeat recordings were completed from each MEA.

5.2.4 Polypyrrole based trilayer actuator

5.2.4.1 Construction of the trilayer actuator

The PPy trilayered actuators were kindly provided by our collaborators at the Intelligent polymer institute. The PPy trilayered actuators were kindly provided by our collaborators at the Intelligent Polymer Research Institute, University of Wollongong. Flexible substrates were prepared by sputter coating platinum (Magnetron sputter coater SC 100MS) on to both sides of a poly(vinylidene fluoride) (PVDF) membrane (Millipore 110mm thickness). P-toluene sulfonic acid (PTSA, 95%) and deionized water were used. Pyrrole (Fluka, 98%) was
distilled before use. The polymerization solution contained 0.1 M pyrrole and 0.1 M PTSA mixed in water containing 1 wt% of deionized water. Before the reaction commenced, the solution was purged with nitrogen gas for 20 min at room temperature. Polypyrrole was prepared via continuous electrochemical polymerization methods. Samples were electrochemically deposited with a galvanostatic process at a constant current density of 0.1 mA/cm² for 8 hours at room temperature. Stainless steel mesh was used for two counter electrodes and potentiostat/galvanostat (EG&G Princeton Applied Research Model 363) was used to generate the constant current. The membranes were coated with polypyrrole on both sides. For initial studies assessing the effect of pores on the biocompatibility of the actuator strips, 150µm pores spaced by 300µm gaps were cut into the strips with laser after polypyrrole coating. For later studies that studied the effect of pore cutting on difference phases of the manufacturing process on polypyrrole breakdown, the pores were either cut after polypyrrole polymerization or before it.

5.2.4.2 Implantation of polypyrrole trilayer actuator

All experiments and treatment of animals were in accordance with the institutional ethics guidelines.

New Zealand white rabbits were anaesthetized with Isofluorane gas (1-3%) in oxygen and laid on their back before both hind-limbs were shaved and decontaminated. Under sterile conditions a skin incision was made over the tibialis anterior on the hindlimb. The tibialis anterior muscle was isolated and longitudinal pockets created within the muscle using blunt dissection. The polypyrrole actuator strip was placed into the pocket and the pocket closed with sutures. For the first study aimed at characterizing the biocompatibility of porous PPy
strips, one hindlimb of each rabbit was implanted with two non-porous strips and the other hindlimb was implanted with two porous strips. The non-porous strips were treated as the control. In the second study the effect of polypyrrole coating before or after strip fenestration on polypyrrole breakdown was examined. One hind limb was implanted with two strips that were first coated with PPy and then cut with pores while the other hindlimb was implanted with two strips that were cut with pores then coated with PPy. The strips that were first coated with PPy and then cut with pores was made the control group in the second study. Following recovery from surgery, the rabbits were sacrificed at three days, two weeks, 4 weeks and 8 weeks post-surgery. A total of three rabbits were used for each time point.

5.2.4.3 Histology

The tibialis muscles were harvested at three days, two weeks, 4 weeks and 8 weeks for histological analysis. The tissues were processed and stained by staff at the Biomedical Sciences Histology Facility at the University of Melbourne. The tissues were fixed in 10% neutral buffered formalin for 24 hours and further dehydrated and embedded in paraffin wax as per following protocol (table 5-1):
<table>
<thead>
<tr>
<th>Solution</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>60</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>60</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>60</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>90</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>90</td>
</tr>
<tr>
<td>100 % ethanol</td>
<td>90</td>
</tr>
<tr>
<td>ethanol/xylene</td>
<td>90</td>
</tr>
<tr>
<td>xylene</td>
<td>90</td>
</tr>
<tr>
<td>xylene</td>
<td>90</td>
</tr>
<tr>
<td>paraffin wax</td>
<td>60</td>
</tr>
<tr>
<td>paraffin wax</td>
<td>60</td>
</tr>
<tr>
<td>paraffin wax</td>
<td>60</td>
</tr>
<tr>
<td>paraffin wax</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 5-1 protocol for tissue dehydration and paraffin wax embedding.
5µm paraffin sections were cut from the paraffin embedded specimens. The sections were stained with eosin and haematoxylin as follows (table 5-2):

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>3 x 3 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Harris haematoxylin</td>
<td>4 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td>1 minute</td>
</tr>
<tr>
<td>Scott's tap water</td>
<td>1 minute</td>
</tr>
<tr>
<td>tap water wash</td>
<td>3 minutes</td>
</tr>
<tr>
<td>0.1% eosin</td>
<td>4 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 x 30 seconds</td>
</tr>
<tr>
<td>Xylene</td>
<td>3 x 2 minutes</td>
</tr>
</tbody>
</table>

Table 5-2 Protocol for haematoxylin and eosin staining
For Mason’s Trichrome staining of sections, the slides were processed as follows (table 5-3):

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>3 x 3 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Bouin's fixative</td>
<td>60 minutes at 60°C</td>
</tr>
<tr>
<td>tap water wash</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Iron haematoxylin</td>
<td></td>
</tr>
<tr>
<td>(Weigert's A (1 part) Weigert's B (1 part))</td>
<td>2 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td></td>
</tr>
<tr>
<td>1% Ponceau 2 R (2 parts)</td>
<td></td>
</tr>
<tr>
<td>1% Acid fuchsin(1 Part)</td>
<td>5 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td></td>
</tr>
<tr>
<td>1% Phosphomolybdic acid</td>
<td>3 minutes</td>
</tr>
<tr>
<td>tap water wash</td>
<td></td>
</tr>
<tr>
<td>2% light green</td>
<td>5 minutes</td>
</tr>
<tr>
<td>wash and leave in</td>
<td></td>
</tr>
<tr>
<td>1% Acetic acid</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 x 30 seconds</td>
</tr>
<tr>
<td>Xylene</td>
<td>3 x 2 minutes</td>
</tr>
</tbody>
</table>

Table 5-3 Protocol for masons trichrome staining
5.2.4.4 Image Analysis

High definition images of the histology slides were obtained using Olympus Olyvia system (Olympus Microscopy, United Kingdom) at 20X magnification and 3µm z-stacks were generated. The images were analyzed using FIJI software. Areas of interest were defined by regions 300-500µm around the actuator strips, with the total area of interest examined between the different groups was the same. Intensity thresholds were applied so that only the black particles from the polypyrrole were detected, with the total area occupied by the particles calculated using particle analysis. All particles equal to or greater than one µm² were identified. The percentage of the region of interest around each strip occupied by the debris particles was then calculated. The debris value of the two control strips from each rabbit was averaged and all debris values of strips implanted in the same rabbit were normalized to the calculated mean of the control strips.

5.2.5 Statistical Analysis

For experiments performed using cell culture wells, results were expressed as mean and standard error of n=6 wells. For MEA experiments examining the effect of PPy coating thickness on signal recording levels, the waveform data from electrodes with the same PPy coating in each MEA were pooled together to calculate average parameters of the waveforms. A total of 6 MEAs were used for the experiment and each MEA was considered as a separate n for calculations of number of active channels. For biocompatibility studies of actuator strips, each rabbit was implanted with two control strips in one hind limb and two
strips with treatment of interest in the other leg. A total of three rabbits were used for each time point. Each strip was treated as a single n and the values were normalized to the average of the two strips on the control limb. Graphpad Prism (Version 5.0) was used to perform the Student’s paired and unpaired t-test and a one-way ANOVA with Bonferroni’s post-hoc test, to assess statistical significance between treatments.

5.3 Results

5.3.1 Parameters that maximize muscle activity in vitro

In order to optimise MEA recordings, the culture conditions that produced the maximum myotube activity needed to be ascertained for both muscle only and muscle-PC12 co-cultures. The area containing electrodes within an MEA occupy less than 1% of the total well area. Therefore the activity of muscle and neuromuscular cultures must be sufficiently widespread and strong for sufficient data to be collected to arrive at scientifically sound conclusions. In muscle cultures, MEA’s detect electrical activity generated by muscle cells during contraction. In neural-muscle cocultures, PC12 neural cells are used to innervate muscle and our previous experience with the cell line indicate that PC12 cells do not display any detectable spontaneous activity when cultured on an MEA. MEAs were initially not used for the optimization process due to their high unit cost. Instead, glass based plates were used to simulate the MEA environment. Muscle cell contractions were taken as a surrogate marker of cell activity. Myotubes were grown on either glass or plastic tissue culture plates coated with either high or low type 1 collagen at either high or low cell density and in the
presence or absence of PC12 neural cells as detailed in the methods section. The contraction data was recorded at over time.

5.3.1.1 Day 1

A high cell seeding density increased muscle contraction on plastic, regardless of collagen coating, in both muscle mono-cultures and PC12-muscle co-cultures. High cell seeding density on plastic increased contraction in each of; low collagen muscle cultures (63% vs. 13%, p<0.01), high collagen muscle cultures (83% vs. 10%, p<0.001), low collagen co-cultures (87% vs 17%, p<0.001) and high collagen co-cultures (70% vs. 10%, p<0.001) (Figure 5-3A). In cultures on glass substrates, high cell seeding density only increased muscle activity in high collagen cocultures (73% vs. 10%, p<0.001).

Collagen coating thickness had no statistically significant effect on muscle contraction, except for high collagen cocultures on glass where myotube contraction was increased (73% vs 3%, P<0.001) (Figure 5-3B).

Innervation by PC12 cells enhanced contraction in only one group, high collagen cultures grown on glass (73% vs. 0%, p<0.001) (Figure 5-3C). Myotube contraction was increased upon plastic substrates over glass in muscle monocultures seeded at high cell density and grown on both low collagen (63% vs 3%, p<0.001) and high collagen (83% vs. 0%, p<0.001) and in cocultures seeded at high cell density and grown on low collagen surfaces (87% vs. 3%, p<0.001) (Figure 5-3D). Substrate choice had no significant effect on myotube contraction for all other groups.

5.3.1.2 Day 3
Cell seeding density had no significant effect on cultures grown on plastic, with the exception of high collagen co-cultures, where increasing cell density decreased myotube contraction (20% vs. 83%, P<0.001) (Figure 5-4A). High cell seeding density increased contraction in low collagen cocultures on glass substrate (63% vs. 10%, P<0.01), although had no statistically significant effect on myotube contraction on other cultures on glass. There was an insignificant trend towards more contraction in high collagen cocultures seeded at high densities on glass compared to the same culture but seeded at lower densities (47% vs. 13%, NS).

Myotube contraction in muscle monocultures with low cell densities on plastic (33% vs. 90%, p<0.001) and in cocultures with high cell densities on plastic (20% vs. 63%, p<0.05) (Figure 5-4B). Otherwise collagen level had no significant effect on myotube contraction at day three.

Innervation enhanced myotube contraction in high cell density cultures on glass, both with low collagen (63% vs. 3%, p<0.001) and high collagen (47% vs. 0%, p<0.05) (Figure 5-4C). Innervation also enhanced contraction in low cell density, high collagen cultures on plastic (83% vs. 33%, p<0.01) (Figure 5-4C). Otherwise, innervation had no significant effect on cell contraction at day three.

A plastic substrate is more conducive towards myotube contraction than glass for muscle mono-cultures in low collagen conditions for both low (90% vs. 0%, p<0.001) and high (60% vs. 3%, p<0.001) seeding densities (Figure 5-4D). There was an increasing but non-significant trend towards more contractions in high collagen muscle monocultures grown on plastic compared to those grown on glass. In co-cultures, plastic substrates improved contractions in low seeding density conditions for both low collagen (97% vs. 10%, p<0.001) and high collagen (83% vs. 13%, p<0.001) conditions (Figure 5-4D). Plastic had no significant effect on myotube contraction in all other co-culture conditions.
5.3.1.3 Day 5

At day 5, myotube contractions were higher in co-cultures at high cell seeding density grown on glass, regardless of collagen coating level (100% vs. 43% for high collagen, p<0.001 and 93% vs. 57% for low collagen, p<0.01) (Figure 5-5A). Cell seeding density did not change the low level contraction noted in all other cultures grown on glass. Myotube contraction was lower for high cell seeding density in co-cultures upon glass with a high collagen coating (67% vs. 97%, p<0.05) (Figure 5-5A). Cell density had no effect in all other cultures grown on plastic.

Collagen coating had no significant effect on myotube contraction in all cultures grown on plastic. High collagen reduced myotube contraction only in high cell density co-cultures grown on plastic substrates (67% vs. 93%, p<0.05). Collagen coating had no significant effect on all other cultures grown on plastic (Figure 5-5B).

Innervation increased contraction of myotubes in all treatment groups grown on both plastic (97% vs. 17% for low density and low collagen, p<0.001, 93% vs. 27% for high density and low collagen, p<0.001, 97% vs. 23% for low density and high collagen, p<0.001, 67% vs. 7% for high density and high collagen cultures, p<0.001) and glass (57% vs. 13% for low density and low collagen, p<0.001, 92% vs. 3% for high density and low collagen, p<0.001, 43% vs. 0% for low cell density and high collagen, p<0.001, 100% vs. 0% for high density and high collagen, p<0.001) (Figure 5-5C).

Plastic substrate enhanced myotube contraction in low cell density co-cultures regardless of collagen coating (97% vs. 57% for low density and low collagen, p<0.001, 97% vs. 43% for low density and high collagen cocultures, p<0.001) (Figure 5-5D). Plastic substrate reduced myotube contraction in cocultures grown at high cell density and high collagen coating (67%
vs. 100%, p<0.01) (Figure 5-5D). There was an increasing but nonsignificant trend towards more contraction on plastic substrates in muscle mono cultures grown on high collagen coatings regardless of cell density and in muscle cultures seeded at high density but with low collagen coatings.

5.3.2 Trend of activity of cultures over time

To optimise the amount and utility of data harvested throughout the experiment, activity levels needed to be maximised both at each time point as well as across the entire culture period. By plotting the activity of cultures over time these questions could be thoroughly addressed. In cultures grown on glass substrates, co-cultures generally displayed more activity than muscle only cultures at day 5 (Figure 5-5C and 6). Amongst co-cultures upon glass, those with the high cell density and high collagen coating contracted the most at day one (p<0.001) and contracted more than any other co-culture except for those with high cell density and low collagen coating at day three (vs. CoCellLColL, p<0.01, vs. CoCellHColL, p<0.001, vs. CoCellLColH, p<0.01) and 5 (vs. CoCellLColL, p<0.001, vs. CoCellHColL, p<0.001, vs. CoCellLColH, p<0.001) (Figure 5-6A). There was no significant difference in contraction between high seeding density co-cultures with high and low collagen coating at day three and day 5 (Fig 5-6A). Muscle monocultures grown on plastic substrates showed two starkly different trends in myotube contraction over time (Fig 5-6B). Muscle cultures with low cell seeding densities displayed activity profiles that peaked at day three before decreasing at day 5, resembling an inverted V (Fig 5-6B). Those with high cell density showed a decreasing trend in activity from day one to 5. Two different trends in activity profile over time could also be discerned in co-cultures grown on plastic substrates. Co-cultures with high seeding density show activity trending downwards and reaching a trough at day three before picking
up again at day 5, whilst co-cultures with low cell seeding density displayed increasing activity over time from day one to day 5 (Fig 5-6B).
A

Mus - Muscle only culture
Co - PC12 - muscle coculture
Cell - cell density
Col - collagen coating
Pl - plastic culture substrate
Gl - glass culture substrate
L - low
H - high

Day 1 (Cell density)

% of fields with contraction

Low cell density - black bars
High cell density - white bars

B

Day 1 (Collagen coating)

% of fields with contraction

Low collagen coating - black bars
High collagen coating - white bars
Figure 5-3 Myotube contraction at day one after culture in PC12DM. The effect of A) cell density, B) collagen coating, C) innervation and D) substrate on myotube contraction. All 4
graphs contain the same body of data but represented differently for ease of comparison.

Data represents mean ±SEM. Activity levels of each culture were expressed as percentage of
five fields that showed contraction. Legend- Mus- muscle monoculture, Co- PC12 neural-
muscle co-cultures, Col- collagen coating, Pl- plastic culture substrate, Gl- glass culture
substrate, L- low, H-high. ** p<0.01, *** p< 0.001 for Bonferroni’s test.
A

Mus- Muscle only culture
Co- PC12- muscle coculture
Cell- cell density
Col- collagen coating
Pl- plastic culture substrate
Gl- glass culture substrate
L- low
H- high

Day 3 (cell density)

Treatment Groups

% of fields with contraction

B

Low collagen coating- black bars
High collagen coating - white bars

Day 3 (collagen)

Treatment Groups

% of fields with contraction
Figure 5-4 Myotube contraction at day three after culture in PC12DM. The effect of A) cell density, B) collagen coating, C) innervation and D) substrate on myotube contraction. All 4
graphs contain the same body of data but represented differently for ease of comparison. Data represents mean ±SEM. Activity levels of each culture were expressed as percentage of five fields that showed contraction. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Col- collagen coating, Pl- plastic culture substrate, Gl- glass culture substrate, L- low, H- high. ** p<0.01, *** p< 0.001 for Bonferroni’s test.
A

Mus- Muscle only culture
Co- PC12- muscle coculture
Cell- cell density
Col- collagen coating
Pl- plastic culture substrate
Gl- glass culture substrate
L- low
H- high

Low cell density- black bars
High cell density - white bars

B

Low collagen coating- black bars
High collagen coating- white bars

Day 5 (Cell density)

Day 5 (Collagen)
Mus- Muscle only culture
Co- PC12- muscle coculture
Cell- cell density
Col- collagen coating
PI- plastic culture substrate
Gl- glass culture substrate
L- low
H- high

Muscle only- black bars
Coculture - white bars

Day 5 (Innervation)

Day 5 (Substrate)

Treatment Groups
Figure 5-5 Myotube contraction at day 5 after culture in PC12DM. The effect of A) cell density, B) collagen coating, C) innervation and D) substrate on myotube contraction. All 4 graphs contain the same body of data but represented differently for ease of comparison. Data represents mean ±SEM. Activity levels of each culture were expressed as percentage of five fields that showed contraction. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Col- collagen coating, Pl- plastic culture substrate, Gl- glass culture substrate, L- low, H- high. ** p<0.01, *** p< 0.001 for Bonferroni’s test.
Figure 5-6 Myotube contractile activity of cultures grown on A) glass substrates and B) plastic substrates over time. Data represents mean ±SEM. Activity levels of each culture were expressed as percentage of five fields that showed contraction. Legend- Mus- muscle monoculture, Co- PC12 neural-muscle co-cultures, Col- collagen coating, L- low, H-high.
5.3.3 Muscle and Co-cultures on MEA

The experiments detailed in the last section showed that on glass substrates, co-cultures with high collagen and high cell density were most active over time. Given the seeming similarities in surfaces between glass and the MEA we used in our lab, the culture settings deduced from the glass well experiments were directly imported into the MEA. PC12 co-cultures and their respective muscle mono-culture controls were seeded at high densities on high collagen coated MEAs. Muscle mono-culture controls were tested as they were critical for comparison with co-cultures to ascertain the amount of activity in the latter that was neural induced and not due to spontaneous activity by aneural muscle cells.

During initial experiments on MEA’s, high cell density co-cultures upon high collagen delaminated from the MEA substrate within 48 hours after growth in PC12 differentiation media. The respective muscle mono-cultures grown at high cell density and on high collagen coating displayed persistently strong activity from day two to day 4 with most of the electrodes of the MEA detecting cellular activity at all time points. The recorded electrical activity consisted of rhythmic spikes that were synchronized across multiple electrodes (Figure 5-7A-B). The muscle mono-cultures formed a confluent layer of cells covering all the electrodes on the MEA (Figure 5-7C, D). However the presence of widespread muscle activity at day one contrasts with our optimization experiments on glass substrate whereby minimal to no contraction was noted on muscle mono-cultures grown on glass at day one regardless of collagen coating or cell density. The difference in behaviour in both muscle-only and co-cultures on MEA suggest that the MEA substrate is different to the glass substrate used in tissue culture plates. Furthermore, in order to establish an in vitro model of NPI on MEAs,
the cell densities of the co-cultures required optimisation using MEAs instead of glass culture wells.

The delamination of co-cultures from the substrate likely arose from overpopulation and the seeding density needed to be adjusted downwards to retain cells on the MEA surfaces. The density of the co-culture needed to be low enough to prevent delamination but high enough to provide signals across a significant number of electrodes for adequate statistical sampling. As explained before, a muscle monoculture control is required for the NPI in vitro model to show neuromuscular activity. As a result, a muscle monoculture must also be prepared in tandem using the same muscle cell density. Our previous work suggests that the contractile activity of muscle monocultures is usually less than PC12-muscle co-cultures. Therefore there may be fewer active electrodes in a muscle monoculture than a co-culture with the same muscle cell density but co-cultured with PC12 cells. Hence, it is more relevant to gauge the lower limits of cell density using muscle monocultures than PC12-muscle co-cultures to find the minimum density that offers adequate number of active electrodes.

Muscle cells were seeded at three different concentrations (25000 cells/cm², 50000 cells/cm² and 100000 cells/cm²) on MEA coated with high collagen coating (Figure 5-8). To measure the prevalence of cell signals across the electrodes on the MEA, the percentage of electrodes with activity was recorded on a daily basis for a week. None of the muscle monocultures delaminated from the MEA throughout the duration of the experiment. When the seeding density was low density (25000 cells/cm²), minimal electrical activity was detected by the MEA throughout the experiment (Figure 5-8). In cultures seeded at 50000 cells/cm² and 100000 cells/cm², electrical activity increased from day one and peaked before subsiding by day 6 and 7. The level of activity in 50000 cells/cm² culture was similar to 100000 cells/cm² over time. Cultures with high seeding density (100000 cells/cm²) initially show the most activity from day one to three. However medium density cultures (50000
cells/cm²) became the most electrically active group by day 4 before decreasing by day 5. A myoblast seeding density of 50000 cells/cm² produced cultures with adequate activity and of potential utility in PC12-muscle co-cultures.

PC12 cells were subsequently added to muscle cultures seeded at 50000 cells/cm² on MEA. The electrical activity of both co-cultures and muscle mono-culture controls were monitored for 11 days. By day 11, muscle mono-cultures had become electrically silent (Figure 5-9). However co-cultures continued to show activity on multiple electrodes (Figure 5-10). Note that activity in co-cultures is not synchronized across electrodes like those in early muscle mono-cultures (Figure 5-7).
Figure 5-7 Bright field images of muscle cells grown on MEA. A- MEA activity of muscle cells one day after growth in PC12DM. A'- A close up trace of electrical signals recorded by one of the electrodes at day one. B- MEA activity of muscle cells three days after growth in PC12 DM. B'- A close up trace of electrical signals recorded by one of the electrodes at day three. C and D- light microscope images of muscle monocultures on MEA at day three after growth PC12DM.

Figure 5-8 Electrical activity of muscle monocultures at three different seeding densities on MEA, with activity levels expressed as percentage of MEA electrodes active. Green- 25000 cells/cm², Blue- 50000 cells/cm², Red-100000 cells/cm². Data represents mean ±SEM.
Figure 5-9  Day 11 muscle mono-cultures on MEA with seeding density of 50000 cells/cm². A) light microscopy images of cultures. B) MEA activity trace of the monoculture at day 11, note the lack of spikes.
Figure 5-10 Day 11 co-cultures on MEA with myoblast seeding density of 50000 cells/cm² and PC12 density of 25000 cells/cm². A) light microscopy images of cultures. B) MEA activity trace of the co-culture at day 11, note that action potentials are present but not synchronised across multiple electrodes.

5.3.4 Polypyrrole coated MEAs
Having developed a culture model for neuromuscular junction formation, the initial component of an in vitro NPI model, the quality of the cell-electrode interface then required optimisation. PPy has been investigated by other authors as a potential coating material for bio-electrodes due to its favourable biocompatibility, modulus and ease of processing. More importantly the effect of different thickness PPy coatings on the signal transmission across the cell-electrode interface remained to be elucidated. PPy doped with pTS was electrodeposited onto gold electrodes of MEA using cyclic voltammetry. The electrodes on each MEA were coated with three different polypyrrole thickness (2CV, 4CV and 8CV). A total of 12 leads were coated with each thickness of polypyrrole in each MEA and the remaining 23 electrodes were left uncoated (total of 59 electrodes per MEA). The distribution of electrodes with different coating thickness was randomized from one MEA to another.

In order to assess the capacity of different PPy coating thicknesses upon electrodes to transmit biological signals from cells, the number of variables in the experiment had to be minimized. More importantly, the signal emanating from the cells should ideally be reproducible and similar across the electrodes. Judging by the variable rhythm and amplitude of signals in co-cultures on MEA, co-cultures might not be the best platform to compare the performance of electrode coating. As a result, muscle mono-cultures were used to examine polypyrrole coated MEAs instead. Monocultures had the advantage of strong and uniform activity across various electrodes that are sufficiently prevalent. Muscle cells were seeded onto the PPy coated MEA at 100000 cells/cm². This density was used to avoid delamination of cells from the MEA whilst maintaining marginally higher activity at earlier times than 50000 cells/cm² (see section 5.3.3). Due to limitations of the coating process, the electrodes can only be coated in batches of 4. Hence electrodes for each coating thickness needed to be coated in three separate batches. This potentially introduced variability owing
to subtle differences in coating between the batches. This potential confounder was further investigated by pooling together all the activity by electrodes coated in the same batch and comparing it to those coated with the same polypyrrole thickness but from different batches. The electrical activity was gauged by calculating the mean amplitude of the largest 10% of action potentials detected through each electrode. There was no difference in the mean spike amplitude detected by electrodes from different coating batches with the same thickness PPy coating (p>0.05) (Figure 5-11). Having ruled out the batch coating as a possible confounder, the activity from electrodes with each PPy coating thickness from each MEA were pooled together and compared with those with different polypyrrole thickness coating upon the same MEA (Figure 5-12). There was no statistical difference between the amplitude of activity detected by uncoated electrodes and electrodes coated with 2CV, 4CV or 8CV polypyrrole (Figure 5-12). There was a non-significant trend towards higher mean amplitude of the signal being detected in uncoated electrodes and electrodes coated with progressively less polypyrrole. However it was later discovered that due to unforeseen circumstances and faults with the coating process, the uncoated MEA leads were unintentionally coated with a fine PPy layer. As a result the data suggests an inverse relationship between PPy thickness and signal transmission from the PPy to the gold electrode whereby thicker PPy coatings attenuate the detected electrical signal. However the thickness of the PPy coating did not affect its ability to detect large spikes as all the large spikes seemed to be synchronized across all the electrodes (result not shown). This indicates that despite reduced sensitivity to electrical spikes more heavily coated PPy, they still detected the same number of large waves as those with less PPy coating. Hence coating of electrodes with varying PPy thickness did not alter the recorded signal levels significantly.
Figure 5-11 Electrical activity detected by electrodes coated in different batches. PPy was deposited onto gold electrodes on MEA at varying thicknesses—A)-2CV, B)-4CV, C)-8CV. The graphs show the mean +/- SEM of the amplitudes of the largest 10% of action potentials detected by electrodes coated in the same batch (batch A, B and C). Each batch contained n=4 electrodes, and data represents recordings from 5 MEAs.

![Figure 5-11 Electrical activity detected by electrodes coated in different batches. PPy was deposited onto gold electrodes on MEA at varying thicknesses—A)-2CV, B)-4CV, C)-8CV. The graphs show the mean +/- SEM of the amplitudes of the largest 10% of action potentials detected by electrodes coated in the same batch (batch A, B and C). Each batch contained n=4 electrodes, and data represents recordings from 5 MEAs.](image)

Figure 5-12 Detected electrical activity by electrodes coated with different thickness polypyrrole. 0-8CV represent electrodes with different thickness polypyrrole coating with

![Figure 5-12 Detected electrical activity by electrodes coated with different thickness polypyrrole. 0-8CV represent electrodes with different thickness polypyrrole coating with](image)
0CV indicating no coating and 8CV with the thickest coating. Data represents mean +/- SEM. For 0CV group, n= 115, for 2-8CV n= 60 for each group. DIV1-6- days in culture.

5.3.5 Biocompatibility of polypyrrole based trilayer actuator

Thus far, the capacity of PPy as an electrode coating material to detect biological signals from cells has been demonstrated. This opens up the possibility of using PPy to create various bio-sensing prostheses that are able to interface with biological tissues. Recently, our collaborators at the University of Wollongong have developed a polypyrrole based trilayer actuator shaped like thin strips that contracts in response to the passage of an electrical current. Theoretically, it is possible for this PPy based actuator to be directly driven by electrical activity from biological sources. This effectively condenses the role of cell detection and actuation into one construct thus simplifying the design. To enhance the anchorage of the actuator to muscles in vivo, holes were engineered into the actuator strips for tissue ingrowth. The in vivo biocompatibility of both fenestrated actuator strips and strips with no pores was assessed by implanting them into rabbit muscle.

At three days after implantation, fibrin and inflammatory cells surrounded both porous and non-porous strips (Figure 5-13). Porous strips with PPy at the cut edges of the pores partially detached (Figure 5-13 A and B) indicating PPy coating instability. By 14 days, the fibrinous exudate undergo granulomatous changes, with the strips directly surrounded by a layer of giant cells (Figure 5-14). Between the giant cells and the surrounding muscle lies a zone of granulation tissue. In porous strips, significant tissue ingrowth into the pores of the actuator was noted (Figure 5-14 A and B). Polypyrrole debris was found around porous strips while non-porous strips show minimal breakdown (Figure 5-14 C and D). By 4 weeks after
implantation, the granulation tissue continued to organize into collagen tissue, encapsulating the PPy strips (Figure 5-15). Breakdown of PPy coating continued in porous PPy strips with more PPy debris embedded in the collagenous tissue around the strip (Figure 5-15A). Non-porous PPy strips remained stable in muscle with minimal signs of PPy coating delamination. (Figure 5-15B). There was also marked fat infiltration into the collagenous tissue around the strips (Figure 5-15 A). By 8 weeks after implantation, there was further breakdown of PPy debris (Figure 5-16 A and B). The granulomatous tissue layer continued to remodel. There was progressive fat atrophy of the muscle surrounding the strip. (Figure 5-16 A-C). Again, minimal breakdown of PPy was noted in non-porous strips (Figure 5-16C).

Given the marked difference in PPy breakdown between porous and non-porous Ppy strips, the amount of PPy debris in the tissue directly apposing the strip was analysed with image analysis (Figure 5-17). At two weeks after implantation there was a statistically non-significant trend towards more PPy breakdown in porous strips compared to non-porous strips (Figure 5-17A). By 4 and 8 weeks the difference between the two treatment arms had further widened, with porous strips showing 34.7 times (p<0.01) and 18.1 times (p<0.01) more PPy debris compared to non-porous strips at 4 weeks and 8 weeks respectively (Figure 5-17 C and E). There was no significant difference in the area of tissue that was analysed for Ppy debris between the porous and the non-porous strip groups at all time points (Figure 5-17 B, D, F).

Up until this point, all the data presented on the actuators relate to actuator strips that were first coated with polypyrrole then cut with pores. One of the proposed mechanisms underlying PPy coating instability in these porous strips was that the plane between the PPy and the platinum was exposed by the cutting of pores in the strips. This allowed fluid to seep into the planes between PPy and platinum thus pushing the PPy off the platinum. To further
test this hypothesis, PPy actuator strips were either first cut with pores and then coated with PPy or processed traditionally by coating before cutting. The two types of strips were implanted into rabbit muscle for 4 and 8 weeks and the level of PPy debris was compared (Figure 5-18). Breakdown was present in both treatments, however at 4 weeks there was a trend towards more PPy debris in muscles with porous strips that were coated then cut (Figure 5-18A). This was not due to any difference in the area of tissue examined for debris (Figure 5-18B). By 8 weeks, strips that were cut then coated were markedly more stable, with PPy debris reduced by as much as 79% compared to strips coated then cut (p<0.01) (Figure 5-18C). Again this was not due to any difference in area of tissue analysed (Figure 5-18D).
Fibrinous infiltrate

A

Masons Trichrome

Delamination of polypyrrole at edge of pores

B

H&E

C

Masons Trichrome

Blood and fibrinous exudate on concave side of strip

D

H&E
Figure 5-13 Mason’s trichrome (A and C) and H&E stains (B and D) of rabbit muscles with porous (A-B) and non-porous (C-D) PPy actuator strips three days post implantation.

Figure 5-14 Mason’s trichrome and H&E stains of rabbit muscles with porous (A-C) and non-porous (D) PPy actuator strips 14 days post implantation. Note the breakdown of Ppy in porous strips (C), contrasting with the stability of the PPy layer in nonporous strips (D).
Figure 5-15 Mason’s trichrome stains of rabbit muscles with porous (A) and non-porous (B) PPy actuator strips 4 weeks post implantation.
Figure 5-16 Mason’s trichrome and H&E stains of rabbit muscles with porous (A-B) and non-porous (C) PPy actuator strips 8 weeks post implantation.
A 2 week debris level

B Area analyzed (2 weeks)

C 4 week debris level

D Area analyzed (4 weeks)

E 8 week debris level

F Area analyzed (8 weeks)
Figure 5-17 Ppy breakdown in porous and non-porous Ppy strip actuators. Histology slides of muscle specimens from two weeks (A-B), 4 weeks (C-D) and 8 weeks (E-F) were analysed for Ppy debris using image analysis. A zone of tissue 300-500um from the strip was isolated and the percentage area of this zone occupied by PPy debris calculated (A, C and E). The total area of tissue around porous and non-porous strips that was analysed was compared to ensure no bias in analysis (B, D and F). Data from 12 strips (6 porous, 6 non porous) implanted intramuscularly into three rabbits per time point. Column and error bars represent mean +/- SEM. **- p<0.01, unpaired student t-test
Figure 5.18 PPy breakdown in porous strips that were coated before holes were cut (control) and those that were cut with pores before PPy coating. PPy debris level (A and C) and the total area that had been examined for debris presence (B and D) were compared. Data from 12 strips (6 cut before coat, 6 coat before cut) implanted intramuscularly into three rabbits per time point. Column and error bars represent mean +/- SEM. **- p<0.01, unpaired student t-test.
5.4 Discussion

5.4.1 Cell density, Collagen coating, innervation, culture substrate and myotube contractility

In order to establish an MEA based platform for modelling a NPI and for testing electrode coatings for cell detection, the activity from the muscle and its respective PC12-muscle co-cultures need to be maximized. This allows for sufficient data and sample size to be collected to arrive at statistically significant results. From our previous experience with MEAs, the activity detected comes principally from myotube contraction. Even in the case of PC12-muscle co-cultures, PC12 cells were not found to show spontaneous activity when cultured alone on MEAs. Therefore the parameters that are needed to maximize myotube contractions needed to be clarified. In our study we investigated the effect of collagen coating (high vs. low), myoblast seeding density (high vs. low), substrate (glass vs. plastic) and innervation with PC12 (aneural vs. innervated muscle) on myotube contraction.

Innervation was the only factor that consistently increased contractile activity of all cultures regardless of all other parameters. Myotube contraction is a sign of maturity of the muscle excitation/contraction machinery. This result is consistent with the current literature on the importance of neural supply to muscle development. Innervation drives the maturation of multiple components of the excitation-contraction coupling process (173-175, 192, 195, 216, 218, 350). At the level of the acetylcholine receptor, innervation catalyses the switch from the immature receptor subtype containing Y subunit to the mature isoform with the \( \varepsilon \) subunit allowing the receptor to respond to high frequency stimulation (173-175). Innervation also promotes maturation of voltage gated sodium channels from the immature NaV1.5 isoform to NaV1.4 (175, 192, 195). Further downstream, innervation modifies the
intracellular calcium dynamics that underpins muscle contraction (365). In response to neural supply, ryanodine receptors on the sarcoplasmic reticulum become activated independent of calcium influx from the extracellular space via dihydropyridine receptors thus mimicking the behaviour of adult muscle (365). Finally, innervation also modulates the expression of mature subtypes of myosin heavy chains that serve as the cornerstone of the contractile machinery of myocytes (216, 218, 350). The heightened contractile activity observed in innervated cultures in our study further corroborates the maturing effect of innervation on muscles.

Plastic substrates were more conducive towards myotube contraction than glass substrate. The difference between the two substrates was more marked in muscle monocultures than in co-cultures. A possible reason for the difference in cellular behaviour between plastic and glass cultures is the difference in modulus between the two substrates. Cells have an innate ability to sense and respond to mechanical cues from the environment. Mechanical parameters such as substrate stiffness are often transduced through integrin receptors on the cell membrane into intracellular signals driving cell migration, adhesion and differentiation (379-383). Mesenchymal stem cells grown on matrix with modulus that approximates nerve, muscle and bone differentiate into neurons, myocytes and osteocytes respectively (381-383). Recent literature suggests that this mechano-transduction is modulated by the dynamic balance between cytoskeletal contraction and adhesive forces at focal adhesion complexes that bind the cell to the substrate (379, 381). The cytoskeleton of eukaryotes contains non-skeletal myosin and actin filaments that are directly attached to focal adhesion complexes. Focal adhesion complexes are multi-molecular constructs that anchor the cell to the surrounding extracellular matrix (379). The myosin and actin filaments of the cytoskeleton actuate to exert a constant pull on the adhesion complexes tending the cytoskeleton. When grown on compliant surfaces, the surface underlying the adhesion
complexes deform ever so slightly thus reducing the tension of the cytoskeleton (381). This signals the cell to regulate its phenotype. However when cells are grown on stiff substrates, the tension of the cytoskeleton increase, driving a signalling cascade that leads to increase in adhesion complexes and fibre tension, stiffening the cell (380, 384-389). In order for myoblast to fully mature, the modulus of its environment must be neither too soft nor stiff (380). In our experiments, the modulus of plastic substrate more closely approximates the modulus of muscle than glass. This is sensed by the myoblasts leading to greater maturation/contraction of muscle monocultures on plastic than on glass. The effect of substrate stiffness was less marked in co-cultures as the maturing effect of innervation likely made up for some of the deficiencies in glass surface cultures. The effect of culture substrate was more pronounced at earlier time points than at later times. At later time points, the number of cells in the culture continues to increase forming a multi-layered culture. In glass cultures, the cells directly apposing the substrate form a compliant, low-modulus “carpet” for the top layer of myoblasts to grow and conferring greater maturity and function to the top layer of cells. In fact it has been found by other authors that the top layer of myoblasts in a multi-layer culture grown on a stiff substrate are more differentiated than the bottom layer contacting the substrate (379, 380). Interestingly, plastic substrate actually decreased activity in co-cultures with high collagen and high cell density. This suggests that the cultures on plastic substrates are at a later phase characterized by decreasing activity. Myotube contraction is known to naturally plateau and subside with time. Plastic substrates may merely accelerate the natural progression of changes to muscle activity rather than necessarily increasing myotube activity. The time course of these sequential changes may have been accelerated in co-cultures with high collagen, high cell density and grown on plastic.
The effect of cell density varied with the substrate, the innervation of the muscle and the collagen coating. Cell density had no effect on activity of muscle-only cultures on glass. However, in co-cultures on glass, higher cell density increased co-culture contractions. In muscle-only cultures on plastic, high density seeding increases activity in all groups at day one. However after day one, activity in densely seeded cultures decreased. Higher seeding density changed the activity trend of myoblast monocultures on plastic from one that is shaped like a bell shape over time to a decreasing trend over time. In co-cultures grown on plastic, high cell density altered the activity trend over time of the cultures changing from an increasing trend over time to a “U” shaped trend with activity high at day one and 5 and lower at day three. It has been reported by others that high myoblast seeding density enhances differentiation (302-305). In one study, it was found that high density myotube cultures have a more “positive” resting membrane potential owing to greater Na/K ATPase activity, thus have a lower threshold for depolarization (369). The mechanism underlying the effect of cell density on myotube differentiation is likely two fold. Firstly, as explained in the last paragraph, high seeding density leads to multi-layered cultures whereby the bottom layers serve as a “feeder layer” providing a compliant substrate for growth of top layered cells (379, 381). Secondly higher cell density increases cell-to-cell contact and signalling amongst myoblasts. Cell-to-cell contact signalling molecules such as n-cadherins are known to promote myoblast differentiation \textit{in vitro} (306-308, 390). The lack of effect of high cell density on muscle monocultures grown on glass suggests that the effect of modulus is relatively more important for differentiation than cell density.

In a similar manner, the substrate modulus, cell seeding density likely accelerates the natural progression of changes in activity of myotubes rather than increasing contraction indefinitely. High cell density changed the activity trend of high collagen myotube monocultures on plastic from one that is bell shaped to a decreasing trend. It is likely that
the natural activity trend has been moved forward in time by the high seeding density so that the peak in activity is now at day one instead of day three producing the seemingly decreasing curve (Figure 5-19). The U shaped graph of activity over time in co-cultures with high collagen and high density is the addition of the activity of aneural as well as innervated muscle in culture (Figure 5-19). As demonstrated in the last chapter, the activity of aneural myotubes tend to increase, plateau and decrease while the activity of innervated myotubes increase with time. In co-cultures with lower cell density and grown on plastic, the peak in aneural myotube activity coincides with the rise activity in innervated myotubes such that that the summated activity over time shows an increasing trend. Most of the activity during the early phase (day three) is attributed to aneural myotubes while innervated myotubes account for most of the contraction at later time points (day 5) (Figure 5-19A). However when high cell density is added to co-cultures with high collagen and cultured on plastic, the peak in activity of aneural myotubes is brought forward as in monocultures while the innervated myotubes are not affected to the same extent (Figure 5-19B). When the activity trends are superimposed on each other, the overall graph resembles a “U” whereby the early peak in activity at day one is due to the aneural myotubes whose development has been expedited by high cell density and the later peak in activity is attributed to innervated myotubes (Figure 5-17B).
Figure 5-19 Conceptual diagram illustrating the summated contractile activity in co-cultures with A) low cell density and B) high cell density.

In a similar manner to cell density, the effect of collagen coating also depended on substrate, cell density and muscle innervation. On glass cultures, high collagen coating had no effect on activity of muscle-only cultures and co-culture activity except when it increased activity in high density co-cultures at day one, changing the profile of activity over time from one that increases with time to one that is shaped like a “U”. The activity was high initially before subsiding and further rebounding at day 5. On plastic cultures, high collagen coating quietened muscle activity in low density cultures but otherwise had no effect on activity in other groups. In co-cultures on plastic, higher collagen only quietened activity in high density cultures while having no effect on other groups. Collagen has long been used as a standard culture substrate for myoblasts (391-393). There are some studies that have demonstrated enhanced expression of CPK (creatine kinase) in myotubes grown on collagen coated surfaces and have concluded that collagen promotes myoblast differentiation (392). However CPK is only a static measure of differentiation and lacks a functional dimension. No
study has demonstrated the effect of collagen on myotube excitation or contraction. Collagen 1 is an abundant component of the basal lamina of muscles. It contains multiple binding sites for integrins such as α1 β1 and α2 β1 (394, 395). Integrins are known to activate multiple intracellular pathways regulating cell differentiation (197, 395-397). Our results show that the effect of collagen on myotube contraction is by no means consistent and it may interact with substrates, cell density and innervation to either increase or decrease contraction. The changing of activity profile of high density co-cultures on glass from an increasing trend to a ‘U’ trend is likely due to the same mechanism as described earlier in the previous paragraph with high cell density. The collagen interacted with high cell density in the presence of neural cells to expedite aneural myotube maturation leading to an overall U shaped trend. However in other situations it quietened the activity of co-cultures. The nature of these interactions is difficult to ascertain from our experiments and further studies are required to explore the effects of collagen on myotubes under different culture conditions. However such studies are beyond the scope of this thesis.

Apart from innervation, all parameters tested failed to show a consistent effect on myotube contraction under all conditions. This suggests there is significant interaction and even synergism between the parameters tested. Each parameter adds to or detracts from myotube development and the overall activity is a summation of various factors. In some cases the negative effects of one factor drowns out the positive effects of other parameters. In other times, the various culture conditions act synergistically to promote myotube contractility. The final trend of contractility can assume several well defined patterns depending on the number of permissive and detractive culture factors (Figure 20). At one end of the spectrum, myotube grown on harsh environments are silent throughout the experiment. With some improvements in culture conditions, the activity of myotube mono cultures assumes a bell shaped curve from day one to day 5. As the environment becomes
more permissive, the peak of the curve is pushed forward towards day one (figure 5-20). In co-cultures, even harsh conditions will still yield an increasing trend in activity over time. If the conditions are further optimized, the increasing trend becomes a “U” curve for reasons discussed earlier. From the perspective of finding the parameters that maximize activity of myotubes on glass, co-cultures with high collagen and high seeding density produced the most activity over time (Figure 5-20). Given the similarities between glass and the constituent of MEA, these conditions should be exported to MEA experiments to both establish an in vitro model of NPI and to test electrode coatings.

Figure 5-20 Conceptual diagram explaining the relationship between permissive culture conditions and overall contractile activity in myotube monocultures and PC12-myotube co-cultures. As the number of permissive factors increase, the activity of cells undergoes discrete stages of development.
There certain limitations in this experiment. Firstly the measure of contraction was obtained by observing pre-set regions of each well for a certain length of time to observe the presence of absence of contractions. Due to cyclical nature of myotube contractions, the time spent observing the well may not be sufficient to detect dormant myotubes that have just stopped or yet to start contracting. However, if one culture was more contractile than another, the dormant periods between bouts of contraction are likely shorter and therefore contractions are more probable to be detected when being observed. Secondly, the results are limited by the sample size of the experiments. Several of the comparisons seem promising but failed to show any statistical significance. A larger sample size may be required to refine the results. Finally glass substrates are an approximation of the MEA substrate. It is still possible that the optimal conditions on glass cultures may not be optimal for MEA. This issue will be addressed in the next section.

5.4.2 Establishing a NPI *in vitro* model

As explained previously, maximizing myotube activity is a critical component of designing an *in vitro* model NPI. Results from our previous experiment on myotube contractility in glass culture wells revealed a set of cell conditions; innervation, high collagen, high seeding density; that allow for maximal activity of myotubes. It was envisaged that these conditions will allow for sufficient data and therefore sample size to be collected to be able to arrive a statistically sound results. These settings were subsequently transferred to the MEA. However to our surprise, the culture peeled off just two days after PC12 cells were added to the myotubes. This indicates that the MEA substrate is different to glass used in standard culture wells. Further enquiry with the MEA manufacturer revealed that the MEA was made of a form of silicon nitride. However further details were fleeting due to intellectual property
considerations. The phenomenon of myotube culture delamination has been reported by other authors (294, 398, 399). Some studies have even used the phenomenon to generate three dimensional myoids for tissue engineering (294, 398, 399). However no study has explored the mechanism and causes of culture delamination. From first principles, the stability of a contractile culture will depend on the balance between the adhesive forces between the cell and the substrate and the contractile force exerted by the myotubes. The delamination of co-cultures is likely due to a combination of stronger contraction by innervated muscle and weakened adhesion to the MEA substrate. Stiff substrates are sensed by the cells through its adhesion complexes triggering remodelling of its cytoskeleton and upregulating focal adhesions (384, 385). Softer surfaces allow for more dynamic and less stable adhesions to form between the cell and the substrate, allowing the contractile force of the myotubes to delaminate the cells (384, 385). Given that the same culture settings did not lead to delamination in both glass and plastic wells, it can be inferred that the treated MEA surface is even more compliant than glass or plastic culture plates. Interestingly the MEA recorded persistently high activity from the muscle monocultures at one and three days after change into PC12 differentiation media while significantly less activity was detected in similar cultures on glass wells and plastic wells. This suggests that the cultures grown on MEA mature faster than its plastic and glass well counterparts. The modulus of the MEA surface may more closely approximate that of muscle than the glass and plastic wells. As discussed in the previous paragraphs, myoblast differentiate more efficiently when grown in environments with modulus that resemble muscle (381-383). This possibility is further corroborated by the longer period of activity of muscle cultures on MEA compared to those on plastic and glass cultures. Muscle monocultures on MEA show sustained activity for up to 8 days after change into PC12 differentiation media, while similar cultures on both glass and plastic culture wells show minimal activity by day 5 after change culture media. The
heightened and prolonged activity is likely mediated by a combination of weaker adhesion and greater differentiation of the myotubes on MEA.

The muscle monocultures grown on MEA displayed highly synchronized electrical activity across all electrodes. This is reminiscent of the coordinated activity noted in cardiac myocytes. Cardiac myocytes synchronize their activity by forming intercellular gap junctions (400). Gap junctions are hemi-channels consisting of 6 connexin subunits that span the cell membrane. The hemi-channels dock with each other to form intercellular conduits permitting exchange of both ions and electrolytes between cells (401). Unlike heart muscle, mature skeletal muscle do not express gap junctions as they need to ensure selective muscle activity in response to neural stimulation (402-404). However gap junctions have been detected in developing muscle (403-406). Connexin 43 and 39 have been found to be expressed during embryonic development. Both decrease to undetectable levels after birth (403-406). Connexin 43 is found in both cardiac myocytes and in myoblasts (406). It is downregulated in myotubes both in vivo and in vitro (402, 406, 407). Connexin 39 is exclusively expressed in cells of the myogenic lineage (403). It is absent in myoblasts but present in developing myotubes (403). Connexins play an integral role in myoblast differentiation (404, 407-410). The synchronicity observed in the muscle monocultures grown on MEA suggest that myotubes are still at an early stage of development and that connexins may still be expressed in the myotubes facilitating dissemination of pulsatile electrical activity. Such synchronized electrical activity is rather undesirable for a NPI as it precludes the specificity of data transmission. Activity of individual nerve fibres must only activate a discrete group of myotubes and transmitted to only a few electrodes to preserve the specificity of signals. Synchronization of muscle activity will eliminate any difference in the pattern of myotube and electrode activation conferred by different nerve fibres.
Unlike muscle monocultures, PC12-muscle co-cultures displayed asynchronous activity on the MEA. The myotubes in the co-cultures are likely to be more mature and therefore express fewer connexions than their monoculture peers leading to asynchronous activity. The activity recorded by the MEAs in co-cultures likely originates from innervated muscles as the respective muscle monocultures grown on MEAs were silent at the same time point. The difference between the co-culture and the muscle monoculture was unlikely due to paracrine effect as replacement of the original media with fresh new media did not change the activity of the two cultures (results not shown). This indicates that the MEA activity is due to NMJ formation. These results have succeeded in showing two main points. Firstly, through demonstrating the electrical “signature” produced by innervated myotubes, we have produced electrophysiological evidence of NMJ formation on a MEA. Secondly, the PC12-myotube co-culture in conjunction with the MEA serves as a preliminary in vitro model that simulates the working of a NPI. Activity of individual neuromuscular units can be picked up by discrete electrodes that can be fed into an actuator system. There has been no study at the time of writing of this thesis that has shown functional NMJ formation using a MEA or created an in vitro model of NPI. The in vitro NPI will serve as a platform for future research in optimizing the neuromuscular junction as well as the muscle-electrode junction, both critical components of a NPI.

There are few limitations to our experiment. Firstly, due to insufficient sample size, we were not able to measure numerically the difference in activity between the co-cultures and muscle-only cultures on MEA. Instead we opted to show representative traces of co-cultures and myotube monocultures. However given the stark difference in electrical trace between the two different cultures, further experimentation was likely going to arrive at the same conclusion. Secondly, PC12 cell lines were used to innervate myotubes. Although PC12 cells have been used as an in vitro model for studying neural cells, cell lines do not fully
recapitulate the behaviour of normal cells (292). However primary neural cells also have its
drawbacks. Primary neural cell cultures are heterogeneous populations containing varying
proportions of glial cells, introducing unwanted variability to the culture. In contrast PC12
cell cultures are homogenous and display reproducible behaviour, thus removing unwanted
variability from the experiments.

5.4.3 PPy coated electrodes

The in vitro model of NPI we have developed consists of two key interfaces; the
neuromuscular interface and the muscle-electrode interface. The former has been
addressed by previous chapters of this thesis. The muscle-electrode interface is critical to
the fidelity and reliability of signal recording by the NPI. Traditionally metal electrodes have
been employed to record signals from cells in vitro and tissue in vivo. However metal
electrodes have several drawbacks. Firstly, metal electrodes have significantly higher
modulus than the tissues it interfaces (16, 17). This mismatch in moduli leads to
encapsulation of the electrode by fibrous tissue in vivo denigrating electrode-tissue coupling
and signal recording (16, 17). Secondly, metal electrodes have biologically inert surfaces that
are unable to enhance cell-electrode adhesion (16, 17). Coating of metal electrodes with the
conductive polymer polypyrrole has become a viable alternative in improving tissue-
electrode coupling. Unlike metals, polypyrrole has a lower modulus closer to biological
tissues and a higher surface area that both promotes cell attachment and reduces electrical
impedance (17). In this study, we examined the effect of coating the gold electrodes of the
MEA with different thickness of polypyrrole doped with pTS; a biocompatible conducting
polymer; on signal recording at the muscle-electrode interface. We found that the thickness
of polypyrrole coating had no effect on the amplitude of signals recorded by the MEA.
No study has examined the effect of polypyrrole/pTS coating thickness on the ability of MEA to record signals from cells. Based on current literature, increasing the thickness of polypyrrole coating allows the small globules that define PPy surfaces to coalesce forming “cauliflower” shaped structures thus increasing both surface roughness and surface area (237, 250-253). The increased surface area will enhance double layer capacitance at the electrode surface reducing the impedance of the electrode (Figure 5-21)(229, 230, 235, 238, 250). The rougher surface texture will allow for greater protein adsorption from the media, facilitating cell spreading and adhesion (411-424). The cell spreading maximizes the area of the junctional membrane of the cell that apposes the electrode reducing the impedance of the cell membrane (Figure 5-21). The heightened cellular attachment mediated by greater focal adhesions pulls the cell closer to the electrode minimizing signal degradation that is associated with distance (Figure 5-21)(229, 230, 235). It also promotes coverage of electrodes by cells thus increasing seal resistance and reducing current leakage (Figure 5-21) (230, 239-243). However, our results seem to contradict these assumptions as thicker coating failed to show any benefit in signal recording. The apparent discrepancy can be reconciled by consideration three further factors. Firstly, the relationship between polypyrrole thickness and roughness/surface area varies as the thickness increases (253, 254). Initially as thickness increases, surface area and roughness increase and impedance drops exponentially (253, 254). However as more polypyrrole deposits onto the electrode the conformation of the polymer changes to one that is less ‘open’ and more densely organized (253, 254). As a result roughness and surface area plateaus and in some cases reduces marginally leading to some rise in impedance. The exact thickness of polypyrrole deposited onto the electrodes in this experiment cannot be ascertained due to limitations in the manufacturing process. It may be possible that the thickness range tested lies within the ‘plateau’ phase of the thickness to impedance/roughness curve. Secondly, rougher surfaces do not necessarily increase cell adhesion. Cell adhesion may also depend on the nature of
the micro/nanostructure of the surface. Cells grown on rough surfaces with micro-pillars only attached preferentially to the tips of the pillars and overall adhesion did not increase with roughness (425). In another study, the adherence of myoblasts to PPy/pTS films was less for thicker films than thinner films (252). As the thickness of the polypyrrole increases, the surface topography may undergo less predictable changes that may alter the relationship between roughness and cell adhesion. Thirdly, the cell-electrode interface may not be the chief determinant of signal recording. Little attention has been focused on the conduction of charge through the substance of the polypyrrole coating as the thickness rises. Some authors have found that the thickness of PPy coating had no effect on its conductivity (238). Others have noted that the mechanism of charge transfer varies as the thickness of polypyrrole increases. Charges are actually more mobile in thinner films less than 300nm than thicker polypyrrole films (426). This may detract from any gain in signal recording conferred by increased surface area and cell attachment secondary to rise in polypyrrole thickness. As discussed thus far, changes in polypyrrole thickness impact on multiple parameters of electrode-cell electrical coupling equation. To definitively assess the effect of each individual parameter on electrode performance, each variable must be studied in isolation.
Figure 5-21 Schematic diagram of an electrical circuit that represents the cell-electrode electrical coupling on the MEA. The electrode and the cell are separated by a thin film of culture media. Both the junctional membrane and the electrode surface can be represented by a RC (resistor/capacitor) circuit. As the surface area of both the electrode and the junctional membrane increase due to increasing PPy coating and cell spreading respectively, the capacitance of the system also increases thus reducing impedance. As electrical activity travels out of the cell, some activity degrades over the fluid layer between the cell and the electrode. Some current is lost through “leakage”. The amount of current leakage is determined by the ‘seal’ that cell processes develop with the electrode which in turn is determined by the cell adhesion. Image created by author. Information adapted from (229, 230).

The experiment was initially designed to also answer the question as to whether polypyrrole coating enhanced the signal recording of MEA compared to plain gold electrodes. Each MEA contained plain gold electrodes as well as those coated with different thickness polypyrrole. However due to issues with the coating process, the control gold electrodes were inadvertently coated with low levels of polypyrrole. This precludes any comparison between gold electrodes and coated electrodes. Despite a lack of statistical significance, there was a trend towards greater signal amplitude in the “gold” electrode group which further lends support findings by Valaski et al. that thin polypyrrole films have high electrical conductivity compared to thicker ones due to differences in conducting mechanism (426). A large amount of variability in signal amplitude was noted from signals recorded by each electrode and across electrodes on the MEA. The shape and strength of signals recorded by each electrode is a product of the relative orientation and position of the cell to the electrode. In order to minimize the impact of signal variation we have increased the number of MEAs used and randomized the position of the electrodes with different thickness coating on each MEA so
that each thickness group will sample from equal portions of cells with different alignments and positions. Despite these attempts, large variations still exist. Future studies should aim to use even greater sample sizes to minimize the effects of data variability. Muscle monocultures were used to examine the muscle-electrode interface instead of PC12-muscle co-cultures in order to minimize the variability. Addition of PC12 cells to the system adds another level of complexity that will only add to the variable data that characterizes MEA recording of muscle monocultures.

Our results have direct implications in the design of electrodes in NPI. We have shown that polypyrrole coated electrodes can be used to record biological activity and that altering the thickness of the coating does not confer any advantages in signal recording. Future studies should aim to corroborate our findings in vivo by implanting MEA with different thickness of polypyrrole into animal muscle and to assess the EMG recorded by the electrodes.

5.4.4 Biocompatibility of Polypyrrole based trilayer actuator

Signals from a NPI will be used to control an actuator system to restore function to amputated limbs. Our collaborators from the intelligent polymer institute have developed a novel trilayer polypyrrole based actuator that deflects in response to passage of electrical current (288, 427, 428). The polypyrrole coating will allow the strip to sense electrical activity from muscle and utilise it to actuate thus obviating the need for complex gears and external power sources that power current prosthetic limbs. In order for it to be used in muscle augmentation, the strip needs to be anchored to existing tendons, ligaments and muscle. To facilitate attachment of the strip to surrounding structures, we engineered pores
into the strip to allow for tissue ingrowth. In this study, we investigated the feasibility of using these actuators for muscle replacement and augmentation by first examining \textit{in vivo} biocompatibility and the effect of strip pores on the \textit{in vivo} response.

As expected there was a foreign body response to the implantation of the actuator strip. The strip was first surrounded by fibrin, blood and inflammatory cells. Over time the exudate began to organize and form granulomatous tissue. Giant cells enveloped the strips and the surrounding tissue was invaded by new vessels and macrophages. By 4 to 8 weeks, the tissue progressively became more collagenous and less cellular forming a layer of collagen around the actuator strip. Similar results have been reported by others with polypyrrole coated electrodes implanted both subcutaneously and in the CNS of animals (6, 248, 256-259). The tissue responses were comparable and sometimes less than common biological scaffolds currently used for implantation such as Teflon and PLGA (6, 248, 256-259). However, most studies have not examined the stability of polypyrrole \textit{in vivo}. Our results show that the presence of pores in the polypyrrole strip destabilizes the coating causing the polypyrrole to delaminate from the platinum substrate. Studies that have examined the stability of polypyrrole coatings have been conducted \textit{ex vivo} in simulated physiological solutions (260-262). Most cases of polypyrrole delamination occur after chronic electrical stimulation (260-262). It was postulated that the redox reactions secondary to stimulation lead to cyclical volume changes to the polypyrrole predisposing it to peel off from its substrate (261). Other authors have attributed the instability to the significant levels of $\alpha$-$\beta'$ coupling in its chemical structure leading to defect sites on its polymer backbone (263, 264). The defect sites are thought to be primary sites of breakdown in response to oxidation. The propensity for delamination has been linked to the thickness of the conductive polymer coating (429). However these explanations do not apply to our setting as the non-porous polypyrrole strip was relatively stable through the experiment. The key difference between the two
treatment arms was the presence of pores in the group that showed delamination. Furthermore, signs of delamination at the edges of the pores were visible as early as three days after implantation of the strips. The pores were likely related to the delamination mechanism. It was thought that by cutting pores in the strips, the interface between the polypyrrole and the platinum was exposed at the edges of the pores. Overtime, biological fluids, cells will infiltrate between the layers delaminating the polypyrrole. This is supported by the finding that sealing the pore edges by first cutting the pores and then coating with polypyrrole significantly reduced the amount of polypyrrole debris.

There are some limitations to our study. Firstly the quantification of polypyrrole debris was achieved through image analysis. Therefore, the measure is subject to sampling bias. However all attempts were made to sample the same area of tissue for both treatment arms. Secondly, during the image analysis process, the area occupied by polypyrrole debris was distinguished from surrounding regions by its black pigment. However sometimes small amounts of dark coloured cell nuclei may also have been detected by the computer algorithm and incorporated into the debris area measure. However the amount of dark nuclei with intensity levels approaching black is small for each slide image and is unlikely going to impact the large differences noted between the treatment arms. Our results serve to inform future design of polypyrrole actuators and improving the stability of polypyrrole in vivo. Polypyrrole actuators should either avoid producing pores or have the edges of the pores coated with polypyrrole to minimize coating delamination. Other methods that may potentially improve coating stability include roughening the underlying substrate. It has been reported that laser roughening of the metallic substrate augments the stability of the conductive polymer coating (430).
5.5 Conclusion

In this chapter, we have developed a MEA based *in vitro* model of NPI using primary mice myotubes and PC12 neural cells. The model will serve as a platform for future studies on optimizing various components of the NPI before *in vivo* experimentation. We have also demonstrated that polypyrrole coated electrodes in a NPI are capable of detecting electrical signals from muscle cells and that thickness of the coating does not impact on the signal transduction at the cell-electrode interface. This knowledge will be valuable in informing future design of NPI electrodes for *in vivo* implantation. Furthermore we completed preliminary biocompatibility studies on a polypyrrole based trilayered actuator that may potentially be connected to the NPI for actuation. We discovered that fenestration of the actuators with pores should not be attempted unless the edges of the pores can be sealed with polypyrrole as this enhances the stability of the polypyrrole coating preventing delamination. Future design of similar polypyrrole based trilayer actuators should take these experimental findings into account to avoid degradation of the coating and subsequent loss of conductivity and actuation *in vivo*.

Chapter 6

6. Future directions on development of neuromuscular prosthetic interfaces.

Limb amputation due to trauma or disease is a significant source of morbidity for the patient physically and mentally. Advances in material science, engineering and biomedicine have led to development of various bio-artificial devices that can compensate the lost limb. This thesis has explored the feasibility of constructing a neuromuscular prosthetic interface (NPI) for better integration of the prosthesis to the body. It evaluated means of optimizing both
the nerve-muscle and the muscle electrode junction of the NPI. Furthermore the biocompatibility of some of the novel polymer actuators that may one day power artificial limbs was also investigated. The next step in the research should aim to further incorporate these findings of the individual components of the NPI-NMJ, MEJ and actuators- into one construct. However, further research into each of these components is required before the findings can be truly combined. From the NMJ perspective, the thesis explored the use of agrin and laminin to prime muscle for innervation thus enhancing NMJ formation between the nerve and muscle in an NPI. We conducted in vitro experiments to examine the complex relationship between aneural acetylcholine receptor prepatterning and innervation. Our work showed that aneural AChR clustering agents agrin and laminin successfully enhanced innervation of muscle by neural cells forming increasing number of functional NMJ. However, in vivo experiments are required to further evaluate the effects of agrin and laminin treatment in animals. Ideally the animal muscles should be denervated and further treated with a combination of agrin and laminin before being neurotised by an implanted nerve. Demonstration of enhanced functional recovery in treated animals would further shed light on the feasibility of using agrin and laminin treatment in the setting of constructing a NPI.

At the level of the MEJ, we developed an in vitro model of the NPI based on MEAs to be used as a platform for optimization of the MEJ. The effect of conducting polymer polypyrrole coating on the signal transduction at the MEJ was assessed using MEAs. Polypyrrole has high impedance and lower modulus compared to traditional metal electrodes and are amenable to biological modification (6, 7, 246, 247). Our results show that signal transduction at the MEJ did not change with the range of Ppy thickness that we examined. However it does not exclude difference in signal transmission at lower thickness values than those tested in this thesis. In addition to thickness, there are many other parameters of the Ppy coating that may impact on the signalling such as roughness, more specifically the type of micro and
nanostructure. Cells can be specific in their response to varying microscale features of the substrate surface (233, 415). The Ppy electrode coating can also be loaded with biomolecules to enhance cell adherence (246-249). It is unclear how the introduction of these biological cues will impact on the final signal transfer at the MEJ. The enhanced cell adhesion may enhance seal resistance. However the biological molecules may dampen the signal as it transverses an extra layer before reaching the electrode. The efficacy of these electrodes must ultimately be examined in an in vivo environment by direct implantation into muscle. The level of fibrous encapsulation and the durability, fidelity of signal recording over time must then be examined. Finally we tested the biocompatibility of new class of polypyrrole based trilayered polymer actuators. Polymer actuators are capable of generating immense stress and have power densities that greatly exceed muscle (270, 283-285). We found that polymer actuators without pores to help muscle interdigitation showed minimal fibrous encapsulation. In actuator strips with pores, actuators that had pores cut after polypyrrole coating showed more delamination of the polypyrrole compared to those that were cut with pores before polypyrrole coating. We proposed that the difference was due to planes between the polypyrrole coating and the underlying platinum/PVDF membrane being exposed to the tissue fluid in the former. Over time, fluid flows into the potential space in the planes beneath the PPy layer leading to delamination. Despite major improvements in tri-layer actuator design, the mechanical properties of the actuators need to be improved upon before in vivo use. The Ppy actuators tend to suffer from low strain and slow strain rate (267, 268, 270, 281). We also found that the actuators can become friable after prolonged implantation. Given the rapid rate of progress recently in the conductive polymer actuator field, it won’t be long before such materials become available.
The NPI proposed in this thesis suffers from several shortcomings. Firstly it will still have fibrous encapsulation of the electrode when it is implanted into muscle. Compared to direct nerve to electrode interfaces, the muscle in the NPI serves as an intermediate modulus cushion connecting the nerve to the electrode thus minimizing the modulus mismatch between the electrode and tissue. NPI likely reduces the fibrous reaction compared to nerve-electrode constructs, but it doesn’t eliminate it. Over time the growth of fibrous tissue between the electrode and the muscle will eventually detract from the on signal at the MEJ precluding long term use. Secondly, at the NMJ level, the NPI is limited by the amount of free muscle available for neurotisation. It requires neurotisation of muscle that has been de-functioned by the amputation of the limb. Ultimately this limits the number of
neuromuscular units available to drive the prosthetic limb and reduces the complexity of motions possible by the limb. In order to address these drawbacks, we propose a further extension to the NPI model. Instead of using native muscle, the new construct will use tissue engineered muscle that are embedded within the substance of the electrode coating thus eliminating the MEJ (Figure 6-1). The new construct will use a conducting hydrogel coating for the electrodes which can act as a three dimensional scaffold for muscle cell growth. Conductive hydrogels are a composite of hydrogel and conductive polymers (17, 431). Hydrogels consist of networks of polymers held together by chemical crosslinks (17, 431). They contain very high water content and are therefore soft with modulus approaching normal tissue. Blending hydrogel with polypyrrole aims to retain the electroactive performance of conducting polymers while imparting a low modulus environment for muscle tissue engineering (432). Conductive hydrogels have recently gained traction in the neuro-prosthetic community as a new alternative to conductive polymers. They have been shown to be capable of accommodating live cells (432-435). The conductive hydrogel seeded with muscle cells essentially merges the muscle and the electrode coating components of the NPI into one, thus forming a “biological” electrode coating. Similar concepts have been proposed by others in the past (17). The new construct will prevent invasion of fibrous tissue between the cells and the electrode coating. Secondly, the use of tissue engineering ensures a theoretically infinite supply of muscle for neurotisation by the nerve fibres of the amputated limb. This will allow a large number of neuromuscular units to be created supplying data for complex activities by the prosthetic limbs.

Main challenges in creating the new NPI model will mainly rest with the design of the conductive hydrogel electrode coating. Firstly the scaffold overlying each electrode must be electrically isolated from each other to prevent neuromuscular activity from one cluster of NMJ overlying an electrode to be recorded by all the electrodes thus degrading the
specificity of the recording. Secondly, the modulus of the conductive hydrogel must be within the optimal range for muscle differentiation. It must also be capable of sustaining muscle cell growth over long periods. These issues present unique challenges to both material scientists as well as tissue engineers. There is also the problem with interpreting the recorded signal from the new NPI construct. Neurites from one nerve may sprout and innervate multiple muscles. Conduits may be required to guide individual nerves to specific muscle sites. Another solution to the problem is to allow free innervation of engineered muscle by individual nerves and to “learn” the recording patterns associated with each movement. In such a system, the patient will be asked to think about moving and doing various activities with the limb as if the previous limb was there. This activates the specific nerves and associated muscles constructs on the electrodes. The recorded activity is then learned by an external computer and used to interpret future activities from the electrodes. This will allow intuitive usage of the prosthetic limb and obviates the need to map out the function of individual axons within each nerve fibre. The proposed NPI offers hope for better connection between the prosthetic limb and the human body. It can potentially provide high fidelity and volume data transfer between the body and the artificial arm, allowing for complex activities over multiple joints on the prosthesis. Further improvements in the construction and design of the NPI require multidisciplinary collaboration between material scientist tissue engineers, biologists and clinicians.
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