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Regulation of Oligodendrocyte differentiation through Neuroligin-Neurexin signalling

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Regulation of Oligodendrocyte differentiation through Neuroligin-Neurexin signalling

by

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Abstract

The neuroligin (NL) gene family has been well characterized in neurons by virtue of its role as a synaptic cell adhesion molecule, and because mutations in the gene have been associated with Autism Spectrum Disorder (ASD). However much less is known about its role in the glial cell population, where one of the subtypes, neuroligin 3, is expressed. The work presented here investigates NL's role in oligodendrocytes (OLs), a subtype of glial cells responsible for the myelination in the central nervous system. This was accomplished by first determining NL's presence in this cell type in hippocampal dissociated cultures by immunocytochemistry and *in situ* hybridization. It was found that by interfering with the interaction of NL and its pre-synaptic partner neurexin (NX), the developmental profile of OL cells was delayed. Conversely, enhancing the interaction accelerated the developmental profile. Evidence is also presented that glutamate receptors are potentially spatially localized to the contact points between oligodendrocytes and axons in response to the interaction between NL and NX. As mutations in NL have been linked to autism, this provides a new potential mechanism by which the cognitive deficits observed could occur.

Acknowledgements

I would like to thank my supervisor Dr. Michael Colicos for not only allowing me to conduct research in his lab, but also for making me the scientist which I am today. Working with Michael over the past 5 years, I have grown both as a lab technician and as a person. I would also like to thank Dr Campbell Teskey and Dr. Grant Gordon for agreeing to serve on my supervisor committee and for providing insightful comments in regards to my progress as a student.

I would also like to thank Dr. Dustin Proctor for helping me hurdle all the obstacles which I ran into during my research and for being an invaluable reference in the lab. I would like to thank Dr. Robyn Flynn and Dr. Carolina Gutierrez as well for helping me out when I first started in the Colicos lab; giving me tidbits of helpful advice over the past few years has made my life in research that much more understandable and enjoyable. Thank you Dr. Cristiane de la Hoz for all the help on the *in situ* experiments, I would not have been able to do it alone. I would also like to thank all the summer students who have joined us over the past five years. I have learned much from these young minds during their time in the lab.

Dedication

I would like to dedicate this thesis to my family for loving and supporting me throughout all my endeavours in life. To my brothers, for always showing me love, to my dad, for teaching me compassion, and to my mom, I would like to thank you Dr. Kileen Tucker Scott; you have always taught me to strive for excellence and I hope with this body of work I have made you proud.

Finally, I would like to dedicate this thesis is to my beautiful wife, for loving me unconditionally when I know I have made it hard for you to do so. Thank you for giving me the inner strength to keep going, for being my guiding star, for being my good karma.

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List of Figures and Illustrations

List of Symbols and Abbreviations

AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
ASD	Autism Spectrum disorders
BME	Basal Media Eagle
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Caspr	Contactin-associated protein
CaCl	Calcium chloride
CFP	Expression plasmid – Cyan fluorescent protein
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CO_2	Carbon Dioxide
CP-AMPAR	calcium-permeable AMPA receptors
DEPC	diethylpyrocarbonate
DPBS	Dulbecco's Phosphate buffered saline
DS	Donkey serum
EBS	extracellular bath solution
ER	endoplasmic reticulum
E/I	excitatory/inhibitory
FBS	fetal bovine serum
GFP	expression plasmid – Green fluorescent protein
GS	Goat serum
HBSS	Hank's Balanced Salt Solution
HEK cell	Human Embryonic Kidney cell
HEPES	4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid
K^+	Potassium
KCl	Potassium Chloride

KO	knock-out
LiCl	Lithium Chloride
MCS	multiple cloning site
MBP	Myelin Basic Protein
$MgCl_2$	Magnesium Chloride
mRNA	messenger RNA
NaCl	Sodium Chloride
NF	Neurofilament
$NaHPO_4$	Sodium Phosphate
NL	Neuroligin
NISA	soluble NL peptide
NLR473E	soluble NL peptide mutation
NMDA	*N*-methyl-*D*-aspartate
NMDAR	*N*-methyl-*D*-aspartate receptor
NP40	Terigtol
NX	Neurexin
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PFA	paraformeldehyde
PNS	Peripheral nervous system
PSD	post-synaptic density
ROI	region of interest
Rpm	rotations per minute
SCM	Slice culture media
SEM	standard error of the mean
TEA	triethanolamine
TX	Triton X

CHAPTER 1: GENERAL INTRODUCTION

1. Introduction

During normal brain development, the trans-synaptic interaction between pre-synaptic neurexin (NX) and post-synaptic neuroligin (NL) is an essential event for synaptic maturation (Nguyen and Südhof, 1997 & Rao *et al.*, 2000). Although this interaction is not necessary for initial synapse formation, through the use of knock-out (KO) models, it has been found to be essential for proper synapse function in the mature animal (Missler, *et al.*, 2003; Varoqueaux *et al.*, 2006; Chubykin *et al.*, 2007). The interaction between NX and NL is also of great interest due to the fact that mutations in these genes have been associated with autism. The discovery of these mutations led to numerous questions about how neuroligin regulates the transfer of information between neurons, and how these mutations interfere with normal synaptic function. One of the neuroligin subtypes, neuroligin 3, is also expressed in the glial cell population. Although NL has been characterized quite well in neurons, limited research has looked into what role NL plays in these cells.

Clinical imaging has revealed differences in the volume and composition of white matter in the autistic brain compared to normal brains (Courchesne *et al.*, 2001; Sparks *et al.*, 2002; Herbert *et al.*, 2003a). This finding suggests that a difference in myelination could be an underlying factor in the cognitive deficits seen in autistic patients. In this thesis I show NL-NX interactions impact the developmental state of myelinating cells in the central nervous system, supporting the hypothesis that observed autism associated mutations in neuroligin could contribute to a dysregulation of myelination and consequently contribute to the pathology of the disorder.

1.1 Trans-synaptic proteins

Synapses mediate communication between neurons and play an integral role in how information is processed in the central nervous system (CNS). In the CNS there are two fundamental synapse types, excitatory synapses and inhibitory synapses. An excitatory synapse increases the likelihood of an action potential propagating from the pre-synaptic cell to the post-synaptic cell where as an inhibitory synapse decreases the probability of an action potential in the post-synapse. The excitatory and inhibitory balance (E/I) amongst synapses is a highly regulated process which requires recruitment of proteins that dictate the specificity of these synaptic contacts.

1.1.1 Neuroligin

Neuroligins (NL) are post-synaptic, heterophilic cell adhesion, type-1 transmembrane molecules which are capable of inducing de novo synaptogenesis as well as regulate dendritic spine density (Ichtchenko *et al.*, 1995; Ichtchenko *et al.*, 1996). The neuroligin family is composed of five sub-types (NL1-5). These subgroups were discovered in 1995 by Thomas Südhof, who, upon his discovery claimed that he found the key to how synapses form and thus held the answers to the development of the brain (Garber, 2007).

Neuroligin family members 1-4 share roughly 70% homology in their amino acid sequences (**Fig.1.1**) resulting in similar extracellular domains amongst their subtypes (Ichtchenko *et al.,* 1996; Bolliger *et al*., 2001). They are composed of five distinct domains; a cleavable N-terminal hydrophobic domain, a large extracellular esterase homology domain, a linker or EF-hand binding domain, a single transmembrane region and a short cytoplasmic C-terminal tail (Araç *et al*., 2007; Ichtchenko *et al.,* 1996; Nguyen and Südhof 1997). NL1 is localized predominantly at glutamatergic, or excitatory synapses while NL2 is localized predominantly at GABAergic, or inhibitory synapses (Song *et al.* 1999) (**Fig.1.2**). Low levels of NL3 have been reported at both excitatory and inhibitory

synapses as well as in glial cell types, including astrocytes. NL4 has been poorly characterized and its localization has not been well established (Tabuchi *et al.*, 2007; Varoqueaux *et al*., 2006).

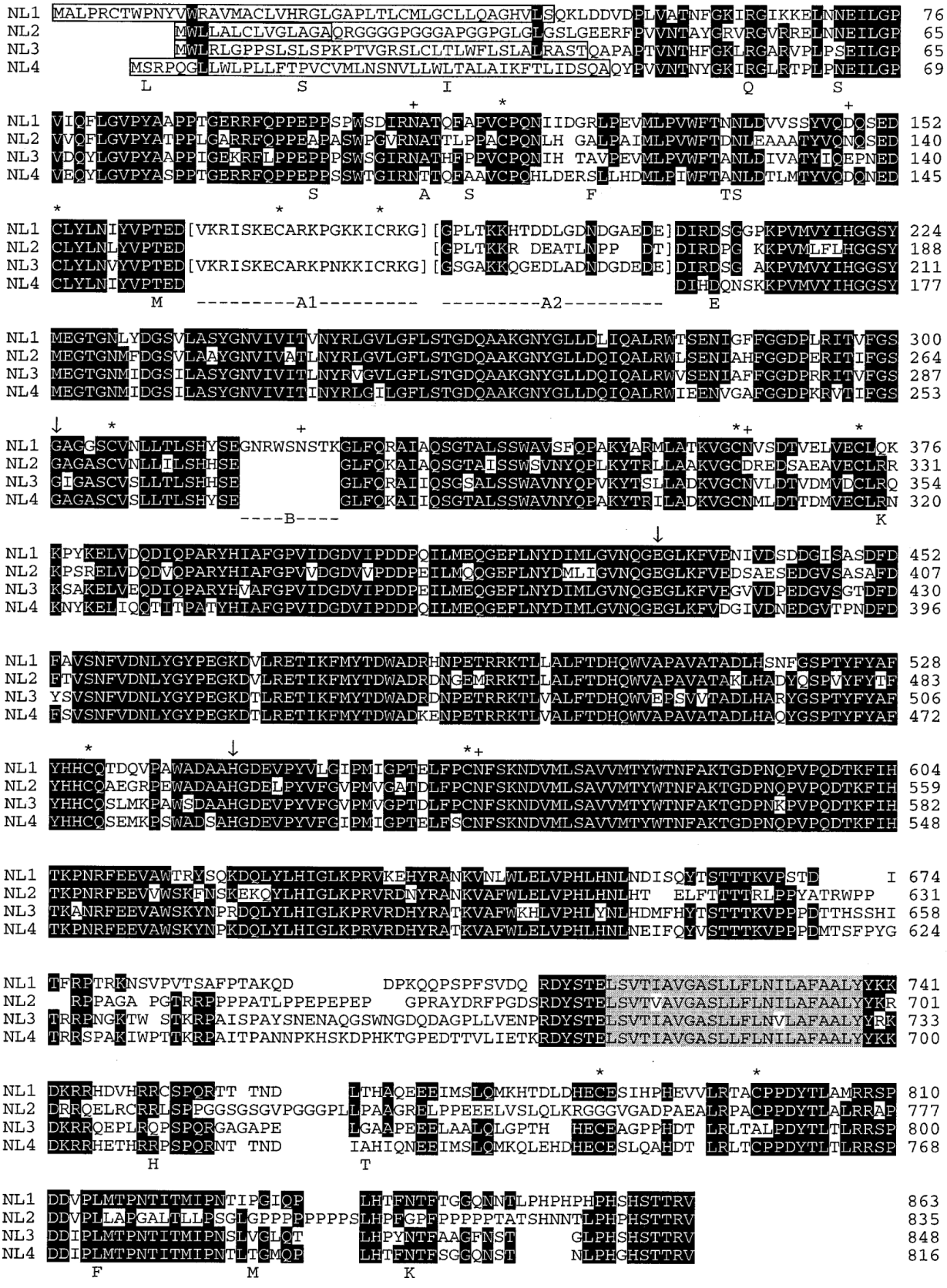
NL1 MALPRCTWPNYVWRAVMACLVHRGLGAPLTLCMLGCLLQAGHVLSQKLDDVDPLVATNFGKIRGIKKELNNEILGP 76
NL2 MWLLALCLVGLAGAQRGGGGPGGGAPGGPGLGLGSLGEERFPVVNTAYGRVRGVRRELNNEILGP 65
NL3 MWLRLGPPSLSLSPKPTVGRSLCLTLWFLSLALRASTQAPAPTVNTHFGKLRGARVPLPSEILGP 65
NL4 MSRPQGLLWLPLLFTPVCVMLNSNVLLWLTALAIKFTLIDSQAQYPVVNTNYGKIRGLRTPLPNEILGP 69
NL1 VIQFLGVPYAAPPTGERRFQPPEPPSPWSDIRNATQFAPVCPQNIIDGRLPEVMLPVWFTNNLLVVSSYVQDQSED 152
NL2 VVQFLGVPYATPPLGARRFQPPEAPASWPGVRNATTLPPACPQNLH GALPAIMLPVWFTDNLEAAATYVQNQSED 140
NL3 VDQYLGVPYAAPPIGEKRFLPPEPPPSWSGIRNATHFPPVCPQNIH TAVPEVMLPVWFTANLLIVATYIQEPNED 140
NL4 VEQYLGVPYASPPTGERRFQPPEPPSSWTGIRNTTQFAAVCPQHLDERSLLHDMLPIWFTANLDTLMTYVQDQNED 145
NL1 CLYLNIYVPTED[VKRISKECARKPGKKICRKG][GPLTKKHTDDLGDNDGAEDE]DIRDSGGPKPVMVYIHGGSY 224
NL2 CLYLNLYVPTED [GPLTKKR DEATLNPP DT]DIRDPG KKPVMLFLHGGSY 188
NL3 CLYLNVYVPTED[VKRISKECARKPNKKICRKG][GSGAKKQGEDLADNDGDEDE]DIRDSG AKPVMVYIHGGSY 211
NL4 CLYLNIYVPTED DIHDQNSKKPVMVYIHGGSY 177
M ---------A1--------- ---------A2--------- E
NL1 MEGTGNLYDGSVLASYGNVIVITVNYRLGVLGFLSTGDQAAKGNYGLLDLIQALRWTSENIGFFGGDPLRITVFGS 300
NL2 MEGTGNMFDGSVLAAYGNVIVATLNYRLGVLGFLSTGDQAAKGNYGLLDQIQALRWLSENIAHFGGDPERITIFGS 264
NL3 MEGTGNMIDGSILASYGNVIVITLNYRVGVLGFLSTGDQAAKGNYGLLDQIQALRWVSENIAFFGGDPRRITVFGS 287
NL4 MEGTGNMIDGSILASYGNVIVITINYRLGILGFLSTGDQAAKGNYGLLDQIQALRWIEENVGAFGGDPKRVTIFGS 253
NL1 GAGGSCVNLLTLSHYSEGNRWSNSTKGLFQRAIAQSGTALSSWAVSFQPAKYARMLATKVGCNVSDTVELVECLQK 376
NL2 GAGASCVNLLILSHHSE GLFQKAIAQSGTAISSWSVNYQPLKYTRLLAAKVGCDRED SAEAVECLRR 331
NL3 GIGASCVSLLTLSHHSE GLFQRAIIQSGSALSSWAVNYQPVKYTSLLADKVGCNVLDTVDMVDCLRQ 354
NL4 GAGASCVSLLTLSHYSE GLFQKAIIQSGTALSSWAVNYQPAKYTRILADKVGCNMLDTTDMVECLRN 320
----B---- K
NL1 KPYKELVDQDIQPARYHIAFGPVIDGDVIPDDPQILMEQGEFLNYDIMLGVNQGEGLKFVENIVDSDDGISASDFD 452
NL2 KPSRELVDQDVQPARYHIAFGPVVDGDVVPDDPEILMQQGEFLNYDMLIGVNQGEGLKFVEDSAESEDGVSASAFD 407
NL3 KSAKELVEQDIQPARYHVAFGPVIDGDVIPDDPEILMEQGEFLNYDIMLGVNQGEGLKFVEGVVDPEDGVSGTDFD 430
NL4 KNYKELIQQTITPATYHIAFGPVIDGDVIPDDPQILMEQGEFLNYDIMLGVNQGEGLKFVDGIVDNEDGVTPNDFD 396
NL1 FAVSNFVDNLYGYPEGKDVLRETIKFMYTDWADRHNPETRRKTLLALFTDHQWVAPAVATADLHSNFGSPTYFYAF 528
NL2 FTVSNFVDNLYGYPEGKDVLRETIKFMYTDWADRDNGEMRRKTLLALFTDHQWVAPAVATAKLHADYQSPVYFYTF 483
NL3 YSVSNFVDNLYGYPEGKDTLRETIKFMYTDWADRDNPETRRKTLVALFTDHQWVEPSVVTADLHARYGSPTYFYAF 506
NL4 FSVSNFVDNLYGYPEGKDTLRETIKFMYTDWADKENPETRRKTLVALFTDHQWVAPAVATADLHAQYGSPTYFYAF 472
NL1 YHHCQTDQVPAWADAAHGDEVPYVLGIPMIGPTELFPCNFSKNDVMLSAVVMTYWTNFAKTGDPNQPVPQDTKFIH 604
NL2 YHHCQAEGRPEWADAAHGDELPYVFGVPMVGATDLFPCNFSKNDVMLSAVVMTYWTNFAKTGDPNQPVPQDTKFIH 559
NL3 YHHCQSLMKPAWSDAAHGDEVPYVFGVPMVGPTDLFPCNFSKNDVMLSAVVMTYWTNFAKTGDPNKPVPQDTKFIH 582
NL4 YHHCQSEMKPSWADSAHGDEVPYVFGIPMIGPTELFSCNFSKNDVMLSAVVMTYWTNFAKTGDPNQPVPQDTKFIH 548
NL1 TKPNRFEEVAWTRYSQKDQLYLHIGLKPRVKEHYRANKVNLWLELVPHLHNLNDISQYTSTTTKVPSTD I 674
NL2 TKPNRFEEVVWSKFNSKEKQYLHIGLKPRVRDNYRANKVAFWLELVPHLHNLHT ELFTTTTRLPPYATRWPP 631
NL3 TKANRFEEVAWSKYNPRDQLYLHIGLKPRVRDHYRATKVAFWKHLVPHLYNLHDMFHYTSTTTKVPPPDTTHSSHI 658
NL4 TKPNRFEEVAWSKYNPKDQLYLHIGLKPRVRDHYRATKVAFWLELVPHLHNLNEIFQYVSTTTKVPPPDMTSFPYG 624
NL1 TFRPTRKNSVPVTSAFPTAKQD DPKQQPSPFSVDQ RDYSTELSVTIAVGASLLFLNILAFAALYYKK 741
NL2 RPPAGA PGTRRPPPPATLPPEPEPEP GPRAYDRFPGDSRDYSTELSVTVAVGASLLFLNILAFAALYYKR 701
NL3 TRRPNGKTW STKRPAISPAYSNENAQGSWNGDQDAGPLLVENPRDYSTELSVTIAVGASLLFLNVLAFAALYYRK 733
NL4 TRRSPAKIWPTTKRPAITPANNPKHSKDPHKTGPEDTTVLIETKRDYSTELSVTIAVGASLLFLNILAFAALYYKK 700
NL1 DKRRHDVHRRCSPQRTT TND LTHAQEEEIMSLQMKHTDLDHECESIHPHEVVLRTACPPDYTLAMRRSP 810
NL2 DRRQELRCRRLSPPGGSGSGVPGGGPLLPAAGRELPPEEELVSLQLKRGGGVGADPAEALRPACPPDYTLALRRAP 777
NL3 DKRRQEPLRQPSPQRGAGAPE LGAAPEEELAALQLGPTH HECEAGPPHDT LRLTALPDYTLTLRRSP 800
NL4 DKRRHETHRRPSPQRNT TND IAHIQNEEIMSLQMKQLEHDHECESLQAHDT LRLTCPPDYTLTLRRSP 768
H T
NL1 DDVPLMTPNTITMIPNTIPGIQP LHTFNTFTGGQNNTLPHPHPHPHSHSTTRV 863
NL2 DDVPLLAPGALTLLPSGLGPPPPPPPPPSLHPFGPFPPPPPTATSHNNTLPHPHSTTRV 835
NL3 DDIPLMTPNTITMIPNSLVGLQT LHPYNTFAAGFNST GLPHSHSTTRV 848
NL4 DDIPLMTPNTITMIPNTLTGMQP LHTFNTFSGGQNST NLPHGHSTTRV 816
F M K

Figure 1.1

Figure 1.1: **Neuroligin homologies across the 4 isoforms, NL1-4**

A comparison of the amino acid sequence of NL isoforms 1-4. Highlighted in black are the homologous regions, conserved across the NL subtypes. The region in gray represents the transmembrane domain (TMS) and below that is the short, intracellular region. There is approximately a 70% homology across NL1-4 (Bolliger *et al.*, 2001).

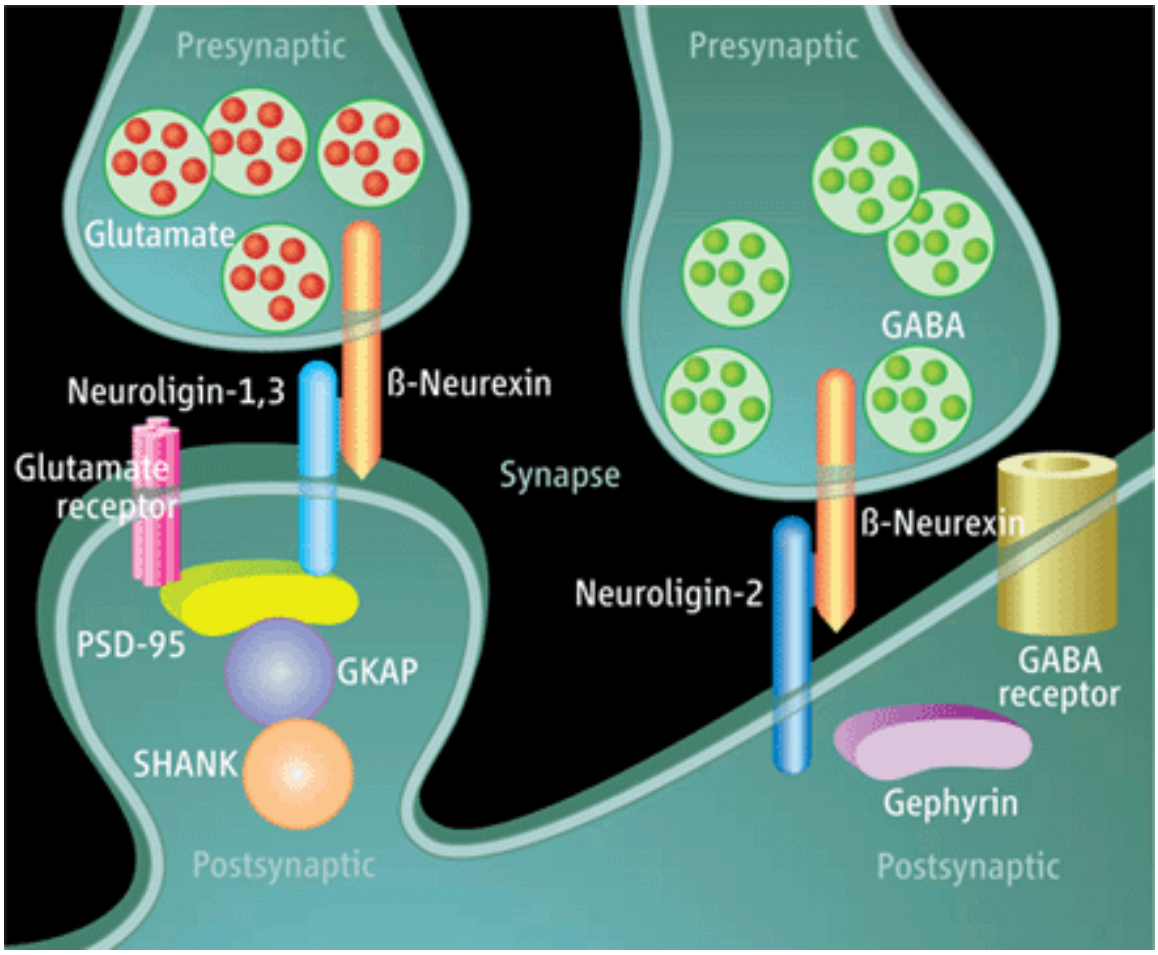
Presynaptic
Glutamate
Presynaptic
GABA
Neuroligin-1,3
ß-Neurexin
Glutamate receptor
Synapse
ß-Neurexin
Neuroligin-2
PSD-95
GKAP
GABA receptor
SHANK
Gephyrin
Postsynaptic
Postsynaptic

Figure1.2

Figure 1.2: **Specific neuroligin isoform localization at specific synaptic types**

Neuroligin isoforms and their interactions with other synaptic and scaffolding proteins. Specific isoforms, or sub-types of NL are expressed in dendrites at different synapse types, i.e. inhibitory and excitatory. It can be seen that NL1 binds to PSD-95 (which is associated with other scaffolding proteins such as GKAP and SHANK) at excitatory synapses while NL2 is associated with gephyrin at inhibitory synapses. These synaptic connections which are formed and regulate excitatory and inhibitory neurotransmission (Hussain and Sheng, 2005).

Previous research has found that NL's presence is not critical for initial establishment of a synapse but is required for normal synaptic function, maturation and development (Missler, *et al.*, 2003; Varoqueaux *et al.*, 2006; Chubykin *et al.*, 2007). Varoqueaux and colleagues at the Institute of Experimental Medicine in Gottingen, Germany, used a NL1-3 knockout (KO) mouse model to determine the role NLs play in synaptogenesis. Selective breeding was performed to generate individual NL KOs (NL1, NL2 and NL3) as well as all combinations of NL KOs (NL1&2, NL2&3 and NL1&3 as well as NL1-3). It was found that all the offspring which had a single or double NL KO were viable, fertile and behaved normally. However, once a triple NL KO was generated, the offspring had lower body weights, displayed irregular breathing movements and died shortly after birth. These findings supported Varoqueaux and colleagues hypothesis that NLs are required for normal synaptic function but are not necessary for the initial establishment of a synapses due to the fact that the pups did not die *in utero*, but died once they were born.

Evidence suggests that intracellular regulated and mediated interactions may also have an influence on the retention of NL at the synapse (Craig & Kang, 2007). It has been found that the C-terminal tails of NLs interact with PSD-95 through PDZ-dependent interactions (Dalva *et al*., 2007). PSD-95 binds to NLs through its third PDZ domain (Irie *et al.*, 1997) and also binds to potassium (K^+) channels (Kim *et al*., 1995), *N*-methyl-*D*-aspartate (NMDA) receptor subunits (Kornau *et al*., 1995) as well as other signalling and scaffolding proteins (Garner *et al*., 2002). These findings suggest that the PSD-95 may play an integral role in regulating the excitatory-inhibitory synaptic ratio, or E/I ratio, by modulating NL localization and function at specific synapse types (Levinson *et al.*, 2005; Dalva *et al*., 2007).

The role NLs play in neuron-neuron signalling has been extensively studied, however even though NL3 is expressed in glia, their role in neuron-glial or glial-glial communication has not been as investigated.

1.1.2 Neurexin

The extracellular domain of neuroligin is associated with its pre-synaptic partner NX (**Fig.1.3)**. This protein interaction has been found to be critical for synapse development and function (Dean & Dresbach, 2006). Over one thousand neurexin isoforms are generated by alternative splicing and these isoforms have been found to be differentially expressed in the nervous system (Missler *et al.*, 1998; Togashi *et al*., 2009). Südhof discovered NX in 1992, three years prior to his discovery of NL by virtue of NX's ability to bind to α-latrotoxin, the venom from the Black Widow spider (Ushkaryov *et al.,* 1992; Scheiffele *et al*., 2000). This toxin binds to pre-synaptic receptors and triggers massive neurotransmitter release (Geppert *et al*., 1997).

NXs and NLs bind to promote adhesion between axons and dendrites. It has been said that these two adhesion molecules are arguably the best characterized trans-synaptic cell adhesion pair (Ushkaryov *et al.*, 1992; Ichtchenko *et* al., 1995). There are four subtypes of the NX protein in the NX family (NX1-4), each of which have two sub-forms, an α form and a β form (Ullrich *et al.*, 1995). The α subtype has a much more elaborate extracellular region compared to the truncated counterpart, the β subtype (**Fig.1.4)**. The α subtype contains six LNS domains, which are composed of a laminin, a nectin and a sex-hormone binding globulin domain while the β subtype only has one of these LNS binding regions (Missler *et al.*, 2003). The α subtype also contains multiple splice sites, which are indicated by the numbered arrows in figure 1.4, compared to the β subtype (Miller *et al.*, 2011). Although their extracellular domains differ greatly, which creates a difference in NL binding efficiency between the two isoforms, the α and β forms share an identical C-terminal region and transmembrane domain (Li *et al*. 2007).

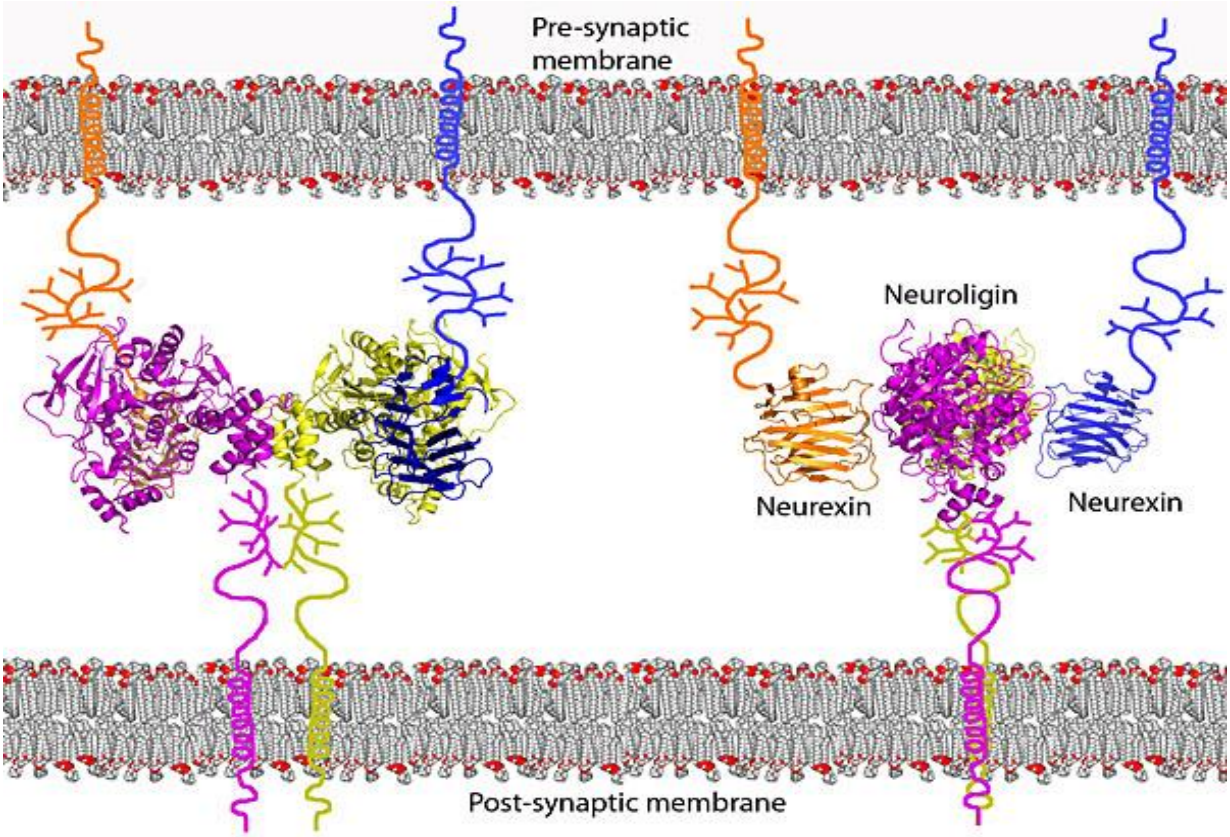
Pre-synaptic membrane
Neuroligin
Neurexin
Neurexin
Post-synaptic membrane

Figure 1.3

Figure 1.3: **Neuroligin and Neurexin binding interaction**

The crystalline structure, illustrating the interaction between neuroligin and neurexin. The interaction between the two proteins forms a trans-synaptic connection. Two molecules of neurexin on the pre-synpase bind to one molecule of NL on the post-synapse (Araç *et al.*, 2007). It is within the binding regions where the autism associated mutation is speculated to interfere with the NL-NX interaction.

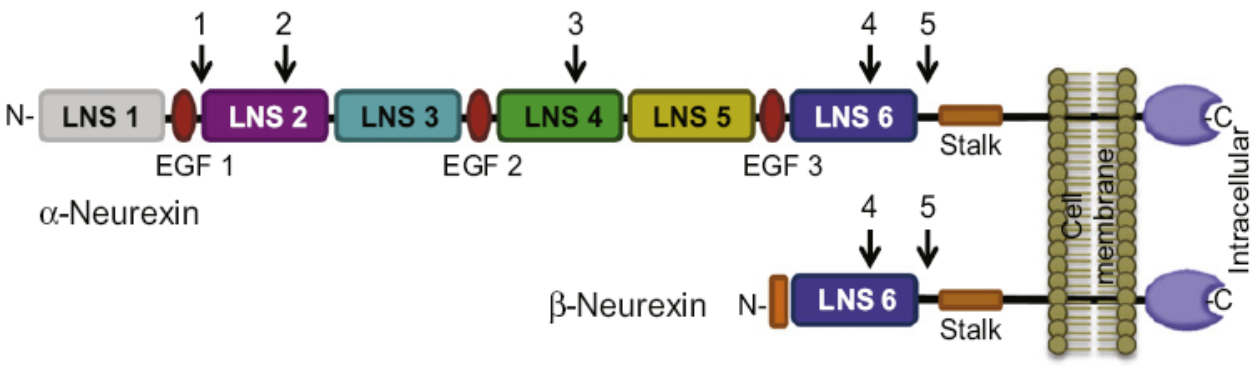
1
2
3
4
5
N-
LNS 1
LNS 2
LNS 3
LNS 4
LNS 5
LNS 6
Stalk
EGF 1
EGF 2
EGF 3
α-Neurexin
4
5
β-Neurexin
N-
LNS 6
Stalk
Cell membrane
C
C
Intracellular

Figure 1.4

Figure 1.4: **Neurexin isoform composition**

A schematic representation of the two different isoforms of neurexin class 1 transmembrane proteins. β-NX is a truncated version of the larger α-NX protein. This difference between the isoforms exists in the N-terminal extracellular region. The α-NXs have a total of six LNS domains while β-NXs have only one, which is identical to the LNS 6 domain found in α-NX. The positions of for alternative splicing sites are indicated with arrows (Miller *et al.* 2011).

It has been demonstrated that NL binds with a higher efficiency to the shorter, β subtype than the longer α form of NX (Chubykin *et al.*, 2005; Miller *et al.*, 2011). Association between NX and NL is Ca^{2+} dependant. A Ca^{2+} ion forms a physical tether between the two molecules aiding in synaptic connectivity (Nguyen and Südhof 1997; Araç *et al.*, 2007) (**Fig.1.5)**.

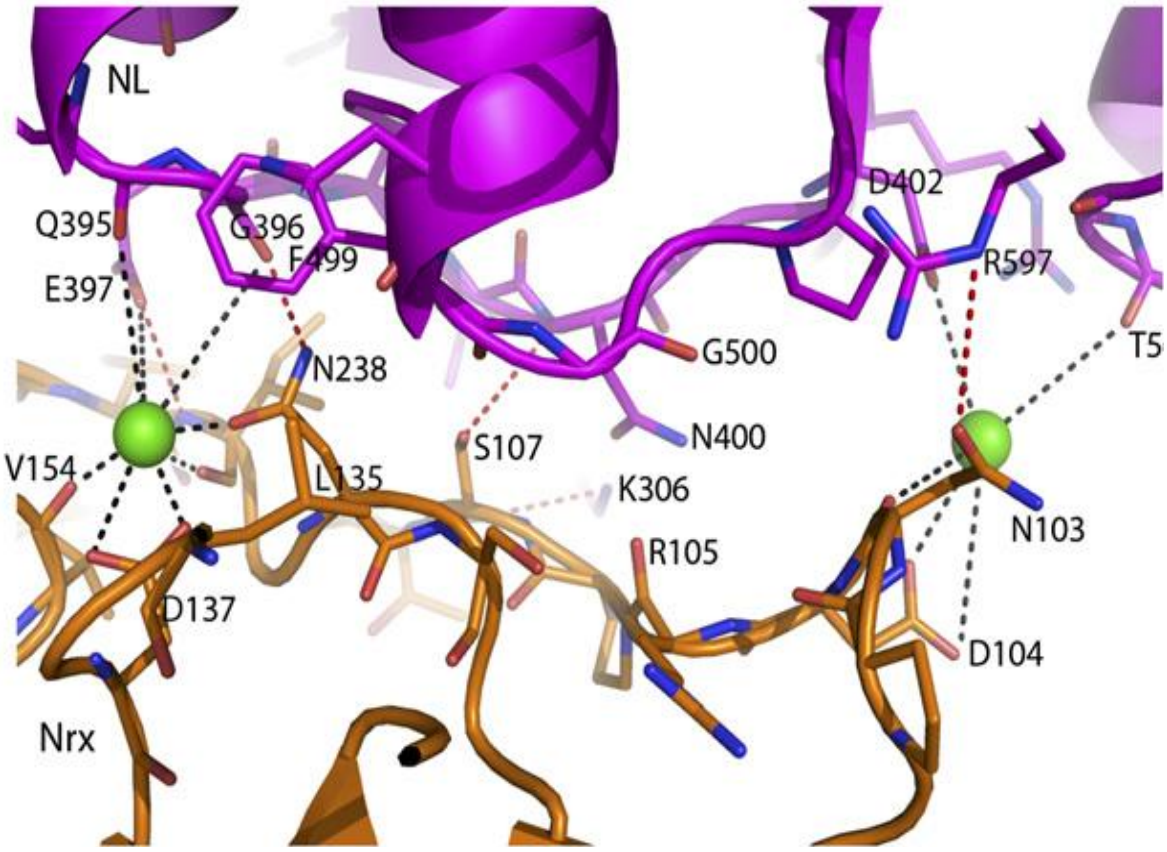
NL
Q395
G396
F499
E397
N238
G500
D402
R597
T5
N400
S107
V154
L135
K306
N103
R105
D137
D104
Nrx

Figure 1.5

Figure 1.5: **Calcium dependent binding of NL and NX**

The interaction between NL and NX has been found to be Ca^{2+} dependent; having a specific Ca^{2+} concentration in the synaptic cleft is required for NL and NX to bind efficiently (Calcium is designated as the green spheres). A close-up view of this interaction reveals that NL and NX bind at amino acid residues Q395, E397, G396, F499, D402, R597 and T569 on the NL side. All of these residues are homologous between NL1 and NL3; however F499 and R597 are conservative changes between NL1 and 3. On the pre-synaptic side, NX binds to NL at residues V154, D137, N238, N103, L135, S107, K306 and D104 (Araç *et al*. 2007). Mutations which arise within amino acid sequences have an influence on how NL and NX bind.

1.1.3 Autism Associated Mutations in Neuroligin

In addition to playing an important role in synaptogenesis, neuroligins have been aggressively studied as a result of their association to autism spectrum disorders (ASD) (Comoletti *et al*., 2004; Khosravani *et al.,* 2005). ASD describes a range of conditions, symptoms of which include communication difficulties, social deficits as well as cognitive dysfunction. This neurological condition affects approximately 1% of the world's population (Baird *et al.*, 2006; Baron-Cohan *et al.*, 2009).

Several mutations in the NL gene family have been identified through genetic screens of patients with autism. One such mutation in NL3 results in a decreased binding capacity to NX, suggesting that disruption of neuroligin-neurexin synaptic signalling could be a causal mechanism contributing to ASD (Comoletti *et* al., 2004; Etherton *et* al., 2011). One theory has suggested that a single point substitution mutation in NL leads to a defective transportation of NL to the cell membrane (Ellegood *et al*., 2011). One such mutation, known as R451C, causes a defect in protein processing which leads to a reduction of synaptogenic activity (Comoletti *et al.,* 2004). The change from an arginine (R) to a cysteine (C) at the 451 residue in NL3 causes a defect in NL trafficking which leads to retention of the protein in the endoplasmic reticulum (ER). The analogous substitution mutation can be engineered in NL1 at the 473 residue, the offset due to differences in other parts of the protein (Tabuchi *et al*., 2007; Comoletti *et al.,* 2004).

Rat and human NL3 amino acid sequences are almost identical, with a 99.8% homology at the amino acid level (Song *et al.,* 1999; Comoletti *et* al., 2004). While research has focused on the effect of this mutation on trans-synaptic, neuron-neuron signalling, it has been found that the autism associated mutations in NL1 and NL2 are less prevalent, and those are the subtypes primarily responsible for mediating excitatory and inhibitory synapses respectively. NL1 and NL2 are predominantly under investigation for their roles in autism due to their ability to change the

excitatory/inhibitory (E/I) synapse ratio (Tabuchi *et al*., 2007). However, it is also important to mention the amount of white matter in the autistic brain differs from that in the normal brain (Courchesne *et al.*, 2001; Sparks *et al.*, 2002; Herbert *et al*., 2003a), bringing that into focus as a potential causal mechanism as well. Moreover, the process of myelination appears to be altered in the autistic brain (Barnea-Goraly *et al*., 2004). The causes of this difference have not been determined and a mechanism to explain these differences has not even been speculated. An underlying hypothesis in this thesis is that mutations in NL3 could be affecting myelination, and consequently this may be the mechanism by which the autistic condition is generated.

1.2 Myelination

Myelination is the process which axons undergo to help insulate their axons in order to increase the speed of conductance (Huxley and Stampfli, 1949). The process of myelination is critical to the physiology and development of the nervous system (Bunge *et al.*, 1962; Bunge, 1968; Barres & Raff, 1993). Failure to myelinate or damaging this lipid-rich layer causes erratic electrical signalling and aberrant myelination has been linked to many different diseases such as multiple sclerosis. Myelin is a spiral structure composed of plasma membrane extensions of oligodendrocytes (OLs) (Bunge *et al.*, 1962). Myelination requires temporally and spatially regulated mechanisms which ensure that the myelin sheath is produced at the appropriate place and time during development (Colello and Pott, 1997; Nave, 2010). Although all myelination in the CNS arises from oligodendrocytes, there are also OLs which are not involved in myelination. These satellite OLs have been speculated to help regulate the microenvironment around specific neurons (Ludwin, 1997).

1.2.1 Oligodendrocytes

Myelination and re-myelination are important for the proper functioning and repair of the CNS. Myelination is mediated by OL-astrocyte-neuron communication in the tripartite synapse (Moore *et al.*, 2011). This interaction indicates that axonal signals may control and influence the timing of OL differentiation (Hardy and Reynolds, 1993; Richardson *et al.*, 2000; Watkins *et al.*, 2008).

OLs have a multitude of functions, which include providing structural support and maintaining ionic homeostasis for neuronal networks. Research found that these glial cells express many of the same receptors for neurotransmitters as neurons as well as have electrophysiological responses to certain neurotransmitters (Sontheimer *et al.*, 1989; Steinhauser and Gallo, 1996). It has been discovered that OLs can also act as both pre-synaptic and post-synaptic elements due to the fact that they are capable of both releasing and detecting certain neurotransmitters (Verkhratsky & Kirchhoff, 2007; Cavaliere *et al.*, 2012). It has been found that OL cell activation may contribute to synapse formation as well as cell development. OL cell activation has also been speculated to contribute to myelination, microcirculation, neuro-protection, regulation of cytokines and some growth factors as well as the modulation of neuronal activity (Thomas *et al*., 2002).

The regulation of OL morphology is critical to allow for efficient and temporally regulated myelination of CNS axons (Richter-Landsberg, 2008; Eyermann *et al.*, 2012). Differentiating OLs undergo extensive cytoskeleton remodelling to enable their processes to extend toward, contact and wrap around axons (Bunge *et al.*, 1962; Richer-Landsberg, 2008). Maturation and differentiation is accomplished by massive alterations in cell morphology as well as their internal physiology (De Biase *et al.*, 2011). Oligodendrocytes begin their development as progenitor cells that quickly mature into oligodendrocyte precursor cells (OPCs). OPCs arise in specific regions of the CNS and begin to migrate across substantial distances to areas where they will differentiate and begin the myelinating process (Barres and Raff, 1993). OPCs can be identified by their few, small processes with little

arborizations. These precursor cells then develop into what is referred to as an immature oligodendrocyte, which can be identified by highly branched and extensive cytoplasmic projections which form in a radial orientation around the cell bodies (**Fig.1.6**). Once these highly branched arborizations make contact with one or more axons, they begin to retract their processes which have not made contact and elongate the ones which have (Thomas *et al.*, 2002). This stage is referred to as the mature, elongated or myelinating form. At this stage oligodendrocytes begin to produce myelin specific proteins such as myelin basic protein (MBP) and marker 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) (Rettig and Old, 1989). OLs are capable of wrapping around upwards of 60 different axons at one time, depending on brain region (Agresti *et al.*, 2005; De Biase *et al.*, 2011). **Figure 1.7** depicts the three states of OL morphology.

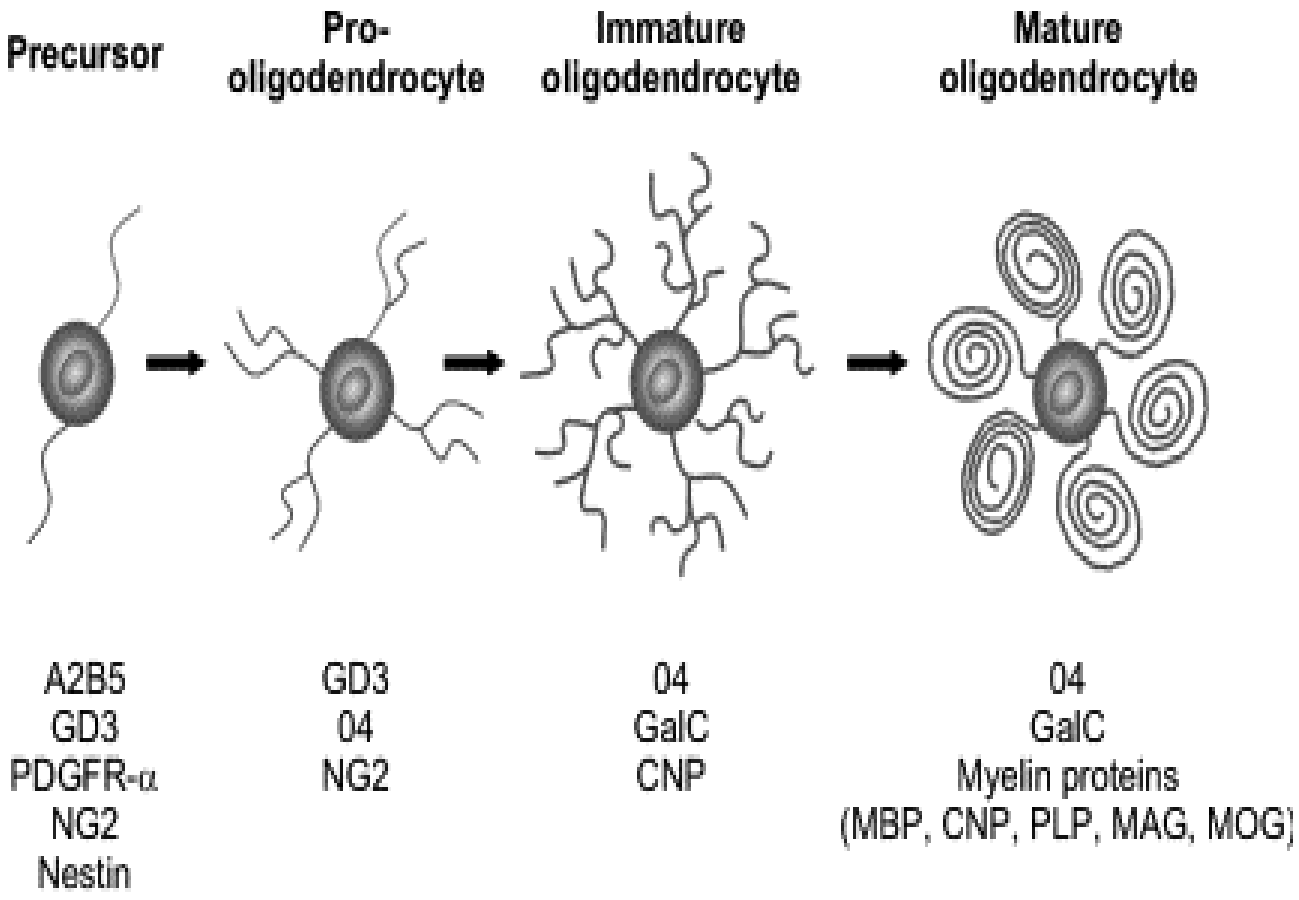
Precursor
Pro-oligodendrocyte
Immature oligodendrocyte
Mature oligodendrocyte
A2B5
GD3
PDGFR-α
NG2
Nestin
GD3
04
NG2
04
GalC
CNP
04
GalC
Myelin proteins
(MBP, CNP, PLP, MAG, MOG)

Figure 1.6

Figure 1.6: **Stages of Oligodendrocyte development and differentiation**

A schematic representation of the progression through the oligodendrocyte lineage. Oligodendrocyte development progresses through specific stages; these stages have unique morphological characteristics as well as unique expression profiles for different antigens. OLs begin their development as progenitor cells which mature into oligodendrocyte precursor cells (OPC). As we can see by the depiction, OPCs are distinguishable by their few, small processes. These cells then develop into immature cells, which are distinguishable by their highly branched processes which form in a radial orientation around their cell body. These cells then develop into mature, myelinating OLs, by retracting processes which have not made axonal contact, and extent processes which have. During their development, from precursor cells to OPCs to mature, myelinating glial cells, OLs lose their migratory and proliferative activity and begin to change their morphology as they differentiate into their myelinating stage (Agresti *et al*. 2005).

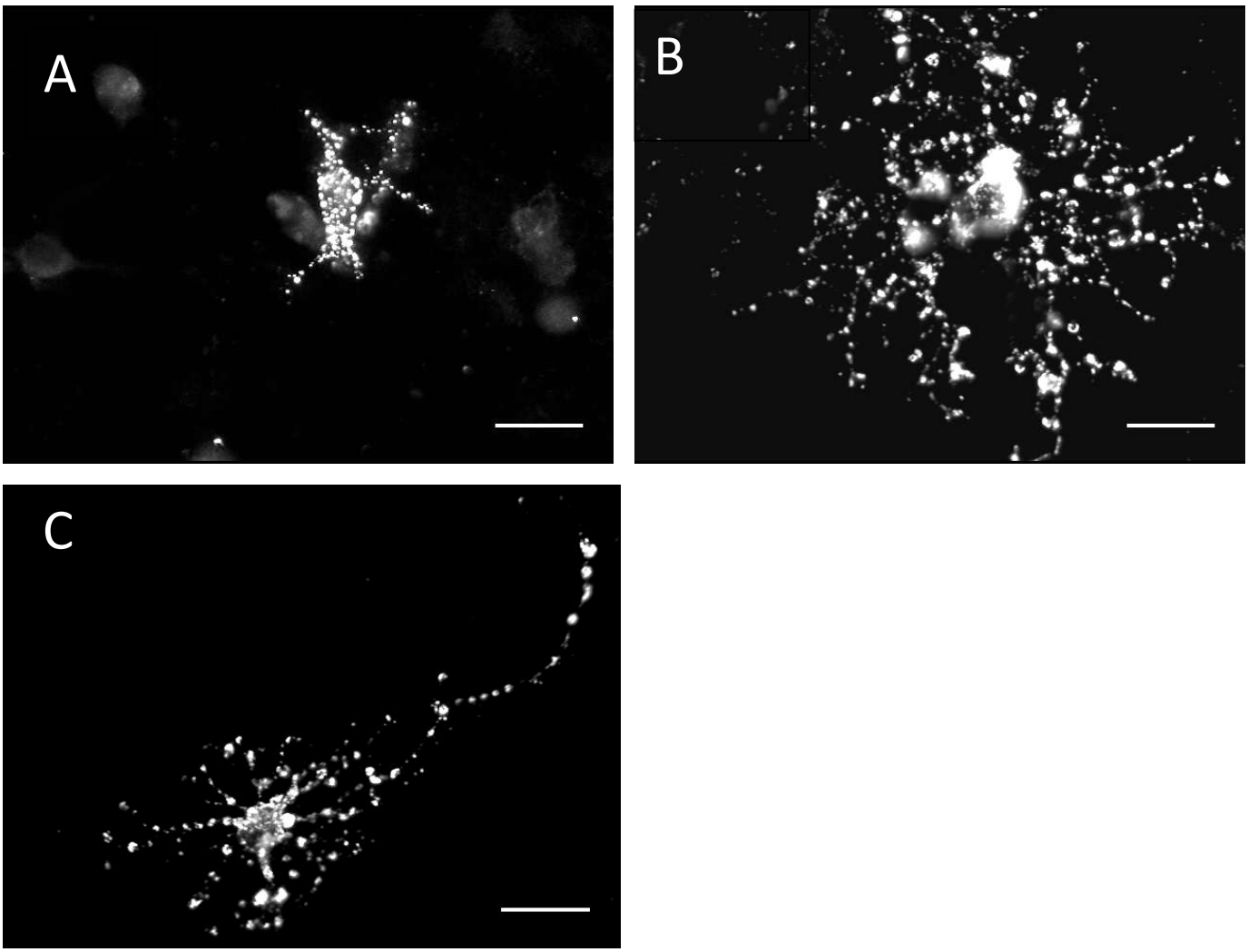

Figure 1.7

Figure 1.7: **Distinctive stages of OL development in an *in vitro* dissociated cell model**

Different stages of OL development. Oligodendrocyte precursor cells (OPC) (**A**) are distinguished by their few processes. Immature OLs, distinguishable by their highly branched pattern (**B**), allow the cell to increase its chances of coming on contact with an axon. Because these are only two-dimensional images, it is hard to get a good representation of how these cells actually look. In a tissue sample these cells would have a homogenous radial orientation in a three-dimensional plane. Once 'immature' OLs come in contact and begin to interact with neighbouring axons, they differentiate into their final, mature stage (**C**) and take on a neuronal-like morphology. In order for cells to be included in the cell counts, they had to fit these specific morphological criteria. If a cell was undistinguishable, it was omitted from the cell count. Scale bar = 25μm

1.2.2 Synaptic glutamate release

Glutamate release from axons acts on glutamate receptors on OPCs, affecting their migration, proliferation and promotes differentiation (Zonouzi *et al.*, 2011; Gallo *et al.,* 1996; Gudz *et al.,* 2006). It has also been speculated that the local release of glutamate could act to initiate production of myelin (Cavaliere *et al.*, 2012). It has been found that OLs express functional α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (McDonald *et al.*, 1998) as well as NMDA receptors (NMDAR) which are capable of inducing intracellular Ca^{2+} transients (Micu *et al.*, 2006). This finding led to the possibility that signalling through AMPA and NMDARs could modulate OL physiology as well as control myelination (De Biase *et al.,* 2011).

1.3 Soluble neuroligin proteins

To test whether the interaction between NL3 expressed in oligodendrocytes and axonal NX regulates OL function and/or development, we used a soluble, truncated version of the NL1 protein, containing the extracellular NX binding domain. For the purposes of this study, we have designated this protein as NISA. This protein was engineered at the University of California by Comoletti *et al.*, in 2003, to have an even greater affinity to bind to NX then endogenous NL. As stated, this soluble protein is a truncated version of endogenous NL, engineered with a stop codon at isoleucine 639, which removed the transmembrane domain. It also has had a glycosylation site (N303) removed allowing for better binding with endogenous NX. Due to the loss of the transmembrane domain, the protein is secreted (**Fig. 1.8**) where it can diffuse and bind to any available NX molecules. By saturating the NL binding sites on NX with NISA, the presumption is that we are blocking the binding of NX with endogenous NL on the oligodendrocyte (**Fig.1.9**). A mutated version of this soluble protein, containing the autism associated R473E substitution was also constructed and for the purposes of this thesis we have designated this mutation as NLR473E. This protein has a single point mutation,

changing the arginine (R) at residue 473 to glutamic acid (E), and has a stop codon introduced at amino acid 692. This makes the NLR473E peptide longer than NISA by 53 residues (**Fig.1.10**). The mutation in NLR473E results in a much reduced capacity to bind to NX compared to wild type neuroligin, and as with NISA the lack of a transmembrane domain allows it to be secreted. To allow for detection of these proteins, a Flag residue sequence (DYKDDDKL) was engineered into the N-terminus of the construct sequence.

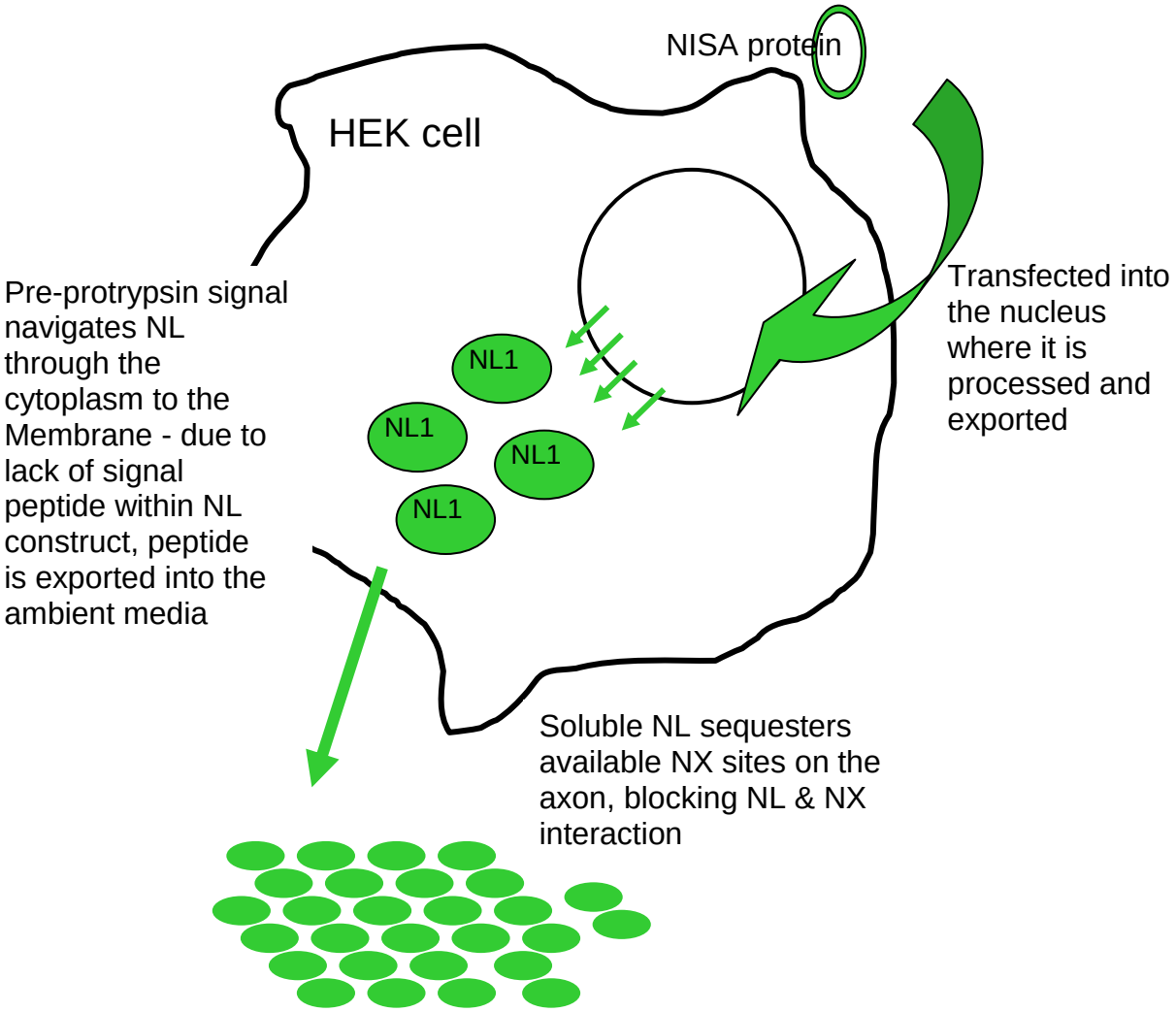
NISA protein
HEK cell
Pre-protrypsin signal navigates NL through the cytoplasm to the Membrane - due to lack of signal peptide within NL construct, peptide is exported into the ambient media
NL1
NL1
NL1
NL1
Transfected into the nucleus where it is processed and exported
Soluble NL sequesters available NX sites on the axon, blocking NL & NX interaction

Figure 1.8

Figure 1.8: **Transfection of soluble protein**

A schematic representation of transfected HEK cells expressing the soluble NL proteins, NISA and NLR473E. Due to a lack of a transmembrane domain, these proteins were exported into the HEK cell media. HEK cells were used to increase the administration of the soluble NL proteins; this was due to the fact that these cell types have a high transfection efficiency. Once the cells were transfected via a Calcium Phosphate protocol (section 2.4) they were provided time to express the soluble proteins. Because of the pre-protrypsin sequence, the protein was vesicularized and shipped to the cell membrane. Due to the lack of a transmembrane domain, the protein was not anchored to the membrane but exported into the HEK cell media. It was my speculation that the soluble NL protein present in the HEK cell media, when added to primary hippocampal cultures, would sequester NX targeting sites, preventing endogenous NL present on the OL surface, from binding to axonal NX.

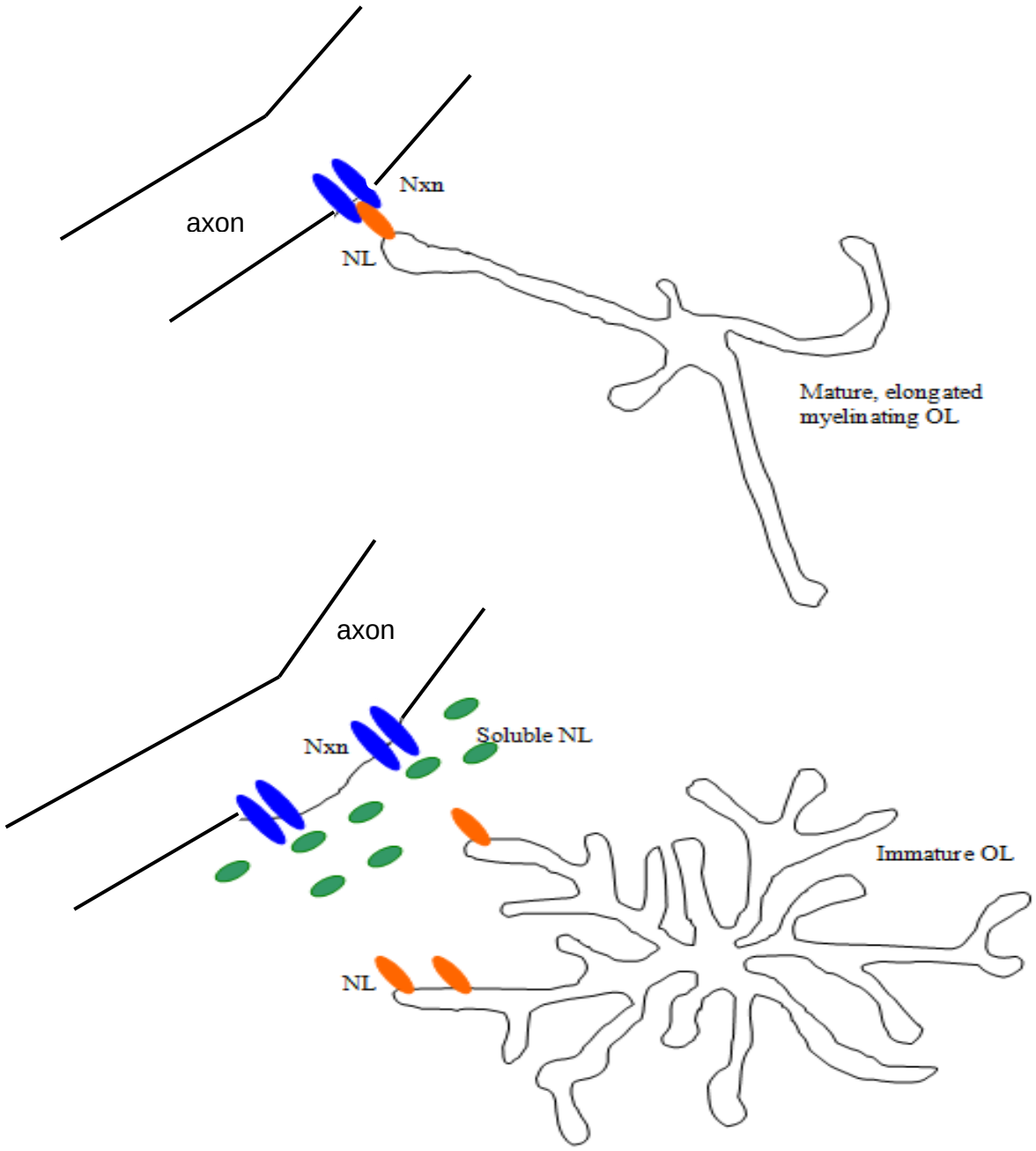
axon
Nxn
NL
Mature, elongated
myelinating OL
axon
Nxn
Soluble NL
Immature OL
NL

Figure 1.9

Figure 1.9: **Blocking the NL and NX interaction with soluble NL peptides.**

A schematic representation of how the soluble NISA binds to available endogenous NX sites on the pre-synapse, interfering with NL-NX signalling. It is this interaction which is speculated to be required in order for cells to develop from their immature, highly branched state into their mature, myelinating cell type. NX, represented by the blue spheres is preset on the axon, where NL, represented by the orange spheres is present on the distal ends of the OL processes. The soluble NL, represented by the green spheres, blocked endogenous NL from binding to its presynaptic counterpart.

Here we can see that if an OL is able to bind to an axon through the NL-NX interaction, it can progress into its myelinating state, as depicted by the reduction of OL processes and the acquisition of bulbous process ends, or feet. It is our speculation that OL NL is present on the ends or process feet as that would be the site of contact with an axon to be myelinated. If, by blocking the NL-NX interaction, by introducing the soluble NL protein, the OL cell would be delayed in the immature cell stage, depicted by the highly branched and arborized processes.

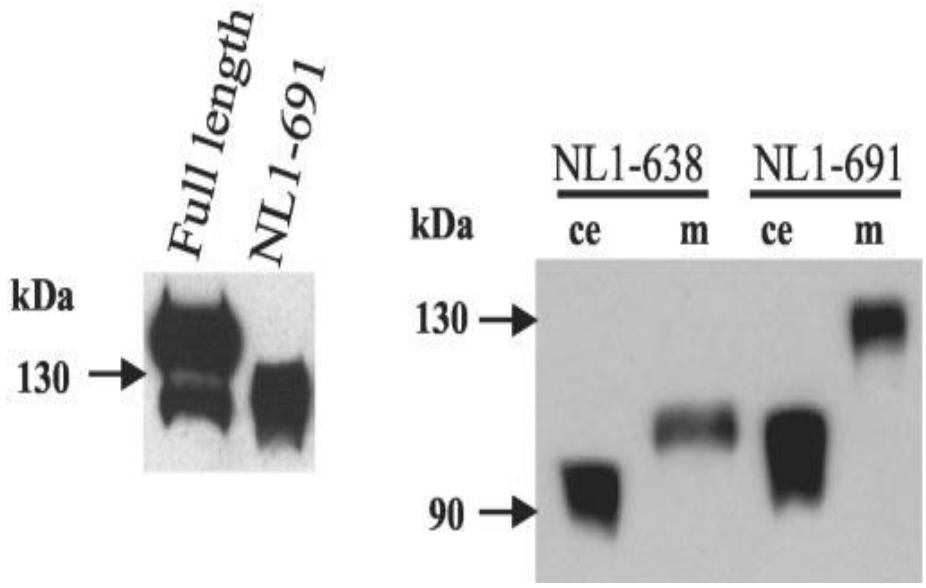

Figure 1.10

Figure 1.10: **Expression of NISA and NLR473E in cell extract and media**

A Western blot from Davide Comoletti *et al.*, 2003 showing the band sizes of the engineered soluble NL constructs. *Left,* a comparison in size between endogenous NL1, purified from cell extract, and NL1-691, purified from cell media. *Right,* comparison in protein size between the truncated proteins NL1-638 (NISA) and NL1-691 with the autism associated mutation (NLR473E) purified from the cell extract (ce) and media (m) (Comoletti *et al.* 2003).

1.4 Glutamatergic receptors

1.4.1 NMDA receptor expression in glia

NMDA receptors (NMDARs) are present in oligodendrocytes and their precursors (Burzomato *et al.*, 2010) and their expression has been documented at different stages of oligodendrocyte development (Karadottir *et al.*, 2005; Paoletti & Neyton, 2007). NMDA receptor distribution in neurons is regulated by their binding to PSD-95 through the PDZ domain. Neuroligin also binds to the PDZ domain of PSD-95 (Irie *et al.*, 1997), and it is through this interaction that neuroligin can modulate NMDAR distribution.

Glial currents are insensitive to extracellular magnesium (Mayer *et al.*, 1984) and it has been found that the predominantly expressed subunit type is NR3a (Verkhratsky & Kirchhoff, 2007). NMDARs which contain the NR3a subunit show a reduced sensitivity to voltage block by extracellular Mg^{2+} ((Henson *et al.*, 2010; Nishi *et al.*, 2001; Sasaki, *et al.*, 2002) as well as a reduced Ca^{2+} permeability (Chatterton *et al.*, 2002; Matsuda *et al.*, 2002). NMDA receptors in glial cells have been found to be concentrated in the cellular processes (Salter & Fern, 2005) and NMDAR regulated responses have been found primarily at distal processes compared to those found in the cell body (Micu *et al.*, 2006).

NMDA receptor (NMDAR) activation influences neurite outgrowth and spine morphogenesis in developing neurons (Ponimaskin *et al*., 2007) Could NMDAR activation have a similar role in developing oligodendrocytes? If NX sites on axons are blocked by the introduction of a soluble NL, this could impact the recruitment of NMDAR to the OL membrane through the post-synaptic density (PSD) pathway. If NMDAR recruitment in OLs is down regulated due to the inability of NL from properly binding to NX, this could influence the morphology of the OL cells due to altered ion influx.

1.4.2 AMPA receptors

OPCs express calcium-permeable GluR2 subunit containing AMPA receptors (CP-AMPARs) (Geiger *et al.,* 1995; De Biase *et al.,* 2010) and these AMPA receptors may provide a channel to activate calcium-dependant signalling pathways in these progenitor cells (De Biase and Bergles, 2011). Activation of OPC AMPARs is thought to be critical in numerous developmental and physiological processes including progenitor proliferation, migration, differentiation and neuron-glia signalling (Gallo *et al.*, 1996; Gudz *et al.*, 2006).

1.5 Neuroligin and Myelination

A potential role for NL3 regulation of myelination has been hypothesized in invertebrate systems. The invertebrate homolog of NL3, gliotactin, is expressed in the glia cells which are responsible for the ensheathing of axons in the *Drosophilia* nervous system. Deletions of gliotactin result in paralysis and eventually death due to a breakdown in the glial-based blood-nerve barrier (Gilbert *et al*., 2001). If gliotactin plays a role in ensheathing cells in the invertebrate model, then perhaps its vertebrate homolog may also be involved in the process of myelination in the mammalian system. Interestingly, it was also found that contactin-associated protein, or caspr, a homolog of neurexin 4, is a key molecule that signals the initiation of myelination. It has also been found that caspr's extracellular domain contains a series of laminin G-like domains, a characteristic similar to the C-terminal of NX (Einheber *et al*., 1997). Caspr and NX have a 30% homology at their amino acid level in their extracellular domains (Einheber *et al*., 1997). Caspr has been speculated to be a critical component of the macromolecular complex involved in the tight interaction between axons and myelinating glial cells in the paranodal regions (Baumann & Pham-dinh, 2001). Perhaps NX, like its homolog caspr, plays a role in myelination.

1.6 Hypothesis and experimental rationale

As stated previously, the interaction between NL and NX has been studied quite intensely at the neuronal synapse; however our preliminary findings have suggested that NL is present in myelinating oligodendrocytes as well.

At the core of this thesis is the hypothesis that NL3 is expressed by the myelinating glial cells in the CNS, the oligodendrocytes, and that a fundamental interaction between NL3 on the surface of oligodendrocytes and NX on axons is a critical signal in the process of oligodendrocyte development, differentiation and myelination.

In the establishment of synaptic connections, post-synaptic NL1 and NL2 interact with the pre-synaptic NX to determine connectivity. Since NL3 can also interact with NX on the axons, yet is present on the surface of glia cells, perhaps the signalling between NL3 and NX regulates axonal and oligodendrocyte interactions and consequently oligodendrocyte differentiation (**Fig.1.11**).

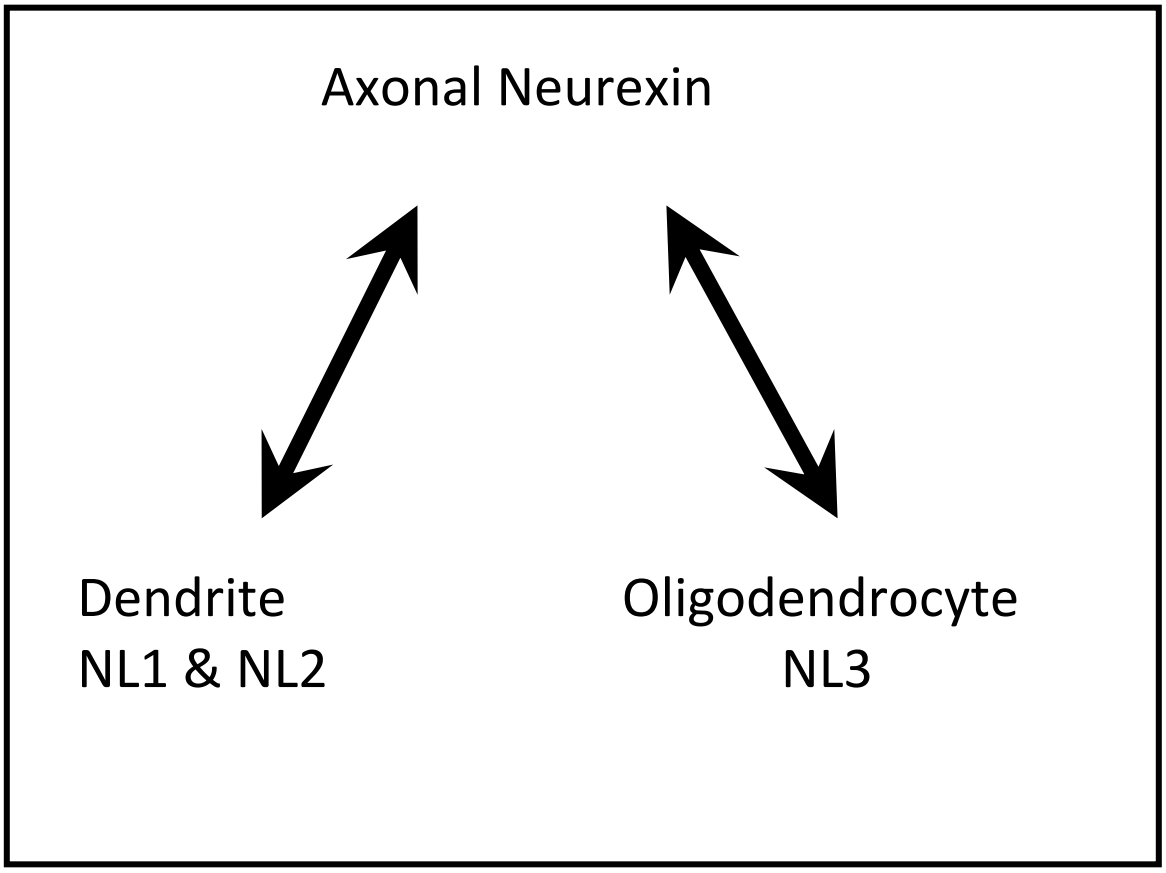

Figure 1.11

Figure 1.11: **Hypothesis for NL regulating synaptogenesis and myelination through convergent interaction with NX**

A schematic representation of my hypothesis; that NL3 on the surface of OL cells interacts with axonal NX and influences or regulates OL development. By interfering with the NL-NX cell adhesion interaction I speculate that the developmental profile of the OLs will be affected. If an OL cell is unable to interact with an axon through the NL-NX signalling pathway, then it will be unable to differentiate into its myelinating cell state. It is known that both NL1 and NL2 are present on dendrites which interact with axonal neurexin, however it is my hypothesis that NL3 is predominantly found on oligodendrocytes, and it is through NL3 that OLs bind to axons and begin myelination.

1.6.1 NL and NX interaction regulates OL differentiation states

Specific Hypothesis 1: *Interfering with the NL-NX cell adhesion interaction will cause a change in oligodendrocyte differentiation states.*

We hypothesize that interfering with NX-NL signalling by the addition of exogenous soluble NISA peptide will impair the ability of oligodendrocytes to differentiate into a myelinating cell.

1.6.1.1 Significance

If disrupting the NL-NX interaction with the soluble NISA protein negatively impacts the ability of OLs to progress through its developmental states, this would demonstrate that the signalling is critical for the differentiation process, specifically the transition from an immature cell to its mature, myelinating state. Alternatively if the soluble neuroligin peptide carrying the ASD associated mutation, NLR473E, is unable to affect differentiation state, this could shed light on how this mutation functions in autism.

1.6.2 Recruitment of Glutamatergic receptors

Specific Hypothesis 2: *The signalling between NL in oligodendrocytes and NX on the axon triggers the recruitment of NMDA and AMPA receptors via mechanisms similar to those found in neurons.*

The recruitment of calcium permeable receptors to the membrane plays an important role in how cells regulate transient Ca^{2+} influxes. If receptor recruitment is in fact modulated by NL/NX contact this could have an impact on the cell's developmental profile as it would alter the amount of calcium that enters the cell. Blocking the ability of OL expressed NL to bind to axonal expressed NX by the addition of exogenous NL peptide would therefore have an impact on calcium influx and therefore potentially modulate oligodendrocyte differentiation.

1.6.2.1 Significance

Co-localization of NMDA or AMPA receptors (NMDAR & AMPAR) and NL has yet to be demonstrated in oligodendrocytes. Although specific NMDAR subtypes are expressed by OLs, co-localization with NL has not been characterized. Similarly AMPARs have also been found to be present in OLs however no co-localization data exists. Determining these interactions will further our understanding of the potential mechanism for any changes in differentiation state regulated by NL/NX binding.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Primary hippocampal co-cultures were prepared from P0 Sprague Dawley rats, strain CD IGS (Charles River, Wilmington, MA, USA), obtained under Animal Protocol number M09008. All dissection procedures were approved by the University of Calgary Ethics board. Pregnant rats were ordered from Charles River so that timing would facilitate them to give birth on day of dissection. This was the case for most of the culture preps, however if a pregnant rat had not given birth by day of dissection, a caesarean section was preformed. The pregnant rats were anesthetised with 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane) (Sigma, B4388), and a cervical dislocation was performed. A 'Y' incision was made along the belly of the rat, at which point the uterus was removed, opened and pups were removed.

2.2 Primary co-cultures

2.2.1 Hippocampal co-cultures

P0 rat pups were placed on ice for five minutes and sacrificed by decapitation. Hippocampi were then isolated, triturated with three decreasing calibre pipettes and plated on poly-D-lysine (Sigma, P7280) and laminin (Sigma, L2020) treated, 1cm by 1cm silicon chip wafers (Silicon Quest International, Reno, NV, USA) as described in Goda & Colicos, 2006. Poly-D-lysine was dissolved in Borate Buffer, consisting of 40mM Boric acid (Sigma, B6768) and 10mM Sodium Tetraborate (Sigma, B3545) at a dilution of 1:100; chips were incubated over night. On day of dissection, the chips were washed three times in 1X Dulbecco's Phosphate buffered saline (DPBS) (Invitrogen, 14190-144) and then Laminin, diluted in DPBS, was added at a concentration of 1:500. The chips were incubated in this solution for three hours and washed three times in 1X DPBS prior to dissection.

These primary hippocampal co-cultures were maintained in basal media Eagle (BME) (Invitrogen, 21010-046) supplemented with 1-4% fetal bovine serum (FBS) (Invitrogen, 16000-044), 2 % B27 (Gibco, 17504-044), Penstrep (Invitrogen, 15070-063), 2mM L-glutamine (Sigma, G8540), 10mM Hepes (Sigma, H7523), 30 μM glucose (Sigma, G8270) and 1X sodium pyruvate (Invitrogen, 11360-070). As the cultures aged, the percentage of FBS was lowered from 4% to 1% over a four week period. Half of the growth media was changed twice a week and replaced with fresh growth media. The cultures were allowed to grow in a 37^{o}C incubator, at 5% CO_2 for 2 to 3 weeks prior to being transfected or fixed for immunocytochemistry staining.

2.2.2 Cerebellar co-cultures

Dissociated cerebellar cultures were also generated using the same dissociated culture protocol as described in section 2.2.1. A one millimetre wide by three millimetre long section of the posterior region of the cerebellum was removed, triturated and plated. This brain tissue was used to confirm that NL3 staining was specific across different brain regions.

2.3 Methods for detecting gene expression

2.3.1 Immunocytochemistry

Immunocytochemistry was performed on our dissociated cultures once they were allowed to mature for two to three weeks. Cells were initially washed in extracellular bath solution (EBS) composed of 135mM NaCl, 10mM glucose, 3mM $CaCl_2$, 5mM KCl, $MgCl_2$, and 5mM Hepes, pH 7.35. This wash removes any proteins from the media which may stick to the chips prior to fixing the cells. Cultures were then fixed in a 15% picric acid (Sigma, P6744) and 4% paraformeldehyde (PFA) (Sigma, P6148) solution in 1X DPBS for 20 minutes at room temperature. The chips were then washed

3 x 5 minutes in 1X DPBS, and incubated in blocking solutions at room temperature for an hour in 5% goat serum (GS), 5% Donkey serum (DS), 0.1% Triton X (TX) and 2% Bovine serum Albumin (BSA) in 1X DPBS. The hippocampal cultures were then stained with specific primary antibodies diluted in blocking solution. The time at which the primary antibody remained on the cultures was antibody specific but usually was left on for an overnight incubation at 4^{o}C. The chips were then washed 3 x 5 minutes in blocking solution and stained with an appropriate secondary, diluted in blocking solution, for an hour at room temperature. Cells were then washed for 3 x 5 minutes in 1X DPBS and imaged on a Olympus BX61WI microscope, with a Watec camera or Nokia D300 camera using HawkVideo or Nikon Camera Control Pro software respectively. Image processing was conducted with Image J and Adobe Photoshop software.

2.3.2 Primary antibodies

The Neuroligin 3 (NL3) antibody used (Antibodies Inc, 75-158), was raised in mouse against fusion protein amino acids 730 to 848. This peptide sequence is found on the intracellular, C-terminus region of rat NL3. A Western blot confirmed that this antibody recognizes a protein band of approximately 110kDA which is the predicted weight of NL3 (**Fig.2.1**).

The OL marker antibody which was used was raised in mouse against O-antigens. O-antigens are sulfatides which function as markers on the surface of OLs. (Sommer and Schachner, 1981). During OL differentiation, O4 is expressed in pro-oligodendrocytes yet has not been found to express in O-2A progenitor cells (**Fig1.6)**. It has been found that O4 is expressed post-natally and is found on the surface of OL cell bodies and processes (Schachner *et al.*, 1981). The O4 antibody used was that of an IgM isotype where the NL3 antibody used was that of an IgG isotype. This made co-staining possible with the proper secondary antibodies. In order to prevent the IgM secondary from binding to the IgG epitope, a sequential staining was performed. This was performed by applying the first

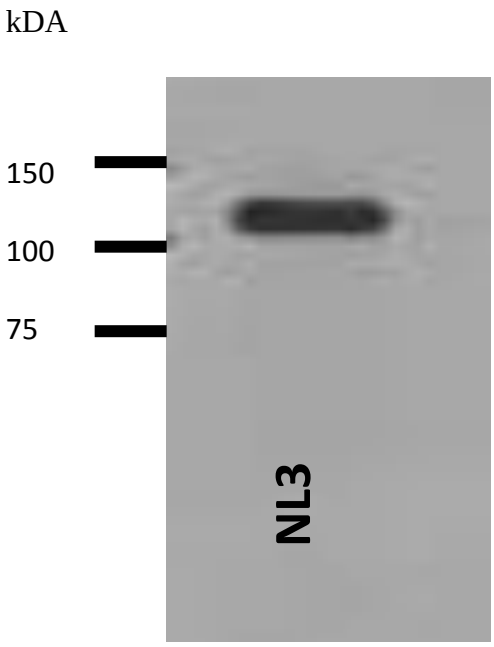

Figure 2.1

Figure 2.1: **Endogenous NL3 expression**

A Western blot on primary hippocampal lysate, showing endogenous expression of Neuroligin 3 (NL3) in this tissue. In order to confirm that the antibody we were using did recognize NL3, we demonstrated that it labelled protein at the expected molecular weight. A Bradford assay was performed on the primary cell lysate and 100μg of protein was loaded into the well prior to running the Western. This was a 10 fold increase in the minimum amount of protein which would be detectable by this antibody as per Antibodies Incorporated product guidelines. The mouse antibody, raised against NL3 (Antibodies Inc, 75-158) recognizes a protein of approximately 110kDA, which is the predicted molecular weight of Rat NL3. This antibody does not cross react with neuroligin isoforms 1, 2 or 4.

antibody, NL3 at a concentration of 1:1000, and allowing it to bind for two days at 4^{o}C. The proper secondary was then added, a Cy3 antibody (Jackson ImmunoRearch, 715-165-150). This secondary is a Cy3 conjugated Donkey- anti-mouse antibody and was applied for 1 hour at room temperature at a concentration of 1:2000. Once this secondary was added, and then washed 3 times in 1X DPBS, and images were taken. The second primary antibody was then added (O4, Millipore, MAB345) at a concentration of 1:2000 for an hour at room temperature. The appropriate secondary was then added (Life Technologies, A21042, Alexa-Fluor 488, goat anti-mouse IgM) at a concentration of 1:2000 and left to incubate for an hour at room temperature. Detection was possible under our Cy2 filter. Images were then taken once again, finding the same cells which were imaged with the initial primary antibody.

For the expression of the NR1 isoform, Rabbit polyclonal NMDAR1 was used (Abcam, ab28669) at a concentration of 1:500. For the NR2A isoform, the Rabbit, polyclonal anti-NR2A was used (Millipore, 07-632) at a concentration of 1:500. For the detection of the NR2B isoform, the rabbit polyclonal NMDA2B antibody was used (Abcam, ab14400) at a concentration of 1:500. And for the detection of the NR3A isoform, the rabbit antiNR3A was used (Millipore, 07-356) at a concentration of 1:1000.

To detect the co-expression of NL3 and the AMPA subunit GluR1, a rabbit polyclonal antibody was used (Millipore, ab1504) at a concentration of 1:500. To detect the expression of the isoform GluR2/3, the rabbit polyclonal antibody, ab 1506 was used (Millipore) at a concentration of 1:750.

The Flag antibody used to detect HEK cell expression of the NISA and NLR473E constructs (Sigma, F3165) was raised in mouse against Flag fusion proteins. This M2 clone recognizes a Flag sequence (N-DYKDDDK-C) inserted at any point in the protein.

2.4 Transfection protocol

The DNA constructs expressing NISA and NLR473E were obtained from Davide Comoletti, from the University of California, and were transfected into our HEK 293 cells via a calcium phosphate protocol. HEK cells were split at 1:10 and allowed to grow until roughly 60% confluent. Fresh HEK cell growth media composed of DMEM (Invitrogen, 11965-092), 10% fetal bovine serum (FBS) (Invitrogen, 16000-044) and 1% Penicillin Streptomycin (Penstrep) (Invitrogen, 15070-063) was added prior to transfecting the constructs. Six micrograms of DNA per 10 cm Petri dish was added to 450μL of 250mM calcium chloride (CaCl) solution. This was then added drop-wise to 500μL of 2x Hepes solution (254mM NaCl, 40mM Hepes, 12mM dextrose, 10mM KCl, 1.4mM $NaHPO_4$, pH 7.0). These two solutions were mixed and added to the fresh HEK cell media in a drop-wise fashion. The DNA was incubated for 5-6 hours in a 37^{o}C incubator before the HEK cell media was aspirated and replaced with fresh growth media. The transfection efficiency with this protocol yields approximately 40-50% of transfected cells via detection of GFP labelled constructs which are approximately the same size as our NISA and NLR473E peptides.

2.5 Western Blot analysis of transfected HEK cells

To test whether or not the HEK cells were expressing the transfected constructs, a Western Blot analysis was performed. The NLR473E and NISA transfected HEK cell cultures were washed with 1XDPBS. The DPBS was then aspirated and the cells were harvested with a lysis buffer consisting of 150mM NaCl, 50mM Tris, pH 7.4, 1% Triton and 1% NP40. Harvesting was performed by gently scrapping the HEK cells, grown in 10cm Petri dishes, with a cell scrapper. The lysates were then run on a 10% Acrylamide gel for an hour at 100 volts. Transferring of the gel to a nitrocellulose membrane was done for an hour at 120 volts in a 4^{o}C cold room. The blots were then blocked for an hour at room

temperature in 4% milk suspended in 1X DPBS, 0.1% Tween (Sigma, 93773). The monoclonal antibody, anti-Flag, raised in mouse (Sigma, F3165) was then added to the blocking solution at a concentration of 1:2000 and rotated overnight at 4^{o}C. The next day, blots were washed three times in 1X DPBS, 0.1% Tween. An ECL anti-mouse IgG, horseradish peroxidase secondary (GE Healthcare, NA931V) was added to the block solution at a concentration of 1:5000 and incubated for one hour at room temperature. Detection was made possible by adding 1mL of ECL Plus Western blotting detection system solution per blot (GE Healthcare, RPN2132). Blots were then detected using Kodak BioMax light film (Sigma, Z370371) or using a Chemidoc MP apparatus (Bio-Rad, Universal Hood III) (**Fig.2.2)**. A Bradford assay was performed on the transfected HEK cell lysate prior to running the samples in order to confirm concentration levels were approximate. This was done by using Thermo Scientific standards as per manufactures' guidelines.

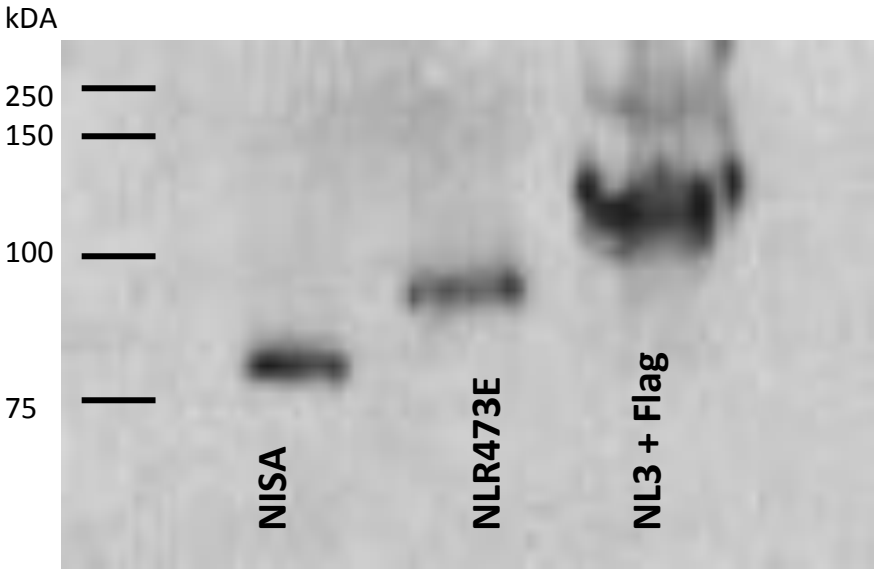

Figure 2.2

Figure 2.2: **Expression of NISA and NLR473E in transfected HEK cells**

To confirm that our HEK cell cultures were expressing the soluble proteins, a Western blot was run on the lysate of Ca^{2+} phosphate transfected HEK cells. The cells were given two days to express the transfected constructs and harvested at which point they were run on a gel. A nitrocellulose blot was probed with a Flag antibody and expression was detected using a Chemidoc station (Bio-Rad, Universal Hood III). The NISA peptide is approximately 90kDA. Due to the truncation of this protein, engineered with a stop codon at isoleucine 639, this is the expected protein size. The molecular weight of the NLR473E protein was larger due to its truncation, at tyrosine 692. The molecular weight of NLR473E is approximately 100kDa. These band sizes correspond to the original engineering of the constructs by Comoletti *et al.*, depicted in Figure 1.10. Lane three depicts a control, a Flagged labelled NL3 construct, provided by Dr. Robyn Flynn, University of Calgary. The NL3-Flag tag protein is approximately 110kDA. The control lane produces a band which is not as uniform as the experimental lanes. This could be due to the fact that the NL3-Flag control construct was much larger then the soluble NL constructs and the HEK cells, which the proteins were being expressed in, had a more difficult time expressing the larger construct.

2.6 Supplementation of the hippocampal cultures with NISA and NLR473E peptides from HEK cell conditioned media

Initial attempts to purify HEK cell expressed NISA and NLR473E peptides produced low yields even though the level of expression in the media was quite high. Therefore in order to have sufficient concentrations of NL peptides we chose to use NISA and NLR473E conditioned HEK cell media to administer the peptides into the primary cultures.

Following transfection, HEK cells were grown for two days in HEK cell growth media, in a 37^{o}C, 5% CO_2 incubator. This provided sufficient time for the cells to export large amounts of the soluble NL proteins into their ambient media. 50μL of conditioned HEK cell media was added to the 650μL of media present in the primary hippocampal co-cultures. These HEK cell media treated hippocampal cultures were treated for four days. A blank pcDNA vehicle was transfected into the HEK cells and this media was used as a control.

2.7 NX expressing HEK cells and hippocampal co-cultures

To produce an environment in which there was an increased number of NX targets for NL to bind to, HEK cells were transfected with a NX-pIRES construct containing a GFP reporter. These constructs were graciously provided by Dr. Robyn Flynn who engineered the GFP-ires-NX1β construct and Markus Missler who provided the GFP-ires-NX1α construct. The addition of the GFP tag provided confirmation that the HEK cells were expressing NX. After transfection, HEK cells where incubated for a period of two days, harvested and transferred to primary neuronal cultures. These co-cultures were incubated for four days. After this time point, cultures were fixed, stained and imaging was performed.

2.8 Oligodendrocyte identification and quantification

OL cell types were categorized into three stages; OPCs, immature OLs, and mature OLs (**Fig.1.7**). OL identification was performed using the antibody described in section 2.3.2. Cells were categorized based on the morphological characteristics previously described in section 1.2.1. A cell count for each of the different developmental OL stages was preformed. This quantification was performed by two lab members to prevent potentially bias counting. Inter-observer reliability had to be considered and each member who participated in the cell count was instructed on the strict set of parameters which were employed to differentiate the cell stages. The member's scores were averaged and normalized by creating ratios of cell stage to total number of cells in each region of interest (ROI). On each chip, ten ROIs were quantified and used for analysis. Media from HEK cells, transfected with an empty pcDNA vector, was used as a control.

Visualization was performed by using an upright Olympus BX61WI microscope under a 20x objective. If a specific cells' morphology could not be categorized under the established criteria to be placed into one of the three categories, i.e. OPC, immature and mature, then the cell was omitted from the total cell count. To normalize the cell counts, ratios of each developmental state to total cell count for each region of interest (ROI) were used for analysis. These cell type ratios for each ROI were averaged and plotted in Microsoft Excel.

2.9 *In situ* hybridization probes

In order to confirm OL cells produce the messenger RNA for NL3, *in situ* hybridization techniques were used. This was done by creating a plasmid which would then be used to make both sense and antisense probes against NL3. The Bluescript II SK (-) (Stratagene) vector was used as a backbone due to its T7 and T3 primer sites on either side of the Bluescript multiple cloning site (MCS). By using a pre-existing NL3 construct, a unique sequence fragment of approximately 293 base

pairs in length was isolated. Using restriction enzymes Hind III and EcoRV, this unique NL3 sequence was removed via a restriction digest which was performed overnight in a 37^{o}C water bath. This digest was run on a 1% agarose DNA gel and extracted using a Qiaquick Gel Extraction Kit (Qiagen, 28704) as per manufacturer's guidelines. DNA was then run once again on an agarose gel to confirm band size (**Fig.2.3)**. The Bluescript vector was also digested with the same restriction enzymes. Once the digests were complete, the NL3 fragment was ligated into the MCS of the Bluescript vector. To confirm proper orientation of the insert in the Bluescript vector, TELT mini preps were done. This protocol required the bacteria cells to be grown overnight in a 37^{o}C shaker. Bacteria cells were then spun for 30 seconds at 12,000 rpm; supernatant was then removed. The bacteria cell pellet was then re-suspended in 100μL of TELT buffer, consisting of 50mM Tris, pH 8.0, 62.5mM EDTA, pH 8.0, 2.5M LiCl and 4% TX. Once the pellet was suspended, 100μL of 25:24:1 Phenol/Chloroform/Isoamyl alcohol was added and vortexed for 15 seconds. This volume was then centrifuged for one minute at 12,000 rpm. The supernatant was then removed and added to 200μL of 95% ethanol. This solution was then centrifuged for five minutes is a 4^{o}C centrifuge. Supernatant was then removed and the DNA pellet was air dried. This pellet was re-suspended in 20μL of UltraPure distilled water (Invitrogen, 10977-015). The mini preps were then digested using restriction enzymes Kpn I and BamHI. These enzymes were used because they would cut into a region of the vector as well as the insert, yielding unique band sizes. These digests were run on an agarose gel and band sizes confirmed that the insert was present and in the correct orientation.

2.10 *In situ* hybridization

Primary hippocampal cultures were fixed for 20 minutes in 4% PFA in DPBS, and washed three time for five minutes in fresh DPBS. They were then rinsed in DEPC-water and mounted on tissue slides with inert grease. Cultures were then put through an acetylation step where they were submerged in 0.25% acetic anhydride in TEA for 10 minutes and washed twice for five minutes. The cultures were then placed in prehybridization solution, consisting of saline-sodium citrate buffer (SSC) (3.0M NaCl, 0.3M sodium citrate, 50% formamide. This step was done for a total of two hours at room temperature. Hybridization was performed by diluting the probes, both sense and antisense, at 1 to 200 in hybridization solution consisting of 1% Ficoll, 1% PVP, 2% BSA, 200μg per mL of salmon sperm DNA and 5x SSC and 50% formamide. The hybridization solution was first heated to 85^{o}C for 10 minutes and placed on ice for 5 minutes. The cultures were then incubated at 62^{o}C over night. A stringency wash was then performed by washing the cells in 5x SSC for 5 minutes, followed by a 1 minutes wash in 2x SSC, a 30 minute wash in 0.2x SSC and 50% formamide and 5 minute wash in 0.2x SSC. Immunocytochemistry was then performed on the cultures by washing the cells for 5 minutes in Buffer 1, consisting of 100mM Tris and 150mM NaCl. They were then blocked for 1 hour in 1% Blocking Reagent (Roche, 11096176001) suspended in Buffer 1. Cultures were then washed for 5 minutes in Buffer 1 and incubated with anti-DIG-Rhodamine Fab (Roche, 11207750910) in 1% BSA, 5% sheep serum and 0.3% TX in Buffer 1 for an hour. Cells were then washed twice for 15 minutes in Buffer 1, followed by a 5 minute wash in Buffer 2, consisting of 100mM Tris, 100mM NaCl and 5mM $MgCl_2$. Finally cells were washed in DPBS and images were taken.

2.11 Statistics

A one way ANOVA was run on the data using SPSS (IBM). Analysis was performed within groups, comparing cell ratio counts within cell stages. A homogeneity of variance test was performed as well as a normality test. These tests confirmed that my data was normal and 3 one-way ANOVAs were performed for the HEK cell expressing soluble NL proteins; a one-way ANOVA for each cell stage. A single one-way ANOVA was performed for the NX expressing HEK cell and hippocampal culture quantification.

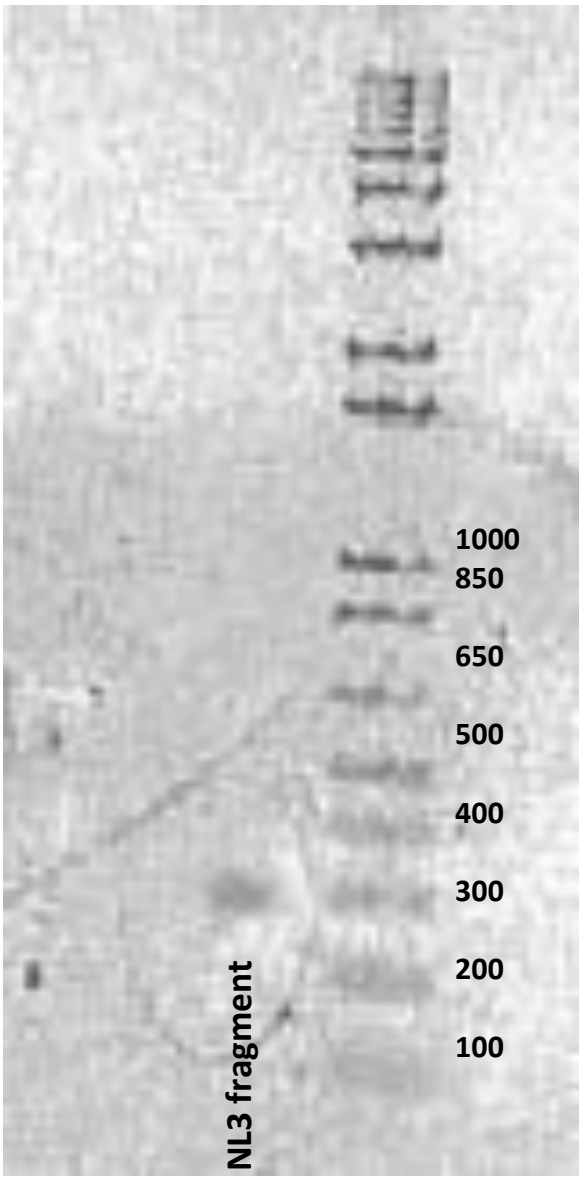

Figure 2.3

Figure 2.3: **Isolated NL3 DNA fragment**

This region of NL3's sequence was isolated due to being unique to NL3, and not present in the NL1 or NL2 sequences. This region of NL3's sequence, of 293 base pairs, was digested out of a NL3-Flag construct, provided my Dr. Robyn Flynn, by using restriction enzymes Hind III and EcoRV. It was then gel extracted and purified and run on an agarose gel to confirm band size. This figure shows the only band, of approximately 293 base pairs after the digest was complete, confirming that the NL3 insert was of predicted size. The Bluescript vector was also digested using the same restriction enzymes. The NL3 insert was then ligated into the Bluescript vector, creating the NL3-Bluescript sequence. This engineered construct was then used by Dr. Cristiane de la Hoz as a template to synthesize the NL3 probes which were later used for *in situ* hybridization experiments (as explained in section 2.10).

CHAPTER 3: EXPERIMENTAL RESULTS

3.1 Expression of neuroligin in oligodendrocytes

3.1.1 Immunocytochemistry

The first step in investigating this hypothesis was to confirm that NL3 is expressed in oligodendrocytes. This was demonstrated by immunocytochemistry; staining neuronal cultures with anti-NL3 antibody (UC Davis Antibodies Inc., 75-158), while co-staining for the OLs with the OL specific marker O4 (Millipore, MAB345). This marker is useful because it binds to proteins which are expressed through much of the OL developmental profile (refer to Fig.1.6). Expression of NL3 can be visualized in O4 positive OL cells. Increasing the strength of our signal, by incubating our antibody for a longer period of time, facilitated the detection of NL3 in the myelinating population of glial cells (**Fig.3.1**). This trend was seen across multiple dissociated hippocampal cell preparations (N=6).

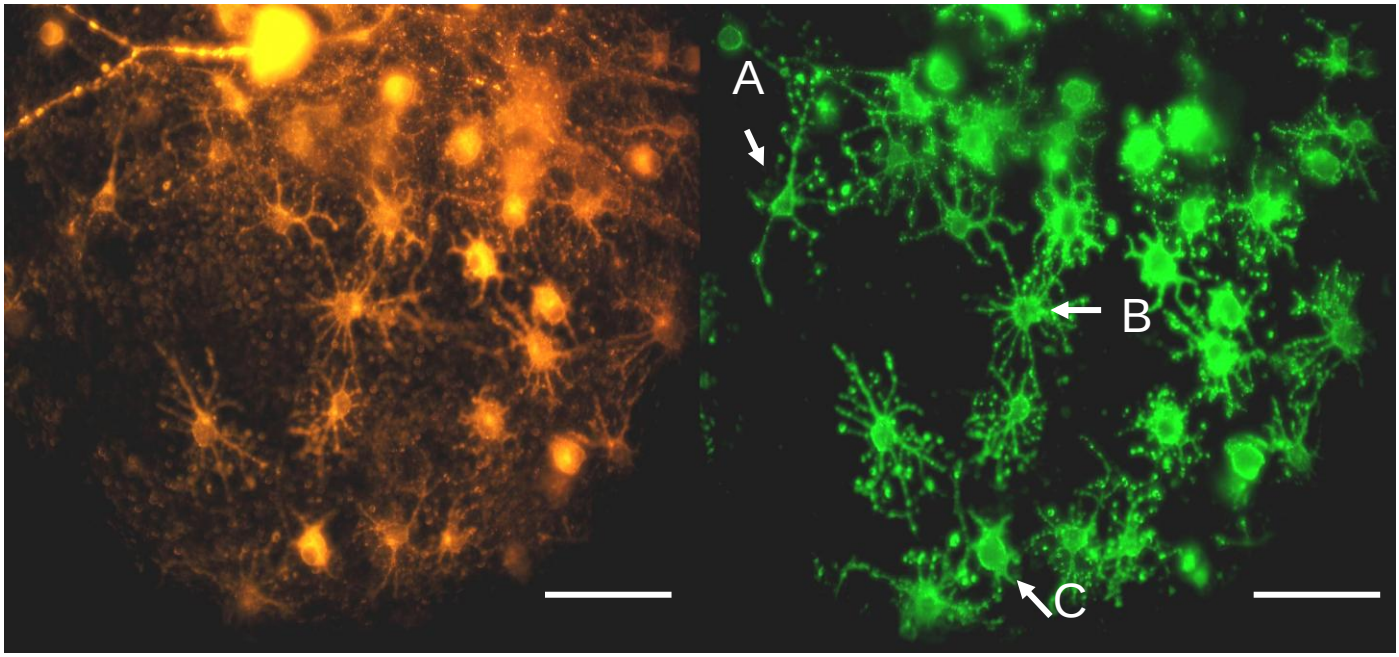

Figure 3.1

Figure 3.1: **Co-expression of NL3 and O4 in dissociated cultures**

Neuroligin 3 (NL3) expressing oligodendrocytes. Neuroligin (red) can be seen in all three of the established morphological states labelled in green with an OL distinguishing antibody, O4; mature cells (**A**), immature cells (**B**) and OPCs (**C**). NL3 antibody from Antibodies Inc., O4 antibody from Millipore. Scale bar = 50μm.

3.1.2 In situ Hybridization

In order to confirm my initial immunocytochemistry results, an *in situ* hybridization protocol was performed on two week old dissociated cultures. Due to the difficulty of these experiments, Dr Cristiane de la Hoz optimized the protocol and created NL3 probes off of the NL3-Bluescript construct which I engineered, as described in section 2.9. By using Digoxigenin-labelled UTPs, and sequentially labelling with an Anti-NG2 Chondroitin Sulfate Proteoglycan antibody (Millipore, AB5320) to confirm OL cell types, she successfully demonstrated the expression of NL3 in NG2 positive oligodendrocytes. The NG2 antibody was used rather than the O4 antibody as it gave superior results following the *in situ* hybridization procedure. Results showed that NG2 positive OPCs were positive for the NL3 messenger RNA and no signal was obtained form the control NL3 sense probe (**Fig.3.2**).

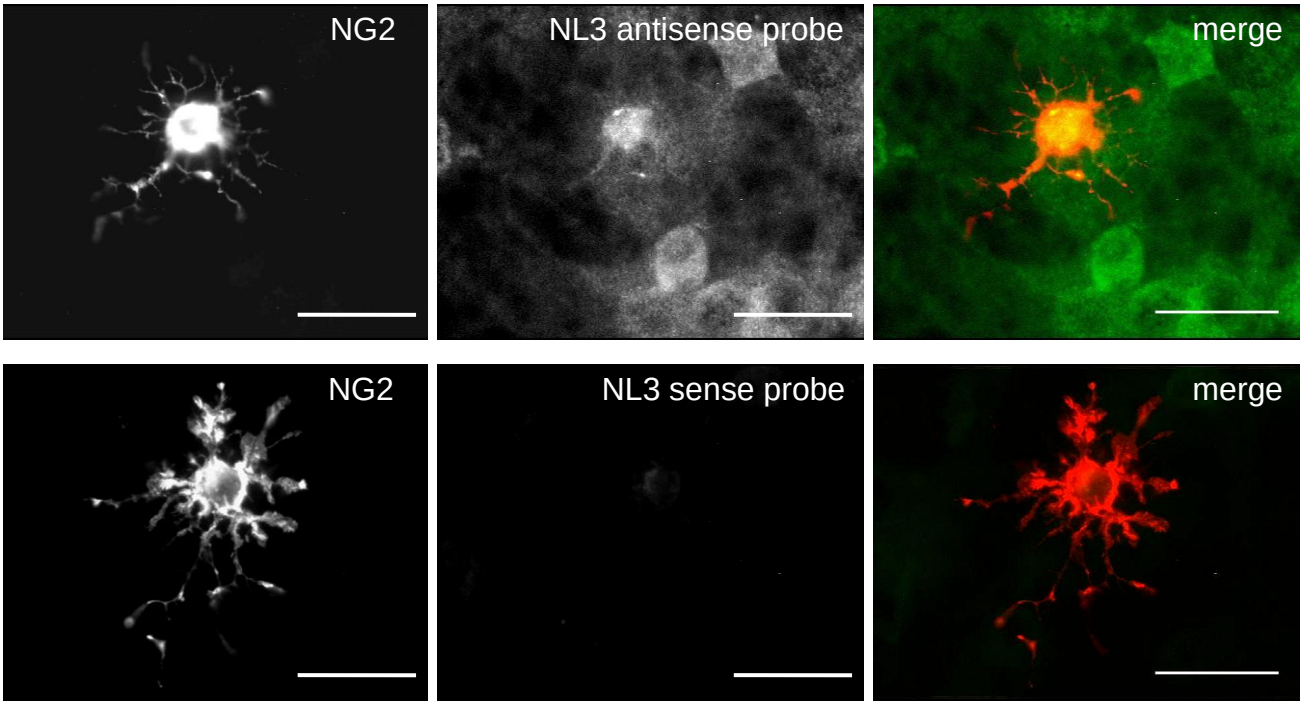

Figure 3.2

Figure 3.2**: Expression of mRNA in oligodendrocyte cells**

In situ hybridization results in NG2 positive cells. NG2 was used to identify the oligodendrocyte precursor cells (OPCs). Here we can see that the antisense probe did bind effectively to the NL3 mRNA however the sense probe did not. These probes were created using the constructs which were made, as explained in section 2.9. Dr. Cristiane de la Hoz created the probes and performed the *in situ* experiments. It is evident to see the NL3 antisense probe bound to what appears to be neurons as well as the OL. Scale bar = 25μm.

3.1.3 NL3 positive staining in dissociated cerebellum cultures

In order to confirm that OLs, across different brain areas were expressing NL3, dissociated cerebellum cultures were stained for NL3 & O4 (**Fig.3.3**). The cultures were given two weeks to mature and then fixed as per our protocol as described in section 2.3.1. We also found that there is co-expression of O4 and NL3 in cerebellum culture preparation. This confirms that OLs amongst different brain tissues do express this NL isoform.

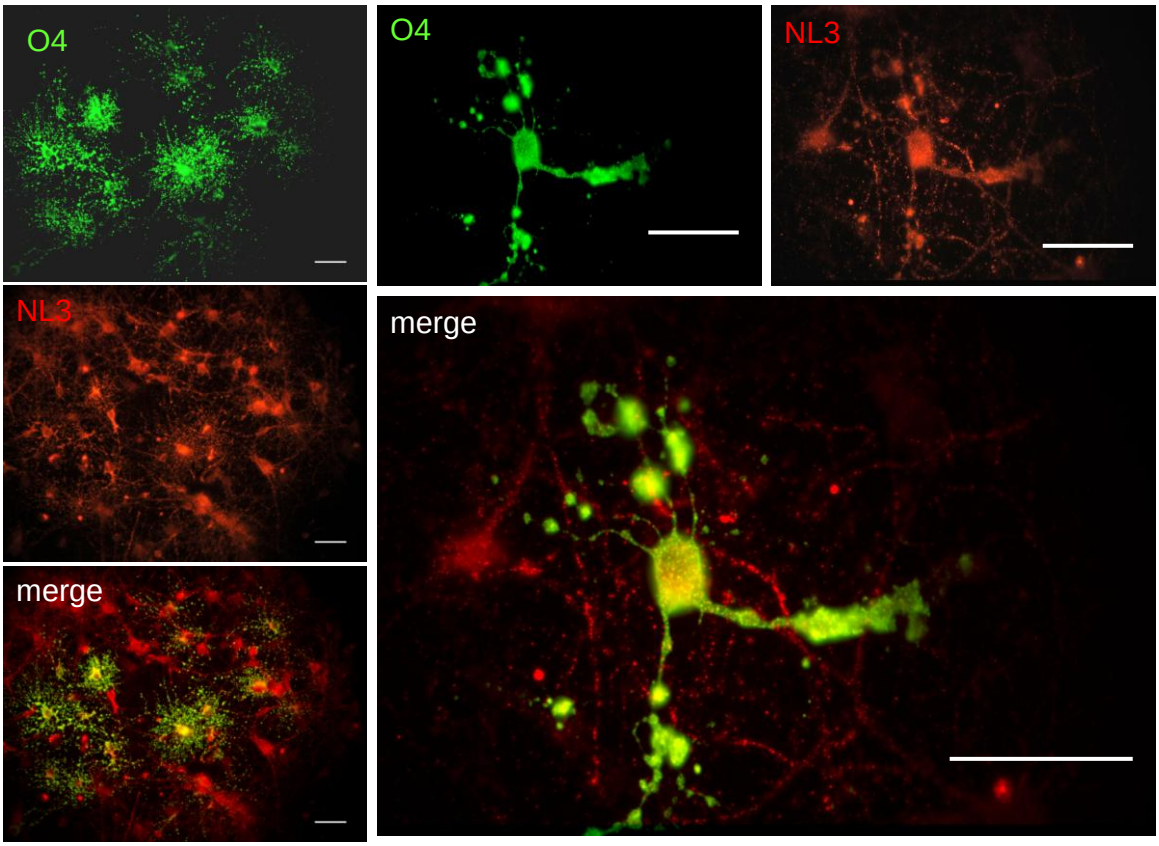

Figure 3.3

Figure 3.3: **NL3 presence in cerebellum oligodendrocytes**

Expression of NL3 in O4 positive cells from cerebellum dissociated cultures. These dissociated cultures were generated to confirm that NL3 is expressed in OLs from different brain tissues. Cerebellum OLs and hippocampal OLs have different developmental expression profiles, however we can still see NL3 positive staining in this glial subtype. Scale bars = 50μM.

3.2 Regulation of oligodendrocyte differentiation

3.2.1 Blocking NL/NX signalling with exogenous peptides

By treating primary hippocampal cultures with HEK cell media, conditioned with either NISA and NLR473E peptides or a vehicle control, we investigated the effect of interfering with normal NL/NX signalling due to the presence of exogenous NX binding protein in the environment. We found that the NISA media treatments lead to a higher percentage of cells in the immature, highly branched cell state whilst the NLR473E and vehicle control conditions yielded a greater population of OL cells in the mature, elongated, myelinating forms compared to the NISA treated cultures (**Fig.3.4**).

These results strongly suggest that OL differentiation state is perhaps modulated by the NX/NL interaction. The transfected HEK cells expressing NISA and NLR473E peptides are producing a soluble NL protein which is being exported into the media, and once this media is added to the hippocampal cultures it is presumed to bind to endogenous NX sites on the axon. This would prevent endogenous NL, expressed on the surface of the OL cells, from binding to its synaptic counter-part. This ‘blockage’ ultimately leads to OLs which do not differentiate into their myelinating cell form.

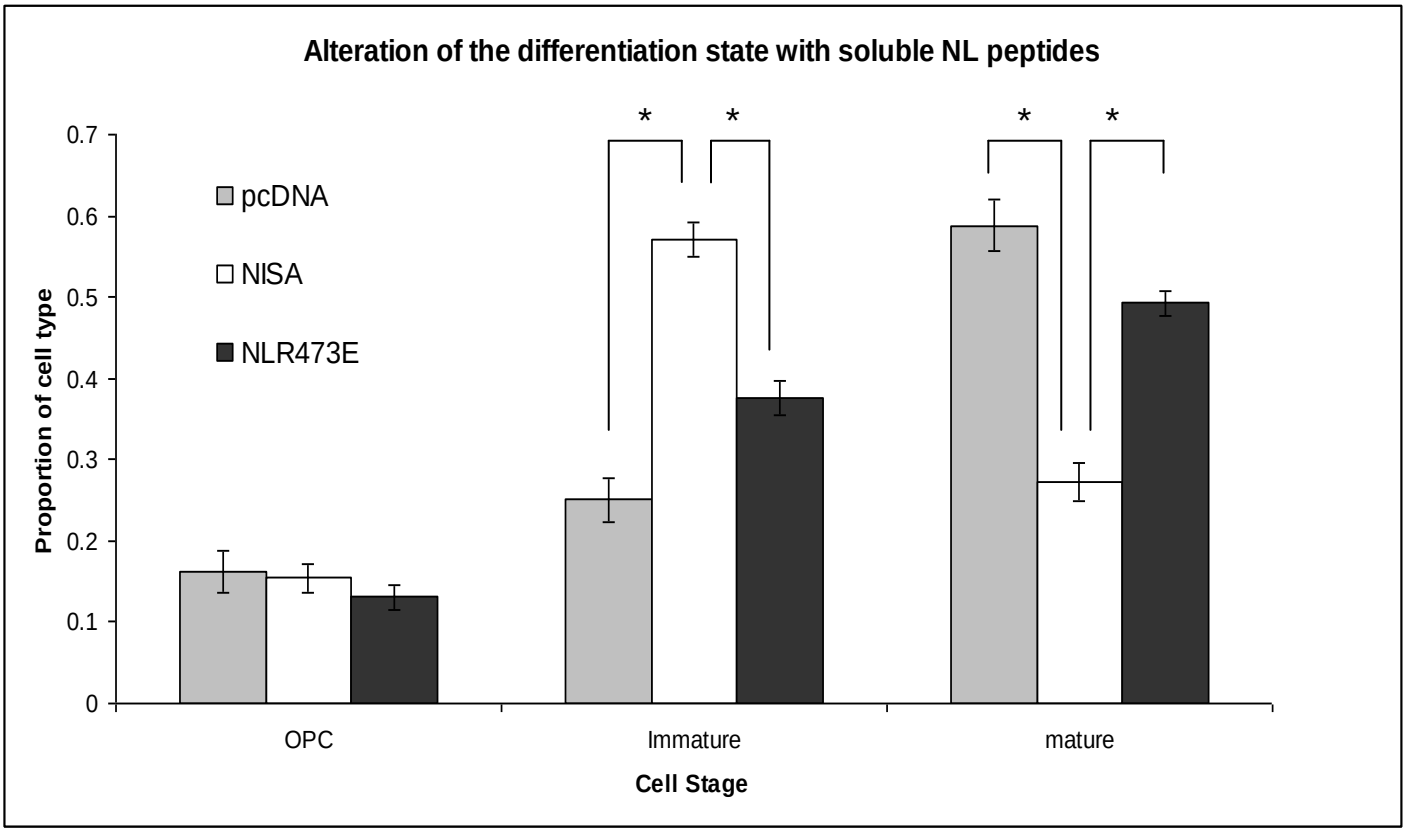
Alteration of the differentiation state with soluble NL peptides
Proportion of cell type
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
pcDNA
NISA
NLR473E
*
*
*
*
OPC
Immature
mature
Cell Stage

Figure 3.4

Figure 3.4: **Quantification of OL morphological state with respect to neuroligin peptide treatment.**

HEK cells were transfected, and given time to express the exogenous NISA and NLR473E peptides. After 2 days, media was collected and 50μL was added to 2 week old hippocampal cultures. These cultures were incubated with the HEK cell media for 4 days and fixed for O4 staining. Error bars show standard error of the mean (SEM). A One-way ANOVA was run on the groups; OPC ($F(2,159) = 0.784$, $p = 0.459$, Immature ($F(2,159) = 39.53$, $p < 0.001$, Mature ($F(2,159) = 50.343$, $p < 0.001$ (N=3). Asterisks represent a significant difference, $P < 0.001$.

3.2.2 NX expressing HEK cell and primary hippocampal co-cultures

In order to perform the corroborating experiment to the application of NX binding NL peptides, we expressed NX on the surface of HEK cells and co-cultured them with neurons. The logic behind this was to provide the culture environment with an excess of NX targets for the NL3 on oligodendrocytes to interact with. Our hypothesis was if blocking the interaction between NX and NL stalled the developmental state of OLs, by providing more NX targets perhaps we could enhance the transition of the OLs to the elongated, myelinating form.

To see if having additional NX sites present in the primary culture would have an impact on OL morphology, we over expressed NX and quantified developmental changes in OL cells. Quantification revealed a significant difference in morphological states between the NX1α expressing HEK cells and CFP expressing control (**Fig.3.5**). An image showing a typical ROI which was used for this analysis (**Fig.3.6**).

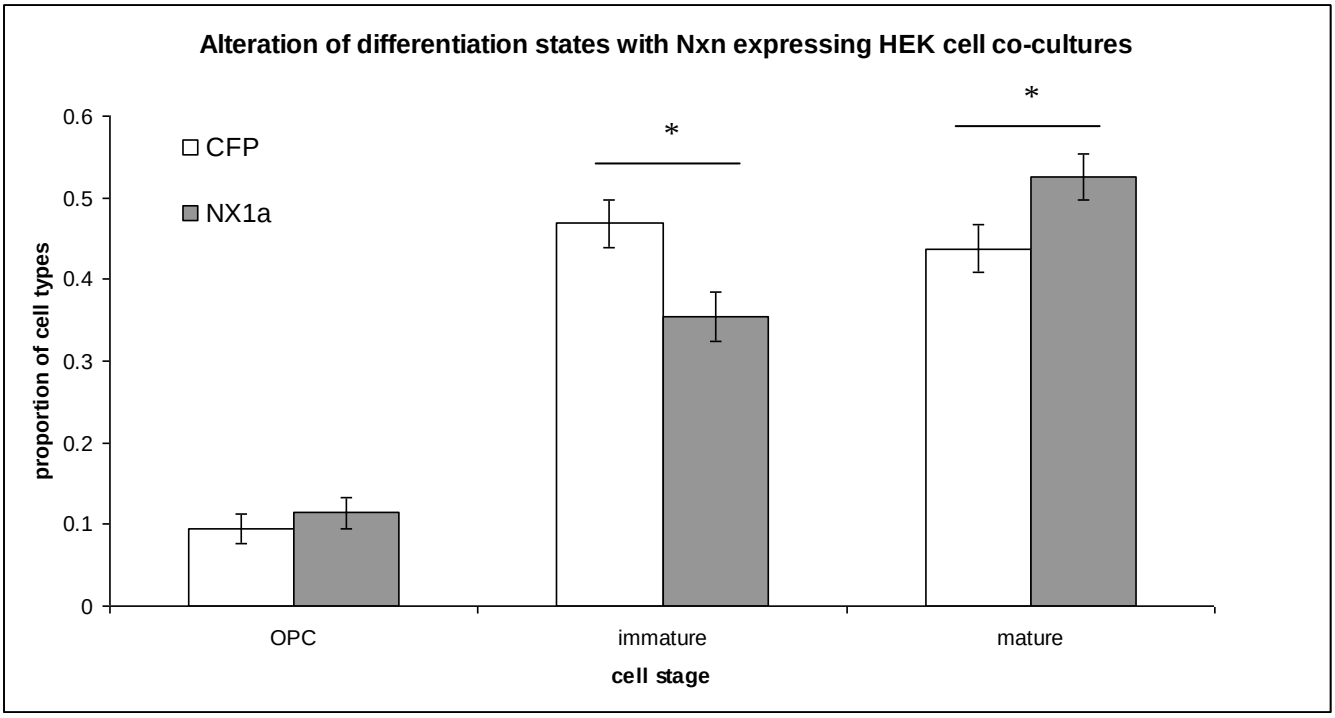
Alteration of differentiation states with Nxn expressing HEK cell co-cultures
proportion of cell types
0.6
0.5
0.4
0.3
0.2
0.1
0
CFP
NX1a
*
*
OPC
immature
mature
cell stage

Figure 3.5

Figure 3.5: **Quantification results from O4 positive OL cells in NX transfected HEK cell co-cultures.**

Quantification of NX transfected HEK cell and primary hippocampal co-cultures. HEK cells were transfected, and given time to express the exogenous GFP tagged NX-α and CFP proteins. After 2 days, cells were fixed, stained with O4 and OL quantification was performed. Here we can see an increase percentage of mature OL forms in the NX-GFP co-culture compared to the CFP vehicle. Standard error bars depict standard error of the mean (SEM). A One-way ANOVA was run on the groups; OPC ($F(1,109) = 0.542$, $p = 0.463$, Immature ($F(1,109) = 7.206$, $p = 0.008$, Mature ($F(1,109) = 4.641$, $p = 0.033$. Asterisks represent a significant difference of $P < 0.05$.

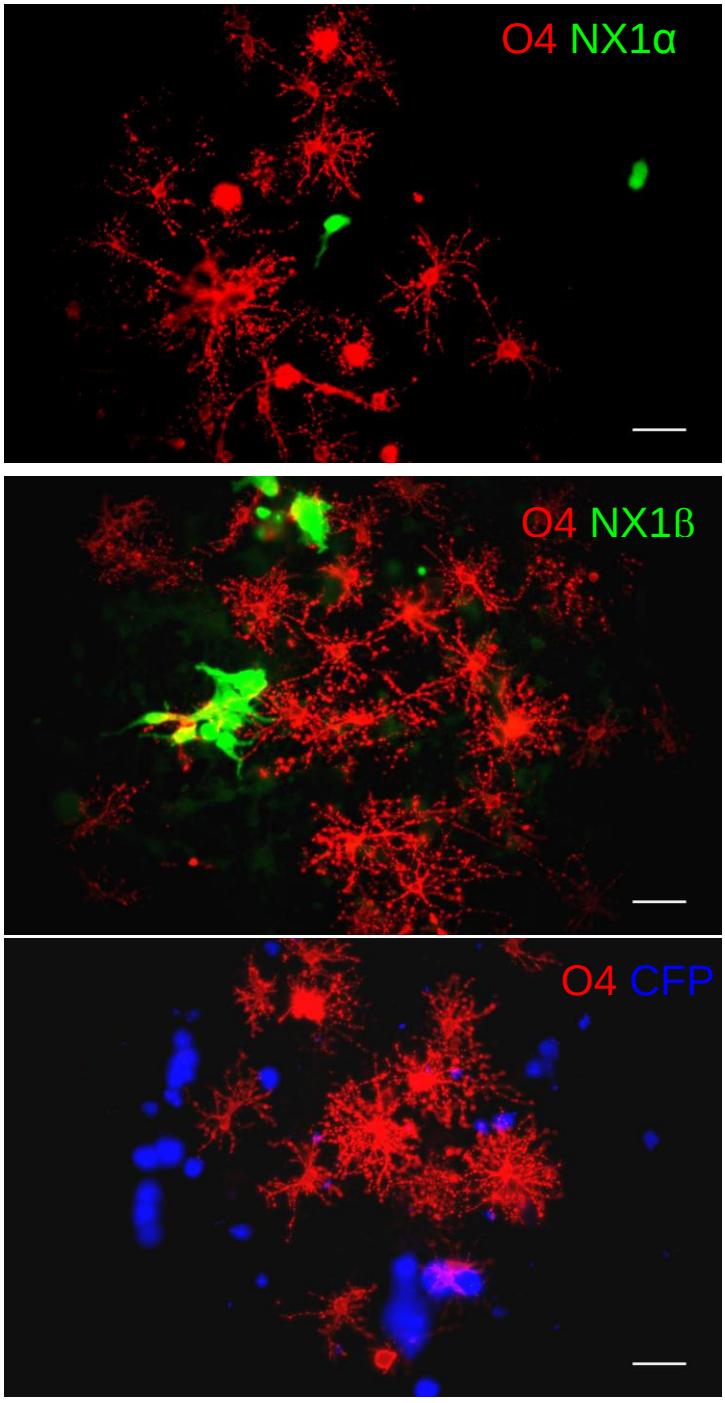

Figure 3.6

Figure 3.6: **Range of oligodendrocyte morphologies in the presence of NX transfected HEK cells**

A depiction of the summary from Figure 3.13. This is an example of a region of interest (ROI) which was used to quantify OL differentiation states. Ten ROIs per chip were used for our statistical analysis. The GFP tag was used to confirm that the HEK cells which were added to the primary hippocampal cells were expressing the NX construct. Scale bar = 50uM.

3.2.3 Direct visualization of oligodendrocyte interactions with NX expressing HEK cells

To further validate these corroborating experiments, one would expect to be able to visualize an interaction between the oligodendrocytes and the NX expressing HEK cells. To test this, HEK cells were transfected with a NX1α-IRES-GFP construct or a NX1β--IRES-GFP construct via a calcium phosphate protocol described in section 2.4. HEK cells are good candidates for this experiment as their non-neuronal cell properties would not have an impact on any direct interaction which was observed. A CFP construct was transfected into the HEK cells as a control. HEK cell and primary hippocampal co-cultures were generated as previously described and immunocytochemistry was performed as summarized in section 2.2. Cultures were stained with the OL marker O4 and images were taken (**Fig.3.7**). OL processes form contacts with HEK cells over-expressing the NX constructs.

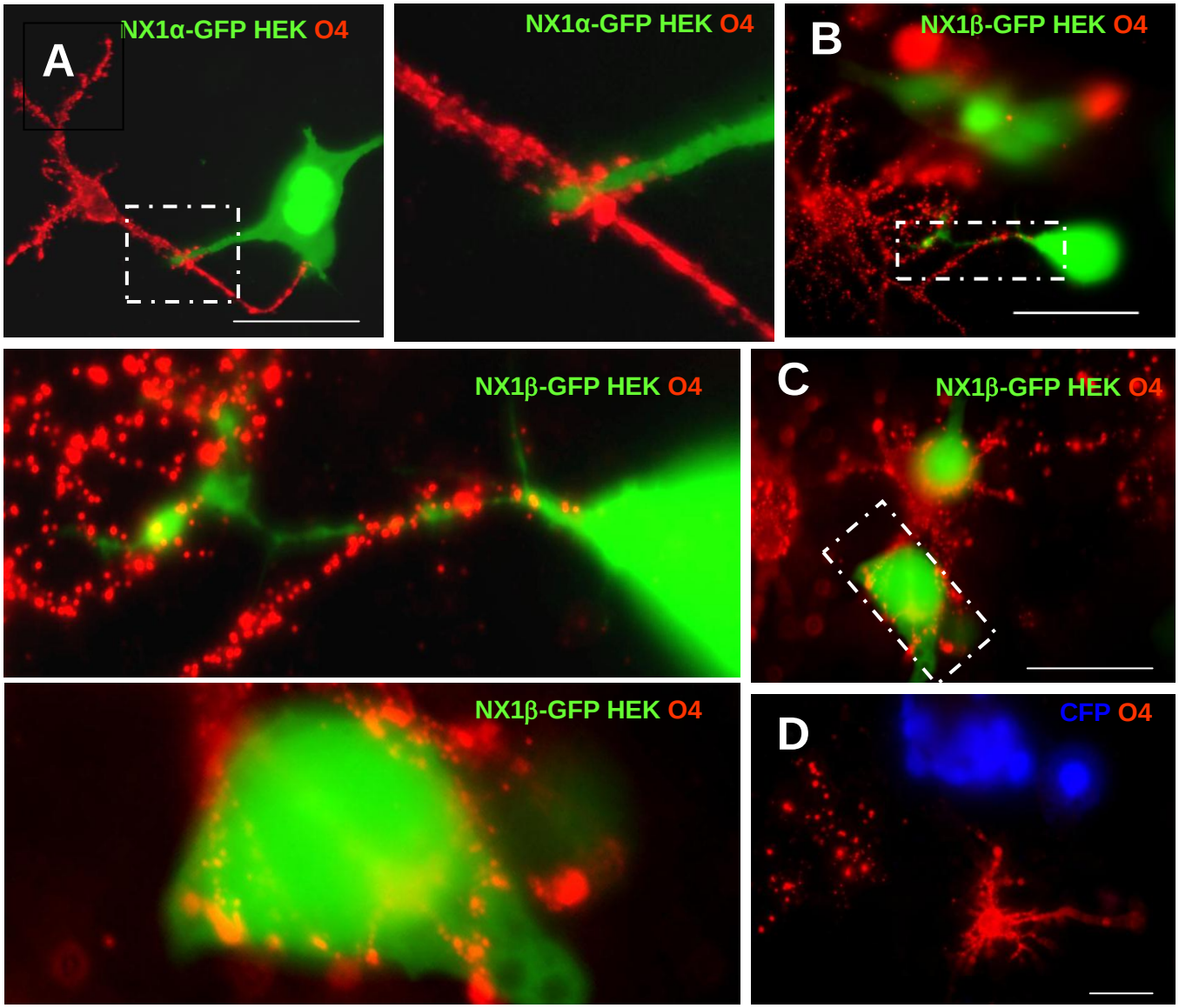
A
NX1α-GFP HEK O4
NX1α-GFP HEK O4
B
NX1β-GFP HEK O4
NX1β-GFP HEK O4
C
NX1β-GFP HEK O4
NX1β-GFP HEK O4
D
CFP O4

Figure 3.7

Figure 3.7: **Hippocampal OLs interacting with Neurexin-GFP (NX-GFP) expressing HEK cells**

Representative images of O4 positive OLs interacting with HEK cell over-expressing NX in a co-culture model. HEK cells were transfected with a GFP tagged, NX1 constructs. (**A**) Evident interactions with the O4 expressing OL and the GFP tagged NX1α expressing HEK cell. The OL process actually changed its growth direction and appears to be enwrapping the HEK cell process. Although we can not prove that it is myelinating this process, an evident enwrapping can be seen. (**B**) This immature OL cell appears to be enwrapping its processes around a HEK cell extension (as seen in the enlarged image). In panel **C**, we can see multiple OL processes enwrapping around of the GFP tagged NX1β expressing HEK cell, as seen in the enlarged image. (**D**) Here we can see that HEK cells expressing our CFP vehicle do not have an evident interaction with the OL cells. Scale bar = 25uM.

3.3 Receptor subunit expression on oligodendrocytes

3.3.1 NMDAR in OLs

The expression of *N-methyl-D-aspartate* receptors (NMDAR) in OLs has been reported in tissue slice models. However the expression profile of NMDAR in dissociated hippocampal cultures is less known. Furthermore, the localization of NMDAR with regards to NL3 expression had not been studied in the OL cell population. Therefore, we next set out to characterize NMDAR expression in our culture system and examine the relationship of NMDAR to NL3 expression in cultured OLs. Staining cultures with NMDAR antibodies against NR1, NR2A, NR2B and NR3a, we confirmed the presence of NMDA receptor subtypes in O4 positive OL cells in accordance with previously reported data in slice preparations (**Fig.3.8-3.10**). Co-labelling OLs with NL3 and NMDAR revealed selective co-localization of NL3 with the NR3A subunit. NL3-NR3A positive puncta were present in both immature and mature, myelinating forms of OLs. This expression was present on the surface of the cell body as well as in the distal processes of O4 positive cells.

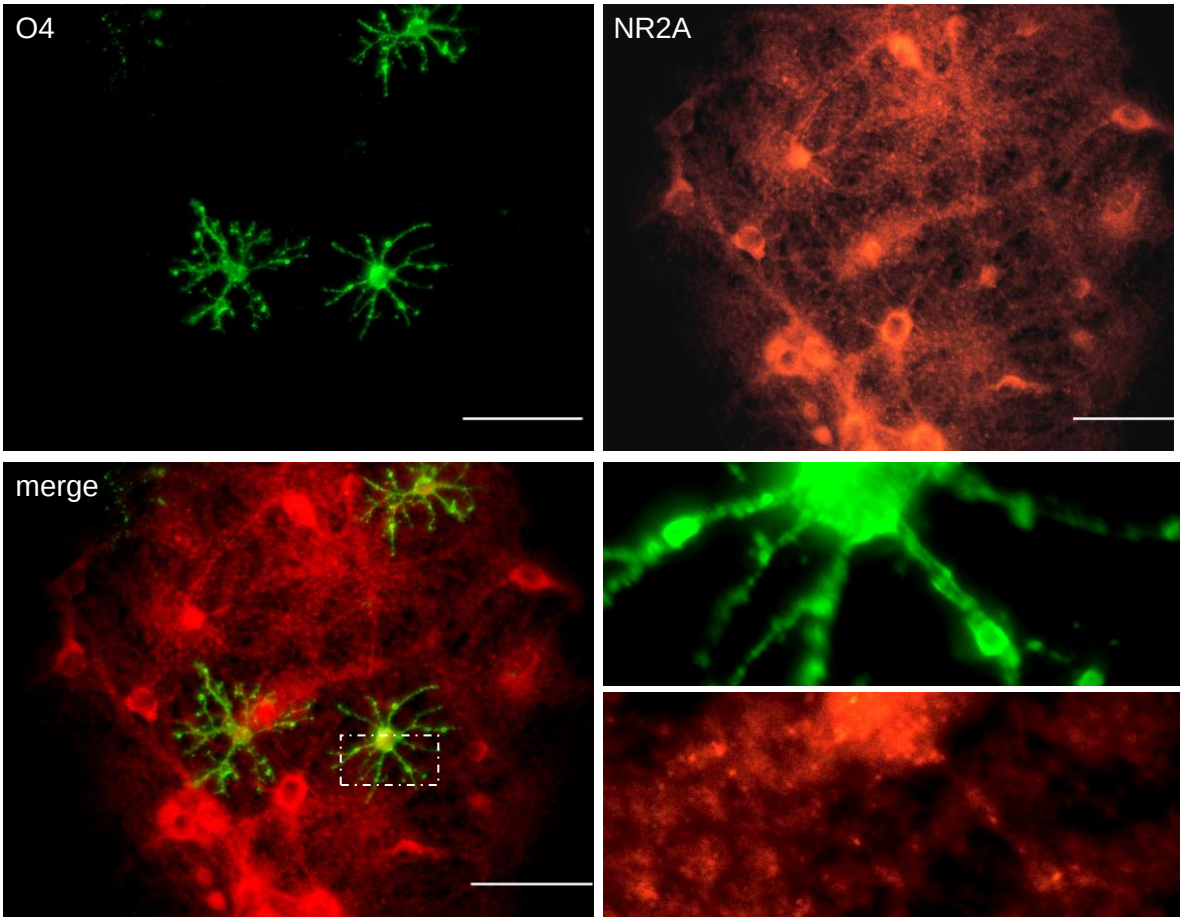

Figure 3.8

Figure 3.8: **Presence of the NMDA receptor NR2a within O4 positive OL cells**

Expression of the NMDA receptor 2a (NR2a) in O4 positive OL cells. NR2A expression can be seen in the cell body as well as O4 positive processes. If given more time to optimize the antibody conditions, the level of background fluorescence could be reduced. This would provide a better 'signal to noise' ratio for these specific images. Although the expression levels of the NR2a are weak, it is evident to see positive staining on the cell body of the OL cell. Scale bar = 50uM.

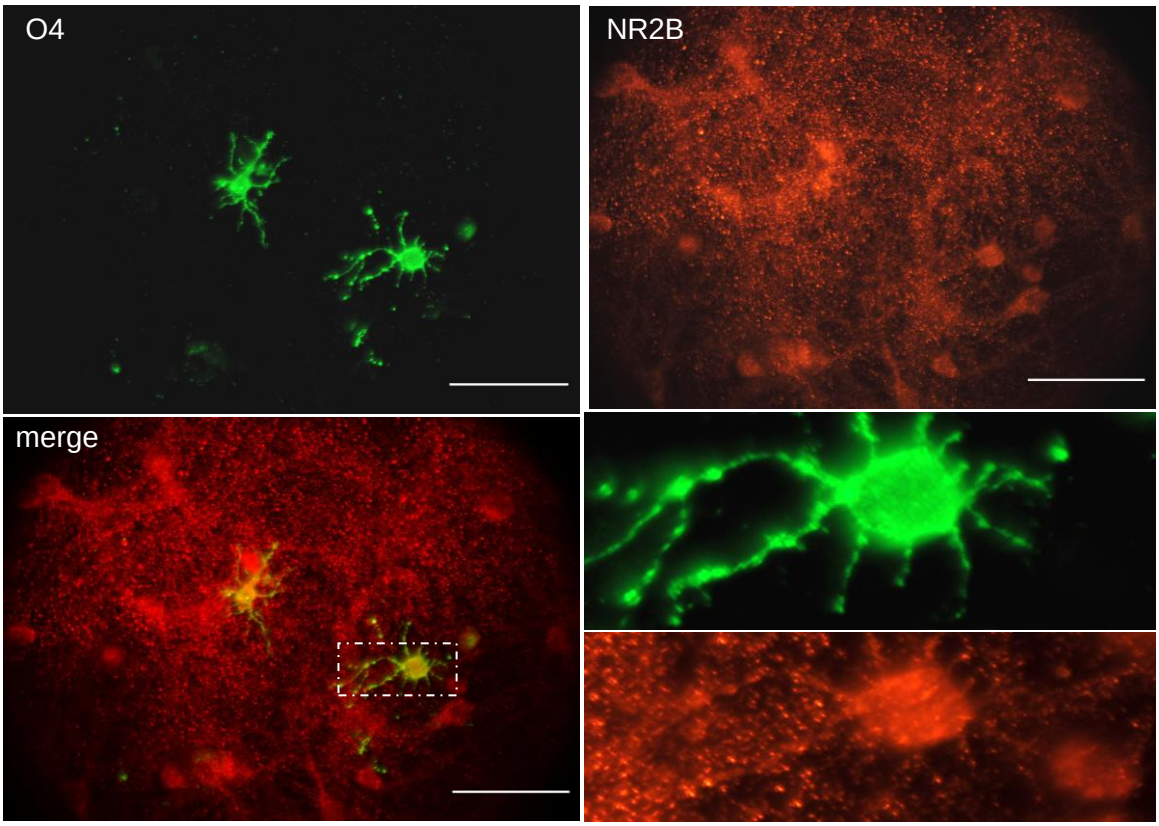

Figure 3.9

Figure 3.9: **Presence of the NMDA receptor NR2b in O4 positive OL cells**

Expression of the NMDA receptor 2b (NR2b) in O4 positive OL cells. NR2b expression can be seen in the soma as well as O4 positive processes. Although it would appear that OL processes are labelled, it appears that only distal processes seem to be expressing the NR2b subunit. This staining produced a high level of background fluorescence, and if time permitted, optimizing the antibody conditions may produce a lower background level. Scale bar = 50uM.

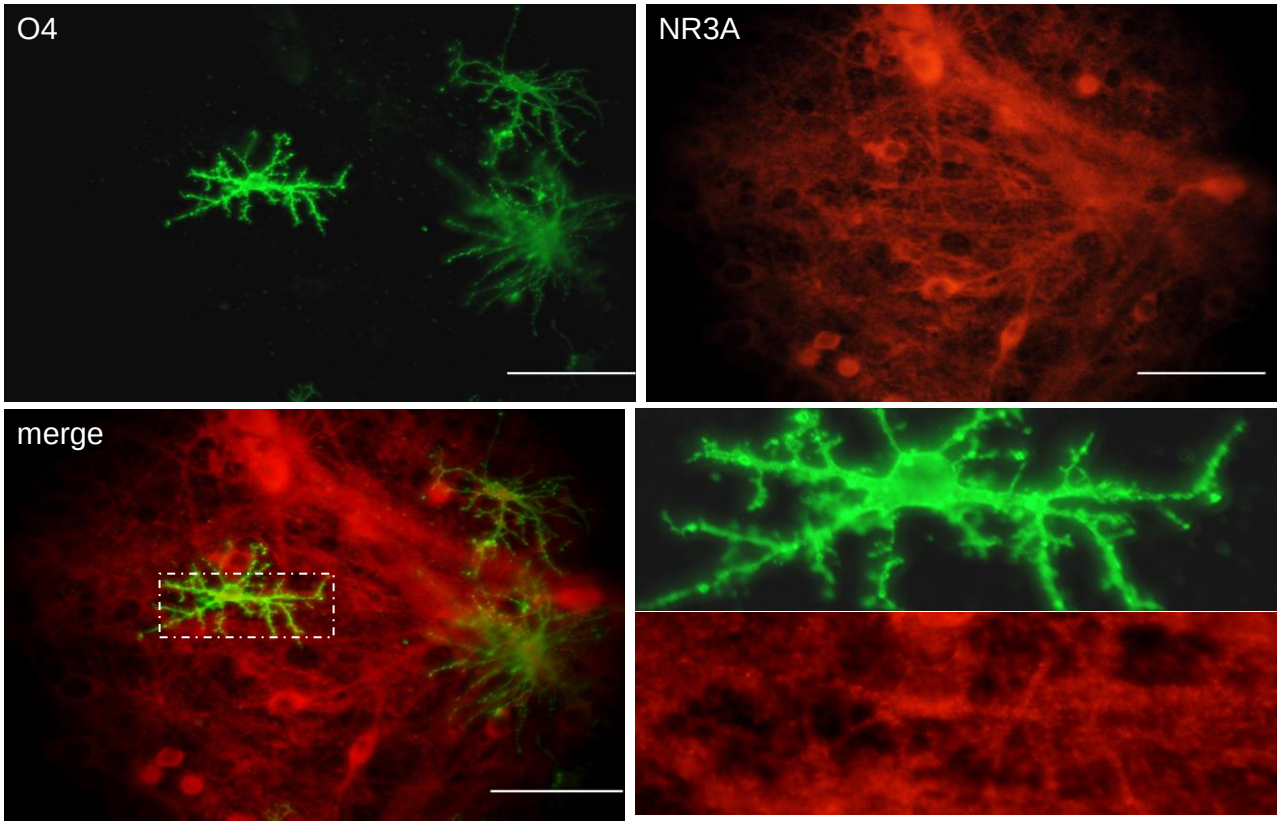

Figure 3.10

Figure 3.10: **Presence of the NMDA receptor NR3a in O4 positive OL cells**

Expression of the NMDA receptor 3a (NR3a) in O4 positive OL cells. NR3A puncta are located in the distal ends of the OL processes. It is also evident to see neuronal cell bodies and processes are expressing the NMDAR subunit NR3a. The high level of background fluorescence may be due to the expression of this subunit in numerous cell types, i.e. astrocytes and neurons. Staining in the cell bodies as well as neuronal processes is visible. If time permitted, optimizing the antibody conditions may reduce the 'signal to noise' ratio. Scale bar = 50uM.

3.3.2 NMDA and NL3 co-localization

Although the co-localized expression of NMDARs and NL3 has been well documented in neuronal cell types, less extensively studied is the co-localization of these proteins in the OL cell population. Labelling with all the NMDAR antibodies shed light on the fact that there is really only a good representation of co-localization amongst the NR3A isoform and NL3 in O4 positive cells (**Fig. 3.11**). Although this image does not include a stain for O4 to confirm that the cells highlighted are OLs, the morphology and arborization, plus the size of the cell body when compared to neuronal cell bodies, strongly suggest that they are OL cells.

3.3.3 AMPAR and NL3 co-expression

In congruence with previous studies' findings (Brand-Scheiber and Werner, 2003), I was not able detect AMPAR GluR1 isoform expression in OL cells (**Fig.3.12**), however, after staining for the GluR2/3 subunit we detected modest expression of this receptor subtype in the OL population. (**Fig.3.13**).

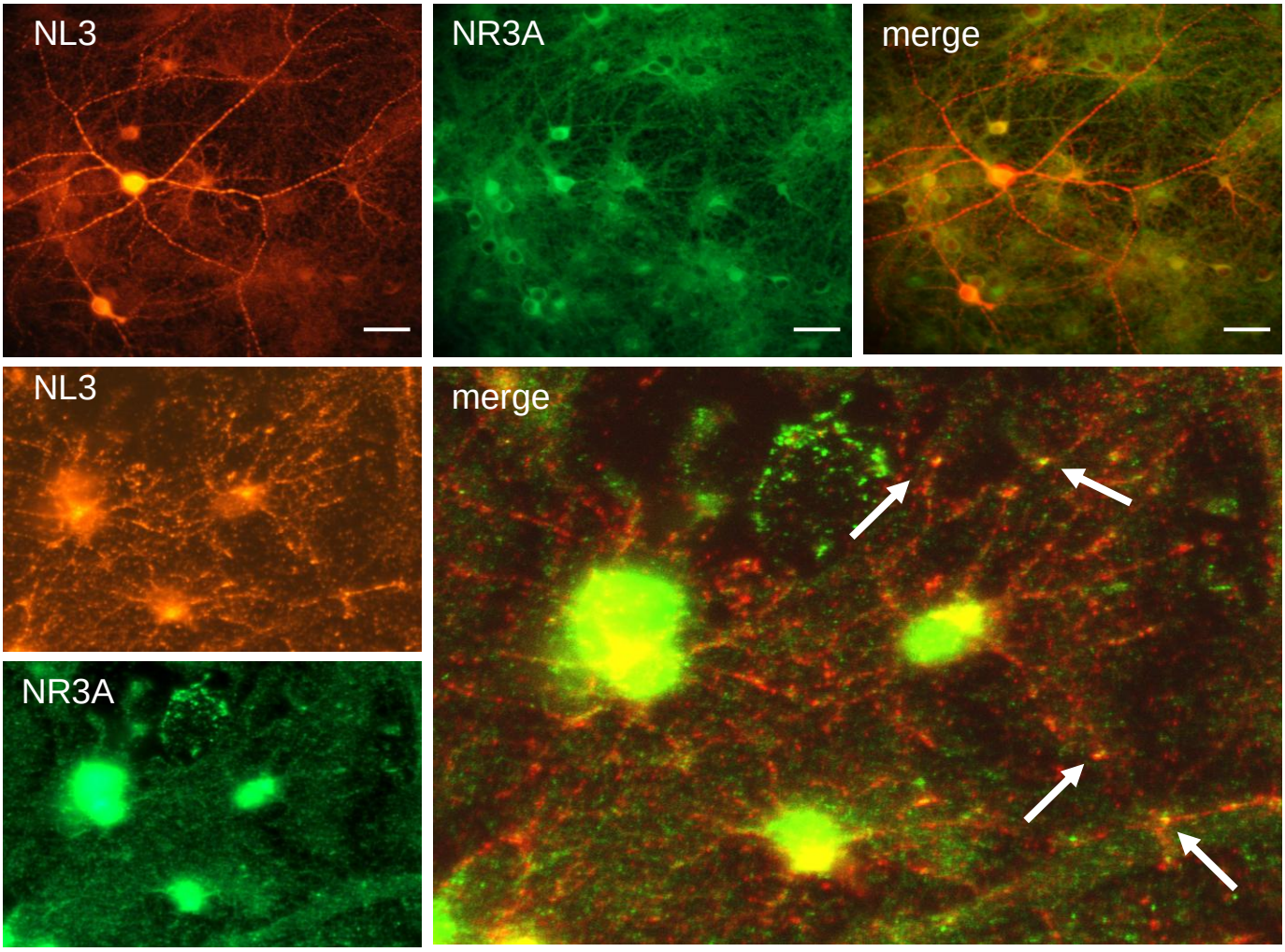

Figure 3.11

Figure 3.11: C**o-localization of NL3 and the NMDA subunit NR3a**

Here we can see the co-localization as well as co-expression of NL3 and the NMDA receptor 3a. The high level of background fluorescence is due to the expression of this subunit in numerous cell types, i.e. astrocytes and neurons. Although we do not have an O4 stain present to confirm that these are OL cells, the size of the cell body as well as the arborization of the processes identifies these cells as oligodendrocytes. The top panels show a good representation of co-expression with NL3 and NR3a and the bottom panels depict co-localization in the distal feet of the OL processes. Here we can see co-localization occurring at the 'feet' of the OL processes. It is at this point where axonal contact is made and myelination occurs. Scale bar = 25uM.

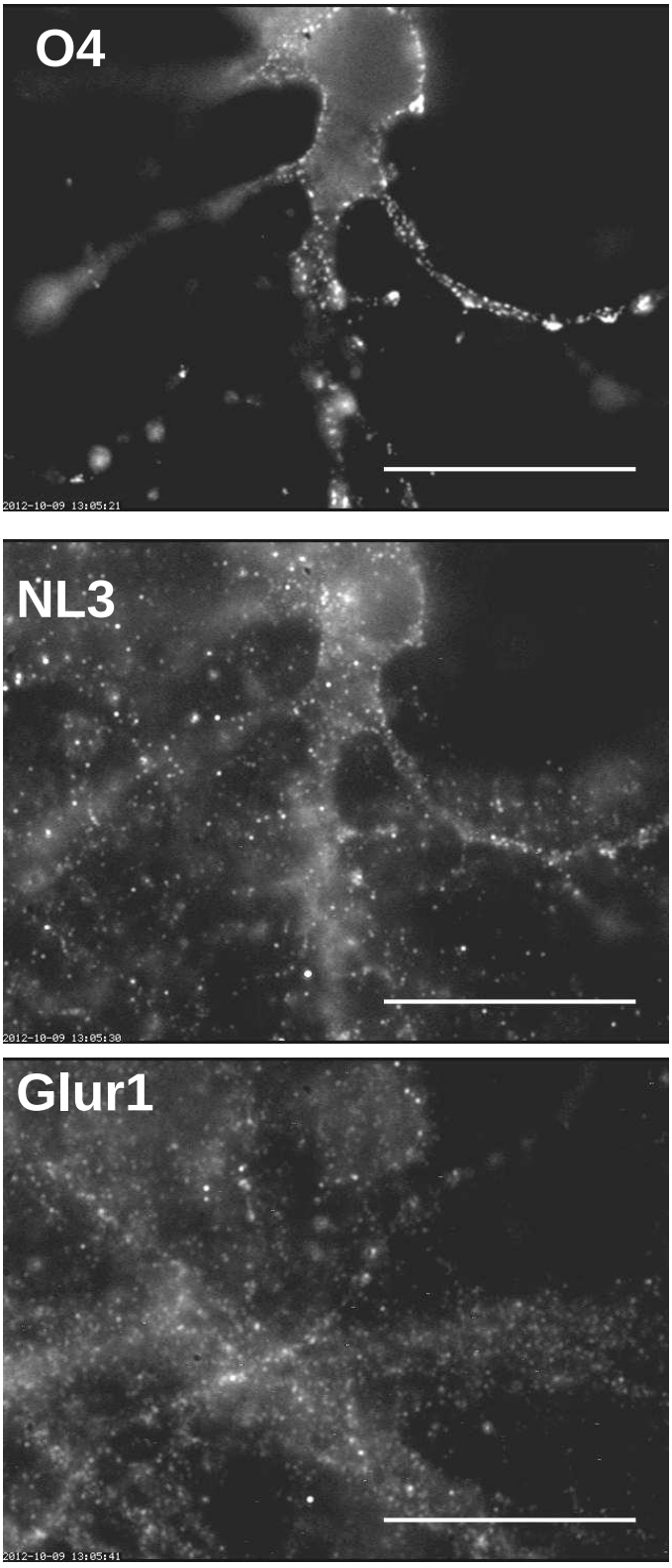
O4
2012-10-09 13:05:21
NL3
2012-10-09 13:05:30
Glur1
2012-10-09 13:05:41

Figure 3.12

Figure 3.12: **A Triple stain image depicting the lack of the AMPA receptor, GluR1 in O4 positive OL cells**

OLs express NL3; however no AMPAR GluR1 was detected in these cell bodies or processes. Multiple dissection preps were performed and immunocytochemistry was done on a total of N of three culture preparations. No GluR1 staining was detected in O4 positive cellular processes across the three trials. This finding corresponds to tissue preparations which have found no GluR1 AMPAR in OL cells. Scale bar = 25μM.

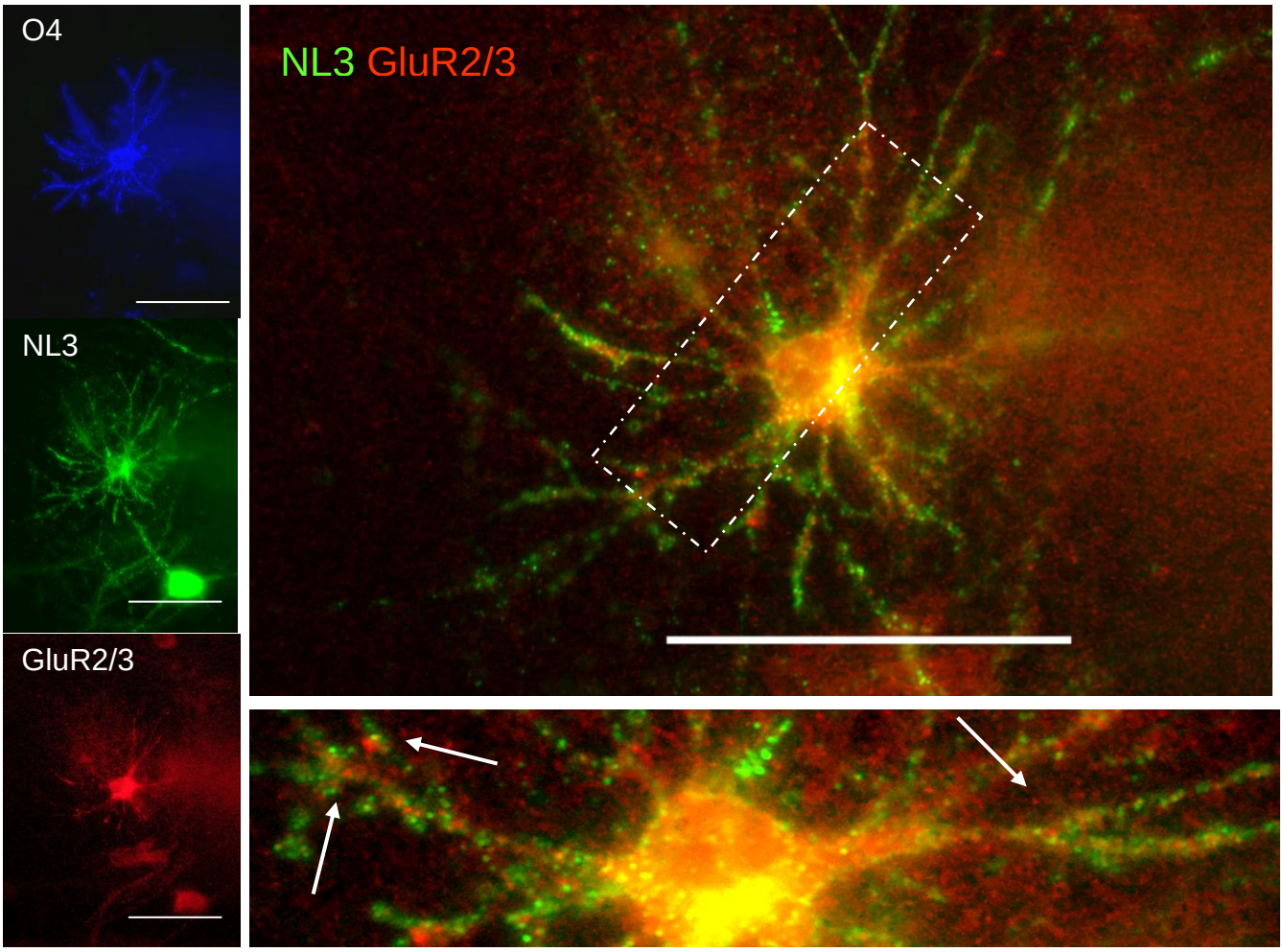

Figure 3.13

Figure 3.13: **The co-expression of NL3 and GluR2/3 in O4 positive OL cells**

GluR2/3 receptors in O4 positive cells. Although co-localization can only be seen within the cell body (depicted in yellow), only juxtaposed expression of NL3 and GluR2/3 can be seen in the distal processes of the O4 positive OL cells. Co-expression of NL3 and the AMPA receptor GluR2/3 is not speculated to be observed as these two proteins do not directly interact. It is thought that AMPAR insertion is due to the activation of NMDAR which are associated with NL through their interaction with PSD-95. The presence of the AMPAR GluR2/3 is also found in tissue preparations and their presence in the myelinating cells of the CNS may shed light as to what these receptor's functions are within the OL cell type Scale bar = 50uM.

CHAPTER 4: GENERAL DISCUSSION

In this thesis I have investigated the role of NL/NX signalling in the regulation of oligodendrocyte differentiation. The first step was to determine whether oligodendrocytes expressed NL3, which was accomplished by the use of immunocytochemistry and *in situ* hybridization techniques. Then, using soluble neuroligin peptides which would bind to neurexin, I demonstrated that in an *in vitro* neuronal culture paradigm the presence of these peptides caused a change in the differentiation state of the oligodendrocytes; stalling them at an immature state, implying that signalling between these molecules plays a regulatory role in this process. This strategy was then reversed by adding HEK cells expressing neurexin to the cultures, which resulted in an increase in the number of mature myelinating oligodendrocytes. The ability of HEK cells expressing neurexin to be recognized by OLs as a target for myelination was demonstrated by direct visualization of HEK cells being wrapped by the processes of an oligodendrocyte. Since the fundamental hypothesis is that NL3 on the OL is analogous to NL1 and NL2 in the dendrite of neurons, we hypothesized that the functional mechanism for signal transduction could also be similar in OLs to their neuronal counterparts. Since neuroligin, through interaction with PSD-95, can recruit and cluster NMDA receptors, the distribution and expression of the different receptor subunits was investigated. Images were obtained that suggested NL3 and the NMDA subunit 3a were co-localized at the tips of OL extensions. Since this is the point of contact with the axon, it raises the exciting possibility that there in fact could be a parallel mechanism between OLs and neuronal dendrites to communicate with axons through neuroligin and neurexin signalling

4.1 Neuroligin 3 expression in oligodendrocytes

4.1.1 Immunocytochemistry of neuronal cultures

Neuroligin expression in glial cells has been documented in the literature as being primarily in astrocytes and not in oligodendrocytes. This conclusion was drawn based on the fact that tissue sections, when stained with an antibody to NL3 showed no significant staining in the white matter tracts. As staining of myelinated fibres is known to be difficult in terms of antibody access, we further pursued the characterization of the expression profile in a dissociated cultures model. By increasing the stringency and length of incubation of our primary NL3 antibody we were able to readily detect NL3 in the OLs. Co-staining of the cells with an O4 antibody confirmed their identity as oligodendrocytes.

4.1.2 Immunocytochemistry of cerebellar cultures

As a consequence of the large amount of white matter present, the cerebellum contains a large number of oligodendrocytes. In order to extend our investigation of the expression of NL3 to different brain regions, we also investigated its expression in cerebellar cultures. The expression of NL3 positive OLs in dissociated cerebellum cultures was shown in Figure 3.3. We also observed that the O4 positive cells in these cultures greatly outnumbered those of the dissociated hippocampal cultures. This was in part the rationale for not using cerebellum cultures for the peptide experiments as the large number of O4 positive cells made it hard to differentiate the cells morphology due to the high density of positive staining. In section 2.8 I stated that if a cell was undistinguishable that it would be omitted from the counts. It was for this reason why I chose to use dissociated hippocampal cultures as their OL density was much lover, making cell morphology much more distinguishable.

Because this was the first demonstration of the expression of NL3 in oligodendrocytes, we further confirmed that the protein detected was from transcription in the OLs itself. After I generated

the required constructs to make the RNA probes, this aspect of the project was performed by Dr. Cristaine de la Hoz. By successfully demonstrating the presence of mRNA transcripts for NL3 in the OLs, we can firmly conclude that the protein is in fact expressed in this cell type.

4.2 Manipulation of neuroligin – neurexin signalling

4.2.1 Application of exogenous neurexin binding peptides

In pilot studies for this project, it was found that transfecting primary hippocampal cultures with NISA peptide expressing constructs led to higher populations of OLs in the immature, highly branched morphological state in comparison to cultures transfected with the NLR473E autism associated mutation or the control plasmid. These results showed subtle differences amongst the treatment groups. Consequently we designed a strategy of expressing the peptide constructs in HEK cells and using the media to supplement the neuronal cultures.

Western blot analysis was used to prove that the HEK cells which were transfected with the solubilized proteins were in fact expressing the constructs, as depicted in Figure 2.2. In order to detect these proteins in the HEK cell media, a protein pull-down with large volume of media would have to be performed with anti-Flag M2 agarose beads. Sufficient volumes of transfected media would have to be used, as per Comoletti *et al.*, 2003 to allow for proper binding in the pull-down assay. This was attempted, unsuccessfully, most likely due to the small amount of starting material that was used. As we had already demonstrated that the HEK cells were expressing the proteins, and the differentiation states of the oligodendrocytes being effectively altered, we did not feel it necessary to pursue this further.

This change of strategy for protein administration led to a significant difference in OL morphology amongst the three media treatments (**Fig.3.4**). The NLR473E, as well as the vehicle control media treatments yielded a greater population of OL cells in the mature, elongated, myelinating

forms compared to the NISA media treated cultures; the NISA media cells had a significant increase in the amount of immature, highly branched OL cells. We speculate this phenomenon occurs because the exogenous NL present in the media was sequestering the available NX sites therefore blocking NL in the OL population from binding, as depicted in Figure 1.9. This data builds a substantial case for the importance of NX/NL signalling in the determination of the differentiation state of oligodendrocytes.

4.2.2 Enhancing the number of neurexin targets

Experiments in which we added exogenous secreted neuroligin proteins to sequester the neurexin binding sites demonstrated that without the signal from neurexin, oligodendrocytes fail to proceed to the mature state. This is intuitively logical, in that one would expect that an OL should make contact with the target axon before beginning myelination. Conversely, one would expect to see an increase in the number of mature OLs if the number of targets was increased. To simulate this situation we transfected HEK cells with neurexin and co-cultured them along with hippocampal neurons. For these experiments, as we can see in **Figure 3.5**, NX1α-GFP expressing HEK cell and hippocampal co-cultures had a significant increase in mature cells when compared to the immature state and had a significant increase in the ratio of mature cells compared to the CFP control. This data further supports the hypothesis that the presence of neurexin on the target to be myelinated interacts with the OL through NL on its surface.

4.2.3 Visualization of the interaction between oligodendrocytes and an artificial neurexin expressing target

As we have the ability to fluorescently label both the OL and HEK cells expressing neurexin, we took advantage of this to visualize the interaction that we hypothesize between a mature OL and a cell expressing NX on its surface. NX-IRES-GFP expressing HEK cell and hippocampal co-cultures were

imaged in conjunction with O4 immunocytochemistry (**Fig.3.7**). In the bottom of panel **A** in this figure, it would appear that the OL process has identified a NX expressing HEK cell and it appears that the OL is beginning to wrap around the HEK cell processes in the enlarged image of panel **A**. This interaction was also seen with the NX1β-GFP expressing HEK cell and hippocampal co-cultures. Panel **B** and **C** depict the immature OLs which seem to be wrapping the NX1β-GFP positive HEK cell. Because there is no MBP staining on these images, one cannot say that the OLs are 'myelinating' the HEK cell, however it is clear to see that there is a robust interaction occurring between the two cells. Importantly, this interaction was not seen in the CFP expressing HEK cell control, with no processes enwrapping the CFP positive cells, depicted in panel **D**.

4.2.4 Future directions

An experiment which would further confirm that the NX/NL interaction does play an integral role in OL differentiation would be to use a soluble NX construct transfected into HEK cells. In the same way that the NL peptide experiments were done, by adding media from the cells to primary neuronal cell culture, I would be able to alter the amount of endogenous NX present in the environment. If my hypothesis is correct, one would expect to see an increase in the ratio of myelinating cells compared to OPCs or immature cells due to an increase in NL signalling in the OLs. If time permitted, I could contact Dr. Peter Scheiffele and request his soluble β-NX protein or begin creating a NX1β construct myself and remove its transmembrane domain to allow cells to secret the protein into the ambient environment. Removing some of its glycosylation sites, similar to how Comoletti *et al.*, 2003 did, we could create an exogenous protein which would bind to NL more efficiently then endogenous NX. Characterization of the binding profile would have to be done in

order to find out which specific glycosylation sites would have to be removed to create this phenomenon.

Recent articles published in Neuron, have addressed the activity dependent cleavage of neuroligin isoforms. These studies have looked at the mechanisms which are responsible for NL cleavage with neuronal cell types. This post-synaptic protein has been found to undergo ectodomain shedding to generate secreted forms of NL as well as membrane-tethered C-terminal fragments (Suzuki *et al.*, 2012; Peixoto *et al.*, 2012). It has been found that ADAM-10 and γ-secretase release NL's extra and intracellular domain fragments respectively. It was also found that ADAM-10 mediated shedding of NL is regulated in an activity-dependant manner through NMDAR activation or, alternatively, by binding to secreted forms for NX (Suzuki *et al.*, 2012). This would mean that NMDAR activation is required for soluble NL to be generated at specific synaptic types. These studies have looked at how proteolytic processing results in a decrease of NL expression in the cell's surface. Interestingly, the production of cleaved NL is very similar to our experiments with our NISA and NLR473E peptides as we mimicked the cleaving process by introducing the peptides into our primary cultures. Instead of looking at how these soluble peptides impacted neuronal cell types, we observed morphological changes with OL cells, potentially via related mechanisms.

4.2.5 Implications for autism

The results obtained in this study demonstrate that there is an important difference between NL proteins carrying the autism associated mutation and the wild type sequence. Our data implies that the point mutation critically affects the signalling process between NL and NX. There is a wide-spread consensus that individuals with ASD have a different brain anatomy compared to that of the 'normal' brain (Baird *et al.*, 2006). It has been speculated that there is a significant difference in the white

matter content and composition in ASD patients (Courchesne *et al.*, 2001; Piven *et al.*, 1996); with some research stating that autistic brains have much more white matter compared to normal brains (Herbert *et al.*, 2003b; Herbert *et al.*, 2004). However, conflicting data has placed researchers on both sides of the fence regarding the difference of autistic brains; with some saying that there is no difference in the volume of the white matter content but more in the composition of the white matter itself (Piven *et al.*, 1996). The fact that this mutation appears to play a critical role in the signalling process for myelination could suggest a mechanism by which this is occurring.

Moreover, being able to manipulate how myelinating cells develop in the CNS could potentially lead to drug therapies for de-myelinating diseases. This advancement in understating how the process for myelination occurs could also potentially help in understanding how tripartite synapses are formed. Because we have found that the population of myelinating OLs can be increased by the presence of exogenous NX targets, creating a peptide-based therapeutic strategy is possible.

4.3 Glutamatergic receptors and NL3 co-localization

4.3.1 NMDAR and NL3 co-localization

Previous studies have shown that NMDAR signalling in OPCs is not required for oligodendrogenesis (De Baise *et al*., 2011). However, we did observe that NMDARs, more specifically NR3A, does seem to localize with NL3 at the tips of extended processes. One could speculate that, as is found in the dendrites of neurons, once NL binds to NX, NMDAR are clustered through interactions with PSD-95 to the site of contact. This is an exciting possibility, as it would be the start of our understanding of the mechanism through which the signal transduction between NX and NL is propagated in the cell. By recruiting NMDARs to the point of contact between the OL and the axon, a mechanism for letting calcium into the OL is created. Since many cellular mechanisms are

regulated by calcium influx, it is possible that not only the differentiation state of the OL but many other aspects of its physiology are affected as well.

4.3.2 AMPAR and NL3 co-localization

Through triple immunocytochemistry staining, we have shown that the AMPAR GluR2/3 does co-express with NL3 in O4 positive cells (**Figure 3.14**). This brings up the question, could NL3 play a role in AMPA insertion in these cell types? While we did not observe any co-localization of AMPAR and NL3 as we did with the NMDAR, the presence of AMPAR in these cells leaves open the possibility of an LTP like mechanism being evoked at some later stage in maturation of myelinated axons. If there is in fact a dynamic communication between the axon and its myelin sheath through a synapse-like structure, it is possible that similar mechanisms to neuronal synapses exist there as well. The will be an exciting avenue for future investigation.

4.4 Myelination signals and NL

While much is already known about the mechanisms and signals which regulate the complex process of myelination, the complete process has yet to be described. We understand sequential steps; that OLs must migrate towards axons which are to be myelinated, that OL processes must then adhere to these axons, and finally the initiation of myelination spiralling which occurs around the axon. We also know a large repertoire of molecules which are critical for those processes. However many questions remain to be answered. The fact that axons and not dendrites are myelinated presents the question as to what signals are being produced by dendrites which inhibit the myelinating process. It would seem obvious that axons produce different chemo-attractants compared to their dendritic counterparts; research has found that OLs are capable of producing a form of un-compacted myelin or myelin-like structures in dissociated, pure OL cultures, i.e. in the absence of neurons (Sarlieve *et al.*,

1983; Wood and Bunge, 1986). This would mean that although axons do produce a ‘myelinating signal’, there must be some signalling cascade present in OLs which permits the production of myelin-like structures without exogenous signals from neighbouring axons.

4.5 Significance

Neuroligin has been widely studied in the neuronal cell population because of its function during synaptic development, and due to their association of NL mutations with ASD; however neuroligin’s role in the glial cell population has not been elucidated. My results show that NL3 does play an important role in OL differentiation and potentially in the process of myelination and that this occurs through signalling to its synaptic partner, neurexin.

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