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SYNAPTIC FUNCTION IN THE HIPPOCAMPUS
IN NEURODEGENERATIVE DISEASE

Chantelle Fourie

A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy. Department of Physiology and Centre for Brain Research.
The University of Auckland, 2013
ABSTRACT

At glutamatergic synapses in the brain the ionotropic glutamate receptors N-Methyl-D-Aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, are bound to membrane-associated guanylate kinases (MAGUKs). Together they regulate important brain functions such as synaptic transmission, plasticity and learning and memory. MAGUKs are the central organisers of excitatory synapses where they are responsible for the architecture of the postsynaptic density (PSD), glutamate receptor trafficking and activating downstream signalling molecules. Neurodegenerative diseases are known to directly affect synapses (Wishart et al., 2006, Raymond et al., 2011) and studying the early changes that occur at the level of the synapse is of the essence for future therapeutic strategies. The work in this thesis has examined the changes that occur in glutamate receptor subunits and MAGUK expression in postmortem human brain tissue from Huntington’s disease (HD) and Parkinson’s disease (PD) patients as well as synaptic function in an animal and cell model of HD.

The first part of this thesis aimed to investigate the changes that occur in glutamate receptor subunit and MAGUK expression in the human brain in HD and PD in both the hippocampus and striatum. We specifically investigated changes that occur in the expression of synapse associated protein 97 kDa (SAP97), postsynaptic density protein 95 kDa (PSD95), the GluA2 subunit of the AMPA receptor and the GluN1 subunit of the NMDA receptor. Our immunohistochemical data revealed that these synaptic proteins are significantly and differentially altered in the hippocampus vs. striatum of HD and PD cases. We hypothesise that the hippocampal changes in synaptic protein expression underlie the cognitive deficits in HD and PD patients (Diamond et al., 1992, Emre, 2003, Ziemssen and Reichmann, 2007, Paulsen et al., 2008). Alongside these changes in the human brain we show no changes in any of the glutamate receptor subunits or MAGUKs in the hippocampus of the YAC128 animal model of HD at 12 months of age. These data indicated that unique changes are occurring in the hippocampus in the human HD brain compared with the YAC128 animal model of HD, correlating with the relative sparing of the hippocampus in the YAC128 model (Slow et al., 2003, Van Raamsdonk et al., 2005a) as opposed to the reported cell loss in the human hippocampus (Spargo et al., 1993, Rosas et al., 2003).

The second part of this thesis aimed to examine synaptic vs. extrasynaptic NMDA receptor signalling and synaptic plasticity in HD in the hippocampus. These experiments utilised acute brain slices from the YAC128 animal model of HD as well as a hippocampal cell culture model of HD. We studied the YAC128 animal model at the early age of 1 month to detect early changes in synaptic function at an
age where we have shown significant cognitive decline to occur (Kim, J., Fourie, C. et al, manuscript in preparation) before classic motor symptom onset. Our electrophysiology data show for the first time that hippocampal synaptic transmission through AMPA and NMDA receptors are normal in the YAC128 slices as compared to slices from their wild type (WT) littermates. Electrophysiology in hippocampal slices further revealed that extrasynaptic NMDA receptor currents were normal in YAC128 slices unlike that reported in the striatum (Milnerwood et al., 2010, Milnerwood et al., 2012). Furthermore, we established a dissociated hippocampal culture model system of HD by transfecting hippocampal neurons with mutant or WT huntingtin. Similarly, electrophysiology experiments using this model also revealed normal synaptic and extrasynaptic glutamate receptor signalling unlike in dissociated striatal neurons from the YAC128 animal model (Milnerwood et al., 2012). These results indicate that extrasynaptic NMDA receptor signalling is unaltered by mutant huntingtin in the hippocampus in our study as compared to increased signalling reported in the striatum. These data correlate with the relative sparing of hippocampal neurons in the YAC128 animal model in contrast to striatal neurons in animal and cell models of HD (Slow et al., 2003, Van Raamsdonk et al., 2005a, Milnerwood et al., 2010, Milnerwood et al., 2012). Furthermore, we also show for the first time a significant deficit in long term potentiation (LTP) in YAC128 hippocampal slices at the young age of 1 month and suggest that this underlies the cognitive and memory impairment. Our results indicate that this is not due to altered presynaptic function or an increase in the threshold for LTP but involves postsynaptic changes. Altogether our results have important consequences for future studies of aberrant synaptic function in neurodegenerative disease and future therapeutic strategies.
Above all I thank my Father in Heaven, Jesus Christ, for giving me strength, courage and peace at all times and for Your favour and grace that rests upon me. You have made me bold and stout hearted. All glory goes to God!

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| Nature of contribution by PhD candidate | The PhD candidate performed all experiments including all analysis for this work, produced all the figures and contributed towards the writing of the manuscript |
| Extent of contribution by PhD candidate (%) | 60% |

### CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
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<tbody>
<tr>
<td>J. Wong</td>
<td>Performed preliminary experiments (2.5% contribution)</td>
</tr>
<tr>
<td>H. Waldvogel</td>
<td>Co-supervisor of PhD candidate provided technical support, human brain tissue and reviewed the manuscript (2.5% contribution)</td>
</tr>
<tr>
<td>A.L. McGregor</td>
<td>Provided the YAC128 animal tissue and technical advice (2.5% contribution)</td>
</tr>
<tr>
<td>R.L. Faull</td>
<td>Co-supervisor of PhD candidate provided technical support, human brain tissue and reviewed the manuscript (2.5% contribution)</td>
</tr>
<tr>
<td>J.M. Montgomery</td>
<td>Supervisor of PhD candidate provided technical support, writing and editing of the manuscript (30% contribution)</td>
</tr>
</tbody>
</table>

### Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<td>[Signature]</td>
<td>15/05/2013</td>
</tr>
<tr>
<td>A.L. McGregor</td>
<td>[Signature]</td>
<td>15/05/2013</td>
</tr>
<tr>
<td>R.L. Faull</td>
<td>[Signature]</td>
<td>15/05/2013</td>
</tr>
<tr>
<td>J.M. Montgomery</td>
<td>[Signature]</td>
<td>17/05/2013</td>
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<th></th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
</tr>
<tr>
<td></td>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td></td>
<td>AchE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td></td>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td></td>
<td>APV</td>
<td>D-(-)-2-Amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>B</td>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>C</td>
<td>CaMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td>Caudate nucleus</td>
</tr>
<tr>
<td></td>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td></td>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>D</td>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td></td>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td></td>
<td>DLG</td>
<td><em>Drosophila</em> tumor suppressor gene</td>
</tr>
<tr>
<td>E</td>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
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<td></td>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td></td>
<td>EPSC</td>
<td>Excitatory postsynaptic current</td>
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<td></td>
<td>EPSCaTs</td>
<td>Excitatory postsynaptic calcium transients</td>
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<tr>
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<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
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<tr>
<td>F</td>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G</td>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td></td>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td></td>
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<td>Glutamate transporter-1</td>
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<tr>
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<td>GP</td>
<td>Globus pallidus</td>
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<tr>
<td></td>
<td>GPe</td>
<td>External segment of the globus pallidus</td>
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<tr>
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<td>GPI</td>
<td>Internal segment of the globus pallidus</td>
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<tr>
<td></td>
<td>GRIP</td>
<td>Glutamate receptor interacting protein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
<td></td>
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<tr>
<td>-------</td>
<td>------------</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HD</td>
<td>Huntington's disease</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<tr>
<td>I</td>
<td>Current</td>
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<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
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<tr>
<td>LTD</td>
<td>Long term depression</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MAGUK</td>
<td>Membrane associated guanylate kinase</td>
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<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
<td></td>
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<tr>
<td>mEPSC</td>
<td>Miniature excitatory postsynaptic current</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium</td>
<td></td>
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<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
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<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>MSN</td>
<td>Medium spiny neuron</td>
<td></td>
</tr>
<tr>
<td>NBM</td>
<td>Neurobasal medium</td>
<td></td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
<td></td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline plus Triton</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
<td></td>
</tr>
<tr>
<td>PICK1</td>
<td>Protein interacting with C kinase 1</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>Post mortem</td>
<td></td>
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<tr>
<td>PPF</td>
<td>Paired pulse facilitation</td>
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</tr>
<tr>
<td>PPR</td>
<td>Paired pulse ratio</td>
<td></td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
<td></td>
</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic density protein 95 kDa</td>
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<tr>
<td>PTP</td>
<td>Posttetanic potentiation</td>
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<td>PV</td>
<td>Parvalbumin</td>
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<td>R</td>
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<tr>
<td>RRR</td>
<td>Readily releasable pool</td>
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S
SAP97 Synapse associated protein 97 kDa
SNARE Soluble N-ethylmaleimide sensitive fusion protein attachment receptors
SNpc Substantia nigra pars compacta
SNpr Substantia nigra pars reticulate
Sst Somatostatin
STN Subthalamic nucleus

T
TARP Transmembrane AMPA receptor regulatory protein
TBOA DL-threo-β-Benzylxyspartic acid
TBST Tris-buffered saline plus Tween
TCA Tricarboxylic acid
TFZ Tebufenozide
t-SNARE Target SNARE
TTX Tetrodotoxin

V
V Voltage
VGLUT Vesicular glutamate transporter
v-SNARE Vesicle associated SNARE

W
WT Wildtype

Y
YAC Yeast artificial chromosome
1.1 GLUTAMATE RECEPTORS

1.1.1 GLUTAMATE AS A NEUROTRANSMITTER

Glutamate and its excitatory effects were discovered in the 1950’s (Hayashi, 1954) but was only recognised as a neurotransmitter in the 1980’s (Fonnum et al., 1981). It was recognised that glutamate fulfils the requirements of a neurotransmitter, i.e. it has a presynaptic location, it is released upon stimulation, it has identity of action with the naturally occurring transmitter and there is a mechanism for the rapid clearance of the neurotransmitter ceasing its action. Glutamate is known as the main excitatory neurotransmitter in the central nervous system (CNS). Glutamate is synthesised from its precursors, glucose and glutamine in the tricarboxylic acid cycle (TCA cycle) (Van den Berg et al., 1966, Hamberger et al., 1979). Astrocytes take up glutamate from the extracellular space and convert it to glutamine, which is released back into the extracellular space and taken up by neurons and reconverted into glutamate (Van den Berg and Garfinkel, 1971, Martinez-Hernandez et al., 1977). Glutamate formed in nerve terminals are taken up into synaptic vesicles in an ATP and temperature dependent manner (Naito and Ueda, 1983).

A glutamate transporter for the uptake of glutamate into presynaptic transmitter vesicles was first cloned in 1994 as a brain-specific plasma membrane sodium-dependent inorganic phosphate transporter (Ni et al., 1994) but was later renamed as a vesicular glutamate transporter (VGLUT1) (Takamori et al., 2000). There are now three such transporters, named VGLUT1-3, which ensure the vesicular uptake of glutamate in CNS neurons. Glutamate is released from these vesicles (see Section 1.2.3) and binds to glutamate receptors on the postsynaptic membrane. The first functional glutamate receptor was cloned in 1989 (Hollmann et al., 1989) and hereafter many other glutamate receptor subtypes were cloned. Glutamate receptors are permeable to cations and there are three types of ionotropic glutamate receptors named after their selective agonists, NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) and kainate receptors.

1.1.2 NMDA RECEPTORS

NMDA receptors consist of a combination of subunits, all of which have been cloned first in rodents and then identified in human: GluN1 (ubiquitously expressed) (Moriyoshi et al., 1991), GluN2 (of
which there are 4 types, GluN2A, B, C and D) (Meguro et al., 1992), and GluN3 (of which there are two types, GluN3A and B) (Ciabarra et al., 1995). All functional NMDA receptors are heteromultimeric complexes that contain the GluN1 subunit (Monyer et al., 1992) and at least one of the GluN2 subunits. The GluN3 subunit can coassemble with GluN1/GluN2 receptors and the GluN1 subunit is necessary for the surface expression of GluN3-containing receptors (Pérez-Otaño et al., 2001). The current amplitude of homomeric GluN1 receptors are small, predicting that NDMA receptors preferentially occur as heteromers (Moriyoshi et al., 1991, Monyer et al., 1992). There are also numerous splice variants for each receptor subunit.

NMDA receptor kinetics are slow as compared to AMPA receptor kinetics. NMDA receptors first start to open 10 ms after glutamate release into the synaptic cleft, upon which they open and close for hundreds of milliseconds until glutamate is unbound, therefore contributing to the slow component of excitatory postsynaptic potentials (EPSPs) (Lester et al., 1990, Dzubay and Jahr, 1996). All functional NMDA receptors have two binding sites for glutamate and two for glycine (Benveniste and Mayer, 1991), where glutamate binds with high affinity to GluN2 subunits (Anson et al., 1998) and glycine binds to GluN1 subunits (Kuryatov et al., 1994). Therefore, glycine acts as a co-factor at NMDA receptors to potentiate the NMDA response through allosteric activation of the receptor (Johnson and Ascher, 1987). The GluN2 subunit has a binding site for NMDA receptor modulators (Zheng et al., 2001) and is also responsible for receptor desensitization, which is seen as a reduced response in the continued presence of glutamate or glycine (Mayer et al., 1989, Lester et al., 1993). NMDA receptors also show calcium (Ca^{2+})-dependent inactivation, which has been recognised by early studies of NDMA receptor function (Mayer and Westbrook, 1985). Further analysis in hippocampal neurons showed that NMDA receptor inactivation can be caused by Ca^{2+} entry through nearby voltage gated Ca^{2+} channels, although Ca^{2+} entry through NMDA receptors was much more effective in inactivating the channel (Legendre et al., 1993). The study by Legendre et al concluded there must be a nearby regulatory protein that is affected by Ca^{2+} transients and capable of altering channel gating. Later it was shown that the GluN1 subunit binds directly to a protein called calmodulin, in a Ca^{2+}-dependent manner in both homomeric GluN1 complexes and heteromeric GluN1/GluN2 complexes and that this binding reduces channel open rate and mean open time (Ehlers et al., 1996). Calmodulin is therefore an intracellular signalling molecule signalling activity-dependent feedback inhibition and Ca^{2+}-dependent inactivation by binding to and regulating NMDA receptors directly. Ca^{2+} dependent inactivation is important to prevent the detrimental effects of excessive NMDA receptor activation, which may lead to excitotoxicity (see Section 1.7). Ca^{2+}-
dependent inactivation is a prominent feature of GluN2A containing receptors but is not significant for GluN2B or GluN2C containing receptors (Krupp et al., 1998).

Unique to NMDA receptors is that they require both a chemical transmitter (glutamate) and a depolarised membrane voltage to open, due to a Mg\(^{2+}\) block on the channel at hyperpolarised potentials, which is released by electrostatic repulsion upon membrane depolarisation (Mayer et al., 1984). NMDA receptors are therefore coincidence detectors of pre- and postsynaptic activity, which is a requirement for Hebbian plasticity (see Section 1.3.1) (Hebb, 1949). In fact, Mg\(^{2+}\) can block the receptor both from the extracellular or intracellular side. The extracellular Mg\(^{2+}\) block is increased with hyperpolarisation of the membrane potential whereas the cytoplasmic Mg\(^{2+}\) block is increased at depolarised potentials (Mayer et al., 1984, Johnson and Ascher, 1990). Both the GluN1 and GluN2 subunits have been shown to be responsible for the voltage dependent Mg\(^{2+}\) block with both extracellular and intracellular Mg\(^{2+}\) (Kupper et al., 1996). Therefore, the subunit combinations are very important in determining specific functional properties of NMDA receptors.

Not only do the different subunits convey differential functionality to NMDA receptors but they also contribute to receptor localisation and their expression and localisation is controlled developmentally. In the hippocampus GluN1 and GluN2B subunits are highly expressed early in development before postnatal day 7 (P7) when synapses are forming while GluN2A expression is low at this point but increases at later stages of development (Monyer et al., 1994). During synapse development, extrasynaptic receptors are composed of GluN1 and GluN2B and outnumber synaptic receptors, whereas mature synapses have decreased numbers of extrasynaptic receptors and synaptic receptors are mainly GluN2A-containing (Rumbaugh and Vicini, 1999, Tovar and Westbrook, 1999). However, NMDA receptors can rapidly move between synaptic and extrasynaptic sites by lateral diffusion (Tovar and Westbrook, 2002). Concluding that synaptic receptors are purely GluN2A and extrasynaptic receptors purely GluN2B containing NMDA receptors is rather ambiguous and is discussed further in Chapter 3.

All NMDA receptor subunit expression levels are regulated developmentally in the hippocampus and differ between hippocampal regions. A study by Ritter et al quantified the expression levels of glutamatergic receptor subunits across different post natal days and areas of the hippocampus (Ritter et al., 2002). GluN1 expression levels are high <P18 and expression is low in the DG and highest in CA2. GluN2A expression levels increases from P0-P21 and is most highly expressed in CA1 and CA2 with low levels in the DG. GluN2B and GluN2C is high early in development and then steadily decline to below birth levels at P35 with expression being particularly low in the DG and CA3 regions. GluN2D levels are higher than birth levels at P35 although overall expression is low in the...
hippocampus. GluN3A expression is highest at P7 only in the CA1 and CA2 regions and declines again after P7 to birth levels. These changes in receptor subunit expression are interesting as they occur at particular times and areas to regulate synaptic organisation and plasticity as the brain develops.

**1.1.3 AMPA RECEPTORS**

AMPA receptors consist of a number of subunits, all of which have been cloned around the 1990’s: GluA1 (Hollmann et al., 1989, Keinänen et al., 1990), GluA2 (Keinänen et al., 1990, Nakanishi et al., 1990), GluA3 (Keinänen et al., 1990, Nakanishi et al., 1990) and GluA4 (Keinänen et al., 1990). Closely related subunits GluA5 (Bettler et al., 1990), GluA6 (Egebjerg et al., 1991) and GluA7 (Bettler et al., 1992) have also been cloned but form kainate receptor complexes. Each of GluA1-4 receptor subunits have two splice variants called flip and flop, creating receptor diversity (Sommer et al., 1990). These splice variants seem to be equally abundant but show differential distribution, e.g. the CA3 pyramidal cells of the hippocampus only express the “flip” form while granule cells of the DG express flop more abundantly and show differential efficacy of glutamate activating the receptor (flip being more efficient) (Sommer et al., 1990). Each of the GluA1-4 subunits form homomers when expressed in oocytes (Boulter et al., 1990) and can also form heteromers consisting of GluA2/3 and GluA1/2 (Boulter et al., 1990, Nakanishi et al., 1990, Sakimura et al., 1990). Most AMPA receptors in the hippocampus are composed of either GluA1/2 or GluA2/3 subunits, although a small fraction are homomeric GluA1 receptors (Wenthold et al., 1996).

AMPA and kainate act as competitive agonists at the same site on AMPA receptors, although AMPA is a partial agonist and kainate a full agonist (Keinänen et al., 1990) with potencies in the order AMPA>glutamate>kainate (Keinänen et al., 1990, Sakimura et al., 1990). Interestingly, kainate does not desensitise AMPA receptors but glutamate does, as shown in hippocampal neurons (Kiskin et al., 1986). AMPA receptors have lower affinity for glutamate (EC50 ~500 µM for hippocampal cultured neurons) compared to NMDA receptors (EC50 ~2.3 µM) (Patneau and Mayer, 1990).

AMPA receptor excitatory postsynaptic current (EPSC) kinetics and receptor desensitisation (in hippocampal neurons ~10 ms) (Colquhoun et al., 1992) is much faster compared to NMDA receptor kinetics. The GluA2 subunit is unique in that it produces only very small current responses to agonists as a homomeric channel and shows an outwardly rectifying current (I)/voltage (V) relationship whereas other subunits (homomeric) show an inwardly rectifying I/V relationship (Nakanishi et al., 1990). However, if GluA2 is expressed as a heteromer or tetramer with the other subunits, the I/V relation become linear (Boulter et al., 1990, Nakanishi et al., 1990). GluA2 is also
different in that it is the only subunit, which if present in the AMPA receptor complex, abolishes Ca\(^{2+}\) permeability of the channel (Hollmann et al., 1991).

Like NMDA receptors, there is a developmental switch in AMPA receptor subunit composition with regard to the GluA2 subunit. It has been shown in the rat cortex, striatum and cerebellum that the number of Ca\(^{2+}\) permeable AMPA receptors (i.e. receptors lacking GluA2) are high at P4 and then decline with age (Pellegrini-Giampietro et al., 1992). In the hippocampus, the number of Ca\(^{2+}\) permeable AMPA receptors increases from P7 to P21 and then declines with age (Pellegrini-Giampietro et al., 1992). Therefore, there is a progressive decline in Ca\(^{2+}\) permeability of AMPA receptors, which may have important consequences for cell excitability and excitotoxicity. In the rat hippocampus, GluA1 expression remains constant during P1-P35, whereas GluA2, GluA3 and GluA4 have increased expression levels at P7 or P18 and expression levels of all subunits are similar between hippocampal regions (Ritter et al., 2002).

1.2 THE GLUTAMATERGIC SYNAPSE

CNS synapses are composed of a presynaptic bouton (containing vesicles with specific neurotransmitters), a synaptic cleft and a postsynaptic membrane. The postsynaptic density (PSD) is a membrane specialisation of the postsynaptic neuron and is a protein rich area of the membrane. Here, densely clustered neurotransmitter receptors are found alongside a vast array of other proteins involved in the structure, architecture and composition of the PSD. This protein organisation gives the synapse its unique properties and determines (together with the presynaptic neurotransmitter) the type of synapse. Glutamate is synthesised, taken up into vesicles and later released in response to activity into the synaptic cleft. Here glutamate binds to glutamate receptors (see Section 1.1) before being removed from the synapse by a number of mechanisms. These processes will be described here.

1.2.1 STRUCTURE

Excitatory synapses are typically asymmetric synapses characterised by a thick, prominent PSD whereas inhibitory synapses are typically symmetric with only a thin PSD. Most excitatory synapses are formed onto dendritic spines, which are mushroom-shaped protrusions from the dendritic shaft and is only about 1 µm in length at CA1 synapses in the hippocampus (Harris and Stevens, 1989). Dendritic spines contain the PSD (Palade and Palay, 1954), as well as an actin cytoskeleton and the spine apparatus (Spacek, 1985). Spine morphology is dynamically regulated by synaptic transmission and overall activity of the neuron (Matsuzaki et al., 2004). The PSD is located on the postsynaptic
membrane and is directly opposite to the presynaptic active zone. The active zone is a highly specialised region of the cytoplasm in the presynaptic terminal where synaptic vesicles are clustered for transmitter release. Therefore, the site of transmitter release is purposefully located in close proximity (~20nm) to the PSD where the neurotransmitter has access to receptors (Schikorski and Stevens, 1997). Using electron microscopy, it has been shown at excitatory CA1 hippocampal synapses that each bouton most often contains one active zone, there are about 10 docked vesicles per active zone and the total number of vesicles per bouton is about 200 (Schikorski and Stevens, 1997). Interestingly, the number of docked vesicles per active zone is half the number in cultured hippocampal neurons compared to hippocampal slices (Schikorski and Stevens, 1997). Further measurements have shown a vesicle diameter of 35 nm, a synaptic cleft width of 20 nm, a PSD area of 0.07 µm² and a spine volume of 0.07 µm², in excitatory hippocampal synapses in slices (Harris and Stevens, 1989, Schikorski and Stevens, 1997).

1.2.3 GLUTAMATE RELEASE

Classically at a glutamatergic synapse, an action potential in the presynaptic cell causes the fusion of glutamate containing vesicles to the presynaptic membrane upon which they are released into the synaptic cleft. Here in the synaptic cleft the neurotransmitter reaches millimolar concentration as it diffuses across the cleft to bind postsynaptic glutamatergic receptors (Clements et al., 1992). However, there may also be glutamate spillover, which will not only activate postsynaptic receptors but also extrasynaptically located receptors. Glutamate binding to the AMPA-type glutamate receptors opens it and allows Na⁺ influx, thereby depolarising the postsynaptic cell. Only upon postsynaptic depolarisation will the NMDA receptor open to cause an influx of Ca²⁺. Therefore, glutamate binding to the NMDA receptor in a hyperpolarised postsynaptic neuron does not cause receptor opening as NMDA receptors are blocked by Mg²⁺ in this state. It is this calcium influx through the NMDA receptor that causes long lasting changes in synapse strength, referred to as long term potentiation (LTP), and is thought to underlie learning and memory. Glutamate can be released from both neurons and astroglia as astrocytes also contain the machinery for exocytosis of glutamate (Parpura et al., 1994).

The active zone in the presynaptic terminal holds a subset of vesicles that can undergo rapid exocytosis in response to a presynaptic action potential. Synaptic neurotransmitter release requires fusion of the neurotransmitter filled synaptic vesicles with the plasma membrane. Docking of synaptic vesicles at the active zone is the first step in neurotransmitter release. Synaptic vesicles are docked at the active zone in close proximity (within 50nm) to the voltage dependent Ca²⁺ channels (Heuser et al., 1979, Cohen et al., 1991). Once vesicles are docked at the plasma membrane, they
undergo maturation steps that make them fusion competent and ready to fuse with the membrane to release its neurotransmitter – this maturation process is called vesicle priming. Munc13-1 (a presynaptic receptor protein) has been identified as one of the essential proteins in glutamatergic hippocampal neurons for synaptic vesicle maturation (Augustin et al., 1999). Primed synaptic vesicles are then stimulated for fusion and subsequent exocytosis. The fusion is triggered by Ca$^{2+}$ influx, which occurs in response to action potential mediated depolarisation of the presynaptic terminal. The magnitude and duration of calcium influx via voltage dependent calcium channels determines the delay between calcium influx and transmitter release, known to be ~200 µs in the squid giant synapse (Llinas et al., 1981). Synaptic vesicles can also fuse with the membrane spontaneously producing a miniature excitatory postsynaptic current (mEPSC), which is the result of the fusion and release of a single vesicle at a synapse, although this occurs with low probability estimated to be 1-2 vesicles per minute in hippocampal neurons (Geppert et al., 1994, Murthy and Stevens, 1999).

Next, exocytosis is mediated by soluble N-ethylmaleimide sensitive fusion protein attachment receptors (SNAREs), which form SNARE complexes. These complexes consist of vesicle associated SNARE (v-SNARE) proteins synaptobrevin-2/VAMP-2, which interact with target SNARE (t-SNARE) proteins syntaxin-1 and SNAP-25 (Söllner et al., 1993, Sutton et al., 1998). The calcium dependence of this fusion is due to synaptotagmin-1, which is a major component of synaptic vesicle membranes and its calcium binding triggers vesicle binding to the plasma membrane (Geppert et al., 1994, Martens et al., 2007). Synaptotagmin binds to the SNARE complex to act as the calcium sensor for exocytosis (Chapman et al., 1995). After neurotransmitter release, the empty vesicles are internalised via endocytosis by clathrin-coated pits and are recycled (Heuser and Reese, 1973). The recycled vesicles are refilled with neurotransmitter and transported back to the active zone – the entire vesicle cycle taking ~1 min as measured in the frog neuromuscular junction (NMJ) (Betz and Bewick, 1992) or 30 – 60 sec as measured in the hippocampus (Ryan et al., 1993).

Therefore, there are three pools of synaptic vesicles: the reserve pool, the recycling pool and the readily releasable pool (RRP). The reserve pool of vesicles is not released under physiological activity and is only recruited when the recycling pool is depleted or under high stimulation frequencies as shown in the frog NMJ (Richards et al., 2003). The reserve pool contains most of the vesicles, whereas the recycling pool contains about 20% of the vesicles (Richards et al., 2003). The recycling pool of vesicles is released upon physiological stimulation and is refilled by new recycled vesicles as shown in the frog NMJ (Richards et al., 2003) and hippocampal neurons (Harata et al., 2001). The RRP of vesicles are those that are docked at the active zone, primed and ready to be released.
are depleted quickly by high frequency electrical stimulation or a few milliseconds of depolarisation as shown in the frog NMJ (Richards et al., 2003) and the calyx synapse (Schneggenburger et al., 1999).

### 1.2.4 GLUTAMATE UPTAKE

After glutamate is released from the presynaptic neurons and has bound to its receptor targets (located both postsynaptically and presynaptically), it continues to diffuse out of the synaptic cleft and into surrounding extrasynaptic spaces. However, diffusion alone is a slow process and glutamate must be actively removed from the synapse. This is done by Na+-dependent high affinity glutamate transporters called excitatory amino acid transporters (EAATs), which transport glutamate from the extracellular space back into the cytosol. Genes encoding three such transporters have been identified and named GLAST, GLT-1 and EAAC1 and later three human glutamate transporters were discovered and named EAAT1, EAAT2 and EAAT3, corresponding to the rodent transporters respectively (Kanai and Hediger, 1992, Pines et al., 1992, Storck et al., 1992, Arriza et al., 1994). There are two more mammalian subtypes, EAAT4 and EAAT5 (Fairman et al., 1995, Arriza et al., 1997). EAAC1 (EAAT3) is specifically localised in certain neurons and is expressed in the cortex, hippocampus and striatum, GLT-1 (EAAT2) is localised to astroglia throughout the brain and GLAST (EAAT1) is found in both neurons and astroglia and is expressed in the cortex and hippocampus (Rothstein et al., 1994). However, studies in the forebrain have shown that GLT-1 is also expressed in neurons in neuronal cultures, specifically in the presynaptic terminal and dendritic shafts (Chen et al., 2002). Certainly later this was also shown to be the case in hippocampal neurons where GLT-1 is expressed in axons, spines and dendrites of excitatory synapses (Chen et al., 2004).

### 1.2.5 ARCHITECTURE OF THE POSTSYNAPTIC DENSITY

At the PSD, densely clustered neurotransmitter receptors (e.g. NMDA and AMPA) are found alongside other synaptic proteins, such as synapse associated proteins (SAPs) and post synaptic density proteins (PSDs) involved in organisation of the PSD (Figure 1). One important family of PSD proteins, called membrane associated guanylate kinases (MAGUKs) binds to glutamatergic receptor subunits to regulate their function and location at the synapse. The MAGUKs are therefore the central organisers of the synapse. Much research has been done on these proteins because their location and interaction with glutamatergic receptor subunits are altered in neurodegenerative diseases, leading to unique synaptic pathologies.
Figure 1. Architecture of the postsynaptic density at excitatory synapses.
The main glutamate receptors (NMDA and AMPA) and MAGUKs are shown here in relation to other PSD proteins, although the PSD architecture is far more complex than that portrayed here (Montgomery et al., 2004).

Common to all MAGUK proteins is a PDZ domain, an SH3 domain and a guanalyte kinase domain. The PDZ domain binds short peptide sequences often found at the C-terminal domain of proteins such as glutamate receptors to form protein – protein interactions (Saras, 1996). The PDZ domain confers binding partner specificity due to amino acid substitutions within the PDZ domain and the C-terminal peptide motif within the PDZ ligand (Piserchio et al., 2002). The SH3 domain is also a site for protein-protein interactions and the SH3 domain in MAGUKs can interact with their own GUK domains to hold the SH3-GUK domain in a closed state (McGee and Brecht, 1999). This may act to regulate the “where and when” of binding of specific partners to the MAGUK structure and thereby regulate the assembly of protein complexes. The GUK domain is very similar in structure to guanylate kinases, which are enzymes that convert GMP into GDP with the use of ATP. However, GUK domains in MAGUKs do not have any enzymatic activity but has been found, by crystallography, to be a protein binding module in MAGUKs (Kuhlendahl et al., 1998, Li et al., 2002).
The *Drosophila* tumor suppressor gene (DLG) subfamily of MAGUKs all contain three PDZ domains, an SH3 domain and GUK domain and was the first to be characterised (Woods and Bryant, 1991). Four vertebrate homologs of DLG have been found, namely PSD95 (also known as SAP90) (Cho et al., 1992), SAP102 (Muller et al., 1996), SAP97 (Muller et al., 1995) and PSD93 (also known as Chapsyn-110) (Brenman et al., 1996, Kim et al., 1996). PSD95 and PSD93 are highly enriched at the PSD due to their palmitoylation (El-Husseini et al., 2002), whereas SAP97 and SAP102 are distributed in the cytoplasm as well as at synapses in dendrites and axons. The protein CASK, is part of another subfamily of MAGUKs and contains a single PDZ domain, an SH3 domain, a GUK domain and an N-terminal domain homologous to Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) (Hata et al., 1996).

PSD95 has been shown to bind to the C-terminus of the GluN2A/B subunit of the NMDA receptor in hippocampal neurons (Kornau et al., 1995) and the yeast two-hybrid system (Niethammer et al., 1996) as well as binding to Shaker-type K⁺ channels (Kim et al., 1995) and inwardly rectifying K⁺ channels (Nehring et al., 2000). Surprisingly, mice lacking PSD95 still show normal clustering of NMDA receptors, suggesting that PSD95 is not necessary for the scaffolding of NMDA receptors at the PSD (Migaud et al., 1998). Nevertheless, these mice display increased synaptic plasticity (LTP) but impaired spatial learning, indicating that PSD95 is crucial for this form of activity. On the other hand, over-expressing PSD95 also does not affect the number of postsynaptic NMDA receptors but increases the number of AMPA receptors at the PSD, as shown in hippocampal neurons (El-Husseini et al., 2002, Schnell et al., 2002). This is achieved by the association of PSD95 and AMPA receptors with the protein Stargazin. Stargazin is a type of transmembrane AMPA receptor regulatory protein (TARP) and its deletion in stargazer mutant mice leads to a disruption of AMPA receptor localization at the synapse in hippocampal pyramidal neurons (Chen et al., 2000). Disrupting the localization of PSD95 at the synapse, also reduces the number of synaptic AMPA receptors (El-Husseini et al., 2002).

SAP97 binds specifically to the GluA1 subunit of the AMPA receptor (Leonard et al., 1998), to the GluN2A subunit of NMDA receptors in a CaMKII phosphorylation dependent manner (Gardoni et al., 2003) to voltage gated K⁺ channels (Kim and Sheng, 1996) and inwardly rectifying K⁺ channels (Leonoudakis et al., 2004). Importantly, SAP97 knock-out mice studies are not possible as these mice do not survive due to feeding problems as a result of craniofacial deformities (Caruana and Bernstein, 2001). The N terminus of SAP97 has a myosin VI binding site and direct binding between myosin VI, SAP97 and the AMPA receptor subunit, GluA1, may be involved in trafficking AMPA receptors to and from the postsynaptic plasma membrane (Wu et al., 2002). CaMKII phosphorylates...
SAP97 at two sites: one at the N-terminus (Ser39) and the other at the PDZ1 domain (Ser232). Phosphorylation of SAP97 (as a result of intracellular calcium e.g. through NMDA receptor activity) at the Ser39 site causes the SAP97/GluN2A complex to be released from the endoplasmic reticulum and trafficked to the PSD (Mauceri et al., 2006). Phosphorylation at the Ser232 site causes GluN2A insertion at synaptic membranes (Mauceri et al., 2006). Therefore CaMKII phosphorylation of SAP97 at two distinct sites regulates GluN2A trafficking and insertion at the synapse. SAP97 is also important for the targeting of NMDARs to dendritic spines in hippocampal neurons through a unique secretory pathway (Jeyifous et al.).

Interestingly, there is a developmental change in the expression of certain MAGUKs. In the hippocampus, SAP102 is highly expressed at P2 whereas PSD95 and PSD93 levels are relatively low (Sans et al., 2000). SAP102 expression levels then remain stable until P35 and are reduced at 6 months of age, whereas PSD95 and PSD93 levels increase from P2 through to 6 months of age (Sans et al., 2000). This study also showed that the high expression level of SAP102 at P2 coincides with a high expression level of GluN2B subunits at CA1 hippocampal synapses. The later increase in expression of PSD95 and PSD93 coincides with high levels of GluN2A subunits (Sans et al., 2000). Furthermore, coimmunoprecipitation studies have shown that there is a preference for GluN2A/PSD95/93 complexes and GluN2B/SAP102 complexes (Sans et al., 2000), therefore these MAGUKs preferentially bind specific NMDA receptor subunits. Another interesting element of MAGUKs is that there may be redundancy amongst some members. Mice genetically lacking either PSD95 or PSD93 show little defect in synaptic transmission but when both proteins are absent in double knock-out mice, there are clear defects, indicating redundancy between these two proteins (Elias et al., 2006). Furthermore, in double knock-out mice of PSD95 and PSD93, SAP102 is upregulated and is responsible for the synaptic targeting of remaining AMPA receptors and residual synaptic transmission (Elias et al., 2006).

1.3 THE HIPPOCAMPUS

The hippocampus forms part of the limbic system of the brain and is an important structure for learning and memory. Descriptions of the structure of the hippocampus date back to the 1500s where it has been compared to the shape of a sea-horse, a caterpillar, a silk-worm, a dolphin and ram’s horns (Lewis, 1923). The term “hippocampus” was first introduced in the late 1500s by Julius Caesar Arantius, a student of the famous anatomist Andreas Vesalius. The hippocampus has been studied extensively due to its involvement in memory and learning, which is thought to result from
use-dependent increases in synaptic strength (Hebb, 1949). The mechanism underlying this increase in synaptic strength is thought to be LTP (Bliss and Lømo, 1973, Bliss and Collingridge, 1993).

There are three main cell types in the hippocampus: the granule cells of the dentate gyrus (DG), the pyramidal cells of the CA3 area and the pyramidal cells of the CA1 area. However, Lorente de No described the hippocampus as consisting of four fields and named them CA1 - CA4 (Lorente de Nó, 1934). The anatomy and cell types of the hippocampus have been well described in the famous anatomical studies of Cajal and Lorente de No (Lorente de Nó, 1934, Ramon y Cajal, 1968). The CA1 area contains pyramidal cells with triangular shaped somata and are relatively scattered in distribution (Dam, 1979). The CA2 area has large cells with oval shaped somata and are relatively densely packed (Dam, 1979). The existence of the CA2 area in various species is debatable but is clearly seen in the primate and human hippocampus (Blackstad, 1956, Amaral et al., 1984). The CA3 region has a curved shape and it contains cells with pyramidal somata, less densely packed than CA2 cells (Dam, 1979). An obvious feature of the CA3 field is also the appearance of fine, non-myelinated fibers, called the mossy fibers, which originate from the DG granule cells and surround the pyramidal cells of the CA3 field forming the stratum lucidum (Lorente de Nó, 1934, Blackstad, 1956, Ramon y Cajal, 1968). The CA4 region is situated in the concavity of the DG and contains cells with large oval shaped somata that are few in number (Dam, 1979). Interestingly, these four fields of the hippocampus have different sensitivity to hypoxia. The CA1 field is the most vulnerable field (Sommer, 1880), the CA3 field is relatively resistant (Spielmeyer, 1927) and the CA4 region is of medium vulnerability (Bratz, 1899). These four areas and their respective layers are shown in Figure 2.

The four areas of the hippocampus described above are connected via glutamatergic synapses (see Section 1.2) expressing glutamatergic receptor subunits in a developmentally regulated manner (see Section 1.1). There are two excitatory input pathways to the hippocampus: the indirect perforant path (also called the trisynaptic pathway) and the direct intrahippocampal pathway (Lorente de Nó, 1934, Blackstad, 1956, Ramon y Cajal, 1968). Cortical input to the perforant path comes from the posterior parietal association cortex projecting to the entorhinal area. The perforant path then starts with axons from layer II of the entorhinal cortex giving input to the DG. The termination of these axons occurs in the molecular layer mainly on the dendritic spines of the granule cells but some also terminate on interneurons (Nafstad, 1967). From the DG the axons of the granule cells (called mossy fibres) project to the pyramidal cells in the CA3 region. The axons of the CA3 pyramidal cells (called Schaffer collaterals) then connect to the CA1 region from where the projections go to the subiculum and then to the entorhinal cortex. Therefore, the subiculum and entorhinal area are the outputs of
the hippocampus, which connect the hippocampus to other cortical areas (such as the posterior cingulate cortex) via the fimbria. Cortical input to the direct pathway comes from the inferior temporal association cortex (via the perirhinal cortex) to the entorhinal area. The direct pathway has projections from layer III of the entorhinal cortex directly to the CA1 pyramidal cells (Doller and Weight, 1982) from where the projections go to the subiculum and to the entorhinal cortex again (Knowles and Schwartzkroin, 1981). From here there are cortical output pathways that reach e.g. the temporal pole and the prefrontal cortex. The polysynaptic pathway is mainly involved in episodic and spatial memory, whereas the direct pathway is involved in semantic memory.

As described above, the CA1 pyramidal cells receive input from the CA3 area and the cortex but there are also various gamma-aminobutyric acid (GABA) inhibitory interneurons that synapse onto CA1PCs. Specifically, there are two groups of interneurons: those that synapse onto pyramidal cell dendrites and those that synapse onto pyramidal cell soma and proximal dendrites (perisomatic). There are two perisomatic interneuron types: the basket cells, which contact the soma and proximal dendrites of pyramidal cells (Seress and Ribak, 1990), and the axo-axonic (Chandelier) interneurons, which contact the axon initial segment of pyramidal cell (Somogyi et al., 1983, Gulyas et al., 1993). Both perisomatic and dendritic GABAergic interneurons may be further subclassified according to

Figure 2. The hippocampal network.
The perforant path is shown here originating at the entorhinal cortex travelling towards the DG then to the CA3 and the CA1 fields of the hippocampus. The direct path is shown that starts in layer III of the entorhinal cortex and travels directly to the CA1 field of the hippocampus. Adapted from (Neves et al., 2008).
their expression of various histochemical markers as well as their anatomical features and their location in particular hippocampal layers (Ramon y Cajal, 1968, Klausberger, 2009). Such classifications are not completely unambiguous as interneurons overlap in many of the aforementioned characteristics. However, among the perisomatic targeting interneurons, only a subset of basket cells contain the vesicular glutamate transporter 3 (VGLUT3) (Somogyi et al., 2004). On the other hand, the dendritic-targeting interneurons, specifically those expressing parvalbumin (PV), (bistratified and OLM cells, see Figure 3) also contain somatostatin (SST), which is not expressed by perisomatic contacting interneurons (Katona et al., 1999, Maccaferri et al., 2000). Therefore, perisomatic and dendritic-targeting interneurons can be separated on the basis of their selective expression of VGLUT3 and somatostatin, respectively.

Figure 3. GABAergic interneuron innervation of CA1 pyramidal cells in the hippocampus. Twelve different types of GABAergic interneurons are shown here with their respective innervation patterns onto a CA1 pyramidal cell in the hippocampus. CB, calbindin; CR, calretinin; LM-PP, lacunosum-moleculare–perforant path; LM-R-PP, lacunosum-moleculare–radiatum–perforant path; m2, muscarinic receptor type 2; NPY, neuropeptide tyrosine; PV, parvalbumin; SM, somatostatin; VGLUT3, vesicular glutamate transporter 3. (Somogyi and Klausberger, 2005)
1.3.1 LONG TERM SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

There are several forms of LTP but most of the research has focussed on NMDA receptor dependent LTP at hippocampal CA1 synapses as the hippocampus is known to underlie learning and memory – (Bliss and Collingridge, 1993). LTP in mossy fibre synapses on the other hand are NMDA receptor independent (Harris and Cotman, 1986). LTP is measured as a persistent increase in the postsynaptic evoked response and is typically induced by tetanic stimulation of presynaptic inputs. More physiological stimulation protocols have been described, such as theta-burst stimulation (Larson et al., 1986). The early phase of LTP lasts less than three hours, whereas a late phase of LTP lasts longer than eight hours and is dependent on protein synthesis (Krug et al., 1984). LTP has three important properties; cooperativity, associativity and input-specificity. Cooperativity occurs among afferents for the induction of LTP where tetanic stimulation of more fibres produces greater potentiation due to an intensity threshold for the induction of LTP (McNaughton et al., 1978). LTP is also associative because if tetanic stimulation is given to both a “strong” pathway that induces LTP and to a “weak” pathway that does not induce LTP, it can lead to potentiation of the weak pathway (McNaughton et al., 1978). Lastly, LTP is input specific as it occurs only when the input is active at the same time as the tetanus (Andersen et al., 1977). These three properties are explained by experiments that validated the fact that LTP will only occur if presynaptic stimulation occurs in combination with postsynaptic depolarisation (Kelso et al., 1986).

The expression and maintenance of LTP relies on AMPA receptors that are rapidly inserted to the synapse upon LTP induction or rapidly removed from the synapse upon long term depression (LTD) induction. For AMPA receptors to be rapidly inserted to the synapse there must be a pool of non-synaptic AMPA receptors close to the synaptic location. AMPA receptors cycle in and out of the synaptic membrane at a rapid rate and undergo activity-dependent endocytic sorting (Lüscher et al., 1999, Ehlers, 2000). Dendritic recycling endosomes have been shown to be the pool from which AMPA receptors are recruited to the plasma membrane during LTP (Park et al., 2004). Indeed, nonsynaptic AMPA receptors far outnumber synaptic AMPA receptors (Shi et al., 1999). Using recombinant AMPA receptors (GluA1-GFP) expressed in hippocampal CA1 neurons, it has been shown that AMPA receptors are mostly (88%) located intracellularly and are only delivered to dendritic spines upon tetanic stimulation and require NMDA receptor activation (Shi et al., 1999).

Protein kinases and AMPA receptor phosphorylation are also critical for LTP. During LTP CaMKII is activated by Ca\(^{2+}\) influx through NMDA receptors, which triggers autophosphorylation of CaMKII (Barria et al., 1997b). Activated CaMKII then phosphorylates the GluA1 subunit of AMPA receptors and enhances AMPA receptor responsiveness (McGlade-McCulloh et al., 1993, Barria et al., 1997b).
However, mutations in GluA1 to prevent its phosphorylation by CaMKII does not prevent AMPA receptor insertion at the synapse but only when a PDZ binding domain in GluA1 is mutated is receptor insertion prevented (Hayashi et al., 2000). It was later shown that PSD95 is required for receptor insertion at the synapse (Ehrlich and Malinow, 2004). The opposite happens when the PKA phosphorylation site on GluA1 subunits are mutated, i.e. this prevents the delivery of AMPA receptors to the synapse by active CaMKII or LTP (Esteban et al., 2003). Again phosphorylation of GluA1 by PKC is also critical for LTP expression (Boehm et al., 2006). GTPases have also been shown to be important for regulating receptor numbers at the synapse as Ras (a GTPase) mediates activity-dependent synaptic insertion of AMPA receptors but Rap mediates the removal of AMPA receptors during synaptic depression (Zhu et al., 2002). Interestingly, the AMPA receptor subunit composition is also an important determinant of receptor insertion as GluA1/2 receptors are inserted into the synapse during LTP but GluA2/3 receptors continuously replace existing synaptic receptors, both of which requires a PDZ domain interaction (Shi et al., 2001).

As described above, the induction of LTP at CA1 synapses requires Ca$^{2+}$ influx through NMDA receptors as blocking NMDA receptors prevents LTP induction (Collingridge et al., 1983). NMDA receptors have a voltage dependent Mg$^{2+}$ block, as described in Section 1.1. The NMDA receptor therefore acts as a coincidence detector as it will only pass current if the postsynaptic cell is depolarised at the same time as presynaptic glutamate release. Importantly, the influx of Ca$^{2+}$ underlies the induction of LTP as intracellular injections of a calcium chelator (EGTA) blocks LTP (Lynch et al., 1983). Indeed it has been shown that LTP induction elevates Ca$^{2+}$ in spines but not in the parent dendrite (Muller and Connor, 1991), therefore spines are the anatomical correlate for LTP induction and expression.

The locus of LTP expression, has been a long debate and is still controversial to some extent. The expression of LTP can be 1) due to a presynaptic mechanism that results in increased transmitter release, 2) a postsynaptic mechanism that involves increased receptor number or a change in their function, 3) an extrasynaptic mechanism that leads to increased transmitter availability to receptors or 4) morphological changes in spines.

1.3.1.1 Presynaptic Mechanisms

Early studies showed that perforant path NMDA receptor dependent LTP is associated with increased glutamate release (measured as an increase in extracellular glutamate) (Dolphin et al., 1982). A quick and easy test for a presynaptic mechanism is to use paired pulse facilitation (PPF), where two presynaptic stimuli within tens of milliseconds from each other causes a larger synaptic response upon the second stimulus due to residual Ca$^{2+}$ in the presynaptic terminal from the first
stimulation (McNaughton, 1982). However, this test has produced varying results even within the same experimental setup, showing either a reduction in PPF or no change after LTP induction at CA1 synapses (Palmer et al., 2004).

More evidence for a presynaptic mechanism of LTP comes from imaging studies that have shown an increase in transmitter vesicle fusion to the presynaptic membrane with LTP both in hippocampal cell cultures (Ryan et al., 1996) and in hippocampal slices at CA1 Schaffer collateral synapses (Zakharenko et al., 2001). Furthermore minimal stimulation experiments (activating only one or a few synapses) in acute hippocampal slices have shown that the fraction of stimuli that produce a postsynaptic response (called synaptic reliability) increases with LTP while the average peak postsynaptic current excluding failures (called synaptic potency) is unaltered, arguing for a presynaptic mechanism at CA1 synapses (Stevens and Wang, 1994). An elegant study by Enoki et al showed that excitatory postsynaptic calcium transients (EPSCaTs) measured at individual CA1 spines are a reliable measure of release probability and that the number of successful EPSCaTs increases with LTP induction (Enoki et al., 2009). They also estimated that the EPSCaT producing spines contributed to ~40% of the average excitatory postsynaptic potential (EPSP) (recorded at the cell body), therefore there are indeed synapses that can sustain LTP by a presynaptic mechanism in the hippocampus (Enoki et al., 2009).

1.3.1.2 Postsynaptic Mechanisms

In further searching for the locus of LTP expression, opposing findings have come from studies using a use dependent NMDA receptor blocker, MK-801, which blocks open channels only. If LTP expression is via a presynaptic mechanism due to an increase in presynaptic release probability then the rate of EPSC decline with MK-801 should increase with LTP. In organotypic hippocampal slices one group showed that there was no increase in the rate of decline (Manabe and Nicoll, 1994), while another group showed that there was an increase in the rate of decline in acute hippocampal slices (Kullmann et al., 1996). A very direct measurement of changes in presynaptic release probability can be made by measuring synaptically activated glutamate transporter currents in astrocytes, which transport glutamate away from the synaptic cleft (see Section 1.2.4) (Bergles and Jahr, 1997). Consistent results using this method came from different laboratories that showed LTP does not alter CA1 astrocyte transporter currents, i.e. there is not an increase in presynaptic release (Diamond et al., 1998, Lüscher et al., 1998). Interestingly, another study showed that while synaptically induced glial depolarisation is not significantly different during LTP (as compared to before LTP) at Schaffer collateral CA1 synapses, it is increased significantly at mossy fibre CA3
synapses (Kawamura et al., 2004). This indicates a postsynaptic mechanism for Schaffer collateral CA1 synapses but a presynaptic mechanism for mossy fibre CA3 synapses.

Furthermore, silent synapses have been studied to argue for a postsynaptic mechanism. Silent synapses have no detectable EPSC at resting membrane potential but are “unsilenced” during depolarisation, indicating that they lack functional AMPA receptors but express functional NMDA receptors at the synapse (Kullmann, 1994). Again consistent results have been obtained showing that silent synapses are “unsilenced” when subjected to LTP induction, indicating that LTP in CA1 synapses is due to a postsynaptic modification via insertion of AMPA receptors (Isaac et al., 1995, Liao et al., 1995, Montgomery et al., 2001). This has lead to numerous studies looking at changes in AMPA receptor trafficking during LTP. In cultured hippocampal neurons mobile pools of AMPA receptors are recruited and trapped at activated synapses when NMDA receptors are activated due to Ca$^{2+}$ influx and subsequent activation of CaMKII (Opazo et al., 2010). CaMKII activation leads to phosphorylation of Stargazin (responsible for AMPA receptor localisation at the synapse, see Section 1.2) and its binding to PDZ domain scaffold proteins, thus trapping AMPA receptors at the synapse (Opazo et al., 2010). This immobilisation of AMPA receptors and its trapping at synapses during LTP expression is therefore both CaMKII and Stargazin-dependent. AMPA receptor exocytosis increases 5-fold during LTP at CA1 synapses in organotypic hippocampal slices and has been shown to be CaMKII independent (Patterson et al., 2010).

Early studies have shown morphological changes in spines during LTP, indicating a physical postsynaptic change during the expression of LTP. Stimulation of perforant path fibers causes swelling of dendritic spines of granule cells (Van Harreveld and Fifkova, 1975) as well as an increase in the PSD length and total surface area (Desmond and Levy, 1986). However, stimulation of Schaffer collaterals and examination of CA1 spines during LTP does not produce any changes in the number of synapses on spines, length of PSD or the surface area of spines but does produce a significant increase in the number of synapses on dendritic shafts in acute hippocampal slices (Lee et al., 1979). More recent studies investigating the structural basis of LTP in CA1 neurons have found that glutamate release causes enlargement of activated spines, which is associated with increased AMPA receptor currents and requires NMDA receptor activation and CaMKII (Matsuzaki et al., 2004).

1.4 THE STRIATUM

The striatum is the main input to the basal ganglia circuit in the brain and is an important structure for movement. The basal ganglia is often thought of as only being concerned with motor function but it is also involved in procedural learning and working memory tasks. More specifically, the dorsal
The striatal complex consists of the caudate nucleus (CN), putamen (Put) and globus pallidus (GP, dorsal striatum) and the nucleus accumbens and olfactory tubercle (ventral striatum). However, the neostriatum (simply referred to here as the striatum) consists of the CN and the Put. The caudate nucleus is an elongated, curved mass of tissue and is in contact with the lateral ventricle. The caudate nucleus, similar to the hippocampus, is often divided into three regions: the head region which extends into the anterior horn of the lateral ventricle, the body region which is dorsolateral to the thalamus and the tail region which extends into the temporal lobe. A very prominent feature of the striatum is the internal capsule, which divides the striatum into the caudate nucleus and putamen. However, the CN and Put remain connected as there are cell bridges along the internal capsule. Another such striking feature is the striosome – matrix structures seen in the CN and Put, which are regions of low acetylcholinesterase (AChE) content (striosomes) and AChE-rich regions (matrix) (Graybiel and Ragsdale, 1978).

Cell types of the striatum are divided into subgroups based on their neurochemical contents and firing patterns. There are two classes of striatal neurons, namely spiny and aspiny neurons. The spiny neurons are of medium sized diameter (10-20 µm), referred to as medium spiny neurons (MSNs), and are GABAergic. The MSNs make up more than 95% of the striatal cell types (Kemp, 1968) and their name refers to the dense distribution of spines on their dendrites. In addition to GABA, they also synthesise substance P and dynorphin (direct pathway MSNs that project to the internal GP) or enkephalin (indirect pathway MSNs that project to the external GP) (Haber and Elde, 1982, Bolam and Smith, 1990, Reiner et al., 1999). The MSNs also contain calbindin, a calcium binding protein (Gerfen et al., 1985). MSNs also express glutamate receptor subunits GluA2/3 (Chen et al., 1996), GluA2/3/4c (Tallaksen-Greene and Albin, 1994), GluA5/6/7 (Chen et al., 1996), GluN1 (Chen et al., 1996) and GluN2A/GluN2B but not GluA1 or GluA4 (Chen et al., 1996). The D1 class of dopamine receptors are expressed on MSNs that contain substance P (forming part of the direct circuit), whereas D2 receptors are expressed on MSNs that contain enkephalin (Surmeier et al., 1996).

The aspiny neurons have been identified as GABAergic interneurons of the striatum (Bolam et al., 1985). There are three classes of aspiny neurons in the striatum, based on their neurochemical contents. The large (20 - 50 µm diameter) aspiny neurons contain acetylcholine (Vincent et al., 1983), the medium (10 – 35 µm diameter) contain GABA and the calcium binding protein parvalbumin (Gerfen et al., 1985, Cowan et al., 1987) and there is another group of medium aspiny
neurons that contain somatostatin (Vincent et al., 1983), nitric oxide, neuropeptide Y and GABA as well as the calcium binding protein calbindin (Bennett and Bolam, 1993, Figueredo-Cardenas et al., 1996). The parvalbumin containing interneurons in the striatum express the GluA1, GluA2/3, GluA2/3/4c, GluA5/6/7 and GluN1 glutamatergic receptor subunits (Tallaksen-Greene and Albin, 1994, Chen et al., 1996). The somatostatin-containing and cholinergic interneurons express GluA5/6/7 and GluN1 (Chen et al., 1996) but not GluA1 or GluA2/3/4c (Tallaksen-Greene and Albin, 1994). One study has shown that GluA4 is not expressed in the rat striatum (Tallaksen-Greene and Albin, 1994) while another study shows that GluA4 is expressed in parvalbumin containing interneurons in the rat striatum (Chen et al., 1996). Apart from the MSNs and interneurons in the striatum, there are also dopaminergic neurons found here (Betarbet et al., 1997, Cossette et al., 2005).

The MSNs of the striatum receive glutamatergic inputs from the cerebral cortex and the thalamus (Fonnum et al., 1981, Somogyi et al., 1983) and dopaminergic input from the substantia nigra pars compacta (Kubota et al., 1986) as well as serotonergic fibres from the raphe nucleus (Clements et al., 1985). The substance P, dynorphin containing MSNs receive excitatory input from the cortex and project to the external (GPe) and internal (GPI) segments of the globus pallidus as well as the substantia nigra pars reticulata (SNpr) and substantia nigra pars compacta (SNpc). The enkephalin containing MSNs also receive excitatory input from the cortex and project to the GPe.

There are two pathways or fibre connections in the striatum, namely the direct and indirect pathways. In the direct pathway, the cortex projects to the CN and Put MSNs to provide excitatory input to these regions (Fonnum et al., 1981). From the striatum efferent fibres project to the GP and from here fibres project from the GPI to the SNr and this projection is topographically organised (Parent et al., 1984). Specifically, MSNs synapsing onto the GPe contain enkephalin and those synapsing onto the GPI contain substance P and dynorphin. The pathway described above indicates that the GPI and the SNr are the main output structures of the striatum. From the SNr, cells project to the ventral anterior and the lateral part of the thalamic nucleus (Ilinsky et al., 2004). The cells from the GPI also project to the thalamus and both the pallidothalamic and nigrothalamic projections are known to be GABAergic (Francois et al., 1984, Penney and Young, 1986). Finally, from the thalamus, glutamatergic cells project back to the cerebral cortex in layers I and III mainly and also the striatum (Kemp and Powell, 1971). Hence, the direct pathway is a positive feedback loop as glutamatergic input from the cortex increases activity in MSNs (inhibitory GABAergic), which leads to decreased activity in the GP and SNr. In turn, this inhibition of the inhibitory cells of the GP and SNr, leads to increased activity in the thalamus and the cortex (i.e. disinhibition).
On the other hand, the indirect striatal circuit starts again with excitatory input from the cortex to the striatum and GABAergic projections from here to the GPe. From here, the GABAergic cells of the GPe project topographically to the subthalamic nucleus (STN) (Carpenter et al., 1981a, Carpenter et al., 1981b). The STN glutamatergic neurons in turn project back to the GPe, GPi and SNr, providing excitation to these regions (Carpenter et al., 1981b, Carpenter et al., 2004). From here, the circuit is completed by projecting to the thalamus and back to the cortex. The STN itself receives glutamatergic input from the cortex. Hence, the indirect pathway is a negative feedback loop as glutamatergic input from the cortex increases activity in MSNs (inhibitory GABAergic), which leads to reduced output of the GPe, leading to disinhibition of the STN neurons. This in turn causes an increase in the glutamatergic excitation of the GPe/SNr and this reduces activity in the thalamus and hence the cortical neurons.

The aspiny neurons receive excitatory input from the cortex and the large aspiny neurons additionally receive input from the thalamus. These aspiny neurons in turn project to other interneurons in the striatum. Medium sized GABAergic striatal interneurons that contain parvalbumin, give input to the MSNs, which receive input from the cortex. The somatostain, neuropeptide Y, nitric oxide containing interneurons terminate on the soma and terminal dendrites of the MSNs, which receive input from the cortex.

1.5 THE NORMAL AGING BRAIN

Before introducing the pathogenesis of neurodegenerative diseases, it is necessary to look at how the brain normally ages and the changes that occur at glutamatergic synapses as a result of normal aging. Normal aging involves senescent neurodegeneration, which leads to symptoms like memory loss, poor cognitive function, rigidity in movements etc. It is important to distinguish this from pathological neurodegenerative processes in the aging brain.

Contrasting results have been obtained in the aging human brain with regard to cell loss in the hippocampus as a result of physiological aging using stereological techniques. Significant neuron loss in the subiculum (50% loss) and the hilus (30% loss) of the hippocampus with no loss in any other regions of the hippocampus with age has been shown (West, 1993). This lack of cell loss in CA1 is unique to the normally aging brain as there is indeed significant cell loss in the CA1 are of Alzheimer’s cases (West et al., 1994). In contrast, another study found a loss of CA1 pyramidal neurons with age in the human brain with no loss in the hilus region (Simic et al., 1997). Furthermore, in humans the dendritic fields of pyramidal neurons in the CA1, CA2 and CA3 regions of the hippocampus are not altered with age but the DG granule cells have a 40% reduction in apical
dendrite length with normal aging (Flood et al., 1987a, Flood et al., 1987b). Human autoradiography studies show no significant change in NMDAR or AMPAR binding in aged individuals in the hippocampus (Johnson et al., 1996). However there is controversy around the interpretation of autoradiography studies with regard to receptor number.

Normal aging in non-human primates has been shown not to be related to any cell loss in the CA1 area of the hippocampus, although a decreasing trend is observed in the subiculum despite recognition memory deficits in these animals (West et al., 1993). In a more extensive study on non-human primates, a preservation of neurons in the subiculum, CA1, CA2, CA3, hilus and DG has been shown despite showing age related memory deficits (Bachevalier, 1993, Keuker et al., 2003). In the aged non-human primate hippocampus, there is a 30% loss of the GluN1 subunit of NMDA receptors only within distal dendrites of DG cells and not in their proximal sites (Gazzaley et al., 1996). This change in NMDA receptor GluN1 localisation occurs without any changes in dendritic morphology or synaptic density (Gazzaley et al., 1996).

Again contrasting results have been obtained in the aging rodent with regard to neuronal cell loss in response to normal aging. One study has shown a decrease in neuronal density with aging in the CA3 region of the rat hippocampus (Landfield et al., 1981), while other studies show preserved neuron number in various areas of the hippocampus despite learning and memory deficits in aged rats (Rapp and Gallagher, 1996, Rasmussen et al., 1996). Furthermore, a consistent decrease in NMDA receptor number has been shown through autoradiography studies in different strains of rat and mice in the hippocampus and the striatum as a result of aging (Magnusson and Cotman, 1993, Nicolle et al., 1996). Alongside this decrease in receptor number there are also changes in NMDA receptor function in rodents in the hippocampus and striatum, which is reported as a decrease in NMDA receptor mediate responses (Gonzales et al., 1991) and increased affinity of the NMDA receptor for glutamate (Cohen, 1992). Western blotting has confirmed a significant decrease in the number of GluN2B and GluN1 subunits in the hippocampus without any changes in synapse density in rodents (Magnusson et al., 2002).

1.6 NEURODEGENERATIVE DISEASE

1.6.1 HUNTINGTON’S DISEASE

Huntington’s disease is an inherited autosomal dominant neurodegenerative disease associated with an expanded trinucleotide (CAG) repeat which is an unstable expansion of the Huntingtin gene on chromosome 4 (MacDonald et al., 1993). More than 36 CAG repeats is classified as Huntington’s
disease and the repeat length is also inversely related to the age of onset (Duyao et al., 1993). The disease is classified by neurodegeneration specifically in the basal ganglia and cerebral cortex, which results in the classical symptoms of involuntary movements, cognitive decline and behavioural symptoms (Vonsattel and DiFiglia, 1998). The medium spiny neurons (GABAergic output neurons of the striatum) are mostly affected in HD as they die with disease progression and show early morphological changes (Lange et al., 1976, Graveland et al., 1985, Vonsattel and DiFiglia, 1998).

George Huntington, in 1872, first named the disease “Chorea” which means “dancing” (Huntington, 1872). A lot of research has followed this first description of the disease and it was subsequently named after the man who discovered it. George Huntington described that the symptoms started with twitches of the face muscles and eyebrows and then later on the limbs also had involuntary muscle movements. He described that patients with Huntington’s disease made rolling movements with their hands and that all their movements were irregular. Sometimes he also saw the muscles of respiration being affected and colleagues also reported a change in heart sounds from patients with chorea. Commonly the muscles displaying chorea were paralysed later. George Huntington and colleagues also noticed that the disease was more frequent in females and that the chorea started in adult life (about 30 to 40 years of age). The disease was reported to be an epidemic in some schools of Long Island and they also knew that the disease could be inherited (Huntington, 1872).

In the time of George Huntington, the disease was classified as a disease of the cerebellum as one of the patients affected by chorea had an abscess in the cerebellum and they also knew that the cerebellum was responsible for movement and the coordination thereof. They also reported that “cold baths” and “plenty of exercise in the fresh air” were successful treatments (Huntington, 1872). Treatment today has come a long way based on these pioneering observations. It was first recognised in the 1800’s by Anton and Lannois that there are significant changes in the striatum and globus pallidus in Huntington’s disease. In 1985 Vonsattel introduced the grading system still used today, which is a neuropathological scale (from 0 to 4) that rates the severity of neurodegeneration at different stages of Huntington’s disease (Vonsattel et al., 1985). These grades were developed using microscopic and histopathological methods. The grades are determined by the severity of atrophy in the caudate nucleus, putamen and globus pallidus, the amount of neuronal cell loss and astrocytic gliosis (Vonsattel et al., 1985). Vonsattel described the grades as follows: Grade 0: no microscopic evidence for HD although there is significant clinical evidence for the disease. Grade 1: evidence for astrocytosis, mostly in the CN, but neuronal number is unaffected. Grade 2: atrophy of the CN and some atrophy of the putamen, astrocytosis in the CN and putamen and neuronal cell loss. Grade 3: smaller CN, globus pallidus and putamen, enlarged lateral ventricle, thinned internal
capsule and severe neuronal cell loss and astrocytosis in the CN and putamen. Grade 4: CN extremely shrunken, enlarged lateral ventricle, reduced width of internal capsule, severe atrophy in the putamen and globus pallidus (half the size of control), astrocytosis in CN, putamen, globus pallidus and nucleus accumbens (also reduced in size) (Vonsattel et al., 1985).

As well as the classical motor symptoms that occur in HD as described above, cognitive decline in HD patients are known to occur many years before motor symptom onset and can predict time of clinical diagnosis and motor symptom onset (Diamond et al., 1992, Foroud et al., 1995, Paulsen et al., 2008). Cognitive deficits include dementia, abnormalities in the retrieval of stored information, learning disabilities, deficits in working memory and depression, which are functions associated with the temporal lobe (Diamond et al., 1992, Nehl et al., 2001, Snowden, 2002, Paulsen et al., 2008). Therefore, although the striatum is widely studied in HD, the early detection of such cognitive deficits renders studies in brain structures such as the hippocampus of importance to detect changes that occur early in the disease progression.

The gene for HD, called interesting transcript 15 (IT15) was isolated in 1993 and predicted to encode the protein huntingtin, which is a ~350 kDa protein expressed widely in all tissues but has particularly high expression in the brain and has a neuronal expression pattern (Huntington’s Disease Collaborative Research Group, 1993, Li et al., 1993). During this pioneering time it was also found that the expression of huntingtin was not reduced in the brain of HD patients and the widespread expression of huntingtin did not explain the restricted distribution of neuronal cell loss in HD (Li et al., 1993). It is still puzzling as to what the function of the normal huntingtin protein is. Huntingtin is a cytoplasmic protein and has been shown to have the same distribution pattern as vesicle membrane proteins in biochemical studies and is located at the cell body and dendrites in immunohistochemical studies of the human and rat brain (DiFiglia et al., 1995). Huntingtin interacts with various other proteins including transcription factors, trafficking and endocytosis proteins, signalling molecules and metabolic proteins. Interestingly, the synaptic scaffolding protein PSD95 (see Section 1.2) binds to huntingtin through its SH3 domain and an expanded CAG repeat in the mutant huntingtin protein causes a decrease in this interaction (Sun et al., 2001). Therefore, normal huntingtin sequesters PSD95, which reduces clustering of NMDA receptors at the synapse, however mutant huntingtin does not bind to PSD95 which is then free to cluster NMDA receptors at the synapse and mediate excitotoxicity. Indeed in the YAC animal model (see Section 1.6.1.1) of HD the association between PSD95 and the GluN2B subunit of NMDA receptors are increased in the striatum in a polyQ length dependent manner (Fan et al., 2009). Normal huntingtin is also linked to the production of brain derived neurotrophic factor (BDNF), which is important for the survival of
striatal neurons (Nakao et al., 1995). Indeed there are reduced BDNF levels in both animal models of HD as well as the human post mortem HD brain (Zuccato et al., 2001).

The glutamate excitotoxicity hypothesis (see Section 1.7) has been established to explain the selective MSN cell death in HD. Over exposure to glutamate or any other excitatory amino acid leads to excessive influx of ions and water into the cell, leading to cell death. Alongside there is also calcium influx which is neurotoxic and leads to downstream degradation of proteins. The earliest study showed that prolonged exposure of the striatum in a live animal to glutamate leads to excitotoxic cell death similar to that seen in HD (McBean and Roberts, 1984). The NMDA receptor therefore seemed a likely target in HD as it binds to glutamate and when activated allows calcium to flow into the cell. It is also known that MSNs express glutamatergic receptors such as the AMPA and NMDA receptors (see Section 1.4). However, there are currently no disease modifying treatments and treatment is based on relieving the symptoms of HD. NMDA receptor antagonists in human clinical trials have been found to have no benefit in relieving symptoms in HD (Murman et al., 1997, Landwehrmeyer et al., 2007) although memantine, an open channel blocker may be promising in relieving symptoms and providing neuroprotection (see Chapter 3)(Ondo et al., 2007).

1.6.1.1 Animal models of Huntington’s Disease

There are mainly three types of mouse models of HD: those that contain a fragment of a mutant transgene, those that contain a full length of the mutant transgene and those that are knock-in models. Although there are various animal models in each of the three categories, an overview of the most studied models is given here. The first transgenic animal model of HD was created in 1996 by inserting a fragment of a human HD patient’s huntingtin gene (carrying about 130 CAG repeats) into the mouse genome (Mangiarini et al., 1996). There were various lines of this model with different CAG repeat lengths and two of them, the R6/1 and R6/2 lines, are widely used in HD animal model studies today. In the R6/2 line the age of symptom onset is 9 – 11 weeks with death at 10 – 13 weeks but occurs later in the R6/1. They display motor symptoms such as resting tremor, involuntary movements and dyskinesia (Mangiarini et al., 1996). They also display non-motor symptoms such as learning disabilities and pathological hallmarks such as reduced brain volume, changes in glutamate receptor expression and reduced LTP (Cha et al., 1998, Murphy et al., 2000). The learning deficits occur before motor symptom onset (Lione et al., 1999). However, these mice do not display the typical striatal cell loss seen in HD (Mangiarini et al., 1996) and their rapid progression of the disease and early death are limiting factors.

Another very popular animal model of HD is the yeast artificial chromosome (YAC) mice which express full-length mutant human huntingtin with expanded CAG repeats e.g. YAC72 and YAC128
(Hodgson et al., 1999). They have much later symptom onset and age of death than the R6 lines, with the YAC128 showing motor symptoms at 6 months of age and the YAC72 at 16 months of age but both have a normal life span (Hodgson et al., 1999, Slow et al., 2003). The motor symptoms are highly correlated with striatal neuronal neurodegeneration which is age-dependant and starts at 9 months of age (Slow et al., 2003). Pathological hallmarks include reduced brain volume, striatal and cortical cell loss, reduced LTP and huntingtin aggregates (Slow et al., 2003). As in the R6 mouse model, the YAC128 shows learning deficits at 2 months of age before motor onset (Van Raamsdonk et al., 2005b).

There is also the N171-82Q model that contains an N-terminal fragment of huntingtin with 82 CAG repeats and was created to investigate intranuclear inclusions of huntingtin which react only with N-terminal huntingtin antibodies (Schilling et al., 1999). This model shows motor symptom onset at 3 months of age but does not reproduce the neuron loss in the striatum or cortex that is known to occur in HD. A knockin model that contains a full length mutant huntingtin (HdH) was created to study the behavioural deficits in HD specifically (Shelbourne et al., 1999). These mice have abnormal social behaviour but do not show any neuron loss in any brain region although they do have a reduced brain volume.

1.6.1.2 Electrophysiological changes in HD Animal Models

There have been a few key pioneering studies that have investigated the altered synaptic plasticity that occurs in animal models of HD. These electrophysiological studies have been performed both in the striatum (where the classical motor symptoms of the disease arise) and the hippocampus (where the cognitive decline arises). In dissociated MSNs of the YAC128 mouse model striatum, a biphasic NMDAR current response was observed where there was an increase in current density presymptomatically (1.5 months) and a reduction at a symptomatic stage (7 months) of the disease (Graham et al., 2009). Similarly in striatal slices, the NMDA receptor mediated current (evoked synaptically) also show this biphasic response with respect to the stage of the disease (Graham et al., 2009). Interestingly the increased peak current in the presymptomatic mice is only visible during low stimulation intensities (0.2 mA and 0.4 mA) but the reduced peak current in the symptomatic mice is visible at all stimulation intensities (Graham et al., 2009). There was also no change in passive membrane properties in the presymptomatic mice but a significant increase in input resistance (a measure of channel function at rest), capacitance (a measure of cell size) and the membrane time constant in the symptomatic mice (Graham et al., 2009).

In striatal slices from YAC72 mice (3 – 4 weeks old), an increase in NMDAR mediated EPSCs have been measured across a range of stimulation intensities but there was no difference in the AMPAR
mediated EPSCs (Li et al., 2004). Interestingly, this group used an extracellular glycine concentration that activates GluN2B containing NMDA receptors and not GluN1/GluN2A receptors (Li et al., 2004). Blocking GluN2B containing receptors specifically revealed that triheteromeric receptors are the major contribution to striatal NMDAR mediated EPSCs (Li et al., 2004). This work suggested that it is GluN2B containing receptors (specifically triheteromeric receptors) that show increased EPSCs as a result of HD. We also know that extrasynaptic receptors are mostly composed of GluN2B subunits (Tovar and Westbrook, 1999). This work is interesting in the light of later studies that showed that these extrasynaptic receptors lead to cell death (Hardingham et al., 2002, Okamoto et al., 2009) and are upregulated in HD (Fan et al., 2007, Milnerwood and Raymond, 2010).

Synaptic plasticity in the hippocampus has also been studied given its importance in learning and memory. Three major studies were published within 2 years, showing that synaptic plasticity is reduced in animal models of HD. Field recordings of the EPSP in the CA1 region of the hippocampus was recorded during stimulation of the Schaffer collaterals. At 10 months of age, hippocampal slices from the YAC46 and YAC72 mice are unable to undergo LTP induction (Hodgson et al., 1999). High frequency stimulation resulted in depression of the EPSP and not potentiation in the YAC72 mice indicating that these neurons cannot keep up with high frequency stimulation (Hodgson et al., 1999). When investigating the cause of this loss of synaptic plasticity, it was found that there was no calcium influx in response to glutamate in the mutant slices, which in turn was due to a higher resting calcium level and reduced calcium buffering capacity in these slices (Hodgson et al., 1999). While this was not due to presynaptic release mechanisms during paired stimulation (as there was no change in PPF), the YAC mice showed reduced posttetanic potentiation (PTP), i.e. the neurons were unable to sustain presynaptic transmitter release during HFS (Hodgson et al., 1999). In another animal model, the Hdh model, the same experimental setup was used to assess synaptic function. This study showed no difference between Wt and mutant slices in their input-output curves, i.e. there is no difference in the density of synapses or their function in response to single stimulations (Usdin et al., 1999). During LTP experiments, the mutant mice showed reduced potentiation and required twice the intensity of stimulation to show potentiation similar to WT slices (Usdin et al., 1999). Again this Hdh model, like the YAC 46 and 72 models, showed impaired PTP but unlike the YAC models also showed reduced PPF (Usdin et al., 1999). Further experiments assessing presynaptic function using the MK-801 open channel NMDA receptor blocker during intracellular recording showed that at high frequencies mutant slices show a slower rate of glutamate release, i.e. they are unable to sustain high frequency activity (Usdin et al., 1999). In yet another animal model of HD, the R6/2 mouse, results were opposite to that of the Hdh model in that the R6/2 model shows no change in input-output curves and no change in PPF (Murphy et al., 2000). However, like the other
models, the R6/2 mouse also shows reduced LTP of the EPSP (although LTP of the population action potential was normal) and reduced PTP (Murphy et al., 2000). Altogether these studies have shown that LTP is reduced in various animal models of HD alongside a reduced ability to sustain transmitter release at HFS. These functional changes in HD mice are likely to underlie the learning deficits present in these mice.

1.6.1.3 Altered expression levels of glutamate receptors and synaptic scaffolding proteins in HD animal models

Animal models of neurodegenerative disease have shed light on the changes in synapse structure and function that occur in response to diseases such as HD and PD. Although no single animal model entirely reflects the human state of the disease, they have been vital in leading the way in our understanding of the molecular mechanisms that govern synapse function, structure and ultimately behavior. Animal models of HD have shown conflicting results with regards to the changes in expression in glutamatergic receptor subunits and PSD proteins. These conflicts arise from the differences between animal models, the different stages of the disease (i.e. symptomatic vs. presymptomatic), differences in whether specific cellular fractions are investigated or the total protein content, and the different experimental approaches taken to assess these changes. When comparing the studies from animal models, these differences must be kept in mind.

In the striatum of the presymptomatic and symptomatic R6/1 mouse model of HD, western blotting analysis has shown a decrease in total SAP97 expression as well as a decrease in the cytosolic fraction while there was no change in the synaptic membrane fraction (Torres-Peraza et al., 2008). Similarly for PSD95, the presymptomatic and symptomatic R6/1 mouse model of HD has shown a decrease in total striatal levels of PSD95 as well as in the cytosolic and synaptic membrane fractions of the striatum (Torres-Peraza et al., 2008). The N171-82Q animal model has confirmed this loss of total PSD95 expression levels (Jarabek et al., 2004). There were also significant decreases in PSD93 and SAP102 total expression levels in the R6/1 mouse model striatum (Torres-Peraza et al., 2008).

For the GluA2/3, GluA4 and GluA1 subunits of the AMPA receptor, no change in total expression levels has been reported as well as no changes in the synaptic membrane and synaptic vesicle fractions as assessed by western blotting in the striatum of the R6/1 (Torres-Peraza et al., 2008) and YAC128 models (Benn et al., 2007). The studies investigating NMDA receptor subunits in the striatum have been more variable. Total levels of the GluN1 subunit has been shown to be increased in the R6/1 and R6/2 mouse models (Cepeda et al., 2001, Ariano et al., 2005), whereas another study in the same model has shown no change (Torres-Peraza et al., 2008). The N171-82Q mouse model has been reported to show no change in total levels of GluN1 in presymptomatic and symptomatic
animals (Jarabek et al., 2004). In yet another model, the YAC128 and YAC72, no change in total GluN1 expression levels has been reported using immunocytochemistry in young animals. For the GluN2A receptor subunit no change in total expression levels has been shown in the R6/1 and N171-82Q mouse models using western blotting (Jarabek et al., 2004, Torres-Peraza et al., 2008). However, the R6/2 mouse model has shown a decrease in total GluN2A subunit expression levels in the striatum using IHC. Again no change in GluN2A expression in synaptic membrane and synaptic vesicles have been shown in the R6/1 mouse (Torres-Peraza et al., 2008) and YAC128 mouse model (Benn et al., 2007). Interestingly, one study has shown that GluN1, GluN2A and GluN2B are increased in synaptosomes from YAC128 striatum specifically at non-PSD sites, whereas only GluN2B was reduced at the PSD (Milnerwood et al., 2010). The importance of the GluN2B receptor subunit in synaptic transmission is discussed later (See Chapter 3).

Although fewer studies have been done in the hippocampus of HD mouse models, a decrease in PSD95 has been shown in the plasma membrane of the R6/2 model (Luthi-Carter et al., 2003). For AMPA receptors an increase in GluA1 and GluA2 in the YAC128 model synaptic membrane and synaptic vesicle fraction has been reported (Benn et al., 2007). On the other hand, the GluN1 subunit has been shown to be decreased in the R6/2 plasma membrane (Luthi-Carter et al., 2003) and no change to occur in the YAC128 synaptic membrane and synaptic vesicle fraction (Benn et al., 2007). GluN2A and GluN2B has been shown to be changed in parallel, where an increase has been shown for both in the YAC128 synaptic membrane and synaptic vesicle fractions (Benn et al., 2007) and a decrease has been shown for both in the R6/2 plasma membrane (Luthi-Carter et al., 2003).

1.6.1.4 Altered expression levels of glutamate receptors and synaptic scaffolding proteins in human HD

To date, there have been few human studies looking at changes in synaptic proteins. The excitotoxicity hypothesis has been widely used to explain the selective loss of MSNs in the striatum of HD patients and animal models. Earlier studies in human tissue have employed autoradiography and in situ hybridization methods, whereas later studies have employed immunofluorescence and western blotting. The human studies discussed here have been performed in the striatum. Using ligand binding in autoradiography experiments, it has been shown that there is a decrease in ligand binding to NMDA and AMPA receptors thought to indicate a loss of these receptors (Young et al., 1988, Dure et al., 1991). This was interpreted to show that MSNs that contain high levels of NMDA receptors are most vulnerable to excitotoxic damage and are lost early in the disease. Using in situ hybridization methods, it has been shown that there are decreases in GluN1, GluN2B and GLT1 (glutamate transporter) mRNA levels in postmortem striatal tissue of HD patients (Arzberger et al.,
This is thought to occur in an attempt to protect neurons from glutamate neurotoxicity by downregulating glutamate receptors. Immunofluorescence and western blotting has shown decreases in BDNF and GLT1 in the human HD striatum (Ferrer et al., 2000, Faideau et al., 2010). This data has contributed to the idea of providing surplus BDNF for treatment in HD as BDNF is a neurotrophic signalling factor. For synaptic scaffolding proteins, PSD95, PSD93 and SAP102, decreases have been shown with the use of western blotting and is thought to occur to compensate for the overactivation of glutamate receptors leading to excitotoxicity (Torres-Peraza et al., 2008).

1.6.2 PARKINSON’S DISEASE

Parkinson’s disease is characterised by a loss of dopaminergic cells in the substantia nigra pars compacta (SNc) leading to a depletion of dopamine in the striatum presenting with both motor and cognitive symptoms (Riederer and Wuketich, 1976). The disease is named after the London physician James Parkinson, who was the first to describe the motor symptoms of PD in 1817 in “Essay on the Shaking Palsy”. The mean age of onset is 70 years, although a small percentage of patients have early onset of the disease before the age of 50 (Van Den Eeden et al., 2003). Only about 2-3% of late onset cases are of genetic mutation origin whereas 50% of early-onset PD is due to genetic mutations (Farrer, 2006, Schiesling et al., 2008). PD is more prevalent in males than females and is more prevalent amongst people of Hispanic origin (Van Den Eeden et al., 2003). Typical symptoms are resting tremor, muscular rigidity, akinesia and bradykinesia, difficulties walking and speaking, masking of facial expressions as well as dementia (Fahn, 1988). The onset of dementia is usually of late onset and is due to cortical Lewy bodies (see below) (Braak et al., 2005).

The neuropathology of PD includes dopaminergic cell loss in the SNc as well as the presence of Lewy bodies (protein intracellular inclusions). Whether Lewy bodies are neuroprotective or pathogenic remain to be confirmed (Chen and Feany, 2005). The major protein in Lewy bodies and Lewy neurites is aggregated α-synuclein (a presynaptic protein) (Spillantini et al., 1997) and is not considered a normal feature of ageing (Braak et al., 1995). It is not fully known as to why the neuromelanin-containing dopaminergic neurons in the SNc are particularly vulnerable. Post-mortem human studies have shown that the neuromelanin content in the SNc dopamine cells is related to their vulnerability (Hirsch et al., 1989, Kastner et al., 1992). Their susceptibility has been linked to their dopamine metabolism, which produces highly reactive oxygen species that increase oxidative stress and impair mitochondrial function (Gluck and Zeevalk, 2004). Other studies have suggested that the vulnerability is linked to their reduced capacity for calcium buffering (Esteves et al., 2010) and calcium-mediated toxicity through Cav1.3 channels (Chan et al., 2007).
Braak and colleagues have introduced a staging system to characterise the evolution of neuropathology during PD based on α-synuclein staining in the human post-mortem brain. Stage 1 and 2 mostly involves the medulla oblongata and at this stage the projection neurons of the coeruleus-subcoeruleus are the first neuromelanin-containing neurons to develop Lewy bodies and Lewy neurites (Braak et al., 2003). Stages 3 and 4 involve the brainstem and it is also at this stage that the melanin-containing neurons of the SNc are affected (Braak et al., 2003). Stages 5 and 6 involve a myriad of other brain regions and damage in the subcortical and mesocortical areas increase with the SNc almost depleted of melanin-containing neurons (Braak et al., 2003).

Mutations in α-synuclein cause autosomal-dominant familial PD (Polymeropoulos et al., 1997) and the mutations in leucine-rich repeat kinase 2 (LRRK2) has also been linked to PD (Zimprich et al., 2004). Various other protein mutations have been discovered and linked to PD including mutations in the parkin gene originally identified in Japanese families with autosomal recessive, juvenile parkinsonism (Kitada et al., 1998) and homozygous mutations in the PINK1 gene originally identified in early onset PD (Hatano et al., 2004). While only about 10% of PD cases are of genetic origin (Dauer and Przedborski, 2003), most cases are idiopathic and environmental causes are unknown, although 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) causes specific neurodegeneration in the SNc and is used most commonly in primate and rodent models of the disease respectively (Ungerstedt, 1968, Burns et al., 1983). Agricultural chemicals such as the pesticide, rotenone, has also been shown to reproduce PD when injected systematically (Betarbet et al., 2000).

Again, there is no cure for PD, although treatment improves quality of life. Interestingly consumption of coffee, tobacco and nonsteroidal anti-inflammatory drugs reduces the risk of PD (Powers et al., 2007). In the late 1950’s the drug L-3,4-dihydroxyphenylalanine (levodopa) was discovered to reverse PD akinesia and showed a marked benefit to treat patients with PD (Carlsson et al., 1957, Birkmayer and Hornykiewicz, 1961). As dopaminergic cells die in the SNc, the best treatment still is the use of levodopa, which acts to increase synaptic dopamine transmission. However, new treatment strategies are of the essence as levodopa has debilitating side effects such as dyskinesias, which include chorea and dystonia, first recorded in 1974 in levodopa treated patients (Duvoisin, 1974). Another treatment strategy is deep brain stimulation (DBS), where stimulation electrodes are implanted permanently in specific brain regions and electrical stimulation delivered via an external programmable unit. DBS was first pioneered in the 1980’s and was shown to be effective in reducing movement disorders in PD when the thalamus was stimulated (Benabid et al., 1987). Currently, the
GPi or STN is targeted for DBS and treats motor symptoms especially during “off” periods of levodopa and reduces dyskinesia and motor fluctuations due to levodopa (Anderson et al., 2005).

1.6.2.1 Animal models of Parkinson’s Disease

There are both toxic and genetic models of PD where the classic PD model is the 6-hydroxydopamine (6-OHDA) model. Injection of 6-OHDA into the SNpc causes 60% of tyrosine hydroxylase containing neurons to die (Ungerstedt, 1968). The 6-OHDA toxicity is relatively specific for dopaminergic neurons while non-dopaminergic neurons are spared, although it does not produce the pathological hallmark of Lewy bodies (Luthman et al., 1989). 6-OHDA is normally injected unilaterally and causes turning behaviour in the animal which is used to rate the extent of the lesion and test the efficacy of various treatment strategies. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was accidentally discovered in 1979 when it produced parkinsonian syndromes in young drug abusers of this substance (Davis et al., 1979). In these drug abusers MPTP produced the biochemical, neuropathological and clinical features of PD with cell loss in the SN, although the pathological hallmark of Lewy bodies was absent (Langston et al., 1983). MPTP is most commonly used today in non-human primate animal models of PD. Another neurotoxin, rotenone, is also a herbicide and pesticide which causes oxidative stress by inhibiting the mitochondrial electron transport chain (Betarbet et al., 2000). Intravenous administration reproduces features of PD and causes neurotoxicity in dopaminergic neurons of the SN and unlike 6-OHDA and MPTP, it causes the formation of α-synuclein aggregation and Lewy body formation (Betarbet et al., 2000).

Genetic models of PD include a transgenic mouse that expresses wilt-type human α-synuclein and has been shown to develop PD motor symptoms as well as Lewy bodies (Masliah et al., 2000). LRRK2 overexpression has been studied in Drosophila and C.elegans where it causes loss of dopamine neurons as well as age-dependent reductions in locomotor activity (Liu et al., 2008, Saha et al., 2009). There is also a LRRK2 transgenic mouse that has a mutant form of LRRK2 and shows age-dependent and levodopa-responsive motor abnormalities as well as axonal pathology in SN dopaminergic neurons although there is no extensive neurodegeneration as seen in PD patients (Li et al., 2009). The Parkin-Q311X transgenic mouse contains a truncated human mutant parkin in dopamine neurons and presents PD-like motor deficits and age-dependent dopaminergic neuron terminal loss in the striatum as well as progressive α-synuclein accumulation in dopaminergic neurons (Lu et al., 2009). PINK1 knock-out mice fail to show dopaminergic neuron loss although they show altered dopamine neurotransmission and synaptic plasticity in the striatum (Kitada et al., 2007). Therefore PINK1 has a critical role in synaptic plasticity and dopamine transmission and these knock-out mice can be used for studies of these features.
1.6.2.2 Altered expression levels of glutamate receptors and synaptic scaffolding proteins in PD animal models

The PD animal models discussed here have implemented the use of subcellular fractionation and western blotting to look at the changes in glutamate receptor subunits and PSD scaffolding proteins in the striatum specifically. In the 6-OHDA model of PD, 2 weeks after the lesion, it has been reported that there is no change in the total expression level of GluA2 AMPA receptor subunit and GluN2A NMDA receptor subunit and neither is there any change in the membrane fraction (Dunah et al., 2000). However, for the GluN1 and GluN2B subunits, a decrease has been shown in the membrane fraction along with no significant change in the total fraction (Dunah et al., 2000). This study also showed no change in PSD95 in the total homogenate (Dunah et al., 2000). However, another study using the same model but looking at a later stage of the disease (6 weeks after lesion), reported a decrease in total PSD95 expression levels, alongside a decrease in the synaptic membrane fraction and an increase in intracellular vesicles (Nash et al., 2005). This study showed the exact same pattern of change for SAP97 (Nash et al., 2005). This shows that both PSD95 and SAP97 are reduced in the 6-OHDA model and that remaining protein is redistributed away from the synapse and into vesicular compartments. Looking at an even later stage of the 6-OHDA model (10 weeks after lesion), a decrease in both SAP97 and PSD95 has been shown in the PSD fraction, while there were no changes in the GluN1 and GluN2A subunits (Gardoni et al., 2006). Finally, in a primate model (MPTP-lesioned), a decrease in GluN1 and GluN2B has been shown in the synaptic membrane, while there was no change in GluN2A in the synaptic membrane (Hallett et al., 2005). The studies discussed here agree that SAP97 and PSD95 both decrease at the synapse/PSD, although their results are different for the glutamatergic receptor subunits.

1.7 NMDA RECEPTOR EXCITOTOXICITY

More than 50 years ago it was discovered that glutamate injections in the mammalian cortex produced seizures and it was also postulated that glutamate causes Na⁺ ion influx into neurons (Hayashi, 1954). The phenomenon of glutamate toxicity was first demonstrated in the mouse retina (Lucas and Newhouse, 1957) and hereafter, Olney J.W. was the first to show that excessive glutamate exposure causes lesions in the mouse developing brain, and he termed it glutamate excitotoxicity (Olney, 1969). The mechanism of this excitotoxicity is mediated via calcium influx, which consequently leads to neurodegeneration, and has been named the common pathway to cell death.
Choi and colleagues determined two distinct phases of this cell death, the first being an acute phase marked by neuronal swelling and is dependent on extracellular Na\(^+\) and Cl\(^-\), the second (late) phase is marked by gradual neuronal death and is dependent on extracellular Ca\(^{2+}\) (Choi, 1987). The first phase could be mimicked by high K\(^+\) exposure (no glutamate) and the second phase could be mimicked by exposure to NMDA. Furthermore, the neurotoxic entry of Na\(^+\) was shown not to be through voltage dependent Na\(^+\) channels as tetrodotoxin (TTX, a blocker of these channels) did not block cell death (Choi, 1987). Similarly, Ca\(^{2+}\) entry through NMDA receptors is specifically neurotoxic and not Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels as experiments showed a significant increase in neuronal cell death when intracellular calcium transients were evoked with glutamate (250 µM) application, as compared to calcium transients produced by high K\(^+\) (Tymianski et al., 1993). The late phase of neurotoxicity in Choi’s experiments could also be blocked by D-(−)-2-Amino-5-phosphonopentanoic acid (APV, an NMDA channel blocker) (Choi, 1987, 1988).

Treating anoxic cultured hippocampal neurons with Mg\(^{2+}\) also prevents cell death by blocking synaptic activity (Rothman, 1983). The high Mg\(^{2+}\) may block Ca\(^{2+}\) entry through at least two pathways: Mg\(^{2+}\) blocks Ca\(^{2+}\) entry by blocking NMDA receptors and higher Mg\(^{2+}\) shifts the local field gradient across the membrane opposing Ca\(^{2+}\) entry. Similarly removing extracellular Ca\(^{2+}\) attenuates excitotoxicity induced neuronal loss in cortical (Choi, 1985) and hippocampal (Rothman and Olney, 1987) cultures. In slices it has also been shown that blocking Ca\(^{2+}\) entry with high Mg\(^{2+}\) and zero extracellular calcium allows evoked responses in the hippocampal slice preparation to recover to 65% after a period of anoxia (Kass and Lipton, 1982) and similarly slices were protected against hypoxic damage if the NMDA receptor was blocked (Lobner and Lipton, 1987).

These experiments led to the hypothesis that NMDA receptor antagonists could be a therapeutic target in stroke and traumatic brain injury. However, the evidence that chronic neurodegenerative diseases (such as HD, PD and AD) were candidates for neuroprotective therapy with NMDA receptor blockers was weak. Albin and Greenamyre then defined a new hypothesis of excitotoxicity where specific populations of neurons would be vulnerable to excitotoxicity by having abnormal glutamate receptor expression and/or impaired cellular energy metabolism (Albin and Greenamyre, 1992). For example, in the HD striatum of animal models both an increase in extrasynaptic NMDA receptor signalling (Milnerwood et al., 2010) and reduced uptake of glutamate via GLT-1 (Huang et al., 2010), renders the MSNs vulnerable to excitotoxic damage. Indeed previously, NMDA receptor antagonists were shown to be effective in animal models of hypoxic/ischemic brain damage (Simon et al., 1984, Ikonomidou et al., 1989). Many clinical trials were performed to test the efficacy of NMDA receptor antagonists but have failed (Lee et al., 1999, Morris et al., 1999) and were considered unsuccessful.
for the treatment of traumatic brain injury and stroke by the year 2001. However, looking back at studies performed prior to these clinical trials is a key as to why these trials failed. It has been shown that raising intracellular Ca\(^{2+}\) (nM range) inhibits neuronal apoptosis (Koike, 1991) and although this may seem contradicting, the Ca\(^{2+}\) levels to induce toxicity have been found to be much higher (µM range) (Hyrc et al., 1997). This suggests that there is a balance of intracellular Ca\(^{2+}\) concentration where too much or too little is neurotoxic. Therefore, the benefit of NMDA receptor antagonists may be counteracted by the deleterious effects of having too little calcium.

Furthermore, it was shown that blocking NMDA receptors in the developing brain leads to apoptosis (Ikonomidou et al., 1999). In another study in the developing rat brain looking at traumatic brain injury, NMDA receptor antagonists increased the severity of secondary apoptotic damage while being neuroprotective at the site of impact (Pohl et al., 1999). Similarly, enriching the environment of rodents (which stimulates synaptic activity) increases neurogenesis, reduces spontaneous apoptosis in the hippocampus and protects against excitotoxic injury (Young et al., 1999). Hence, there is also a balance for NMDA receptor activity, where too much or too little is detrimental.
1.8 WHERE TO FROM HERE?

Research has made great progress from when neurodegenerative diseases such as HD and PD were first documented and investigated. However, there are still gaps in our knowledge of the molecular determinants underlying such diseases and this is evident in the lack of disease modifying treatments since current therapies only improve quality of life (Anderson et al., 2005, Landwehrmeyer et al., 2007). The role of excitotoxicity and glutamatergic transmission in the brain has been reviewed here and is still today an attractive pathway to study for the development of new treatment strategies. Few studies have been performed in the human postmortem brain of HD and PD patients at the molecular or subcellular level (Torres-Peraza et al., 2008). It is therefore not known if glutamate receptor changes that are reported in animal models of these diseases are representative of the human state. While animal models have contributed hugely to our current knowledge of disease processes and will continue to be of great aid, they are models of the human disease and studies in human cases are of the essence.

Nevertheless, the YAC128 model of HD is one of the best HD models as it most reliably mimics the human HD condition. The YAC128 as reviewed here shows age-dependent neuronal degeneration (unlike many other models), HD-like motor abnormalities, cognitive decline before the onset of motor systems, pathological hallmarks of the disease and presents many practical advantages such as a normal life span and late onset motor abnormalities (Hodgson et al., 1999, Slow et al., 2003). Motor symptoms and cognitive decline have been elegantly described for this model (Van Raamsdonk et al., 2005b). However, very little functional or electrophysiological data is available for this model, especially in the hippocampus. While a sophisticated study has been performed in the YAC128 striatum investigating extrasynaptic NMDA receptor function (Milnerwood et al., 2010), the hippocampus remains less well studied even though cognitive decline and learning abnormalities occur before motor symptom onset (Van Raamsdonk et al., 2005b). It is therefore of great interest to study the early changes that occur in the hippocampus of the YAC128 mouse model as this may lead to treatment strategies targeted at early intervention.

Many electrophysiological studies of neurodegenerative diseases, such as HD, utilise transgenic animal models and are performed in acute brain slices (Hodgson et al., 1999, Usdin et al., 1999, Murphy et al., 2000). However, acute brain slices pose some limitations to the use of molecular biology to manipulate the expression of proteins of interest in order to probe the molecular mechanisms that underlie disease. Such manipulations can be performed with relative ease in cell culture model systems such as primary dissociated hippocampal neuronal cultures (Goetze et al., 2004, Jiang and Chen, 2006). It is therefore of great interest to study diseases such as HD in a cell
culture model system where flexible and rapid investigations can be performed to study synaptic function in disease. It is known that dissociated striatal MSN cultures made from the YAC128 animal model show increased extrasynaptic NMDA receptor activity and is thought to underlie the extensive MSN death in HD (Hardingham et al., 2002, Milnerwood et al., 2012). It is not known if this also occurs in hippocampal neurons expressing mutant huntingtin. It would also be of great interest to investigate the molecular mechanisms underlying this mislocalisation of receptors in the striatum and/or hippocampus by manipulating protein expression in a transient cell model of HD. As each system, post-mortem human brain tissue, acute slices from transgenic animals or transient cell models has its own advantages and disadvantages, this thesis combined all three systems to investigate the changes that occur in neurodegenerative disease in the hippocampus and striatum, with particular emphasis on the HD hippocampus.
1.9 GENERAL AIMS

Glutamate receptors are involved in glutamatergic signalling at excitatory synapses in the brain and have been implicated in neurodegenerative diseases due to their involvement in excitotoxicity. The subcellular location of glutamate receptors are regulated by MAGUKs, which also have important roles in the architecture of the PSD, synaptic plasticity and synaptic transmission. However, human brain studies are few in number and it is not known if important MAGUKs and their bound glutamate receptor subunits are altered in the human brain in neurodegenerative disease. Our study is therefore the first to investigate changes in glutamate receptor subunit and MAGUK expression in the human brain in HD and PD. Furthermore, the localisation of NMDA receptors has been implicated in neuroprotective vs. cell death pathways depending on the synaptic or extrasynaptic location of the receptors respectively (Hardingham et al., 2002, Okamoto et al., 2009). This has been studied in the striatum in the YAC128 animal model of HD and increased extrasynaptic NMDA receptor signalling has been reported (Milnerwood et al., 2010, Milnerwood et al., 2012) but it is not known if this also occurs in the hippocampus in HD. Our study is therefore the first to investigate if such changes occur in the hippocampus in both an animal model of HD (YAC128) and a hippocampal cellular model of HD. Given the gaps in our knowledge we aimed to investigate the following:

1. To investigate the changes that occurs in glutamate receptor subunits and MAGUK proteins in the human postmortem hippocampus and striatum in HD and PD.

2. To investigate if such changes in glutamate receptor subunits and synaptic scaffolding proteins also occur in the YAC128 mouse hippocampus at a symptomatic age.

3. To investigate the pathophysiological changes that may occur in the YAC128 hippocampus with regard to glutamate receptor function and synaptic plasticity with the use of electrophysiology in acute brain slices from YAC128 animals.

4. To investigate the pathophysiological changes that may occur in dissociated hippocampal neurons transfected with mutant huntingtin protein with regard to glutamate receptor function with the use of paired recordings using electrophysiology.

For the first time the data here will reveal specific changes that occur in the human brain in HD and PD with regard to MAGUKs and glutamate receptor subunit expression. It will also reveal if the hippocampus has altered synaptic vs. extrasynaptic NMDA receptor signalling as has been reported in the striatum (Li et al., 2004, Milnerwood et al., 2010, Milnerwood et al., 2012). It is hoped that
these data will shed light on the molecular mechanisms that underlie neurodegenerative disease and potentially lead to better therapeutic approaches.
CHAPTER TWO: ALTERATIONS IN POSTSYNAPTIC DENSITY PROTEINS IN THE HUMAN BRAIN IN RESPONSE TO NEURODEGENERATIVE DISEASE

The results in this chapter have been submitted for publication to the journal *Neuroscience*. Title: Differential changes in postsynaptic density proteins in post-mortem Huntington’s and Parkinson’s Disease human brains. Authors: C. Fourie, J. Wong, H. Waldvogel, A.L. McGregor, R.L. Faull, J. Montgomery. Additional results are presented here (Section 2.4.3) that are not part of the original manuscript submitted for publication.

2.1 ABSTRACT

N-Methyl-D-Aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors and their bound membrane-associated guanylate kinases (MAGUKs) are critical for normal brain function including synaptic transmission, plasticity, learning and memory. MAGUKs act as scaffolding molecules and are responsible for maintaining the structure of synapses, trafficking of receptors and activating signalling molecules. These proteins may also play a role in the changes in synapse function that occur in the neurodegenerative diseases Huntington’s disease (HD) and Parkinson’s disease (PD). We performed immunohistochemical and synaptosomal analysis of human brain tissue to determine changes in the expression of synapse associated protein 97 kDa (SAP97), postsynaptic density protein 95 kDa (PSD95), the GluA2 subunit of the AMPA receptor and the NR1 subunit of the NMDA receptor (GluN1) in human control, Huntington’s and Parkinson’s disease postmortem hippocampus and striatum tissue. Immunohistochemical analysis revealed significant increases in SAP97 in the HD and PD hippocampus, whereas PSD95 was differentially altered in the hippocampus (upregulated in HD and PD) and the striatum (downregulated in HD). Alongside this, we found a significant increase in GluN1 in the HD hippocampus and a decrease in GluA2 levels in diseased striatum. Synaptosomal fractionation of the human hippocampus revealed that the hippocampal changes in expression levels occurred away from synapses. Parallel immunohistochemistry experiments in the YAC128
mouse model of HD showed no change in the hippocampal expression levels of any of these synaptic proteins. Therefore our human data show that major changes occur in glutamatergic proteins with neurodegenerative disease, particularly in the hippocampus. Moreover, these changes differ from those occurring in HD mouse models, suggesting that unique changes occur at a subcellular level in the diseased human hippocampus.
2.2 INTRODUCTION

Huntington’s disease (HD) and Parkinson’s disease (PD) are neurodegenerative diseases that present with unique motor and cognitive symptoms. HD is an autosomal dominant inherited disease caused by the expansion of a polyglutamine repeat sequence in the huntingtin gene, which results in a progressive loss of medium spiny neurons in the striatum (Vonsattel et al., 1985). PD is a sporadic neurodegenerative disease, although there are rare familial cases. It is marked by the loss of dopaminergic neurons of the substantia nigra pars compacta, which leads to abnormal basal ganglia circuitry, resulting in motor symptoms (Gibb, 1991, Braak et al., 2003). Treatments for these diseases are symptomatic and new therapeutic targets are of the essence. Emphasis is now being placed on the changes that occur at the synapse and the processes that underlie cognitive dysfunction as it has been shown that synaptic and cognitive dysfunction occurs long before the onset of clinical symptoms in the human (Foroud et al., 1995, Lawrence et al., 1998, Paulsen et al., 2008).

Glutamate receptors are currently viewed as valuable therapeutic targets in both HD (Milnerwood and Raymond, 2010) and PD (Johnson et al., 2009). N-Methyl-D-Aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) -type glutamate receptors and their bound postsynaptic density - membrane associated guanylate kinases (PSD-MAGUKs) are critical for synapse development and plasticity (Montgomery et al., 2004). MAGUKs act as scaffolding molecules and are responsible for maintaining the structure of synapses, trafficking of receptors and activating signalling molecules. PSD95 targets AMPA receptors to the synapse through its interaction with stargazin (Schnell et al., 2002) and also binds directly to NMDA receptor subunits (GluN2A and GluN2B) for synaptic targeting (Kornau et al., 1995, Niethammer et al., 1996). SAP97 binds directly to the GluA1 subunit of AMPA receptors to traffic them to the PSD (Leonard et al., 1998) and the GluN2 subunits of NMDA receptors (Bassand et al., 1999) and together with CASK traffic NMDA receptors through a unique secretory pathway to the PSD (Jeyifous et al., 2009).

It is evident that MAGUKs could play a role in the pathogenesis of neurodegenerative disease (Gardoni et al., 2009; Gardoni et al., 2010). Normal huntingtin is associated with NMDARs via PSD95 but mutant huntingtin impairs the interaction between PSD95 and huntingtin, leading to excitotoxicity through increased NMDA receptor activity (Sun et al., 2001), which is a key feature of neurodegenerative disease (Zeron et al., 2002; Shehadeh et al., 2006; Fan et al., 2007). In the striatum of YAC128 HD model mice, increased levels of PSD-95 as well as increased PSD-95-GluN2B interactions are observed in extrasynaptic regions (Fan et al., 2009; Fan et al., 2012). A reorganisation of postsynaptic density proteins, including a switch of PSD-93 by PSD-95 in the striatum of the R6/1 HD mouse model, has also been described (Torres-Peraza et al., 2008). In the
N171-82Q transgenic HD mouse model, a decrease in striatal PSD-95-like proteins was observed (Jarabek et al., 2004). In the striatum of 6-OHDA lesioned PD animal models, it has been shown that there is a reduced interaction between NMDARs and MAGUKs (Gardoni et al., 2006), as well as a change in the subcellular distribution and levels of PSD-95 and SAP97 (Nash et al., 2005).

To date, these animal models of HD and PD have provided valuable information on how synaptic structure and function may be altered in these human diseases; however the results with respect to changes in the glutamatergic synapse vary between studies and between models [for review see (Fan et al., 2007, Gardoni et al., 2010)]. Here we have used postmortem human brain tissue to investigate which synaptic proteins are altered in response to human neurodegenerative disease and thereby may play an important role in the changes in synapse function that occur in disease. The hippocampus and striatum were investigated, with a focus on the hippocampus as many HD and PD patients have dementia, depression, cognitive decline and other non-motor symptoms as well as the classic well characterised motor symptoms. We show that changes occurring in MAGUK and glutamate receptor subunit expression in the human hippocampus differ from changes in animal models of disease, revealing that unique changes occur in the human brain in response to neurodegenerative disease that vary across different brain regions.
2.3 EXPERIMENTAL PROCEDURES

2.3.1 HUMAN BRAIN TISSUE

Human tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank (Centre for Brain Research, University of Auckland). The consent and research protocols used in this study were approved by the University of Auckland Human Participants Ethics Committee. The post-mortem human brain tissue was processed and dissected as described elsewhere (Waldvogel et al., 2007). Briefly, brains were perfused via the basilar and carotid arteries with phosphate buffered saline (1% sodium nitrite) and then with 15% formalin in 0.1M phosphate buffer (pH 7.4). Brains were dissected into the different functional parts including blocks of the striatum and hippocampus. The blocks were then cryoprotected in 20% sucrose 0.1M phosphate buffer with 0.1% sodium–azide and frozen at -80°C. The hippocampal and striatal frozen tissue blocks were cut into 50 µm coronal sections on a microtome and stored in PBS-azide at 4°C until used for immunohistochemistry. For hippocampal studies, a total of 34 human brains were examined, 12 control cases, 11 HD cases and 11 PD cases (see Table 1). For the striatum, a total of 21 brains were examined, 7 control cases, 8 HD cases and 6 PD cases (see Table 2). Numbers are presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Age (years)</th>
<th>Sex</th>
<th>PM Delay (hrs)</th>
<th>Pathology</th>
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<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>67.1 ± 15.7</td>
<td>9 males 3 females</td>
<td>17.3 ± 4.7</td>
<td>Normal</td>
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<tr>
<td>HD</td>
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<td>65.9 ± 9.8</td>
<td>9 males 2 females</td>
<td>12.7 ± 4.3</td>
<td>Grade 1-4</td>
</tr>
<tr>
<td>PD</td>
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<td>79.6 ± 6.1</td>
<td>8 males 3 females</td>
<td>16.0 ± 9.3</td>
<td>Parkinson’s Disease</td>
</tr>
</tbody>
</table>

Table 1. Summarised details of cases used for the hippocampus immunohistochemistry.
<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Age (years)</th>
<th>Sex</th>
<th>PM Delay (hrs)</th>
<th>Pathology</th>
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<td>20.2 ± 3.2</td>
<td>Normal</td>
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<tr>
<td>HD</td>
<td>8</td>
<td>67.0 ± 12.0</td>
<td>6 males 2 females</td>
<td>14.5 ± 6.9</td>
<td>Grade 1-4</td>
</tr>
<tr>
<td>PD</td>
<td>6</td>
<td>80.0 ± 5.9</td>
<td>4 males 2 females</td>
<td>11.1 ± 5.4</td>
<td>Parkinson’s Disease</td>
</tr>
</tbody>
</table>

Table 2. Summarised details of cases used for the striatum immunohistochemistry.

2.3.2 IMMUNOHISTOCHEMISTRY IN HUMAN BRAIN SECTIONS

Free-floating tissue sections were first incubated in PBS-Triton (PBST, 0.2%) overnight at 4°C. Tissue was washed in citric acid buffer (pH 4.5) and sections then heated in the microwave for 30 seconds on high power for antigen retrieval if needed. The sections were cooled to room temperature and washed with PBS-T (3 x 10 min). The sections were then incubated in 50% methanol, 0.9% hydrogen peroxide solution for further antigen retrieval and for blocking endogenous peroxidise activity. Sections were incubated for 72 hours (4 °C) in primary antibodies against mouse GluA2 (Neuromab) 1:500, rabbit PSD95 (Sigma, HPA010122) 1:300, rabbit SAP97 (ABR, PA1-741) 1:1000 and mouse GluN1 (Millipore, MAB363) 1:300. Sections were then washed and incubated overnight at room temperature with the respective biotinylated secondary antibodies, goat anti-mouse (Sigma, B7264) 1:500 and goat anti-rabbit (Sigma, B7389) 1:1000. Sections were again washed and incubated in the tertiary antibody, extravadin peroxidase (Sigma, E2886) 1:1000 for 4 hours at room temperature. The chromogen was 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, D5637) and 0.01% H₂O₂ in 0.1 M phosphate buffer, pH 7.4 for 10-20 min. Sections were mounted, dehydrated and cleared in xylene before being coverslipped and imaged. Omission of the primary antibody resulted in no significant immunoreactivity (not shown).
2.3.3 TOTAL HOMOGENATE PREPARATION

Western blotting was performed to assess the specificity of the antibodies used in this study based on methods in our previous studies (Waldvogel et al., 2007). Both human and rat hippocampal lysates were used for western blotting experiments. Fresh frozen normal human hippocampus tissue was collected and homogenised (150mM sucrose, 15mM HEPES pH 7.9, 60mM KCl, 5mM EDTA, 1mM EGTA) with protease inhibitor cocktail (1 tablet/10ml, Roche). Homogenates were centrifuged to remove blood and then resuspended in lysis buffer (homogenisation buffer plus 2% Triton) and incubated on ice for 1 hour. The sample was then centrifuged (800 x g, 15min, 4°C) and the supernatant resuspended and centrifuged again (1000 x g, 15min, 4°C) and the supernatant stored at -80°C. For rat hippocampal lysates a similar procedure was followed. Four hippocampi were dissected from P7 Wistar rats and lysed in lysis buffer (50mM Tris-HCl, 1mM EDTA, 1% Triton) with protease inhibitor cocktail (1 tablet/10ml, Roche) on ice for 30min. The same centrifuge steps were used as for the human lysates. Protein concentrations were assessed using the Bio-Rad Dc protein assay.

2.3.4 WESTERN BLOTS

Proteins were denatured in laemmli loading buffer (Sigma, S3401) at 95°C for 5min. Proteins (30 µg per sample) were separated by gel electrophoresis (NuPAGE 4-12% Bis-Tris gel; Invitrogen, NP0335) and electrophobbed to a PVDF membrane (Amersham RPN303F). After blotting, the membranes were washed with water and TBS-T (0.05% Tween-20) and nonspecific binding of the primary antibodies was blocked with 5 % non fat dry milk in TBS-T for 1 hour. The membrane was then washed 3x10 min in TBS-T and incubated in the primary antibody overnight diluted in 1 % non fat dry milk, 4°C. The membrane was then washed in TBS-T and incubated in horseradish peroxidase (HRP)-labelled anti rabbit (Millipore, AP322P) 1:2000 or anti-mouse (Millipore, AP326P) secondary antibody 1:2000 diluted in 1 % non fat dry milk for 2 hours at room temperature. Unbound antibody was again washed off and bands were detected using chemiluminescence (ECL Plus; Amersham, RPN2132). The bands were visualised with the Fuji Film LAS-4000 scanner and software.

2.3.5 IMMUNOHISTOCHEMISTRY IN YAC128 BRAIN SECTIONS

Similar immunohistochemistry procedures were followed for the YAC128 hippocampus sections. Free-floating tissue sections were first incubated in PBS-Triton (0.2%) overnight at 4°C. The sections were then incubated in 50% methanol, 0.9% hydrogen peroxide solution for further antigen retrieval and for blocking endogenous peroxidise activity. Hereafter the sections were incubated in 5%
normal goat serum in PBST for 1 hour at room temperature. Sections were then incubated for 72 hours (4°C) in primary antibodies against mouse GluA2 (Alomone) 1:200, rabbit PSD95 (Sigma) 1:200, rabbit SAP97 (ABR) 1:1000 and mouse GluN1 (Neuromab) 1:300. Procedures for secondary and tertiary antibody incubation were exactly as for the human brain sections, except that a 1:500 dilution was used. DAB procedures were as for human brain sections.

2.3.6 IMAGE J DENSITOMETRY

Densitometry analysis was performed using Image J (NIH USA, public domain). Densitometry analysis was performed for all MAGUK and glutamate receptor subunit immunostaining. Images were first collected on a Nikon TE2000 inverted microscope in brightfield mode. The imaging conditions were optimised for each antibody but kept constant for all control and diseased cases immunostained with each antibody. Z-stack images (1280x960 pixels), 10 images at 2µm apart, were taken at 40x magnification, allowing the cell bodies, apical and basal dendrites to be clearly visible. For the hippocampus, images were collected in the dentate gyrus, CA3 and CA1 regions. In the striatum images were collected in the putamen and caudate nucleus. The z-stack images were converted to z-projections in Image J and the coloured images converted to grey scale images (8-bit). Background was measured on each z-projection image individually and automatically subtracted for each image. The image was then inverted so that density measurements were made in arbitrary units where a value of 255 is complete transparency and a value of 0 is complete darkness. A similar method for analysis of DAB immunohistochemistry in the human brain using Image J has been used successfully (Leuba et al., 2008). Densitometric values for each immunohistochemistry set were normalised to the average of the normal cases in that set to get the relative change in intensity (independent of experimental set). Intensity values for HD and PD are presented as a ratio of the grey value divided by the control (non-diseased tissue) density value ± SEM. Statistical analysis was performed with SPSS (IBM Corporation 2010). Data are presented as mean ± SEM and one way analysis of variance (ANOVA) was used to compare the densitometric measurements between control and diseased groups. Differences were considered statistically significant at p < 0.05.

2.3.7 CRUDE SYNAPTOSOME PREPARATION OF HUMAN HIPPOCAMPUS BRAIN TISSUE

To investigate the changes that occur at the synapse specifically a crude synaptosomal fraction (P2) and a cytosolic fraction (S2) was prepared from fresh frozen human control and HD hippocampus tissue (Dunkley et al., 2008, Sokolow et al., 2012). Human cases used for synaptosome preparation are summarised in Table 3, showing the mean post-mortem (PM) delay, age and sex of cases. The P2
crude synaptosomal fraction contains presynaptic and postsynaptic components of the synapses including synaptic vesicles, mitochondria, the cytoskeleton, the postsynaptic membrane and the PSD (Dunkley et al., 1986). Fresh frozen human hippocampus tissue was collected in homogenisation buffer: 0.32 M sucrose, 10 mM Tris, 2 mM EDTA, 2 mM EGTA, protease inhibitor cocktail (1 tablet/10 ml, Roche), pH 7.4. To homogenise the samples, 0.5 mm glass beads (GB05, Next Advance) were used and the samples homogenised for 3 min, at 4°C in the bullet blender homogeniser (Next Advance). The samples were then centrifuged at 1000 x g for 10 min and the supernatant (S1) was further centrifuged at 10,000 x g for 20 min. The supernatant (S2, cytosolic fraction) was stored at -80 °C and the pellet (P2, crude synaptosomal fraction) was resuspended in homogenisation buffer and stored at -80 °C. Protein concentrations were assessed using the Bio-Rad Dc protein assay. Western blots were performed as per Section 2.3.4. Antibodies were as follows: anti-synaptophysin from Dako (A 0010) 1:100, anti-LDHA (lactate dehydrogenase A) from SantaCruz (sc 27230) 1:100, anti-SAP97 from Enzo (ADI-VAM-PS005) 1:400, anti-PSD95 from Sigma (HPA010122) 1:500, anti-GluA2 from Neuromab (75-002) 1:300, and anti-GluN1 from Millipore (MAB363) 1:300. Ponceau S (Sigma) was used as a loading control (Romero-Calvo et al., 2010). The efficiency of the subcellular fractionation for all human tissue fractions was confirmed with the enrichments of the synaptosomal fraction with the synaptic marker synaptophysin and the cytosolic fraction with the cytosolic marker LDHA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Age (years) Mean ± SD</th>
<th>Sex</th>
<th>PM Delay (hrs) Mean ± SD</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8</td>
<td>66 ± 10.9</td>
<td>4 males 4 females</td>
<td>18.7 ± 6.8</td>
<td>Normal</td>
</tr>
<tr>
<td>HD</td>
<td>8</td>
<td>53 ± 11.5</td>
<td>5 males 3 females</td>
<td>15 ± 7.5</td>
<td>Grade 2-4</td>
</tr>
</tbody>
</table>

Table 3. Summarised details of cases used for the hippocampal synaptosome preparation.

**2.3.8 IMAGE J SYNAPTOSOME DENSITOMETRY**

The gel analyser was used in Image J (NIH USA, public domain) to analyse western blots of the cytosolic and crude synaptosomal fraction. The gel analyser takes into account the intensity of the band of interest as well as the size of the band. Protein loading was normalized to total protein levels in each lane using the gel analyser for Ponceau S staining in each lane (Romero-Calvo et al.,
Density values for each protein set were normalised to the average of the normal cases in that set to get the relative change in density (independent of experimental set). Statistical analysis was performed with SPSS (IBM Corporation 2010). Data are presented as mean ± SEM and one way analysis of variance (ANOVA) was used to compare the density measurements between control and HD groups. Differences were considered statistically significant at $p < 0.05$. 
2.4 RESULTS

2.4.1 HUMAN BRAIN IMMUNOHISTOCHEMISTRY REVEALS DIFFERENTIAL CHANGES IN MAGUK EXPRESSION IN THE DISEASED HUMAN BRAIN

To determine whether altered expression of synaptic proteins and glutamatergic receptor subunits occur in HD and PD in the human brain, quantitative immunohistochemistry was performed on post-mortem human hippocampal and striatal tissue. The expression of PSD95, SAP97 and glutamate receptor subunits GluN1 and GluA2 were examined in the dentate gyrus, CA3 and CA1 regions of the hippocampus, and in the caudate nucleus and putamen of the striatum. Immunohistochemistry and imaging criteria were kept constant for all control and diseased cases to enable detection of changes in expression levels of the different synaptic proteins.

We first investigated expression changes in PSD95 in the human hippocampus and striatum (Figure 4 A-E). Strong PSD95 immunostaining was evident in the cell bodies and in the dendrites in both control and diseased hippocampal and striatal neurons (Figure 4 C,D). In HD postmortem tissue, a significant 1.52 ± 0.26 fold increase in PSD95 expression was observed in area CA3 of the hippocampus (n = 6, control n = 8; p < 0.05) and in the dentate gyrus (HD DG: 1.45 ± 0.22, n = 6, control n = 8, p = 0.05). No significant change was measured in area CA1 (HD CA1: 1.45 ± 0.31, n = 6, control n = 8, p = 0.13). In PD post-mortem tissue, a significant 1.37 ± 0.19 fold increase in the dentate gyrus (PD n = 6, control n = 8; p < 0.05) and a significant 2.44 ± 0.73 fold increase in area CA1 (PD n = 6, control n = 8; p < 0.05; Figure 4 A) regions were also observed. However, PSD95 levels in the CA3 region in PD were not significantly different from control levels (0.99 ± 0.12; PD n = 4, control n = 8). In the striatum we observed stark differences in PSD95 expression levels. In the HD striatum the expression of PSD95 was significantly decreased in both the caudate nucleus (HD mean = 0.40 ± 0.12, n = 3, control n = 4; p < 0.05) and putamen (HD mean = 0.51 ± 0.09, n = 3, control n = 3; p < 0.05; Figure 4 B). However, there were no changes in PD caudate nucleus (1.02 ± 0.25; PD n = 4, control n = 4) or putamen (1.16 ± 0.22; PD n = 4, control n = 4). Specificity of the PSD95 antibody is shown by Western blot analysis in Figure 4 E.
Figure 4. PSD95 expression in the human HD and PD hippocampus and striatum.
A. PSD95 is significantly increased in DG and area CA3 in HD and in the DG and area CA1 in PD. *p<0.05. B. PSD95 is significantly decreased in the HD striatum in both the CN (caudate nucleus) and Put (putamen). ** p<0.005. C. Representative images of PSD95 immunostaining in the hippocampus (area CA1) and striatum (CN). Scale bar 25 µm. D. High power example image of PSD95 immunolabelling showing the somatic and dendritic localisation pattern. E. Example western blot showing a ~82kDa band for PSD95 in both the rat and human hippocampus.
We next examined whether similar changes also occur in SAP97 expression and observed that the changes in SAP97 were different to those observed for PSD95. Increases in SAP97 expression were found to occur in both the PD and HD human hippocampus but not in the striatum (Figure 5 A-C). SAP97 immunostaining was evident in the cell body layers and dendritic regions throughout the hippocampus and striatum (Figure 5 C,D). Consistent with its role in trafficking receptor complexes through the secretory pathway and along dendrites (Sans et al., 2001; Jeyifous et al., 2008), SAP97 appeared diffusely along dendrites (Figure 5 D). SAP97 expression in each hippocampal region was found to increase to a similar degree in both HD and PD, and occurred in both cell body and dendritic regions of the hippocampus. In the dentate gyrus SAP97 was increased 2.06 ± 0.25 fold in HD (n = 6; p < 0.005) and similarly increased 1.91 ± 0.22 in PD (n = 9; p < 0.05) above control levels (n = 6). In the CA3 region, SAP97 was increased 2.22 ± 0.51 fold in HD (n = 5; p < 0.05) and 1.62 ± 0.26 fold in PD (n = 10; p < 0.05) above control (n = 6). In the CA1 region SAP97 was significantly increased 2.15 ± 0.31 fold in HD (n = 8; p < 0.005) and 1.88 ± 0.35 in PD (n = 6; p < 0.05) as compared to control (n = 6). In contrast, in the striatum there were no significant changes in the expression of SAP97 in the caudate nucleus in HD (n = 6) or PD (n = 5), nor in the putamen in HD (n = 6) or PD (n = 5) as compared to control (caudate n = 5, putamen n = 4; Figure 5 B). Specificity of the SAP97 antibody is shown by Western blot analysis in Figure 5 E. Overall these results indicate that SAP97 expression is significantly altered throughout the hippocampus in human HD and PD, but not in the striatum. Moreover, these data show that the MAGUK proteins PSD95 and SAP97 are differentially affected by neurodegenerative diseases in the human brain and that these changes can differ in different brain regions.

We next aimed to determine whether the observed changes in PSD95 and SAP97 are reflected in changes in glutamate receptor levels in the human hippocampus and striatum. In the hippocampus, the majority of AMPA receptors are composed of GluA1/2 subunits or GluA2/3 subunits (Wenthold and Roche, 1998). We therefore examined whether AMPAR expression is altered in PD and HD in the human hippocampus and striatum by examining the expression of the GluA2 subunit common to both these receptor subtypes (Figure 6 A-D). GluA2 immunostaining was again evident in the hippocampal and striatal cell bodies and the dendritic regions in both control and diseased human tissue (Figure 6 C,D). No significant changes in GluA2 subunit expression were observed in the HD or PD hippocampus dentate gyrus, area CA3, or area CA1, or in the striatal caudate nucleus (HD n = 4, PD n = 5, control n = 5; p > 0.05 in all cases; Figure 6 A-C). However, significant decreases in GluA2 expression were observed in the putamen (HD mean = 0.52 ± 0.07, n = 4; PD mean = 0.67 ± 0.08, n = 5; Figure 6 A-C; p < 0.05).
Figure 5. SAP97 expression in human HD and PD hippocampus and striatum.

A. Significant increases in SAP97 expression in the post-mortem human hippocampus in HD and PD patients. The significant increase in SAP97 expression occurs in all hippocampal regions examined: DG, area CA3 and area CA1. * p<0.05, ** p<0.005. B. No significant changes in SAP97 expression were observed in HD or PD striatum in either the caudate nucleus (CN) or the putamen (Put). C. Representative images of SAP97 immunostaining in the hippocampus (CA3) and striatum (Put). Scale bar 25 µm. D. High power example image of SAP97 immunolabelling, showing the expected somatic and dendritic localisation. E. Example western blot showing a ~140 kDa band for SAP97 in both the rat and human hippocampus.
Figure 6. GluA2 expression levels in human postmortem HD and PD hippocampus and striatum.
A. No significant changes in GluA2 expression in HD or PD hippocampal regions. B. GluA2 specific changes occur in the putamen (Put) of HD and PD tissue. C. Representative images are shown for GluA2 in the hippocampus (CA1) and striatum (Put). Scale bar 25 µm. D. High power example image of GluA2 immunolabelling, showing the somatic and dendritic localisation. E. Western blot showing a ~98kDa band for GluA2 in both the rat and human hippocampus.
We also examined potential changes in the expression of GluN1, the obligatory subunit of the NMDA receptor (Figure 7 A-D). Strong GluN1 immunostaining was evident throughout the somatic and dendritic regions in both hippocampal and striatal neurons (Figure 7 C,D). We observed significant increases in GluN1 expression levels in the HD hippocampus (Figure 7 A-C). Specifically, a significant 2.49 ± 0.62 fold increase occurred in the dentate gyrus (HD n = 8, control n = 8, p < 0.05) and a significant 3.17 ± 0.89 fold increase in area CA1 (HD n = 8, control n = 7, p < 0.05). No significant change was observed in area CA3 (p > 0.1). No changes occurred in PD hippocampus in either the dentate gyrus, area CA3, or area CA1 (PD n = 6, control n = 7). In the striatum, no change in GluN1 expression was observed in either disease in the caudate nucleus or putamen (HD n = 4, PD n = 4, control n = 4; Figure 7 B; p > 0.05 in all cases). For both glutamate receptor subunits, specificity of antibody staining is supported by Western blot analysis showing bands at the correct molecular weight (Figure 6 E and Figure 7 E). Together these data show that different changes in NMDA and AMPA receptor subunit expression levels occur in the hippocampal and striatal human brain regions in response to HD and PD.
Figure 7 GluN1 expression levels in human postmortem HD and PD hippocampal and striatal tissue.
A. GluN1 is significantly increased in the DG (dentate gyrus) and CA1 region in HD but no changes in GluN1 expression occurs in PD hippocampus in any region. B. No significant changes in GluN1 expression levels were observed in HD and PD striatum in either the caudate nucleus (CN) or the putamen (Put). C. Representative images are shown for GluN1 in the hippocampus (area CA1) and striatum (CN). Scale bar 25 μm. D. High power example image of GluN1 immunolabelling, showing the somatic and dendritic localisation. Western blot analysis showing a ~110kDa band for NR1 in both the rat and human hippocampus.
2.4.2 SIMILAR CHANGES IN MAGUK EXPRESSION DO NOT OCCUR IN THE HIPPOCAMPUS OF HD ANIMAL MODEL YAC128

We were particularly intrigued by the significant changes in the expression levels of glutamatergic synaptic proteins in the human hippocampus. Although HD is considered a motor disorder, there is significant evidence that cognitive effects appear before the motor symptoms suggesting a role of hippocampal changes in the disease (Vonsattel et al., 1985; Lawrence et al., 1998; Montoya et al., 2006; Giralt et al., 2012). In animal models of HD, striatal changes in glutamatergic synapse structure and function have been widely addressed (e.g. Fan et al., 2007, 2009, 2012; Zeron et al., 2002; Graham et al., 2006), however the hippocampus in HD model mice has been shown to be spared of atrophy and degeneration (Van Raamsdonk et al., 2005; Carroll et al., 2011). We wanted to determine whether the changes in MAGUK and glutamate receptor expression we observed in the human hippocampus in HD were also occurring in the hippocampus of the YAC128 mouse model of HD. Immunohistochemistry was performed on hippocampal sections from YAC128 mice at 12 months of age, when the animals are at the end stage of HD and therefore most comparable to tissue from post-mortem human HD patients. As expected, SAP97, PSD95, GluA2 and GluN1 immunostaining was observed in the cell body layers of the hippocampus and also strongly in the dendritic regions of area CA1, CA3 and dentate gyrus (Figure 8 A-D). However, densitometry quantification revealed that none of the changes in SAP97, PSD95, GluA2 and GluN1 that we observed in human post-mortem HD hippocampal tissue occurred in YAC128 mice hippocampal tissue. We observed no significant changes in expression levels for any of these synaptic proteins compared to control tissue in either dentate gyrus, area CA1 or area CA3 of YAC128 hippocampus (Figure 8). To ensure that our immunohistochemical analysis could detect changes in protein expression levels in YAC128 tissue, we examined the expression changes of DARPP-32 to act as a positive control in control and YAC128 striatal tissue. DARPP-32 is a marker of dopaminergic neurons in the striatum and has been reported to decrease in YAC128 striatal tissue (Van Raamsdonk et al., 2005a,b,c; Van Raamsdonk et al., 2006). Indeed we observed that DARPP-32 levels were significantly decreased in YAC128 striatum to 0.42 ± 0.12 of wildtype control levels (p < 0.01; Figure 8). Therefore our data suggest that different subcellular hippocampal changes are occurring in human HD compared to animal models of the same disease.
Figure 8. Quantitative immunohistochemistry of SAP97, PSD95, GluN1 and GluA2 expression in YAC128 hippocampal sections.

Sections were prepared from symptomatic 1 year old YAC128 mice to provide a comparison of the end stage of human HD. A - D. Top: Quantification of (A) SAP97, (B) PSD95, (C) GluA2, (D) GluN1 expression levels in dentate gyrus (DG), area CA3 and area CA1. Below: example immunohistochemical staining for each glutamatergic synaptic protein in the hippocampal CA1 region in control (wildtype) and YAC128 mice. E. Immunohistochemical quantification of DARPP-32 expression in wildtype and YAC128 striatum (caudate putamen, CPu).
2.4.3 HUMAN HIPPOCAMPAL SYNAPTOSOMES SHOW NO SIGNIFICANT CHANGES IN MAGUKS OR GLUR EXPRESSION

To assess the subcellular location of the changes in GluRs and MAGUKs, we created subcellular fractions of human hippocampus tissue that consisted of a cytosolic S2 fraction and a crude synaptosomal P2 fraction. The efficiency of the subcellular fractionation was indicated by enrichment of the P2 fraction by synaptophysin (a synaptic marker) and an enrichment of the S2 fraction by lactate dehydrogenase (LDHA, a cytosolic marker) (Figure 9A). As expected, SAP97 was present in both the P2 and S2 fractions (Muller et al., 1995), whereas the other proteins PSD95, GluA2 and GluN1 were expressed only in the P2 crude synaptosomal fraction. It is known that PSD95 is present in the PSD as well as presynaptic locations (Cho et al., 1992, Kistner et al., 1993) and SAP97 is present in cytosolic compartments as well as glutamatergic terminals (Muller et al., 1995). Quantitative analysis revealed that there was a trend (although not significant) for SAP97 to be reduced in HD in the crude synaptosome fraction but increased in HD in the cytosolic fraction (P2: control mean = 1.00 ± 0.40, n = 3; HD mean = 0.65 ± 0.14, n = 3, p = 0.45; S2: control mean = 1.00 ± 0.15, n = 8; HD mean = 1.25 ± 0.20, n = 8, p = 0.33). For PSD95 there was no significant change in the crude synaptosome fraction (control mean = 1.00 ± 0.31, n = 4; HD mean = 1.40 ± 0.29, n = 4, p = 0.43). For glutamatergic receptor subunits there was also no significant change in the crude synaptosome fraction for GluA2 (control mean = 1.00 ± 0.31, n = 4; HD mean = 1.40 ± 0.42, n= 4, p = 0.43) and GluN1 (control mean = 1.00 ± 0.06, n = 4; HD mean = 1.37 ± 0.23, n =4, p = 0.17). We also assessed whether presynaptic protein expression was altered in HD in the hippocampus in the P2 fraction by measuring the expression of synaptophysin (a presynaptic marker) (Figure 9 D). There was no significant change in the expression of synaptophysin in HD tissue as compared to control tissue (P2: control mean = 1.00 ± 0.055, n = 8; HD mean = 1.15 ± 0.06; p = 0.086).
Figure 9. Quantitative intensity analysis of SAP97, PSD95, GluA2, GluN1 and synaptophysin expression in human synaptosome and cytosolic fractions of the hippocampus.

(A) Representative blots showing the differential distributions of synaptic proteins in synaptosomal (P2) and cytosolic (S2) fractions. As expected, synaptophysin was present only in the P2 fraction, LDHA was present only in the cytosolic fraction, SAP97 was present in both fractions, PSD95 was present only in the P2 fraction and both glutamate receptor subunits were present only in the P2 fraction. (B) Graphs showing quantitative analysis of the protein expression levels in control vs. HD human hippocampus fractions, all normalized to the control group. (C) Representative protein bands of the data in (B) is shown. (D) Presynaptic protein expression is not altered in HD in the hippocampus.
2.5 DISCUSSION

Here we report changes that occur in SAP97, PSD95, GluN1 and GluA2 in the human brain in response to the neurodegenerative diseases HD and PD. Overall, we observed that changes in these glutamatergic synaptic proteins occurred in the human hippocampus, and these changes differed from those occurring in the striatum. These hippocampal data suggest that changes in SAP97, PSD95 and GluN1 are related to the non-motor symptoms of HD and PD such as cognitive decline or dementia (Diamond et al., 1992, Lawrence et al., 1998, Ziemssen and Reichmann, 2007). Moreover our data provide an interesting comparison between cellular changes that occur in human tissue versus data in animal tissue, and suggest that unique changes occur in the human hippocampus with HD.

2.5.1 MAJOR CHANGES IN MAGUKS OCCUR IN HD AND PD HUMAN BRAIN

One of the major changes that we observed in synaptic protein expression in human HD and PD brains occurred in SAP97 levels. Our data suggest a hippocampal-specific role of SAP97 expression changes occurring in HD and PD, as changes in SAP97 expression levels were not observed in the striatum despite other significant subcellular pathology occurring in this region (Vonsattel et al., 1985, Braak et al., 2003). This also suggests that SAP97 expression changes are not involved in the subcellular striatal changes that underlie the motor symptoms of PD and HD, but that the specific SAP97 hippocampal changes may be a pathological hallmark of the cognitive changes seen in HD and PD patients (Diamond et al., 1992, Lawrence et al., 1998, Ziemssen and Reichmann, 2007, Paulsen et al., 2008).

Changes in SAP97 expression are known to alter AMPA and NMDA receptor-mediated postsynaptic currents (Elias et al., 2006, Waites et al., 2009, Li et al., 2011) and NMDA receptor dependent excitotoxicity (Cui et al., 2007). Therefore the significant increases in SAP97 reported here in the HD and PD hippocampus could have effects on the trafficking and/synaptic expression of NMDA and AMPA receptors. Our observed increase in GluN1 expression levels in the DG and CA1 regions in the HD hippocampus suggests increased NMDA receptor expression and is consistent with this effect being mediated by increased SAP97 levels. However no parallel increase in GluA2 expression was observed, suggesting that if the increase in GluN1 is SAP97-induced, that it is a dominant effect on NMDA receptors and not on AMPA receptors.

Similar to SAP97, changes in PSD95 expression levels also occurred in the hippocampus in HD and PD post-mortem tissue, and the lack of change in striatal PD tissue suggests it does not play a role in the
striatal pathology of PD. However, in contrast to SAP97, subcellular changes in PSD95 levels do appear to play a major role in the subcellular striatal pathology of HD as evidenced by a significant downregulation of PSD95 occurring in the HD striatum. This is in agreement with previous western blot analysis of human HD brain (Torres-Peraza et al., 2008). The consequence of this significant decrease is not known, but it may be an attempt to reduce NMDA receptor-mediated excitotoxicity as reduced PSD95 levels reduce NMDA receptor currents and toxicity (Elias et al., 2006, Cui et al., 2007). In addition, given the importance of PSD95 in the synaptic targeting of AMPARs (Schnell et al., 2002), the reduced expression of PSD95 in the striatum may also correlate with the observed striatal reduction in the GluA2 AMPAR subunit.

With regards to changes in glutamate receptor expression, our data show that changes in GluN1 and GluA2 expression levels are differentially affected by hippocampal versus striatal subcellular pathology that occurs in PD or HD human brain. For example, GluN1 was not altered in the human HD striatum, but was significantly increased in the human HD hippocampus, which may reflect a role in cognitive changes in HD. The precise sub-synaptic location of the expression of the obligatory GluN1 subunit will be important to determine in both the human striatum and hippocampus. In the YAC128 animal model of HD, extrasynaptic NMDA receptors in the striatum are upregulated and their blockade reverses the HD-induced signalling and motor learning deficits (Hardingham et al., 2002, Okamoto et al., 2009, Milnerwood et al., 2010). Whether the observed upregulation of GluN1 in the human hippocampus represents an increase in extrasynaptic receptors is not yet known. Our observed lack of change in GluN1 in the YAC128 hippocampus suggests the change in NMDA receptor distribution in HD may be restricted to the striatum, or alternatively that total NMDA receptor levels remain the same but are simply redistributed to extrasynaptic regions. Interestingly, NMDA receptor localisation is differentially regulated by α- and β- isoforms of SAP97, where αSAP97 causes synaptic localisation of NMDA receptors and βSAP97 sequesters NMDA receptors to extrasynaptic sites (Li et al., 2011). It will be of significant interest to determine whether the upregulation of SAP97 observed in human HD and PD brain is specific to α− or βSAP97 isoforms that regulate synaptic versus extrasynaptic NMDA receptors.

With regards to AMPA receptors, most animal studies have focussed on the GluA1 subunit, which forms homomeric GluA1-containing AMPA receptors that constitute a minority of AMPA receptors (Wenthold et al., 1996). Here we measured GluA2 expression as this subunit forms part of both the GluA1/2 and the GluA2/3 heteromeric AMPA receptors, and therefore could reflect changes in either subtype of receptor. In contrast to GluN1, the decrease in GluA2-containing AMPA receptors was restricted to the putamen in both HD and PD postmortem brain tissue suggesting that changes
in AMPAR subunits do not contribute to hippocampal changes in HD and PD. However, a decrease in GluA2-containing receptors in the striatum in PD and HD could alter AMPA receptor-mediated synaptic transmission in the basal ganglia and consequently play a more dominant role in the motor symptoms of PD and HD.

Currently, it is difficult to know whether the observed increases in hippocampal MAGUK and GluR expression levels helps or hinders neuronal survival, synaptic transmission, synaptic plasticity or cognition. That is, whether the changes represent the active decline of neuronal structure and function, or represent proactive changes in an attempt to restore lost synapse function. In this study we utilised quantitative immunohistochemical techniques to enable us to examine changes occurring in the principal neurons. Unfortunately, the use of human tissue precludes quantification of synaptic proteins at synaptic sites via double immunolabelling due to the lipofuscin-induced autofluorescence that interferes with synaptic staining (Double et al., 2008). Reduced synaptic plasticity is routinely seen in Huntington’s disease mouse models (Hodgson et al., 1999, Usdin et al., 1999, Murphy et al., 2000), which are proposed to underlie the observed cognitive changes. This has been attributed to NMDA receptor inactivation (Hodgson et al., 1999), impaired high frequency synaptic transmission (Usdin et al., 1999) and activity-dependent depotentiation (Murphy et al., 2000). Whether similar changes in synaptic plasticity occur in the human HD brain has so far not been able to be determined, but remains of significant interest.

To investigate the subcellular location of the changes in receptor subunits and synaptic proteins in HD, we used subcellular fractionation and western blotting. We could not detect any significant changes in any of the proteins in the crude synaptosomal and cytosolic fractions. However, an interesting trend was observed for SAP97 where it was reduced in the HD synaptosome fraction but increased in the HD cytosolic fraction as compared to control cases. More human brain cases are required to detect a significant change as human tissue presents with significant variability between cases. Nevertheless, redistribution from the synapse to the cytosolic compartment has been observed for SAP97 and PSD95 in the rat striatum in neurodegenerative disease (Nash et al., 2005). Here in our data this redistribution of SAP97 from the synapse to the cytosol may occur in an attempt to counteract excitotoxicity which is known to occur in HD.
2.5.3 COMPARATIVE CHANGES IN SYNAPTIC PROTEIN EXPRESSION IN HUMAN TISSUE VERSUS ANIMAL MODELS

The major changes in glutamatergic synaptic protein expression that we observed in the HD human hippocampus prompted us to examine whether similar changes occur in animal models of HD where previous work has largely focussed on striatal synaptic changes (Zeron et al., 2002; Shehadeh et al., 2006; Fan et al., 2007; Fan et al., 2009; Milnerwood et al., 2010; Fan et al., 2012). By directly comparing the cellular changes that occur in the hippocampus of human HD patients versus animal models of HD, we have found that the changes in expression levels of SAP97, PSD95 and GluN1 in human HD-affected hippocampi do not occur in the YAC128 mouse model of HD. This likely reflects the previous observation that the hippocampus in HD model mice are spared of atrophy and degeneration (Van Raamsdonk et al., 2005; Carroll et al., 2011). Our comparison suggests that MAGUKs are differentially altered in the diseased human versus mouse brain, and that unique changes may occur in the human hippocampus with HD, making further study of human tissue of significant importance.

Differences are not restricted to human versus animal data however, as animal models of the same disease show conflicting data. For example, PSD95 has been shown to decrease in PD animal models (Nash et al., 2005, Gardoni et al., 2006) but not change in another study (Dunah et al., 2000). In addition, while altered AMPAR-mediated synaptic transmission has been hypothesised to contribute to the motor symptoms of PD, animal models have failed to show a consistent trend in pathological changes. For example, reports of increased, decreased, or no change in GluR immunoreactivity levels have been reported in varying animal models of PD (Bernard et al., 1996; Lai et al., 2003; Picconi et al., 2004). Depending on the animal model, the methodology used, and the different symptomatic stage, different changes in total GluN1 levels have been reported in HD model mice (Cepeda et al., 2001, Jarabek et al., 2004, Ariano et al., 2005, Fan et al., 2007, Torres-Peraza et al., 2008). For obvious reasons, in human tissue we can only examine changes at the end stage of these diseases. These changes are likely different from changes occurring in pre-symptomatic and symptomatic animals and therefore this is where a major strength of animal models lies. Another major difference in the human versus animal studies is the inherent variability in the measurements of changes in protein expression evident in data from human brain tissue. This is not occurring in animal studies and is likely due to the use of genetically similar laboratory animals, enabling changes to be more easily deciphered. However, despite the inter-patient variation, our observed changes in SAP97, PSD95, GluA2 and GluN1 were routinely observed across all patients within a disease group, reflecting consistent changes across the spectrum of patients.
2.6 CONCLUSIONS

The overall changes in SAP97, PSD95, GluA2 and GluN1 levels in HD and PD postmortem human brains represent unique changes occurring differentially in discrete brain regions. We hypothesise that the hippocampal changes in SAP97, PSD95 and GluN1 may be a pathological hallmark of the cognitive changes seen in patients (Diamond et al., 1992, Lawrence et al., 1998, Paulsen et al., 2008). A lack of human data has made it difficult to predict therapeutic outcomes in the human and to extrapolate animal model data to the human. However, here we have shown that unique changes in synaptic protein expression occur in the human hippocampus and striatum, which must be considered in future studies.
CHAPTER THREE: ELECTROPHYSIOLOGY OF ACUTE YAC128 BRAIN SLICES

3.2 INTRODUCTION

3.2.1 THE YAC128 MODEL

The YAC mouse model expresses the full length huntingtin protein (Hodgson et al., 1999), unlike some models that express a truncated, N-terminal fragment of the protein (Mangiarini et al., 1996) (see Chapter 1). Initially the YAC model was created with 46 and 72 CAG repeats and they have helped greatly in discovering e.g. reduced BDNF levels in HD (Zuccato et al., 2001) and mitochondrial dysfunction that exists in HD (Panov et al., 2002). However, one important drawback was that these mice exhibited neuronal degeneration only late in life (12 months of age) (Hodgson et al., 1999). Because it is known that CAG repeat length is related to the age of onset of HD (Brinkman et al., 1997), the YAC with 128 CAG repeats was created. In the YAC128 a hyperkinetic phenotype develops by 3 months of age with progressive motor deficits appearing at 6 months of age and striatal cell loss at 9 months of age (Slow et al., 2003). In the YAC128 cognitive and memory deficits occur before motor deficit onset and are evident by 2 months of age, continuing to decline with age (Van Raamsdonk et al., 2005b).

The YAC128 mouse model of HD is one of the best models as it most accurately replicates the pathological hallmarks of HD (reviewed in Chapter 1). However, very few electrophysiological studies have been done in this model and most studies have focused on the striatum as it is the origin of the classical motor symptoms of HD. A recent study in the YAC128 striatum has revealed significant increases in extrasynaptic NMDA receptor signalling at 1 month of age (Milnerwood et al., 2010) but it is not known if such changes occur in the hippocampus also. NMDA receptor signalling in HD is also of particular interest due to its involvement in excitotoxicity (see Chapter 1). Studying the changes that occur at the synapse will be of particular benefit for the development of drug therapies as synaptic dysfunction precedes cell death in the human brain (Schippling et al., 2009) and HD symptom onset occurs before overt neuronal loss (Tobin and Signer, 2000, Levine et al., 2004, Cepeda et al., 2007). Therefore studying early pathophysiological changes in the HD brain is of the essence.
A profound study in 2002 showed that not only is the level of NMDA receptor activity critical for neuron survival but actually the location of the stimulated NMDA receptors are also important (Hardingham et al., 2002). NMDA receptors can be found both at the synapse (at the PSD) and at some region distal from the PSD (extrasynaptic). Synaptic NMDA receptors are defined as those being activated during low frequency synaptic events (evoked or spontaneous), whereas extrasynaptic NMDA receptors are those that are not activated during such events (Harris and Pettit, 2007). Extrasynaptic NMDA receptors can be found at various locations including the dendritic shaft, the cell body and at perisynaptic regions (adjacent to the PSD) (Petralia et al., 2010). Tovar and colleagues have shown that the NMDA receptors have different subunit composition depending on where they are located and confirmed that there is an age dependent change in the subunit composition (Tovar and Westbrook, 1999). Using the noncompetitive antagonist, ifenprodil, which is 400 times more potent at GluN1/GluN2B containing receptors than GluN1/GluN2A receptors (Williams, 1993), the location dependent subunit composition was investigated by Tovar and colleagues. Ifenprodil reduced the whole cell current in days in vitro (DIV) 1 - 7 micro-island neuron cultures to the same extent as whole cell currents in HEK cells transfected with GluN1/GluN2B receptors, showing that at this young age most receptors are GluN1/GluN2B containing (Tovar and Westbrook, 1999). Evoked EPSCs (synaptic receptor stimulation) were less sensitive to ifenprodil block than whole cell currents (synaptic and extrasynaptic receptor stimulation) in the micro-island neurons, indicating that the synaptic receptors are comprised of subunits that are relatively insensitive to ifenprodil. Producing EPSCs and whole cell currents in the same cell (DIV 1 - 7) it was also shown that the peak whole cell currents were 55 times greater than the EPSCs, indicating that there is a lot more extrasynaptic receptors than synaptic ones at this young age. This ratio of extrasynaptic to synaptic receptors was shown to be 3:1. Comparing NMDA receptor-mediated EPSCs at older than DIV 13 with that of DIV 1-7, revealed that the more mature cultures were less sensitive to ifenprodil. Altogether these results indicated that at a young developmental age most NMDA receptors are composed of GluN1/GluN2B, GluN2B containing receptors are located predominantly at the extrasynaptic sites and there is a developmental dependent increase in synaptic NMDA receptors.

However, there is still not a clear distinction between the subunit compositions of synaptic vs. extrasynaptic NMDA receptors. Are there any GluN2B receptors at the synapse or any GluN2A receptors at extrasynaptic sites at all? In the hippocampus it has been shown that both GluN2A and GluN2B receptors are found at the extrasynaptic sites and that these sites are not particularly
enriched in GluN2B containing receptors (Figure 10)(Harris and Pettit, 2007, Petralia et al., 2010). Another important question is how much do extrasynaptic NMDA receptors get activated during normal stimulation in different experimental hippocampal preparations and in WT vs. diseased animals? There also fails to be more specific blockers of GluN2A vs. GluN2B receptors. Nevertheless, the role of extrasynaptic receptors and their possible implication in neurodegenerative disease is a topic of great interest.

Figure 10. Electron micrographs of NMDA receptor locations in the hippocampus.
(A) GluN2A labelling in the adult hippocampus CA1 area at both synaptic and extrasynaptic sites. (B) GluN2B labelling in a hippocampal culture at both synaptic and extrasynaptic sites. Synaptic (PSD) labelling is indicated by the asterisk, arrowheads indicate extrasynaptic and perisynaptic labelling. P, presynaptic terminal; sp, spine. Scale bar is 100nm. From (Petralia et al., 2010)

The first evidence that synaptic NMDA receptors are neuroprotective as opposed to extrasynaptic NMDA receptors came from a 2002 study done in cultured hippocampal neurons (Hardingham et al., 2002). Elegant protocols were used to specifically stimulate synaptic or extrasynaptic receptors. To stimulate synaptic receptors, bicuculline (GABA receptor blocker) was added, which resulted in action potential firing accompanied by global calcium transients (calcium spikes) at a low frequency. To stimulate extrasynaptic receptors only, MK-801 was added after the bicuculline to block all the activated synaptic receptors. Hereafter, bath application of glutamate stimulated calcium influx through extrasynaptic NMDA receptors. Subsequent immunocytochemical analysis showed that synaptic NMDA receptor activity leads to cAMP response element-binding protein (CREB) phosphorylation and BDNF expression, whereas extrasynaptic NMDA receptor activity stopped CREB phosphorylation and reduced BDNF expression (Hardingham et al., 2002). Even more interesting is that ifenprodil (GluN2B antagonist) prevented this CREB block, as hippocampal extrasynaptic NMDA receptors are thought to be predominantly composed of GluN2B subunits (Tovar and Westbrook,
This experiment showed that synaptic NMDA receptor activity resulted in healthy cells with normal mitochondrial membrane potentials, whereas extrasynaptic NMDA receptor activity caused rapid breakdown of the mitochondrial membrane potential and caused cell death (see Figure 11 for a summary) (Hardingham et al., 2002).

A 2010 study was the first to show that the elevated NMDA receptor currents in the YAC128 striatum are due to glutamate spillover and activation of non-synaptic NMDA receptors (Milnerwood et al., 2010). They showed that during paired pulse stimulation, only at high intensities does the peak NMDA receptor current amplitude increase significantly in YAC128 during the first and second pulse. However, NMDA receptor current during spontaneous EPSCs did not change in the YAC128, suggesting that this significant elevation of NMDA currents in YAC128 only occurs when there is glutamate spillover or activation of non-synaptic NMDA receptors (outside the reach of individual spontaneous single release events). Further elegant experiments were done using the glutamate transporter 1 (GLT-1) inhibitor DL-threo-β-Benzyloxyaspartic acid (TBOA), which allowed spillover or activation of extrasynaptic sites to occur as glutamate uptake from the synaptic cleft is prevented using this blocker. In the presence of TBOA, YAC128 mice consistently displayed a slowing of the NMDA receptor current (slower than WT), which indicates that there are either an increased number of extrasynaptic receptors or an increase in extrasynaptic receptor activity. Eliminating synaptic NMDA receptor currents with the use-dependent NMDA receptor blocker MK-801 and then applying TBOA still produced elevated NMDA receptor currents in the YAC128 mice both at a presymptomatic age (1 month of age) and at 1 year of age (Milnerwood et al., 2010). To more specifically investigate which types of extrasynaptic NMDA receptors are underlying this abnormal current, ifenprodil was used to block GluN2B containing receptors (Williams, 1993). When ifenprodil was added to the bath, subsequent TBOA application resulted in no difference in the NMDA receptor current kinetics between WT and YAC128, indicating that the previous increase in extrasynaptic NMDA receptor current is produced by GluN2B containing receptors located at extrasynaptic sites (Milnerwood et al., 2010).
3.2.3 THE NMDA RECEPTOR ANTAGONIST MEMANTINE

Memantine (1-amino-3,5-dimethyladamantane hydrochloride) blocks NMDA receptors and is currently on the market for the treatment of Alzheimer’s disease. Memantine is an analog of amantadine (1-adamantanamine hydrochloride), which has been used as an antiviral agent (Zlydnikov et al., 1981). Memantine is the first NMDA receptor antagonist that is clinically well tolerated. It is a weak voltage-dependent uncompetitive NMDA receptor antagonist, therefore low doses of memantine are thought to block extrasynaptic NMDA receptors only (Parsons et al., 2007, Okamoto et al., 2009). The mechanism of action of memantine was first shown in 1992 in rat cortical and retinal ganglion cell neurons to be an uncompetitive open channel antagonist (Chen et al., 1992). Using electrophysiology experiments this group showed that the degree of block by memantine was voltage dependent and that the steady state inhibition was agonist (NMDA) dependent. This means that memantine is an open-channel blocker as it can only bind when the NMDA receptor is already open. Furthermore, the steady state inhibition also occurred quickly (within 1 sec), was specific for the NMDA receptor subtype and the dissociation constant was slow (seconds). These properties are ideal in a clinical setting where the drug is specific for the target, the onset of therapeutic effect will be fast and the effect will last long after a dose is given. The more
important factor was that it was also shown that low concentrations of memantine prevented the excessive influx of Ca\(^{2+}\) produced by NMDA application but that there was still normal basal levels of NMDA receptor activity and subsequent Ca\(^{2+}\) entry (Chen et al., 1992). This is important because blocking all NMDA receptors to completely stop Ca\(^{2+}\) entry is also toxic to cells (Ikonomidou et al., 1999).

Chen and colleagues also showed that memantine prevented cell death in vitro, resulting from glutamate application and in vivo in forebrain ischemia (Chen et al., 1992). Furthermore, cultured cortical neurons transfected with mutant huntingtin are more vulnerable to glutamate excitotoxicity than non-transfected neurons (Okamoto et al., 2009). These neurons were protected against cell death with the treatment of low concentrations (5 – 10 µM) of memantine (shown to preferentially block extrasynaptic NMDA receptors) (Chen et al., 1992, Chen et al., 1998) or ifenprodil (shown to preferentially block GluN2B containing extrasynaptic NMDA receptors) (Williams, 1993, Hardingham et al., 2002). Interestingly low doses of memantine restore the motor deficits present in the YAC128 mice, supposedly by blocking only extrasynaptic NMDA receptors at such a low concentration (Milnerwood et al., 2010).

### 3.2.4 GLUTAMATE TRANSPORTERS IN HD

Glutamate transporters are vital for the fast removal of glutamate from the synaptic cleft and to prevent excitotoxicity due to excessive glutamate activation of NMDA receptors (see Chapter 1). GLT-1 has been shown by different methods to be reduced in the human HD striatum (Arzberger et al., 1997, Faideau et al., 2010) and glutamate uptake is reduced by 43 % in the human HD prefrontal cortex (Hassel et al., 2008). Furthermore, in the R6/2 mouse model, studies show an age-dependent (from 4 to 12 weeks of age) downregulation of GLT-1 in the striatum without any changes in other glutamate transporters (Liévens et al., 2001, Behrens et al., 2002). These studies suggest that excitotoxicity underlies the MSN death observed in the striatum in HD as it occurs before any neurodegeneration is evident. Upregulation of GLT-1 in symptomatic R6/2 mice with ceftriaxone (a β-lactam antibiotic) reduces HD-like motor symptoms, increases GLT-1 expression in the striatum and reverses the glutamate uptake deficit in the striatum measured with the use of microdialysis (Miller et al., 2008). Interestingly, this 2008 study showed that there is no difference in GLT-1 expression levels between WT and R6/2 at 7 weeks of age but that the GLT-1 transporters are deficient in their function rather than displaying reduced protein levels. More evidence for this came from a study where GLT-1 expression levels in the YAC128 mouse model in the cortex, hippocampus and striatum were comparable to those in WT animals at 1, 3 and 12 months of age (Huang et al., 2010). Although protein levels were unaltered, palmitoylation of GLT-1 (required for its normal function) were
significantly reduced in the YAC128 mice and was reflected in the reduction in glutamate uptake in the YAC128 striatum and cortex at 12 months of age as assessed by a radioactive glutamate uptake assay in crude synaptosomes. Interestingly, glutamate uptake was also significantly reduced at 3 months of age in the striatum only, before motor symptom onset. This study did not measure glutamate uptake in the hippocampus of YAC128 mice.

3.2.5 MISSING PIECES IN THE HD PUZZLE

The YAC128 mouse model of HD most accurately represents HD-like symptoms and pathology (see Chapter 1). While various studies have been done in the YAC128 striatum, few studies have looked at the hippocampus in the YAC128 mouse even though cognitive decline occurs before motor symptom onset and neurodegeneration. Neurodegenerative diseases such as HD are “diseases of the synapse” (Raymond et al., 2011) and it is of interest to identify early changes that occur at the synaptic level in order to develop more specific and new treatment strategies that target the synapse. While NMDA receptor excitotoxicity is thought to underlie the selective MSN death in HD (Okamoto et al., 2009, Milnerwood et al., 2010), early changes in the hippocampus that underlie the cognitive decline in HD has not been identified. Although few, studies in the YAC128 hippocampus have shown that GLT-1 protein levels are not altered at 1, 3 and 12 months of age (Huang et al., 2010), metabotropic glutamate receptor (mGluR) binding is not altered at 12 months of age in the CA1 and CA3 fields while it is increased in the DG (Benn et al., 2007) and GluA2/3, GluN2A and GluN2B are increased in synaptic membranes at 15 months of age while there are no changes in GluN1 (Benn et al., 2007). No electrophysiological studies to probe synaptic function have been done in the YAC128 hippocampus although the YAC72 has been studied (Hodgson et al., 1999). The very imperative topic of synaptic vs. extrasynaptic NMDA receptor function has also been studied only in the striatum of the YAC128 mouse model (Milnerwood et al., 2010, Milnerwood et al., 2012). It is therefore of great interest to study synaptic function and plasticity in the YAC128 hippocampus at an early age as cognitive decline precedes motor deficits and neurodegeneration (Van Raamsdonk et al., 2005b) and may lead to novel treatment strategies early in the disease.
3.3 AIMS

We aim to study for the first time synaptic vs. extrasynaptic NDMA receptor-mediated currents in YAC128 hippocampal slices. Synaptic NMDA receptors have been reported to be neuroprotective, whereas extrasynaptic NMDA receptor activation is thought to lead to cell death pathways (Hardingham et al., 2002). Of particular interest also is to study synaptic plasticity in the YAC128 hippocampus at the early age of 1 month, which will reveal for the first time if there are indeed changes in LTP that accompany the cognitive and memory decline present in this model at this age (Kim, J., Fourie, C. et al, manuscript in preparation). Given these gaps in our understanding of the physiological changes occurring in the presymptomatic YAC128 hippocampus, we aimed to determine the following:

1. To measure synaptic AMPA and NMDA receptor-mediated currents in WT and YAC128 acute hippocampal slices using field stimulation of the Schaffer collaterals and whole cell recording in CA1 pyramidal cells

2. To measure the total NMDA receptor-mediated current induced by application of TBOA in WT and YAC128 acute slices using whole cell recording in CA1 pyramidal cells

3. To measure LTP in WT and YAC128 slices using whole cell recording in CA1 pyramidal cells

The results from these experiments will determine if synaptic transmission is altered in YAC128 hippocampal slices, which will give us insight to possible deficits in basal synaptic transmission in HD in the hippocampus. It will also, for the first time, reveal if extrasynaptic NMDA receptor-mediated signalling is altered in the hippocampus of YAC128 mice as it is in the striatum (Milnerwood et al., 2010). This will help us understand if the pathophysiological changes that occur in the YAC128 mice are the same or different in the hippocampus and striatum. Lastly, the LTP experiments will help us to correlate altered synaptic function with the behavioural phenotype (i.e. memory deficits) that is present in the YAC128 mouse at 1 month of age. Together these experiments will help elucidate the cellular mechanisms that underlie cognitive and memory decline in this HD animal model.
3.4 EXPERIMENTAL PROCEDURES

3.4.1 ANIMALS AND SLICE PREPARATION

The YAC128 mouse model of Huntington’s disease was used in this study and the experimenter was blind to the genotype of all animals at the time of recording. This yeast artificial chromosome (YAC) model, established in 2003, contains the entire human HD gene with 128 CAG repeats (Slow et al., 2003). Transgenic and wildtype (WT) YAC128 male and female mice of 4 – 5 weeks old were used in this study. All procedures were approved by the University of Auckland Animal Ethics Committee (AEC). Mice were killed by cervical dislocation and decapitation. The brain was quickly dissected out and placed in the ice cold cutting solution (in mM: 2.55 KCl, 7 MgSO₄·7H₂O, 0.5 CaCl₂, 1.01 NaH₂PO₄, 26.1 NaHCO₃, 10.98 glucose, 228 sucrose) for approximately 60 sec. Hereafter the brain was quickly trimmed and secured on the vibratome chuck using super glue. 350 µm thick hippocampal sections were prepared on a vibratome (Leica VT 1200 S). All slicing was performed in the same ice cold cutting solution bubbled with carbogen (95 % O₂, 5 % CO₂). Slices were hemi-sectioned and immediately transferred to an incubating chamber in standard ACSF (in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO₄·7H₂O, 2.5 CaCl₂, 1 NaH₂PO₄, 26 NaHCO₃, 11 glucose) at 32 °C for 30 min while being bubbled with carbogen. Hereafter the slices were maintained for approximately 1 hour at room temperature before electrophysiology recording began.

3.4.2 RECORDING SETUP

For electrophysiology recording slices were transferred to a recording chamber (Warner Instruments, RC-26GLP) mounted on a Zeiss AxioScope upright microscope. All recordings were done at room temperature in standard ACSF (bubbled with carbogen) containing 100 µM picrotoxin (Sigma) to block polysynaptic inhibitory (GABA) currents, which may interfere with AMPA receptor-mediated EPSCs. Slices were stabilised with a slice holder. A glass stimulating electrode was placed in the stratum radiatum and stimulation delivered with a Digitimer constant current isolated stimulator (model DS3). Hippocampal slices were visualized using DIC with a 40x dipping objective lens. Membrane currents and potentials were processed with a Multiclamp 700B commander (Axon Instruments, CA, USA) and digitized at 10 KHz (Digidata 1440, Axon Instruments, CA, USA) to convert analogue to digital signals. Events were sampled at 10 KHz and low-pass filtered at 1 KHz. Series resistance (Rs) was measured and recordings with Rs variation greater than 20 % were discarded from the data analysis. Data acquisition and analysis was performed using pClamp 10 acquisition software and Clampfit 10 respectively (Axon Instruments, CA, USA). Whole cell recordings were done in the CA1 pyramidal cells of the hippocampus with patch pipettes of 3-7 MΩ filled with internal
solution (in mM) 120 Cs gluconate, 40 HEPES, 5 MgCl₂, 2 NaATP, 0.3 NaGTP and 5 QX314, pH 7.2, 298 mOsm.

3.4.3 AMPA AND NMDA RECEPTOR- MEDIATED CURRENT MEASUREMENTS

To measure AMPA receptor-mediated EPSCs, the Schaffer collaterals were stimulated at 0.1 Hz while recording from a CA1 pyramidal cell voltage clamped at -65 mV. The stimulator strength was adjusted to minimize failures and to get a consistent postsynaptic AMPA receptor-mediated EPSC. Hereafter, in the same cell, 10 µM 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma) was bath applied to block AMPA receptor-mediated currents. This solution also contained glycine (10 µM, Sharlau), strychnine (2 µM, Sigma) and picrotoxin (100 µM, Sigma). Glycine was used to augment NMDA currents and strychnine to block the associated glycine receptor currents. Neurons were depolarised to +40 mV to record synaptic NMDA receptor-mediated EPSCs. Hereafter, in the same cell, a solution identical to the one above plus 10 µM TBOA (Tocris Bioscience) was bath applied to block glutamate uptake through GLT1/EAAT2. TBOA blocks both glial and neuronal glutamate uptake and has been shown to increase the NMDA receptor-mediated EPSC peak amplitude slightly and significantly slow EPSC kinetics (Diamond, 2001, Arnth-Jensen et al., 2002). TBOA does not interact with NMDA receptors (Jabaudon et al., 1999). The cell was then again depolarised to +40 mV to record total (synaptic and extrasynaptic) NMDA receptor-mediated EPSCs.

3.4.4 LTP

Baseline AMPA receptor-mediated EPSCs were recorded by stimulating the Schaffer collaterals at 0.1 Hz as described previously for an average of 12 sweeps. Regardless of stimulation protocol, LTP was always induced within 10 min of obtaining whole cell mode to prevent LTP wash out (Malinow and Tsien, 1990, Kato et al., 1993). LTP was induced by stimulating the Schaffer collaterals at 1 Hz for 1 min while holding the postsynaptic cell at +10 mV. This pairing protocol is well established and produced NMDA-dependent LTP in the hippocampus at these synapses (Wigström and Gustafsson, 1986, Malinow and Tsien, 1990). The post-LTP AMPA receptor-mediated current amplitude was then measured for at least 30min. Different control experiments were done where either no high frequency stimulation was delivered (i.e. 0.1 Hz stimulation was used for an extended period of time to see if the peak AMPA receptor-mediated current was stable over time) or there was no pairing (i.e. 1 Hz, 1 min stimulation but no postsynaptic depolarization to +10 mV). To depotentiate the synaptic response after LTP induction, neurons were stimulated at 1 Hz for 6 min while holding at -65 mV. Thereafter the baseline recording conditions were resumed to measure the depotentiated response. Because the YAC128 slices often failed to produce LTP, tetanic stimulation was used in an
attempt to induce LTP in a subset of slices; the neuron was stimulated at 100 Hz for 1 sec, held at 0 mV. After LTP induction, neurons were held at -65 mV again and the response recorded for 30 min to determine if LTP could be induced with this more intense stimulation. Paired pulse facilitation (PPF) was induced by stimulating the Schaffer collaterals with two pulses separated by 40 ms and recording the AMPA receptor-mediated current from a neuron voltage clamped at -65 mV. At least 20 sweeps were obtained per neuron.

3.4.5 ANALYSIS

Analysis was performed with Clampfit 10 software. Recordings were filtered offline and the baseline normalized to 0 pA. For all current measurements, an average current was obtained from at least 20 sweeps recorded from a particular neuron. Data are presented as mean ± SEM, n = number of animals. For AMPA receptor-mediated currents, the peak amplitude of the monosynaptic current was measured. For NMDA receptor-mediated currents, both the peak current amplitude and the charge (calculated as the area under the curve) were measured. The peak current amplitude was measured as a 10 ms window at the peak of the transient. The charge was measured from the upstroke of the NMDA receptor-mediated current until 300 ms from the start of the recording. The decay time constant ($\tau_{\text{decay}}$) was measured for the NMDA receptor-mediated current by fitting a standard exponential function from the peak of the current up to 300 ms from the start of the recording. For LTP measurements, the peak monosynaptic AMPA receptor-mediated current amplitude was measured before LTP induction and averaged across at least 12 sweeps. This average was compared to the average peak monosynaptic AMPA receptor-mediated current amplitude after LTP induction, measured during the 28 – 30 min time window after the start of the recording. The data in Figure 16 are normalized within each group to the baseline (before LTP induction). If there was no significant change in the average peak AMPA receptor-mediated current amplitude, the recording was labelled “LTP failure” in Figure 17 and if there was a significant reduction it was labelled “LTD” in Figure 17. For PPF, the peak monosynaptic AMPA receptor-mediated current amplitude was measured and a ratio was obtained by dividing the peak amplitude of the second current response by the peak amplitude of the first current response and an average ratio obtained. Data are presented as mean ± SEM, n = number of animals.

Statistical analysis was performed with Graphpad Prism (version 5.02). The Kolmogorov-Smirnov test for normality and Levene test for homogeneity of variances were used to determine if parametric tests could be used. To determine if changes in peak current amplitudes, NMDA receptor-mediated charge, PPF and LTP were statistically significant the student’s t-test was used where n = number of animals. For measurements of putative extrasynaptic charge, the non-parametric Mann-Whitney
test was used as the WT and YAC128 groups did not have equal variances and the YAC128 data was not normally distributed. To determine if differences in the cumulative probability distribution of peak AMPA receptor-mediated currents post-LTP were significantly different between WT and YAC128, the Mann-Whitney test was used. Differences were considered statistically significant at p < 0.05.
3.5 RESULTS

3.5.1 SYNAPTIC AMPA AND NMDA RECEPTOR CURRENT AMPLITUDES ARE SIMILAR IN WT AND YAC128 HIPPOCAMPAL SLICES

It is known that cognitive decline occurs in human HD and in the YAC128 animal model of HD before motor symptom onset (Foroud et al., 1995, Van Raamsdonk et al., 2005b, Paulsen et al., 2008), and that mutant huntingtin is expressed in the hippocampus both in humans and the YAC128 animal model (Gutekunst et al., 1999, Hodgson et al., 1999). Therefore, we aimed to study synaptic transmission in YAC128 model mice to determine whether mutant huntingtin alters glutamatergic synaptic transmission in the hippocampus. Monosynaptic peak AMPA and NMDA receptor-mediated current amplitudes were assessed in WT and YAC128 animals at 1 month of age. The peak monosynaptic AMPA receptor-mediated current amplitude (Figure 12) was not different (p = 0.53) between WT (n = 26 recordings from 7 animals, mean = -58.9 ± 6.9 pA) and YAC128 (n = 14 recordings from 5 animals, mean = -69.2 ± 16.2 pA) animals. Similarly, the peak synaptic NMDA receptor current amplitude (Figure 12) was not significantly different (p = 0.66) between WT (n = 22 recordings from 8 animals, mean = 39.0 ± 6.8 pA) and YAC128 (n = 16 recordings from 6 animals, mean = 45.6 ± 11.1 pA) animals.

3.5.2 SYNAPTIC AND TOTAL NMDA RECEPTOR CHARGE ARE SIMILAR IN WT AND YAC128 HIPPOCAMPAL SLICES

Since there was no significant difference between YAC128 and WT animals in synaptic NMDA receptor current, it was of interest to see if the total (synaptic plus extrasynaptic) NMDA receptor-mediated current was altered. To measure total NMDA receptor current and compare it to synaptic NMDA receptor current, the charge (measured as the area under the curve) was measured before TBOA application (synaptic current) and after TBOA application (synaptic plus extrasynaptic current). If there is an increase in extrasynaptic NMDA receptors in YAC128 slices we expect to measure an increase in total NMDA receptor-mediated charge. TBOA application slowed the NMDA receptor-mediated EPSC kinetics, measured as $\tau_{\text{decay}}$, (Figure 13 C) in agreement with other studies in the hippocampus (Arnth-Jensen et al., 2002). NMDA receptor EPSC $\tau_{\text{decay}}$ was similar in WT (standard ACSF n = 22 recordings in 8 animals, mean = 128.9 ± 31.6 ms) and YAC128 (standard ACSF n = 16 recordings in 6 animals, mean = 139.8 ± 8.6 ms, p = 0.21) and also in the presence of TBOA (WT: n = 7 recordings in 6 animals, mean = 159.7 ± 12.0 ms; YAC128: n = 9 recordings in 6 animals, mean = 225.3 ± 38.7 ms, p = 0.16).
Figure 12. Peak Synaptic AMPA and NMDA receptor-mediated currents.
(A) Peak AMPA receptor current amplitudes are similar in WT and YAC128 slices. (B) Peak synaptic NMDA receptor current amplitude is also similar in WT and YAC128 slices. Example traces are shown on the right.
For synaptic NMDA receptor charge measurements, there was no significant difference ($p = 0.42$) between WT (n = 22 recordings from 8 animals, mean = 5355.5 ± 988.9 pA.ms) and YAC128 (n = 16 recordings from 6 animals, mean = 6953.2 ± 1815.5 pA.ms) animals (Figure 13 A). Interestingly there was also no significant difference ($p = 0.34$) in total NMDA receptor charge between WT (n = 7 recordings from 6 animals, mean = 6379.5 ± 1601.3 pA.ms) and YAC128 (n = 9 recordings from 6 animals, mean = 9442.62 ± 2737.9 pA.ms) animals (Figure 13 B). However, when examining the distribution of the total charge (Figure 13 D box plot), a greater range in the YAC128 data (20954 pA.ms) compared to the WT data (12060 pA.ms) is readily observed. This data shows that there is a higher variability in the total NMDA receptor-mediated charge in YAC128 compared with WT animals.

In order to obtain a measure of the extrasynaptic NMDA receptor-mediated current, we subtracted the synaptic from the total NMDA receptor-mediated charge (referred to as putative extrasynaptic NMDA receptor charge in (Figure 13). When the synaptic current (obtained in standard ACSF) was subtracted from the total current (obtained in TBOA solution), the difference between WT (n = 7 recordings from 6 animals, mean = 1127.7 ± 214.2 pA.ms) and YAC128 (n = 9 recordings from 6 animals, mean = 3313.27 ± 1095.1) became closer to significance ($p = 0.09$, Figure 13 E). A non-parametric test was used for this analysis as variances between the two groups were not equal (Levene’s test $p = 0.04$) and the data for YAC128 was not normally distributed. This subtraction was done for total and synaptic NMDA receptor current recordings obtained within the same neuron. Again, the difference in the variability and distribution of this extrasynaptic NMDA receptor charge can be seen in the box plots for WT and YAC128 animals (Figure 13 F). There was a greater range in the YAC128 data (10224 pA.ms) compared to the WT data (1295 pA.ms).
Figure 13. Synaptic vs. Total NMDA receptor charge.
Synaptic (A) and total (B) NMDA receptor-mediated charge is similar in WT and YAC128 slices. TBOA slowed NMDA receptor-mediated current kinetics ($\tau_{\text{decay}}$) to a similar extent in WT and YAC128 slices, traces are shown before and after TBOA application (C). (D) Distribution and spread of total NMDA receptor-mediated charge is greater in YAC128 than WT as shown in the box plot in (E). Total (in the presence of TBOA) less synaptic (in standard ACSF) NMDA receptor-mediated charge shows a trend towards an increase in YAC128 slices ($p = 0.09$) and similarly the distribution and spread of the putative extrasynaptic NMDA receptor-mediated charge is greater in YAC128 (F).
3.5.3 LTP IS REDUCED IN HIPPOCAMPAL SLICES FROM YAC128 HD MOUSE MODEL AT 1 MONTH OF AGE

Next, we aimed to determine if LTP is altered in YAC128 mice at this early age (1 month old). First, baseline AMPA receptor-mediated EPSCs were measured for 2-3 min before LTP was induced to prevent the well reported washout of LTP (Malinow and Tsien, 1990). Indeed when the pairing protocol was applied after more than ~10 min of obtaining whole cell mode, LTP did not occur due to cytoplasmic washout of LTP. For all experiments, LTP recordings were maintained for ~30 min including the baseline and induction period.

In WT animals, LTP induction resulted in a long-lasting increase in synaptic strength (e.g. Figure 14 A). Peak AMPA receptor-mediated responses were stable over time and did not increase in the absence of either high frequency stimulation or the pairing protocol (Figure 14 B and C). The potentiated current amplitude after LTP induction could also be depotentiated with an LTD protocol (see Experimental Procedures) (Figure 14 D).

Figure 15 shows that baseline AMPA receptor-mediated current amplitudes were similar (p = 0.74) between WT (mean = -77.5 ± 3.1 pA, n = 13 recordings from 3 animals) and YAC128 (mean = -62.1 ± 1.85 pA, n = 13 recordings from 4 animals) animals before LTP induction. However at 30 min post-LTP the AMPA receptor-mediated current was significantly potentiated only in WT slices (mean = -115.1 ± 26.9 pA, n = 13 recordings from 3 animals, p = 0.04) but not in YAC128 slices (mean = -67.2 ± 15.2 pA, n = 13 recordings from 4 animals, p = 0.40). The cumulative probability plot of the post-LTP AMPA receptor current amplitudes (Figure 15) shows that the current amplitudes from YAC128 animals are shifted to the right (i.e. smaller amplitudes) with respect to the WT amplitudes and this difference in distribution was significant (Mann-Whitney test p<0.005). Figure 16 shows the AMPA receptor-mediated current post-LTP normalised to baseline (pre-LTP) for WT and YAC128 slices. When normalised to the baseline AMPA receptor amplitude, WT showed 177.8 % potentiation at 30 min compared with 116.8 % in YACs.
Figure 14. LTP control experiments.
(A) LTP induction with 1 Hz, 1 sec stimulation (indicated by arrow in graph) produced a long lasting increase in the AMPA receptor-mediated current amplitude. (B) LTP could not be induced without high frequency (1 Hz) stimulation or (C) without pairing presynaptic high frequency stimulation (indicated by arrow) with postsynaptic depolarization. (D) LTP (induction indicated by arrow in graph) could also be depotentiated with 1 Hz, 6 min stimulation protocol. Arrow in current trace indicates monosynaptic peak AMPA receptor current amplitude.
Figure 15. LTP deficit in 1 month old YAC128 mice, non-normalized data.
(A) AMPA receptor-mediated currents measured before and after LTP induction in WT and YAC128 slices. Arrow indicates LTP induction. Pre-LTP current amplitudes are similar (p = 0.74) in WT and YAC128 but post-LTP current amplitudes are potentiated only in WT slices (p = 0.04) but not in YAC128 slices (p = 0.40). (B) Example traces of pre-LTP baseline AMPA receptor-mediated currents and post-LTP currents are shown for WT and YAC128. (C) The cumulative probability function of AMPA receptor-mediated currents post-LTP show that the distribution is significantly different between WT and YAC128 slices (p<0.005).
Figure 16. LTP deficit in 1 month old YAC128 mice, normalized data.
Post-LTP AMPA receptor-mediated current amplitudes normalized to pre-LTP baseline currents in WT and YAC128 hippocampal slices. Arrow indicates LTP induction.
A striking feature of the YAC128 slices was that LTP induction (1 Hz, 1 min) frequently resulted in no significant change in AMPA receptor-mediated EPSC amplitude compared with baseline EPSC amplitude (failure to induce LTP) or in fact the induction of LTD (AMPA receptor-mediated current amplitude smaller than baseline). The failure to induce LTP occurred in approximately half the YAC128 whole cell recordings (mean = 46.2 %, Figure 17 A). LTD occurred in 38.5 % of the YAC128 whole cell recordings. These percentages are high compared to WT recordings where the failure to induce LTP occurred in 23.1 % of cases and LTD occurred in 7.7 % of cases (Figure 17 A). Example traces recorded in YAC128 slices are shown in Figure 17 B where the LTP induction protocol failed to produce LTP as shown by a lack of potentiation of the peak monosynaptic AMPA receptor-mediated current amplitude. In Figure 17 C an example is shown where the LTP induction protocol produced a depressed peak monosynaptic AMPA receptor-mediated current amplitude in a YAC128 slice.

The failure to induce LTP in YAC128 hippocampal slices suggested that a more intense stimulus could be required to increase AMPA receptor mediated current amplitudes. Previously it has been shown in the Hdh mouse model of HD that these animals have a higher threshold for LTP induction (Usdin et al., 1999). Therefore, a stronger induction protocol was trialled in a subset of YAC128 hippocampal slices. Specifically, we applied tetanic stimulation (see Experimental Procedures). Two examples of the outcome of this tetanus protocol are shown in Figure 17 D and E. The tetanic stimulation produced either a slightly potentiated response (Figure 17 D) which was not significantly different from baseline at 30 min (p = 0.22) or it produced a short term potentiation (only up to approximately 10 min) (Figure 17 E) which returned to the baseline (pre-tetanus) AMPA receptor-mediated current amplitude. In total the tetanus induction protocol was performed in 5 different neurons from 3 different YAC128 animals. In only one of these neurons did the tetanus stimulation produce significant LTP.
Figure 17. Failure to induce LTP in YAC128 slices and appearance of LTD.

(A) Failure to induce LTP or the induction of LTD with the LTP induction protocol was a striking feature of YAC128 slices. Example traces of recordings in YAC128 slices are shown where LTP induction failed (B) or produced LTD (C). When tetanic stimulation was used to induce LTP (indicated by arrow) in YAC128 slices there was still no long lasting potentiation of the AMPA receptor-mediated current (D and E). NS, not significant.
3.5.4 PAIRED PULSE FACILITATION IS NOT ALTERED IN HIPPOCAMPAL SLICES FROM THE YAC128 HD MODEL MOUSE

To test whether the loss of LTP in YAC128 slices was due to altered presynaptic Ca\textsuperscript{2+} handling or altered presynaptic glutamate release, we measured the paired pulse ratio, a measure of presynaptic release (Katz and Miledi, 1968, Charlton et al., 1982). If a reduced presynaptic release probability occurred in YAC128 slices, an increased paired pulse ratio would be detected. This is because with a low release probability, the first pulse will create a relatively small current amplitude that is able to be potentiated upon the second pulse due to residual presynaptic Ca\textsuperscript{2+} from the first pulse. If on the other hand release probability is high, the first pulse will deplete the available transmitter vesicles and there is no opportunity for an increase in vesicle release upon the second pulse. However, when PPF was measured, there was no significant difference between YAC128 (mean = 1.78 ± 0.14, n = 11 recordings from 5 animals) and WT (mean = 1.65 ± 0.09, n = 13 recordings from 5 animals) animals (Figure 18).
Figure 18. Paired Pulse Facilitation (PPF) is normal in YAC128. The paired pulse ratio (PPR) is similar in WT and YAC128 slices, example traces are shown on the right.
3.6 DISCUSSION

The experiments performed here aimed to investigate if the presence of mutant huntingtin altered basal synaptic transmission, extrasynaptic NMDA receptor-mediated currents and synaptic plasticity in the YAC128 model of HD. We show for the first time that both synaptic transmission through AMPA and NMDA receptors and extrasynaptic NMDA receptor-mediated currents are unaltered in hippocampal slices of YAC128 animals as compared to WT animals at 1 month of age. This is in contrast to the increased extrasynaptic NMDA receptor-mediated signalling that has been reported in striatal slices of YAC128 animals at this age (Milnerwood et al., 2010). Furthermore, we show for the first time a deficit in LTP in hippocampal slices of YAC128 animals as compared to WT animals at 1 month of age. Our data suggests that this LTP deficit is not due to changes in presynaptic function or a higher threshold for LTP in YAC128 slices but involves postsynaptic changes. This deficit in LTP is thought to underlie the cognitive and memory deficits present in YAC128 animals at this young age (Kim, J., Fourie, C. et al, manuscript in preparation).

3.6.1 SYNAPTIC TRANSMISSION IN HIPPOCAMPAL SLICES FROM YAC128 ANIMALS AT 1 MONTH OF AGE

For the first time we report here that there are no changes in synaptic AMPA receptor-mediated current amplitude or synaptic NMDA receptor-mediated current amplitude and charge in the YAC128 hippocampus at 1 month of age. A lack of change in synaptic AMPA and NMDA receptor-mediated currents suggest that there are no changes in the number of postsynaptic receptors, and/or no changes in presynaptic glutamate release and/or no change in receptor properties. While we did not look at receptor number using immunohistochemistry at this young age, we reported a lack of change in total protein expression of glutamatergic receptor subunits and synaptic scaffolding proteins at 12 months of age as described in Chapter 2. However, given the lack of change in synaptic currents these data do not suggest the measurement of receptors with immunohistochemistry is required. Furthermore, although we show no changes in synaptic transmission, we did not measure these synaptic currents at different stimulation intensities. It has been shown in striatal slices that only at high stimulation intensities in the YAC128 mouse (1 month of age) does NMDA receptor charge increase significantly above that of the WT mice (Milnerwood et al., 2010). Higher stimulation intensity will activate more synaptic receptors and also extrasynaptically located receptors due to the glutamate spillover to extrasynaptic sites (see Section 3.6.2.3). Here in our study, it is unlikely that higher stimulation intensities will reveal a significant difference between WT and YAC128 animals as we show no significant change in total NMDA
receptor-mediated current (see Section 3.6.2). Therefore, the lack of change in synaptic NMDA receptor-mediated currents in the YAC128 measured here agrees with that in the striatum at low stimulation intensity.

3.6.2 A ROLE FOR EXTRASYNAPTIC NMDA RECEPTORS IN THE HIPPOCAMPUS OF YAC128 MICE?

To investigate whether extrasynaptic NMDA receptor-mediated currents are altered in YAC128 mice, we measured synaptic NMDA receptor-mediated charge and total NMDA receptor-mediated charge (synaptic plus extrasynaptic receptors) with the use of TBOA to block glutamate uptake. We used this to obtain a measure of extrasynaptic NMDA receptor-mediated charge by subtracting synaptic from total NMDA receptor-mediated charge. TBOA blocks GLT1, which is present on both glia and neurons (Rothstein et al., 1994, Chen et al., 2004), resulting in glutamatergic activation of synaptic and extrasynaptic receptors (Diamond, 2001, Arnth-Jensen et al., 2002, Milnerwood et al., 2010). Our data showed that there was no significant change in the total or extrasynaptic NMDA receptor-mediated charge, suggesting no change in the amount of extrasynaptic NMDA receptors in the YAC128 hippocampus at 1 month of age.

Our data in the hippocampus are in contrast to that reported in the YAC128 striatum (Milnerwood et al., 2010). Binding of mutant huntingtin to PSD95 is impaired in HD (see Chapter 1) (Sun et al., 2001), which leads to an increased association between PSD95 and GluN2B receptors in the YAC128 striatum at extrasynaptic sites (Fan et al., 2009, Fan et al., 2012). Extrasynaptic NMDA receptor-mediated current is increased in striatal slices from YAC128 animals at the age of 1 month (Milnerwood et al., 2010). There are no studies reporting such an increase in the association between PSD95 and any of the glutamatergic receptor subunits in the YAC128 hippocampus. Our results provide evidence against such an increase as this will increase synaptic and/or extrasynaptic glutamate receptor numbers and we report no changes in synaptic AMPA and NMDA receptor-mediated currents or in extrasynaptic NMDA receptor-mediated currents at 1 month of age.

3.6.2.1 Age of the animal at which TBOA recordings were done

The lack of a significant change in total or extrasynaptic NMDA receptor-mediated charge in YAC128 slices may be due to the large variability in the YAC128 recordings and increasing the number of recordings could be of benefit. However, the conclusion most synonymous in our data is that changes in extrasynaptic NMDA receptor activation do not occur in the hippocampus at this young age. An interesting feature seen in YAC128 slices but not in WT slices with respect to the total and extrasynaptic NMDA receptor-mediated charge is that the YAC128 slices show significant variability.
Our data show that at the age of 1 month this large variability renders extrasynaptic NMDA receptor-mediated charge insignificant from that of the WT. The YAC128 model was originally created because the YAC72 model showed large inter-animal variability in HD related changes (Slow et al., 2003). Different grades/stages of a neurological disease may underlie variability between subjects/animals affected by the disease. Although these grades are not characterised in animal models it has been characterised in the human brain (Vonsattel et al., 1985) and is a significant source of variability between patients affected by the disease. The large variability in the YAC128 hippocampal slices could be due to being on the cusp of a significant change in extrasynaptic NMDA receptor-mediated currents occurring after 1 month of age. It would therefore be very important to determine if extrasynaptic NMDA receptor-mediated charge is altered in later stages of the disease at 2, 6 and 12 months of age in YAC128 hippocampal slices.

Cognitive decline in HD occurs before motor symptom onset in both the YAC128 animal model (Van Raamsdonk et al., 2005b) and humans (Foroud et al., 1995, Lawrence et al., 1998, Paulsen et al., 2008). Increased extrasynaptic NMDA receptor-mediated currents are observed before motor symptom onset in striatal slices of YAC128 animals (at 1 month of age) (Milnerwood et al., 2010). This is thought to underlie MSN death, which is a typical feature of the striatum in HD (Vonsattel et al., 1985, Van Raamsdonk et al., 2005a). It is also thought to underlie the motor symptoms in HD as motor symptoms are reversed by blocking extrasynaptic NMDA receptors with low dose memantine (Okamoto et al., 2009, Milnerwood et al., 2010). Our results show that cognitive decline at 1 month of age in the YAC128 model (Kim, J., Fourie, C. et al, manuscript in preparation) is not related to increased extrasynaptic NMDA receptor-mediated currents in the hippocampus. This difference in the YAC128 hippocampus vs. striatum at 1 month of age may be due to the hippocampus (but not the striatum) being relatively spared in this model (Slow et al., 2003, Van Raamsdonk et al., 2005a) and therefore do not present with increased extrasynaptic signalling, which leads to cell death pathways (Hardingham et al., 2002).

3.6.2.2 The effect of TBOA on synaptic NMDA receptors

The use of TBOA to measure total NMDA receptor-mediated charge and consequently obtain a measure of extrasynaptic NMDA receptor-mediated charge, may not provide a pure “extrasynaptic charge” measurement. We know that synaptic NMDA receptors are not saturated during a single release event; in fact the fractional occupancy for a single release event is estimated to be less than 0.56 in hippocampal slices (Mainen et al., 1999). Therefore, TBOA application to block glutamate uptake 1) may increase synaptic NMDA receptor-mediated current by activating more synaptic NMDA receptors if they were not initially saturated, and/or 2) may increase extrasynaptic NMDA
receptor-mediated current by causing spillover of glutamate to extrasynaptic sites. Therefore, our future experiments should use (5R,10S)-(−)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cycohepten-5,10-imine maleate (MK-801, an activity-dependent blocker of NMDA receptors) to block synaptic receptors during the normal 0.1 Hz stimulation in standard ACSF and thereafter apply TBOA to activate only the remaining extrasynaptically located NMDA receptors. This has been used by another study and shown to reveal receptors which in the absence of TBOA was outside the postsynaptic domain – these could be extrasynaptic receptors or receptors from inactive neighbouring synapses (Arnth-Jensen et al., 2002, Milnerwood et al., 2010).

3.6.2.3 Does glutamate spillover occur in the absence of glutamate uptake inhibitors?

Can we assume that stimulating Schaffer collaterals in the absence of TBOA is in fact only activating synaptic receptors? This is important in light of the results shown in Figure 13 as this may affect the NMDA receptor-mediated current measurements between WT and YAC128. It is thought that only when glutamate transporters are blocked does spillover to extrasynaptic receptors occur (Diamond, 2001). However, in the study by Diamond and colleagues this was measured indirectly with the use of D-amino adipate (D-AA, a competitive NMDA receptor antagonist) which preferentially blocks NMDA receptors that sense low concentrations of glutamate, which is assumed to be due to their location away from the active synapse and being activated by glutamate spillover. In addition it has also been shown that TBOA significantly prolongs the decay of the NMDA receptor-mediated EPSC and that this depends on active synapse density, as reduced stimulation intensity or increased extracellular Mg²⁺ (to block NMDA receptors) reverses the TBOA effect on EPSC kinetics (Arnth-Jensen et al., 2002). However, a prolonged EPSC could also be created in the absence of any glutamate uptake inhibitors in 2 cases: 1) with increased stimulus intensity (but below the action potential threshold of the postsynaptic neuron) and 2) if the frequency of presynaptic action potentials in paired recordings in slices were increased to 100Hz. These results indicate that when many synapses are activated in the absence of glutamate uptake inhibitors, they can cooperate to activate postsynaptic receptors, both extrasynaptic receptors and receptors from non-activated synapses.

Consequently, activation of extrasynaptic receptors in the absence of glutamate uptake inhibitors only occurs during high intensity or high frequency stimulation. In agreement with this are experiments that showed that an LTP protocol that utilizes presynaptic tetanic stimulation induces LTP of NMDA receptor-mediated EPSCs even when LTP of the AMPA receptor-mediated EPSC is prevented (Kullmann et al., 1996). This is because NMDA receptors can participate in “synaptic
cross-talk” as they are able to sense glutamate spillover from neighbouring terminals. In light of the experiments done in our study, stimulation in the absence of TBOA activated mostly synaptic NMDA receptors in WT slices as we did not use high stimulation intensity or frequency (see Experimental Procedures). However, we cannot assume that this is the case in the YAC128 slices due to the presence of mutant huntingtin. If it is not the case in the YAC128 slices (i.e. if stimulation in the standard ACSF activates extrasynaptic receptors), then the NMDA receptor current measured in the presence of TBOA may be underestimated for this group.

3.6.2.4 The effect of temperature on TBOA experiments

Interestingly, when the experiments by Kullmann and colleagues were done at physiological temperatures, this “synaptic cross-talk” was significantly reduced and it is thought to be due to the glutamate uptake mechanisms being much greater at higher temperatures (Wadiche et al., 1995, Asztely et al., 1997). Therefore glutamate transporters minimise spillover and synaptic cross-talk in the hippocampus more efficiently at physiological temperature. Because our experiments were performed at room temperature, we may underestimate the NMDA receptor-mediated current in the presence of TBOA as the glutamate uptake mechanisms are not working optimally before TBOA application. Temperature may also affect receptor saturation (see Section 3.6.2.2) as it has been shown that NMDA receptors are not saturated during a single release event at physiological temperature (Mainen et al., 1999) in slices but it is not known if this is true at room temperature in our experiments. It would be interesting to repeat the experiments at physiological temperature to further investigate extrasynaptic NMDA receptor-mediated currents in the YAC128 hippocampus.

3.6.2.5 The effect of mGluRs on TBOA experiments

Another factor to consider with the use of TBOA is that presynaptic and/or postsynaptic mGluRs will be activated more readily by this spillover glutamate in the synaptic cleft that is not removed by active transport mechanisms. This may have important consequences for the interpretation of experimental results that involve the use of TBOA. Group I mGluRs (mGluA1 and 5) are located at extrasynaptic sites of the postsynapse (Baude et al., 1993, Shigemoto et al., 1993), group II mGluRs (mGluA2 and 3) are located presynaptically remote from the active zone (Luján et al., 1997) and group III mGluRs (mGluR4, 6 and 7) are also located presynaptically but within the synaptic cleft at the active zone (Shigemoto et al., 1996). In CA1 pyramidal cells in acute slices, the widely used group I/II mGluR agonist, 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) suppresses excitatory synaptic transmission and this is developmentally regulated so that this effect is maximal during the first postnatal month (Baskys and Malenka, 1991). However, group I and II mGluR activation by ACPD can also decrease inhibition onto CA1 pyramidal cells (Desai and Conn, 1991). When selective agonists
are used for group II mGluRs, synaptic transmission is inhibited in the hippocampus, indicating a
presynaptic autoinhibitory action of these receptors (Kew et al., 2001). Presynaptic mGluR activation
is thought to inhibit synaptic transmission by its actions on voltage-gated Ca\(^{2+}\) channels (Takahashi et
al., 1996). On the other hand, the use of selective agonists and antagonists for mGluR5 (located
postsynaptically) in the hippocampus, indicates that this receptor enhances NMDA receptor-
mediated currents (Pisani et al., 1997, Mannaioni et al., 2001, Kotecha et al., 2003). It is not known if
and how presynaptic and postsynaptic mGluR activity and its effect on transmission are altered in
the YAC128 hippocampus at 1 month of age. This is especially important since the age at which these
recordings were done is known to have maximal presynaptic mGluR effects on synaptic transmission
in the hippocampus (Baskys and Malenka, 1991). Therefore if the expression and/or activity of any
of the mGluR receptor subtypes are altered in the YAC128 hippocampus, it may affect our
measurement of the total NMDA receptor-mediated current measured in the presence of TBOA.

3.6.3 EARLY DEFICITS IN LTP IN HIPPOCAMPAL SLICES FROM YAC128 ANIMALS

Here we report for the first time a reduction in LTP in the YAC128 hippocampus at 1 month of age.
Previously, cognitive deficits in this model have been reported to appear around 2 months of age
(Van Raamsdonk et al., 2005b). We together with our collaborators have observed a significant
deficit at the early age of 1 month in YAC128 mice in the rotarod and T maze spontaneous alteration
tasks (Kim, J., Fourie, C. et al, manuscript in preparation) and occur together with the deficits in LTP
that we report here. The rotarod is an apparatus used for detecting deficits in motor behaviour
(Dunham and Miya, 1957) while the T maze spontaneous alteration task assesses working memory
and hippocampal dysfunction (Deacon and Rawlins, 2006). The T maze is an enclosed apparatus in
the form of a T where animals start at the base of the T and freely choose one of the arms. Normally
on the second trial rodents will choose the arm not visited before, indicating that the animal
retained memory of the first choice. With the T maze spontaneous alteration task, WT mice at 1
month of age show alteration rates of 75\% whereas YAC128 animals have a rate of 42\% (approximately half the rate) (Kim, J., Fourie, C. et al, manuscript in preparation). This indicates
significant hippocampal memory impairment in the YAC128 animals as young as 1 month of age. The
deficit in LTP observed here is expected to continue to decline with age as the learning disability of
these animals has been shown to progress with age (Van Raamsdonk et al., 2005b). Similar to our
LTP data, a deficit in CA1 LTP has been shown in the YAC72 mouse at 10 months of age as well as the
observation that high frequency stimulation (an LTP induction protocol) causes LTD in YAC72
hippocampal slices (Hodgson et al., 1999), similar to what we described. Altogether our data indicate
that reduced LTP in YAC128 hippocampal slices correlates with the learning and memory deficits and this occurs presymptomatically at 1 month of age.

3.6.3.1 Presynaptic mechanisms for deficits in LTP

An important question is why LTP is reduced in the YAC128 mouse. The reduced LTP is due to presynaptic changes and/or postsynaptic changes. To explore the presynaptic avenue, we used PPF to assess whether there is a change in presynaptic transmitter release. The PPR was not significantly altered between WT and YAC128 slices in our study. This result is similar to the YAC72 mouse at 10 months of age where there is also no change in PPF in hippocampal slices as measured with field recordings (Hodgson et al., 1999). Interestingly, when posttetanic potentiation was measured in YAC72 hippocampal slices it was reduced compared to WT animals. Posttetanic potentiation is also indicative of presynaptic release and like PPF depends on residual presynaptic Ca\(^{2+}\) (Zucker, 1989). While paired pulse facilitation can last up to hundreds of milliseconds, posttetanic potentiation can last up to several minutes (Zucker and Regehr, 2002). Altogether, PPF is not altered in hippocampal slices from YAC72 animals (Hodgson et al., 1999), R6/2 animals (Murphy et al., 2000) and also not in the YAC128 as we report here. Therefore, postsynaptic mechanisms appear to underlie the deficit in LTP that we have measured.

3.6.3.2 Postsynaptic mechanisms for deficits in LTP

A postsynaptic mechanism for a deficit in LTP could be due to changes in postsynaptic glutamate receptor number. Our electrophysiology data show that AMPA and NMDA receptor-mediated currents are normal in YAC128 hippocampal slices. This indicates that there is no change in postsynaptic receptor number or a redistribution of receptors to or from the synapse. In the YAC72 mouse hippocampus at the age of 10 months it has been shown that the Ca\(^{2+}\) buffering capacity is reduced, which leads to higher resting levels of Ca\(^{2+}\) in postsynaptic CA1 neurons and therefore a reduced driving force for Ca\(^{2+}\) influx through NMDA receptors, which is a requirement for LTP (Lynch et al., 1983, Hodgson et al., 1999). However, the LTP deficit that we report is not due to altered postsynaptic Ca\(^{2+}\) influx as synaptic NMDA receptor-mediated currents are normal in YAC128 hippocampal slices compared to WT slices.

Short term potentiation could be induced in some YAC128 slices with a tetanic stimulus. Therefore only when synaptic spillover occurs (during the tetanic stimulation) and when many synapses are activated at the same time could short term potentiation be induced in some YAC128 slices. Therefore, it does not seem that the threshold for LTP is simply altered in the YAC128 model as LTP could not be induced even with higher frequency stimulation. A more complex mechanism must
underlie the LTP deficit. A change in the threshold for LTP was also not reported in the 10 month old YAC72 hippocampus (Hodgson et al., 1999), although it is thought to be the underlying mechanism of a deficit in LTP in the Hdh animal model where enhanced tetanic stimulation could produce LTP in mutant slices (Usdin et al., 1999). Nevertheless, LTP cannot be induced in YAC128 hippocampal slices with a tetanic stimulus.

Furthermore, glutamate receptor trafficking or cycling may be altered preventing AMPA receptor insertion at the synapse during LTP in YAC128 slices. AMPA receptors are rapidly inserted into the synapse during NMDA receptor-dependent LTP in the hippocampus (Shi et al., 1999, Hayashi et al., 2000). AMPA receptors in the hippocampus are mostly composed of GluA1/2 or GluA2/3 subunits (Craig et al., 1993, Wenthold et al., 1996). Specifically, GluA1/2 receptors are added to the synapse during synaptic plasticity and this requires GluA1 binding to PDZ domain proteins (Shi et al., 2001). On the other hand, homomeric GluA2 and GluA2/3 receptors continuously replace existing AMPA receptors at the synapse during baseline synaptic transmission (Shi et al., 2001). Therefore the delivery of GluA1 containing AMPA receptors to the synapse during LTP may be deficient in YAC128 slices while baseline synaptic transmission involving GluA2/3 receptors are normal.

High frequency synaptic stimulation (such as during LTP induction) causes Ca²⁺ influx through NMDA receptors, subsequently activates CaMKII and induces LTP (see Chapter 1). The GluA1 subunit of AMPA receptors are specifically phosphorylated by activated CaMKII, which leads to potentiation of AMPA receptor channel function and underlies the expression of LTP (Barria et al., 1997a, Barria et al., 1997b, Lee et al., 2000). A lack of GluA1 phosphorylation in GluA1 phosphorylation mutant mice produces severe deficits in NMDA receptor-dependent LTP in the hippocampus (Lee et al., 2003). This occurs in the absence of any changes in baseline synaptic transmission or NMDA receptor mediated synaptic responses (Lee et al., 2003), similar to what we have reported here. Therefore, a deficiency in CaMKII-dependent phosphorylation of GluA1-containing AMPA receptors may underlie the LTP deficit in YAC128 slices that we report here while normal baseline synaptic transmission is maintained.

Moreover, MAGUKs play a vital role in trafficking and anchoring glutamate receptors at the synapse (see Chapter 1). SAP97 binds directly to the GluA1 subunit of AMPA receptors (Leonard et al., 1998) and to NMDA receptors (Gardoni et al., 2003), whereas PSD95 binds to AMPA receptors through the transmembrane AMPA receptor regulatory protein (TARP) stargazin (Chen et al., 2000, Schnell et al., 2002) and directly to NMDA receptors (Kornau et al., 1995). The deficit in LTP that we report here may be due to altered MAGUK function that occurs in an activity dependent manner during synaptic plasticity. Baseline synaptic transmission and extrasynaptic NMDA receptor activity is normal in our
study and it is therefore only during high frequency stimulation to induce LTP that we suggest a
deficit in MAGUK dependent receptor trafficking.

Such altered MAGUK function during LTP is likely to involve SAP97. SAP97 but not PSD95 has been
shown to have a fast turnover rate (similar to AMPA receptors) in spines (Nakagawa et al., 2004)
Interestingly, SAP97 interacts with AMPA receptors early in the secretory pathway but dissociates
from AMPA receptors at the plasma membrane, indicating that SAP97 has a role in receptor
trafficking and targeting but not anchoring at the membrane (Sans et al., 2001). In support of this
electron microscopy and biochemical data has shown that SAP97 is predominantly located in the
cytoplasm (although also present at synapses) but PSD95 is concentrated at the PSD only (Sans et al.,
2001). Altogether, SAP97 is responsible for trafficking and targeting of AMPA receptors to the
surface but PSD95 is responsible for receptor anchoring at the PSD. For this reason we suggest that
LTP-related SAP97 trafficking of AMPA receptors may be deficient in YAC128 hippocampal slices.
During baseline synaptic activity, trafficking and anchoring of AMPA receptors are normal as we
report synaptic transmission to be normal in YAC128 hippocampal slices. However, during high
frequency synaptic activity such as during the induction of LTP, there may be a deficiency in SAP97
dependent trafficking of AMPA receptors. This may be either due to deficient association of SAP97
with AMPA receptors or a deficiency in the trafficking per se during LTP.

Moreover, different isoforms of SAP97 and PSD95 exist (see Chapter 1 and 4) due to N-terminal
domain alternative splicing, which results in α-isoforms that are palmitoylated and β-isoforms that
contain an L27 domain (Schlüter et al., 2006). It is also known that αSAP97 is targeted to the PSD
whereas βSAP97 is targeted to non-PSD sites in hippocampal neurons and thereby regulate AMPA
receptor localisation (Waites et al., 2009). Importantly, αSAP97 regulates the synaptic pool of AMPA
receptors whereas βSAP97 regulates the extrasynaptic pools of AMPA and NMDA receptors, which
have important consequences for synaptic plasticity (Li et al., 2011). If SAP97 expression or function
is altered and underlies the deficit in LTP in YAC128 hippocampal slices, it will be important to
determine if it is isoform specific.

Furthermore, TARPS and other AMPA receptor interacting proteins, such as protein interacting with
C kinase 1 (PICK1) and glutamate receptor interacting protein (GRIP) are also important for the
trafficking of AMPA receptors. For example, an interaction between PICK1 and GRIP in the
hippocampus is responsible for the regulation of GluA2-containing AMPA receptor trafficking and if
disrupted results in reduced surface expression of GluA2 (Lu and Ziff, 2005). Similarly, the C-terminus
of stargazin is responsible for binding to PSD95 PDZ domains and to AMPA receptors and if disrupted
results in reduced synaptic AMPA receptors (Schnell et al., 2002). Another TARP, called γ-8 is highly
expressed in the hippocampus and TARP γ-8 deficient mice (γ-8/−) show reduced synaptic (35%) and extrasynaptic (90%) AMPA receptor-mediated currents as well as reduced LTP (75%) (Rouach et al., 2005). The γ-8/− mice show a small but significant reduction in synaptic AMPA receptor-mediated currents but a large reduction in LTP. Therefore, if TARP expression and/or function are altered in the YAC128 hippocampus it may underlie the LTP deficits that we have measured while not affecting synaptic AMPA receptor-mediated currents.

Additionally, breakdown of the actin cytoskeleton may underlie the deficit in LTP as AMPA receptor delivery to the synapse involves actin-dependent mechanisms, which if perturbed could block LTP. Indeed it has been shown that there is a labile pool of AMPA receptors in CA1 pyramidal cells that are sensitive to actin inhibitors and microtubule motor inhibitors which leads to reduced AMPA receptor mediated responses (Kim and Lisman, 2001). At hippocampal CA1 synapses disruption of the actin filament cytoskeleton impairs LTP maintenance while not affecting basal synaptic transmission or PPF (Krucker et al., 2000). As we have found reduced LTP in the YAC128 slices while synaptic transmission and PPF was normal, disruption of the actin filament network at the synapse may be a potential mechanism underlying the LTP deficit seen here. In hippocampal cultured neurons, intense glutamate receptor activity produces a loss of spines as well as a loss of filamentous actin from the synapse in a time and concentration dependent manner (Halpain et al., 1998). However, synaptic transmission in the YAC128 slices is normal in our study and intense receptor activity is therefore not the underlying mechanism of possible actin breakdown at the synapse.

3.7 FUTURE DIRECTIONS

To further explore extrasynaptic NMDA receptor-mediated currents in the hippocampus the use of MK-801 to block synaptic NMDA receptors activated in the absence of TBOA and then applying TBOA hereafter would be useful as to make a clear distinction between synaptic and extrasynaptic NMDA receptors. More complex methods can also help to activate synaptic vs. extrasynaptic NMDA receptors such as local 2-photon uncaging of NMDA at synaptic vs. extrasynaptic sites (Harris and Pettit, 2007). The effect of physiological temperature on extrasynaptic NMDA receptor-mediated currents in WT vs. YAC128 hippocampal slices is of importance. Higher temperatures will cause glutamate uptake mechanisms to be more effective before they are blocked and may reveal a greater difference between WT and YAC128 extrasynaptic NMDA receptor-mediated currents. Furthermore, measuring glutamate uptake in WT vs. YAC128 hippocampus will reveal if there is a deficit in GLT-1 function here as there is in the striatum of YAC128 animals (Huang et al., 2010).
will have important consequences for extrasynaptic glutamate receptor activation, glutamate spillover and excitotoxicity in HD.

We reported increased variability in total and extrasynaptic NMDA receptor-mediated currents in YAC128 slices, which may be due to being on the cusp of a significant change after 1 month of age. It would therefore be important to study synaptic and extrasynaptic receptor-mediated currents at older ages. This will determine whether there is a change in extrasynaptic NMDA receptors in older animals when the disease has progressed and if the extrasynaptic NMDA receptor currents in the hippocampus then follow a similar pattern to the striatum (Milnerwood et al., 2010). If there is a change in NMDA receptor currents in older animals in the hippocampus, it would also be interesting to determine whether these changes are altered in hippocampal cell types other than the CA1 pyramidal cells. Pyramidal neurons in the CA1 field of the hippocampus are particularly vulnerable to cell damage (Sommer, 1880; Davolio and Greenamyre, 1995; Wang et al., 2005), therefore it would be of interest to see if changes in extrasynaptic NMDA receptor-mediated currents correlates with this increased vulnerability and if it is less pronounced in other areas of the hippocampus.

Further investigation of mGluR receptor changes in the YAC128 mouse model will be of great interest as mGluRs modulate NMDA receptor activity in the hippocampus (see Section 3.6.2.5) as well as synaptic plasticity (Lu et al., 1997). Studying the effect of presynaptic mGluR agonists or postsynaptic mGluR antagonists on extrasynaptic NMDA receptor-mediated currents, LTP and animal behaviour in the YAC128 would be important in light of a potential avenue for drug therapy. Lastly, the mechanisms underlying the LTP deficit measured here in the YAC128 hippocampus must be investigated at the postsynaptic level. Such a study would be extensive as many factors will need to be investigated directly including the role of mGluRs, postsynaptic receptor kinetics, MAGUK-dependent postsynaptic receptor trafficking, subunit specific AMPA receptor insertion during LTP and CaMKII-dependent phosphorylation.
3.8 CONCLUSION

Here we have shown that glutamatergic synaptic transmission through AMPA and NMDA receptors is normal in the YAC128 hippocampus. Moreover, we observed no significant changes in extrasynaptic NMDA receptor current in CA1 pyramidal cells in YAC128. Therefore altered hippocampal CA1 extrasynaptic NMDA receptor current does not underlie the learning impairment that is evident at 1 month of age in the YAC128. There was a significant deficit in LTP in the YAC128 animals at 1 month of age and this was not due to presynaptic mechanism or due to a higher LTP threshold in mutant slices. We conclude that this deficit in LTP could underlie the learning impairments observed at this young age in YAC128 animals. Our data suggests that the LTP deficit is not due to a presynaptic deficit and further investigation into postsynaptic mechanisms including AMPA receptor cycling and the role of MAGUKs will be important.
CHAPTER FOUR: ELECTROPHYSIOLOGY IN A TRANSIENT DISSOCIATED HIPPOCAMPAL NEURON MODEL OF HUNTINGTON’S DISEASE

4.1 INTRODUCTION

4.1.1 THE REGULATION OF GLUTAMATE RECEPTOR LOCALISATION AND ITS RELATION TO HD

The importance of synaptic scaffolding proteins and their function in glutamate receptor trafficking, architecture of the postsynaptic density and regulation of synaptic plasticity have been introduced in Chapter 1. Both PSD95 and SAP97 scaffolding proteins have alternative N-terminal domain splicing that gives rise to both a palmitoylated α isoform and a β isoform that contains an L27 domain (Cho et al., 1992, Muller et al., 1995, Chetkovich et al., 2002, Schlüter et al., 2006). While αPSD95 is the dominant PSD95 variant in the brain, βSAP97 is the dominant form of SAP97 in the brain (Muller et al., 1995, Chetkovich et al., 2002). Within α and βSAP97 there are five other alternative splice variants, called I1 to I5 that arise from different insertions located at specific regions within the protein sequence (Lue et al., 1994, Mori et al., 1998, McLaughlin et al., 2002). The α and β forms of SAP97 differentially targets SAP97 to the PSD and non-PSD sites respectively and thereby differentially regulate the localization and dynamics of AMPA receptors as SAP97 directly binds GluA1 subunits of AMPA receptors (Leonard et al., 1998, Waites et al., 2009).

Interestingly, αSAP97 regulates LTP by altering the synaptic location of AMPA receptors, whereas βSAP97 regulates LTP by altering the extrasynaptic location of AMPA and NMDA receptors (Li et al., 2011). This study from our lab showed that overexpression of βSAP97 in dissociated hippocampal cultures reduced baseline synaptic AMPA receptor currents as well as NMDA receptor currents. On the other hand αSAP97 increased synaptic AMPA receptor current but did not change synaptic NMDA receptor currents. However, overexpression of βSAP97 showed significantly increased total (synaptic plus extrasynaptic) NMDA receptor-mediated current, indicating that βSAP97 causes a higher total surface expression of functional NMDA receptors while producing fewer functional synaptic NMDA receptors. Interestingly overexpression of either α and βSAP97 blocked LTP (reduced
the AMPA receptor current) but increased LTD in dissociated hippocampal cultures. When total AMPA receptor-mediated currents were measured after LTP was induced in the cultures and compared to the current before LTP induction, αSAP97 caused a reduction while βSAP97 did not change the total surface current, indicating that βSAP97 causes an increase in the extrasynaptic pool of AMPA receptors with LTP. When the same experiment was done but measuring total surface NMDA receptor currents, αSAP97 had no effect but βSAP97 significantly increased the current and along with other experiments indicated that βSAP97 increased the extrasynaptic pool of NMDA receptors during LTP. Currently it is not known if NMDA receptors are increased at extrasynaptic sites in hippocampal neurons as described in striatal MSNs in HD (Milnerwood et al., 2010, Milnerwood et al., 2012). This extrasynaptic location of NMDA receptors are linked to cell death pathways as it affects neurotoxicity of mutant huntingtin (Okamoto et al., 2009, Milnerwood et al., 2010, Milnerwood et al., 2012). It is also not known if α and βSAP97 expression is altered in HD and if it plays a role in the mislocalisation of NMDA receptors to extrasynaptic sites.

The huntingtin protein interacts with the synaptic scaffolding protein PSD95 by binding to its SH3 domain, however mutant huntingtin disrupts this interaction (Sun et al., 2001). Such an interaction has not been reported for SAP97 but the location of NMDA receptors, which are regulated by SAP97, has been shown to affect mutant huntingtin aggregate formation and neurotoxicity (Okamoto et al., 2009). Interestingly, NMDA receptor activity is required for mutant huntingtin aggregate formation in striatal cell models of HD as blocking NMDA receptors with APV reduces aggregate formation (Okamoto et al., 2009). Blocking synaptic transmission with tetrodotoxin (TTX) also reduces aggregate formation, leading to the conclusion that synaptic NMDA receptors are required for mutant huntingtin aggregate formation. It is also thought that the chaperonin TRiC (T complex-1 (TCP-1) ring complex) detoxifies mutant huntingtin by forming multiple mutant huntingtin inclusions and decreasing toxic soluble forms of aggregates (Pickett, 2006). Blocking synaptic activity (as assessed by blocking with TTX) as well as blocking NMDA receptor activity significantly reduces TCP-1 expression (Okamoto et al., 2009). Therefore, synaptic NMDA receptor activity induces the expression of TCP-1, which then mediates mutant huntingtin aggregate formation.

It is debatable whether huntingtin aggregates are neurotoxic or neuroprotective (see Section 4.1.2). Nevertheless it was shown that activation of both synaptic and extrasynaptic NMDA receptors with exogenous application of glutamate rendered mutant huntingtin neurons vulnerable to cell death, an effect which could be significantly reduced with low doses of memantine or ifenprodil. Low dose memantine has been shown to preferentially block extrasynaptic NMDA receptors (Parsons et al.,
2007, Okamoto et al., 2009) and ifenprodil has been shown to be relatively selective for NR2B containing NMDA receptors (Williams, 1993), which are thought to be extrasynaptically located (Tovar and Westbrook, 1999) (but see (Petralia et al., 2010)). Therefore while synaptic NMDA receptor activity is required for aggregate formation, it is thought to be neuroprotective whereas extrasynaptic NMDA receptor activity causes cell death in mutant huntingtin neurons. If the activation of NMDA receptors with synaptic vs. extrasynaptic locations underlie cell death and neurotoxicity in HD in the hippocampus, it would be of great interest to study the effects of α vs. βSAP97 on synaptic and extrasynaptic glutamate receptor activity.

### 4.1.2 HUNTINGTIN AGGREGATES – TOXIC OR NOT?

Aggregates of the mutant huntingtin protein that contains an expanded poly(Q) length have generally been thought to be toxic to cells. Interestingly, one study showed that it is not the poly(Q) length that influences disease risk but rather the aggregation efficiency of the protein (Yang et al., 2002). This study chemically synthesised poly(Q) peptides that contained a fluorescence tag and were expressed in mammalian cell lines. They show that when poly(Q) aggregates are localised to the cytoplasm they have little impact on cell survival. However, when the poly(Q) peptide was synthesised to also contain a nuclear localisation signal, the aggregates were localised to the nucleus and lead to dramatic cell death in these cultures regardless of the poly(Q) length. Similarly another study showed that in cultured striatal neurons, mutant huntingtin-induced neurodegeneration only occurred if the aggregated protein was expressed in the nucleus and not when it was exported from the nucleus with a nuclear export signal incorporated into the mutant huntingtin transgene for transfection (Saudou et al., 1998). Furthermore, inhibition of polyglutamine oligomerization promotes clearance of polyglutamine repeats in vivo and in vitro (Sanchez et al., 2003). When polyglutamine oligomerization is prevented in the R6/2 mouse model of HD, the pathogenic weight loss, diabetes as well as dyskinesia of the hindlimbs were all significantly reduced (Sanchez et al., 2003). Therefore both the polyglutamine oligomerization and the specific location of aggregates have important implications for cell death.

However, if mutant huntingtin aggregates are toxic, we could hypothesise that they should be found more readily in the cells that are most susceptible to cell death in HD such as the MSNs. Contrary to this notion, HD post mortem human brain tissue show that there are more huntingtin aggregates in the cerebral cortex than in the striatum where most of the cell death occurs (Gutekunst et al., 1999). In fact, only 1-4% of striatal neurons across various grades of HD have nuclear aggregates (Gutekunst et al., 1999). Also, neuropil aggregates are more frequent than nuclear aggregates and are present in great numbers before symptom onset in HD patients (Gutekunst et al., 1999).
Therefore polyglutamine aggregation is possibly not a predictor of cell death in HD and leads to the hypothesis that huntingtin aggregates may be protective against polyglutamine neurotoxicity.

Indeed later studies showed that inclusion bodies (aggregated huntingtin) prolong survival of striatal neurons in culture that are transfected with 47Q length huntingtin (Arrasate et al., 2004). Neurons that formed an inclusion body 4-6 days after transfection survived significantly longer than cells that had no inclusion bodies. Inclusion body formation was also associated with a decreased risk of death compared to WT transfected neurons (17Q). This effect was not specific to striatal neurons as the result could be reproduced in a PC12 cell line. Interestingly, inclusion body formation was correlated with a decrease in diffuse forms of huntingtin and diffuse huntingtin was a negative predictor of cell survival (this opposes the results of (Yang et al., 2002)). Survival analysis showed that both diffuse cytoplasmic and nuclear forms of expanded poly(Q) huntingtin impact neuronal survival. Both neurons with diffuse mutant huntingtin and nuclear inclusion bodies survive longer than cells without any inclusion bodies. Therefore, while some studies show that aggregate formation protects against cell death, others show that it is toxic.

4.1.3 CELL CULTURE MODELS IN HD

Various studies have used cell culture models to study HD either by making primary cultures from HD animal models (P0) or by transfecting cultured neurons with mutant huntingtin. Striatal cultures made from YAC128 mice have been used to show that there are increased GluN2B mediated currents in YAC128 MSNs and that they are sensitized to glutamate-induced apoptosis (Zhang et al., 2008). They also showed that this glutamate-induced apoptosis was due to the loss of mitochondrial membrane potential, which could be prevented by inhibiting GluN2B containing NMDA receptors or mGluR1/5 containing receptors (Zhang et al., 2008). Another study using primary striatal cultures from YAC128 animals showed that NMDA receptor-mediated currents and GluN2B expression are normal in mutant MSNs (Fernandes et al., 2007). However, when co-cultures of cortical and striatal neurons were made from YAC128 animals, it was revealed that the mutant MSNs have increased extrasynaptic NMDA receptor-mediated currents and GluN2B expression, which underlies their vulnerability to cell death (Milnerwood et al., 2012). However, studies have also made striatal cultures from control animals and transfected the neurons with either N-terminal fragments of mutant huntingtin or full length mutant huntingtin (Okamoto et al., 2009). Using this culture model system to study HD they have shown that synaptic NMDA receptor activity induces mutant huntingtin inclusion formation and extrasynaptic NMDA receptor activity causes mutant huntingtin-containing MSNs to be vulnerable to cell death (Okamoto et al., 2009).
Furthermore, the plasmid that we use in our study here (see Experimental Procedures) to express wild type and mutant forms of huntingtin have been used in cell lines to study HD. It has been used in neuronal PC12 cells as a cell-based screen for drugs to treat HD (Aiken et al., 2004). Using the pBWN expression vector they screened the neuroprotective effects of 1040 compounds (each at five different concentrations) against huntingtin-induced cell death. Interestingly, this study revealed that caspase inhibitors and cannabinoids show reproducible protection against cell death and are promising agents for treating HD. This plasmid has also recently been used in an in vitro model to study cannabinoid receptor agonists as neuroprotective agents in HD (Scotter et al., 2010). This study used neuronal PC12 cells expressing the wild type (25Q) or mutant (97Q) huntingtin pBWN construct, induced with 0-1 µM tebufenozide (TFZ) and measured cell death and aggregate formation in cultures that received different treatments including HU210 (a CB1 agonist). Interestingly, they found that the CB1 receptor agonist was protective against cell death through a G-protein alpha subtype i/o (Gi/o) pathway but increased aggregate formation through coupling with Go, which may hinder its use as a therapeutic. Nevertheless, they show that increased TFZ concentration increases the number of cells expressing huntingtin as well as the number of cells with huntingtin aggregates (97Q).

Moreover, cultured dissociated hippocampal neurons as we have used in our study here have been used for many decades as an in vitro model to address a variety of research questions. Dissociated hippocampal cultures were first developed in 1977 for the purpose of morphogenetic studies to investigate factors that are responsible for the unique shape of different types of neurons and to determine the mechanisms that underlie their distinct connectivity (Banker and Cowan, 1977). These first pioneering studies characterised cell adhesion to the coverslip, neurite outgrowth, cell density, cell type, long term cell survival and the morphology of the dissociated hippocampal cells (Banker and Cowan, 1977). Although the intact hippocampal circuit is lost in dissociated cultures, remarkably they could still distinguish between CA1 and CA3 pyramidal cells in culture by silver staining (Banker and Cowan, 1977). Furthermore, morphological comparisons between dissociated hippocampal neurons and those neurons in vivo, indicated that the rate of process formation and the general form of the neurons are remarkably the same (Banker and Cowan, 1979). Hippocampal neurons also develop polarity in culture where at first all processes are short but within a few hours in culture one process starts to grow rapidly and becomes the axon whereas other processes slowly elongate days later and become the dendrites (Dotti et al., 1988). Interestingly, this process does not require intercellular contacts as hippocampal neurons grown in culture at a very low density still express the features of axonal and dendritic differentiation as per normal (Bartlett and Banker, 1984a).
Perhaps more importantly is the synaptic contacts that are established between hippocampal neurons in vitro. In electron microscopic studies of dissociated hippocampal neurons grown in culture, the majority of synapses on dendritic spines are asymmetric (i.e. excitatory) whereas those on the cell body are symmetric (i.e. inhibitory) (Bartlett and Banker, 1984b). Similarly, in electron microscopy studies of hippocampal slices, excitatory synapses are most prominently located on dendrites and inhibitory synapses are located on the pyramidal cell body (Blackstad and Flood, 1963, Andersen et al., 1964, Andersen et al., 1966). Both types of synapses may be observed on dendritic shafts although the asymmetric excitatory synapses are more abundant (Bartlett and Banker, 1984b). Morphological measurements with the use of electron microscopy have also shown similar measurements for cultured dissociated hippocampal neurons and hippocampal slices, for example the spine length is estimated to be 1.15 – 1.5 µm at 2 weeks in culture as compared to adult rat hippocampal slices where the spine length is estimated to be 0.95 ± 0.42 µm (Harris and Stevens, 1989, Papa et al., 1995). Therefore, the types of synapses, synaptic polarity and morphology that form in culture occur very similar to when they develop in situ.
4.2 AIMS

The emerging role of synaptic vs. extrasynaptic NMDA receptors and excitotoxicity in neurodegenerative disease has been reviewed in Chapters 1 and 3. Here we aim to explore the use of dissociated hippocampal cultures to elucidate the mechanisms underlying possible synaptic dysfunction in HD. It is not known if extrasynaptic NMDA receptor activity is altered in the hippocampus in HD as it has been described in the striatum (Milnerwood et al., 2010, Milnerwood et al., 2012). Therefore, in this chapter we describe experiments addressing whether there is a unique phenotype in mutant huntingtin cells as compared to wild type cells by measuring synaptic AMPA and NMDA receptor-mediated currents as well as total NMDA receptor-mediated current (synaptic plus extrasynaptic receptors). Our study here is therefore the first to study synaptic and extrasynaptic changes in NMDA receptor-mediated function in wild type and mutant huntingtin transfected hippocampal neurons in culture. Given the gaps in our knowledge of hippocampal NMDA receptor-mediated signalling in HD and the great experimental potential that lies in a mutant huntingtin cell model, we aimed to investigate the following:

1. To establish a transient cell model using cultured dissociated hippocampal neurons that express both wild type and mutant forms of huntingtin

2. To qualitatively assess mutant huntingtin aggregate formation in this cell model

3. To measure baseline synaptic transmission through synaptic AMPA and NMDA receptors with the use of recordings from synaptically connected pairs of hippocampal neurons

4. To measure total NMDA receptor-mediated currents (synaptic plus extrasynaptic receptors) between pairs of connected hippocampal neurons with the use of TBOA

The establishment of a transient cell model of HD will help us explore if altered synaptic and extrasynaptic glutamate receptor activity underlies altered synaptic function in HD in hippocampal neurons. The experiments proposed aim to establish if mutant huntingtin expression alters synaptic AMPA and NMDA receptor-mediated transmission in hippocampal neurons. It will also reveal if extrasynaptic NMDA receptor-mediated currents are altered by mutant huntingtin and if this is the same or different in our hippocampal neurons as compared to striatal MSNs. It is hoped that these data will lead to future studies addressing the mechanism of receptor displacement in HD, taking into account the advantages this simple cell system brings.
4.3 EXPERIMENTAL PROCEDURES

4.3.1 PLASMIDS

The plasmids used in this study were a kind gift from Dr Eric Schweitzer (Aiken et al., 2004). The inducible pBWN plasmid (Figure 19) is an ecdysone-responsive expression vector, produced by Steve Suhr and Fred Gage at the Salk Institute (Suhr et al., 1998). This plasmid contains a constitutively active CMV-β-actin promoter which directs the expression of the nuclear hormone receptor for ecdysone (EcR). Application of the non-steroidal insect hormone TFZ causes activation and nuclear translocation of a heterodimeric receptor complex composed of the ecdysone receptor and endogenous retinoid X receptors (RXR) (Suhr et al., 1998). This complex then binds to the ecdysone receptor-responsive promoter, inducing gene expression. This plasmid contains a synthetic DNA insert encoding exon 1 of human huntingtin containing either 25 (WT huntingtin) or 97 (mutant huntingtin) mixed CAG/CAA repeats fused to a C-terminal enhanced green fluorescent protein (EGFP) tag (Kazantsev et al., 1999, Scotter et al., 2010). Transgene expression was induced by the addition of 0.5 µM TFZ, an ecdysone (insect hormone) analog (Suhr et al., 1998, Aiken et al., 2004, Scotter et al., 2010). 0-1 µM TFZ has been shown to steeply increase aggregate number in PC12 cells from 24 hrs to 72 hrs (Aiken et al., 2004, Scotter et al., 2010).

4.3.2 BACTERIAL TRANSFORMATION

Aseptic technique was used for all bacterial work. First, 50 µl of E.coli DH5α competent bacterial cells (stored at -80°C, Invitrogen 18265-017) were thawed on ice. To this, 0.5 µl of plasmid DNA was added, gently mixed and incubated on ice for 30 min. The E.coli was then heat shocked to lyse cells
and aid DNA uptake at 37 °C for 40 sec and the bacteria returned to ice for 2 min for membranes to reform. Next, 950 µl of LB broth (Merck 110285) was added to the bacteria and incubated at 37 °C for 1 hour shaking at 225rpm. 100 µl of bacteria was then spread with a glass spreader onto an ampicillin (50 µg/mL, Roche 10835242001) containing agar (Merck 110283) plate to select for the transformed bacteria. Plates were incubated overnight at 37 °C for bacteria to grow. The next day, a well isolated colony of bacteria was selected using a sterile pipette tip and added to 8 ml of LB broth containing 50 µg/mL ampicillin and incubated overnight at 37 °C, shaking at 225rpm.

4.3.3 MINI PLASMID PREPARATION

Mini plasmid preparations were performed with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen K2100-10). 1 ml of the bacterial LB broth was removed and centrifuged at 7000 g for 3 min and all supernatant removed by aspiration. The remainder of bacterial LB broth was stored at 4 °C for later use. The pellet was resuspended in 250 µl resuspension buffer with RNase A (R3: 20 mg/ml RNase A, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) and 250 µl lysis buffer added (L7: 1% SDS, 200 mM NaOH) and the tube gently inverted to mix. After 5 min of lysis, 350 µl precipitation buffer (N4) was added and gently mixed by inversion. This mixture was centrifuged at 12000 x g for 10 min to clarify the lysate from lysis debris. The supernatant was then loaded onto a spin column placed inside a wash tube. This was then centrifuged at 12000 x g for 1 minute after which the flow through was discarded and the column was placed back in the tube. DNA trapped in the column was then washed with 500 µl wash buffer with ethanol (W10) and centrifuged as before. Hereafter the DNA was eluted by placing the column in a clean tube, adding 75 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and centrifugate at 12000 x g for 2 min. A nanodrop was used to determine the DNA concentration and the DNA then stored at -20 °C.

4.3.4 MAXI PLASMID PREPARATION

Maxi plasmid preparations were performed with the PureLink™ HiPure Plasmid DNA Purification Kit (Invitrogen K2100-17). The column was equilibrated with 30 ml of equilibration buffer (EQ1: 0.1 M Na acetate, pH 5.0, 0.6 M NaCl, 0.15% Triton X-100) inserted into the filtration cartridge (inserted into the maxi column). Solution in the column was drained by gravity flow. 400 ml of the overnight LB bacterial culture was centrifuged at 4000 x g for 10 min, 4 °C and all the supernatant removed. 10 ml of resuspension buffer with RNAse A (R3) was added to resuspend cells, then 10 ml lysis buffer (L7) was added and mixed by inversion. After about 5 min at room temperature, 10 ml precipitation buffer (N3: 3.1 M K+ acetate, pH 5.5) was added and mixed by inversion. The precipitated lysate was then transferred into the equilibrated filter column and left to feed through by gravity flow. The
filtration cartridge was discarded and the DNA trapped in the maxi column was washed with 50 ml wash buffer (W8: 0.1 M Na⁺ acetate, pH 5.0, 825 mM NaCl). The DNA was then eluted by adding the elution buffer (E4) and collecting the purified DNA in an elution tube.

### 4.3.5 Precipitating DNA and Gel Electrophoresis

DNA was precipitated using isopropanol and centrifugation. Isopropanol was added to the DNA in the elution tube and centrifuged at 12,000g for 30 min at 4 °C. Hereafter ethanol was used to resuspend the DNA pellet and centrifuged again as before for 5 min. The DNA pellet was air dried and resuspended in TE Buffer. Agarose gel electrophoresis with the use of Syber Safe (Invitrogen S33101) was performed to identify certain molecular weight bands for each plasmid and for future reference (Figure 20). The DNA was mixed with EcoR1 (Invitrogen 15202-013) and BamH1 (Invitrogen 1520-023) restriction enzymes and React 3 buffer (Invitrogen Y90004) and incubated for 1 hour at 37 °C. The digested DNA and 1Kb ladder was loaded onto an agarose gel and run at 80 V for 40 min. The gel was imaged hereafter using the GelDoc system (Bio-Rad). These restriction enzymes produced 2 bands of 5500 bp and 3600 bp for each of the plasmids. The DNA concentration was determined using a Nanodrop and TE Buffer as a blank.

![Example gel of pBWN plasmids.](image)

All three plasmids, pBWN-GFP, pBWN-2SQ and pBWN-97Q produced two main high molecular weight bands of 5500 bp and 3600 bp for all plasmids when digested with EcoR1 and BamH1 restriction enzymes.
4.3.6 DISSOCIATED HIPPOCAMPAL CULTURES

All culturing procedures were done under sterile conditions in a Class II Biological Safety Cabinet (Heraeus). First, 13 mm round coverslips were washed with 70% nitric acid (BDH101686E) and washed in water several times before being stored in 100% ethanol. Next, the ethanol was flamed off the coverslips and 4 coverslips added per well of a 6 x 35 mm well plate (Flacon 353046). To coat coverslips so that cells would adhere, 800 µl of 10 µg/mL poly-D-lysine (Sigma P1149) made in in 1x phosphate buffered saline (PBS; in mM: 136.89 NaCl, 2.68 KCl, 10.15 NaH₂PO₄ and 1.76 KH₂PO₄) was added per well and incubated for 4 - 24 hrs at 37 °C. To remove the poly-D-lysine, coverslips were washed once with sterile PBS. All dissecting tools were sterilised with heat using the tool steriliser (Steri 350: Sigma Z378585) and the hood sterilised with 70% ethanol and UV (20 min).

Postnatal day zero (P0) Wistar rat pups were decapitated in accordance to guidelines set by the University of Auckland Animal Ethics Committee. A scalpel was used to expose the brain by cutting through the skull. The brain was then scooped into a dish with ice cold Hanks' Balanced Salt Solution (HBSS: 9.5 g HBSS, 2.38 g HEPES, pH 7.2 with 5 M NaOH). Any meninges were removed using tweezers and the two hemispheres were gently pushed back with curved tweezers while anchoring the brain at the cerebellum using another tweezer. Next, a cut was made with the tweezers to separate the hippocampus and the surrounding hemisphere from the midbrain. Any meninges and blood vessels surrounding the hippocampus were removed and the hippocampus was removed from the rest of the tissue using the curved tweezers and snipping around the hippocampus. The hippocampi were then placed in fresh ice cold HBSS.

The hippocampi were dissociated using one vial of papain (Worthington Biochemicals LK003178) in 5 ml prewarmed Hank's balanced salt solution (HBSS) and incubated at 37 °C for 15 min while inverting the tube every 5 min. Hereafter, the papain was removed and 5 ml of warm enzyme inactivation solution [4.5 mL Minimum Essential Medium (MEM: Gibco 11090-081) with 0.5 mL Fetal Bovine Serum (FBS: Gibco 10091-148)] was added and incubated for 1 – 2 min. The enzyme inactivation solution was then removed and 1 ml of prewarmed culture media added containing: neurobasal media (NBM, Gibco 21103-049), B27 (Gibco 17504-044) and 1x glutamax (Invitrogen 35050-061). The hippocampi were then triturated with a fine bore, fire polished, glass pipette. The dissociated cell suspension was then added to prewarmed culture media (1 ml cell suspension to 12 ml media) and 2 ml added per well of the 6 well plate. Plates were then transferred and maintained in a 5 % CO₂ incubator (Sanyo) at 37 °C. The day after cultures were made, 0.5 ml of culture media was replaced per well with fresh media.
4.3.7 TRANSFECTION

Dissociated hippocampal neurons were transfected with plasmid DNA via the Ca$^{2+}$ phosphate precipitate method. Using this method the DNA precipitate is taken up by mammalian cells via phagocytosis while the Ca$^{2+}$ phosphate precipitate helps protect against DNA degradation by intracellular nucleases (Loyter et al., 1982). The transfection efficiency with this method is typically 1-5% but can be increased (while maintaining low cell toxicity) to about 60% by manipulating the precipitate formation and size as well as having relatively long incubation periods (Jiang and Chen, 2006). All transfections were done at DIV 8-9. 0.5 ml culture media was replaced with 0.5 ml of fresh media before the transfection. The DNA solution per well contained 6 µg DNA, 60 µl 2x HBS, 7.6 µl 2M CaCl$_2$ and sterile water up to 60 µl. The HBS solution consisted of in mM: 274 NaCl, 10 KCl, 1.4 Na$_2$HPO$_4$·7H$_2$O, 15 D-glucose and 42 HEPES, pH 7.14 with 5M NaOH. The DNA and CaCl$_2$ solution was added to the HBS by tapping the tube to help precipitate formation. The solution was then left in the dark for 20 min. All NBM was removed from the cells and replaced with 1 ml of the transfection cell medium (NBM without B27 plus 50 µM APV and 10 µM CNQX). 120 µl of the DNA solution was then added drop-wise to each well and incubated at 37°C for 25 min. To stop the transfection, coverslips were rinsed 3 times with 1-2 ml of prewarmed HBSS (Sigma H2387, pH 7.2 with 5M NaOH). Hereafter the culture media was returned to each well and the cultures maintained at 37°C until use. To induce the expression of each of the constructs, 0.5 µM TFZ in NBM plus B27, was added to each well at DIV 10-11. 0.5 µM TFZ produced adequate numbers of transfected neurons without producing severe cell death. This allowed us to have an adequate window of opportunity to perform patch clamping experiments on these cells and have sufficient numbers of transfected cells to do paired recordings. Electrophysiology commenced 24hrs after the induction (DIV 11-12).

4.3.8 RECORDING SETUP

For electrophysiology recording dissociated hippocampal neurons grown on coverslips were transferred to a recording chamber (Warner Instruments, RC-26GLP) mounted on a Zeiss AxioScope upright microscope. All recordings were done at room temperature in standard ACSF (in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO$_4$·7H$_2$O, 2.5 CaCl$_2$, 1 NaH$_2$PO$_4$, 26 NaHCO$_3$, 11 glucose) bubbled with carbogen. Hippocampal neurons were visualized using DIC with a 40x dipping objective lens. Membrane currents and potentials were processed with a Multiclamp 700B commander (Axon Instruments, CA, USA) and digitized at 10KHz (Digidata 1440, Axon Instruments, CA, USA) to convert analogue to digital signals. Events were sampled at 10KHz and low-pass filtered at 1 KHz. Series resistance (Rs) was measured and recordings with Rs variation greater than 20% were discarded from the data analysis. Data acquisition and analysis was performed using pClamp 10 acquisition
software and Clampfit 10 respectively (Axon Instruments, CA, USA). Dual whole cell recordings were performed between two connected pairs of neurons with patch pipettes of 3-7 MQ. The postsynaptic recording pipette was filled internal solution (in mM) 120 Cs gluconate, 40 HEPES, 5 MgCl₂, 2 NaATP, 0.3 NaGTP and 5 QX314, pH 7.2, 298 mOsm. The presynaptic recording pipette was filled internal solution (in mM) 120 K gluconate, 40 HEPES, 5 MgCl₂, 2 NaATP and 0.3 NaGTP, pH 7.2 with KOH, 298 mOsm.

**4.3.9 WHOLE CELL PATCH CLAMP ELECTROPHYSIOLOGY**

To measure monosynaptic AMPA receptor-mediated currents, the presynaptic neuron was held in current clamp and stimulated at 0.1 Hz with a 300-700 pA current step of 10 ms duration to induce an action potential. Simultaneously, the postsynaptic cell was voltage clamped at -65 mV. To confirm if the monosynaptic response was an EPSC or inhibitory postsynaptic current (IPSC), the postsynaptic neuron was voltage clamped at -30 mV, and the peak monosynaptic current in response to presynaptic action potential firing was assessed for reversal from an inward current to an outward current. Synaptic responses were judged to be excitatory and monosynaptic if they failed to reverse at -30 mV (but did reverse at 0 mV) and occurred within 5 ms of the peak of the presynaptic action potential (Pavlidis and Madison, 1999). At least 20 AMPA receptor-mediated EPSCs were recorded between each pair of neurons after which 10 µM CNQX (Sigma) was bath applied to block AMPA receptors. This solution also contained glycine (10 µM, Sharlau) and strychnine (2 µM, Sigma), to augment NMDA currents and to block the associated glycine receptor currents respectively. The neuron was then depolarised to +40 mV to record synaptic NMDA receptor-mediated EPSCs. Hereafter, in the same neuron, a solution identical to the one above plus 10 µM TBOA (Tocris Bioscience) was bath applied to block glutamate uptake through GLT1/EAAT2. TBOA blocks both glial and neuronal glutamate uptake and has been shown to increase the NMDA receptor mediated EPSC peak amplitude and significantly slow EPSC kinetics (Diamond, 2001, Arnth-Jensen et al., 2002). TBOA does not interact with NMDA receptors (Jabaudon et al., 1999). Each neuron was then depolarised to +40 mV to record total (synaptic and extrasynaptic) NMDA receptor-mediated EPSCs.

**4.3.11 ANALYSIS**

Analysis was performed with Clampfit 10 software. For all current measurements, the average baseline current was obtained from at least 20 sweeps recorded from each neuron. Data are presented as mean ± SEM. For AMPA receptor-mediated currents, the peak amplitude of the monosynaptic current was measured. For NMDA receptor-mediated currents, both the peak current amplitude and the charge (calculated as the area under the curve) were measured. The peak current
was measured as a 10 ms window at the peak of the transient. The charge was measured from the upstroke of the NMDA receptor-mediated current until 300 ms from the start of the recording.

Statistical analysis was performed with Graphpad Prism software. The Kolmogorov-Smirnov test for normality and Levene test for homogeneity of variances were used to determine if parametric tests could be used. The data presented here did not satisfy the assumptions of a parametric test as well as having small n-numbers in some groups. Therefore, to determine if changes in current amplitudes and charge were statistically significant between groups the Kruskal-Wallis test was used. To determine if changes in current amplitudes and charge were statistically significant between 48 hrs vs. 72 hrs within a particular group (repeated measures), the Wilcoxon signed-rank test was used. Differences were considered statistically significant at p < 0.05.
4.4 RESULTS

4.4.1 RELATIVE DISTRIBUTION OF WT AND MUTANT HUNTINGTIN IN DISSOCIATED HIPPOCAMPAL NEURONS

Because WT and mutant huntingtin have not been examined in hippocampal neurons in vitro, we first assessed the relative distributions of GFP tagged huntingtin protein transfected into dissociated hippocampal neurons. The distribution of GFP and the 25Q huntingtin protein was diffuse throughout the cytoplasm of the neuron as well as in spines, dendrites and the cell body (Figure 21 A, B). Only the 97Q construct produced aggregated mutant huntingtin after 24hrs of expression (Figure 21 C, D) as described previously in PC12 cells (Scotter et al., 2010). These aggregates were often found in the nucleus and occasionally also in the cytoplasm of dendrites and the cell body (Figure 21 C, D). Aggregate size varied greatly between cells with some large intranuclear aggregates and others small (Figure 21C, D). The nuclear aggregates appear similar to intranuclear inclusions described in HD animal models and human HD post-mortem brains (Davies et al., 1997, Becher et al., 1998). Aggregates were not observed in the 25Q or GFP transfected cells at any of the time points examined. The results here parallels features of HD where poly(Q) lengths longer than 36Q causes neurodegeneration (Duyao et al., 1993).

4.4.2 SYNAPTIC AMPA AND NMDA RECEPTOR-MEDIATED CURRENTS IN 25Q AND 97Q TRANSFECTED NEURONS

Next, we determined if synaptic transmission was altered in the presence of mutant huntingtin (97Q) by measuring peak AMPA and NMDA receptor-mediated current amplitudes at 48hrs and 72hrs post-induction. This was performed with the use of paired recordings between two connected hippocampal neurons, where the postsynaptic neuron contained the GFP tagged transfected protein (Figure 22 A). The peak monosynaptic AMPA receptor-mediated current amplitude was similar (p = 0.29) at 48hrs between GFP (mean = -125.63 ± 47.83, n = 10 paired recordings), 25Q (mean = -124.98 ± 31.88, n = 14 cells) and 97Q (mean = -149.24 ± 25.53 pA, n = 15 paired recordings) transfected neurons (Figure 22).

Furthermore, at 72hrs post-induction, there was no significant difference (p = 0.28) between GFP (mean = -216.30 ± 38.35, n = 5 paired recordings), 25Q (mean = -132.40 ± 34.5, n = 11 paired recordings) and 97Q (mean = -164.85 ± 27.1, n = 18 paired recordings) transfected neurons (Figure 22). We also determined if peak AMPA receptor-mediated current amplitudes were becoming significantly larger over time (from 48 hrs to 72 hrs) due to maturation of the neurons (Wu et al.,
For GFP transfected neurons AMPA receptor-mediated current amplitudes significantly increased over time ($p = 0.04$). There was no significant increase from 48 hrs to 72 hrs for 25Q transfected neurons ($p = 0.91$) or 97Q transfected neurons ($p = 0.80$).

We then measured the synaptic NMDA receptor-mediated current amplitudes by blocking the AMPA receptor-mediated current (see Experimental Procedures). For synaptic NMDA receptor-mediated current amplitudes, at 48hrs there was no significant difference ($p = 0.98$) between GFP (mean = 20.43 ± 4.61, $n = 5$ paired recordings), 25Q (mean = 28.36 ± 8.24 pA, $n = 9$ paired recordings) and 97Q (mean = 23.86 ± 4.61 pA, $n = 13$ paired recordings) transfected neurons (Figure 23). At 72hrs, there was again no difference ($p = 0.89$) between GFP (mean = 43.41 ± 17.62 pA, $n = 9$ paired recordings), 25Q (mean = 22.79 ± 4.35 pA, $n = 9$ paired recordings) and 97Q (mean = 29.97 ± 5.38 pA, $n = 16$ paired recordings) transfected neurons (Figure 23).

We also determined if peak NMDA receptor-mediated current amplitudes were becoming significantly larger over time (from 48 hrs to 72 hrs). There was no significant increase from 48 hrs to 72 hrs for GFP transfected neurons ($p = 0.5$), 25Q transfected neurons ($p = 0.95$) or 97Q transfected neurons ($p = 0.92$). Therefore synaptic transmission at all time points was similar for all transfections.
Figure 21. Dissociated hippocampal neurons transfected with GFP, WT (25Q) and mutant (97Q) huntingtin constructs.

(A) GFP and (B) 25Q expressing hippocampal neurons had a diffuse distribution of the transfected protein. (C) 97Q neurons had diffuse and small nuclear aggregates of the mutant huntingtin protein or (D) often large nuclear and dendritic aggregates with no diffuse distribution (transmitted light image of D is shown in E). Arrows indicate nuclear aggregates, arrowheads indicate dendritic aggregates. Scale bar 25µm.
Figure 22. Synaptic AMPA receptor peak current amplitudes in GFP, WT (25Q) and mutant (97Q) transfected hippocampal neurons.

(A) Schematic showing a paired recording between an untransfected presynaptic neuron (Pre) and a transfected postsynaptic (Post) neuron. A monosynaptic EPSC (indicated by arrow) is measured in response to the presynaptic action potential. (B) At both 48hrs and 72hrs post-induction of the plasmid expression, there were no significant differences amongst the different transfections. There was a significant (p = 0.04) increase in AMPA receptor current amplitude from 48 hrs to 72 hrs in GFP transfected neurons only. Example traces of paired recordings for GFP transfected neurons (C), 25Q transfected neurons (D) and 97Q transfected neurons (E) are shown. Polysynaptic excitatory and inhibitory currents were frequently observed in paired recordings. These occurred at a longer latency and did not interfere with the monosynaptic response.
Figure 23. Synaptic NMDA receptor peak current amplitude in GFP, WT (25Q) and mutant (97Q) transfected hippocampal neurons.

At both 48hrs and 72hrs post-induction of the plasmid expression, there were no significant differences amongst the different transfections. Example traces of paired recordings for each transfection are shown.
4.4.3 SYNAPTIC AND TOTAL NMDA RECEPTOR-MEDIATED CHARGE IN 25Q AND 97Q TRANSFECTED NEURONS

We aimed to determine if mutant huntingtin caused altered extrasynaptic NMDA receptor-mediated currents as has been previously observed in striatal neurons (Milnerwood et al., 2010, Milnerwood et al., 2012). To do this we measured total NMDA receptor-mediated charge (consisting of both synaptic and extrasynaptic NMDA receptors) by blocking glutamate uptake in the presence of TBOA. Any changes in total NMDA receptor-mediated charge would provide us with an indication of whether changes in extrasynaptic NMDA receptor-mediated current are occurring.

The synaptic NMDA receptor-mediated charge was similar at 48hrs (p = 0.90) between GFP (mean = 3224.59 ± 1027.21 pA.ms, n = 5 paired recordings), 25Q (mean = 4614.22 ± 1505.64 pA.ms, n = 9 paired recordings) and 97Q (mean = 3869.25 ± 853.46 pA.ms, n = 13 paired recordings) transfected neurons (Figure 24 A). At 72hrs there was still no significant difference (p = 0.86) between GFP (mean = 7308.85 ± 3231.72 pA.ms, n = 9 paired recordings), 25Q (mean = 3696.00 ± 803.52 pA.ms, n = 9 paired recordings) and 97Q (mean = 5054.69 ± 872.99 pA.ms, n = 16 paired recordings) transfected neurons (Figure 24 A).

We also determined if NMDA receptor-mediated charge was becoming significantly larger over time (from 48 hrs to 72 hrs). There was no significant increase from 48 hrs to 72 hrs for GFP transfected neurons (p = 0.69), 25Q transfected neurons (p = 0.86) or 97Q transfected neurons (p = 0.97). Therefore synaptic NMDA receptor-mediated charge was similar at all time points for all transfections.

Since there was no change at any of the time points in synaptic NMDA receptor-mediated charge, we next measured total (synaptic plus extrasynaptic receptors) NMDA receptor-mediated charge. However, there was no significant difference (p = 0.85) between GFP (mean = 5721.53 ± 2246.98 pA.ms, n = 3 paired recordings), 25Q (mean = 3283.87 ± 1436.25 pA.ms, n = 3 paired recordings) and 97Q (mean = 5952.51 ± 2894.387 pA.ms, n = 4 paired recordings) transfected neurons at 48hrs (Figure 24 B). Similarly, at 72hrs there was also no significant difference (p = 0.70) between GFP (mean = 9819.32 ± 7705.73 pA.ms, n = 4 paired recordings), 25Q (mean = 4678.81 ± 1004.23 pA.ms, n = 5 paired recordings) and 97Q (mean= 4307.71 ± 1120.89 pA.ms, n = 3 paired recordings) transfected neurons (Figure 24 B). The average total NMDA receptor charge (Figure 24 B) may be smaller than the average synaptic NMDA receptor charge (Figure 24 A) due to the small increases in charge produced by the application of TBOA within a particular neuron and a greater number of recordings of synaptic NMDA receptor
charge as compared to total NMDA receptor charge. There was no significant increase in total NMDA receptor-mediated charge from 48 hrs to 72 hrs for GFP transfected neurons (p = 0.59), 25Q transfected neurons (p = 1.00) or 97Q transfected neurons (p = 0.59). Therefore total NMDA receptor-mediated charge was similar at all time points for all transfections.

We also aimed to see if the types of connections that formed between pairs of connected neurons were altered by the expression of mutant huntingtin. The types of connections between paired recordings were classified as monosynaptic excitatory, monosynaptic inhibitory or not connected (no monosynaptic connection between pairs of neurons). Both 25Q and 97Q expressing neurons formed elevated numbers of monosynaptic excitatory connections above that recorded for GFP, with 97Q having more than 25Q (15.2% in GFP, 27.53% in 25Q and 43.39% in 97Q pairs; Figure 25). The opposite occurred for inhibitory connections where both 25Q and 97Q pairs had lower numbers of inhibitory connections compared to GFP pairs with 97Q and 25Q having similar percentages (45.65% in GFP, 31.88% in 25Q and 33.96% in 97Q). However, 40.13% of GFP pairs were not connected and a similar number was observed in 25Q pairs (40.58%) but in 97Q pairs this percentage was about half (22.64% of 97Q pairs not connected). These results indicate that on average paired recordings in 97Q transfected cultures had more connected pairs of neurons and more excitatory connections as compared to other transfections.
Figure 24. Synaptic vs. total NMDA receptor charge in paired recordings of GFP, WT (25Q) and mutant (97Q) transfected neurons.

(A) At both 48hrs and 72hrs post-induction of the plasmid expression, there were no significant differences in synaptic NMDA receptor charge amongst the different transfections. (B) Similarly, for total NMDA charge at both 48hrs and 72hrs post-induction of the plasmid expression, there were no significant differences amongst the different transfections. (C) Example traces are shown for paired recordings for each transfection.
Figure 25. Types of connections between pairs of cells in GFP, WT (25Q) and mutant (97Q) transfected cultures.

There were more excitatory connections in 25Q and 97Q cells as compared to GFP and fewer inhibitory connections. Similar percentages of pairs were not connected in GFP and 25Q-expressing neurons but fewer pairs in the 97Q cultures were not connected.
4.5 DISCUSSION

Here we created a transient cell model of HD by transfecting dissociated hippocampal neurons \textit{in vitro} with GFP, wild type huntingtin (25Q) or mutant huntingtin (97Q). We show that transfection with mutant huntingtin results in aggregate formation, with both diffuse and nuclear locations, within 24hrs of inducing protein expression, similar to that found in HD in the human brain (DiFiglia et al., 1997, Becher et al., 1998, Gutekunst et al., 1999). In this study we aimed to determine if there was a unique phenotype in mutant huntingtin transfected hippocampal neurons with regard to basal synaptic transmission between pairs of connected neurons as well as their extrasynaptic NMDA receptor-mediated current. We measured this as total NMDA receptor-mediated currents with the use of TBOA to block glutamate uptake. TBOA blocks GLT1, which is present on both glia and neurons (Rothstein et al., 1994, Chen et al., 2004), resulting in glutamatergic activation of synaptic and extrasynaptic receptors (Diamond, 2001, Arnth-Jensen et al., 2002, Milnerwood et al., 2010). We report here no significant changes in synaptic transmission through AMPA and NMDA receptors and no significant changes in extrasynaptic NMDA receptor-mediated current in mutant huntingtin transfected neurons. Therefore our results indicate that while increased extrasynaptic NMDA receptor-mediated currents and toxicity are reported in dissociated mutant huntingtin MSNs of the striatum (Okamoto et al., 2009, Milnerwood et al., 2012) this does not occur in dissociated mutant huntingtin hippocampal neurons. Therefore, the pathophysiology in the hippocampus in HD is not due to increased extrasynaptic NMDA receptor activity but other mechanisms underlie synaptic dysfunction in the HD hippocampus.

4.5.1 SYNAPTIC TRANSMISSION AND TOTAL NMDA RECEPTOR-MEDIATED CURRENTS ARE UNALTERED IN 97Q MUTANT HUNTINGTIN HIPPOCAMPAL NEURONS

Here we report a lack of any significant change in AMPA and NMDA receptor-mediated currents in mutant huntingtin neurons. This may not be surprising as we also showed no change in these measurements in the YAC128 hippocampal slices in Chapter 3. Therefore our HD hippocampal cell model accurately replicates (at least up to 72hrs post-induction) what occurs in the YAC128 (1 month of age) hippocampus with regard to synaptic and extrasynaptic transmission through glutamate receptors and could therefore be used to elucidate other mechanisms of the pathophysiology in HD. The mutant 97Q transfected neurons present with HD-like aggregates at 24hrs post-induction, hence mechanisms that underlie the pathophysiology in these neurons (e.g. altered extrasynaptic NMDA receptor-mediated currents) should be present within the time frame that we recorded. However, we report a significant increase in AMPA receptor mediated current
amplitude from 48 hrs to 72 hrs in GFP transfected neurons only, which reflects normal neuronal
maturation (Wu et al., 1996). As we do not report this in 25Q or 97Q transfected neurons, changes in
postsynaptic receptor number may only be revealed later than 72 hrs and it will be of interest to
extend this study to a longer time period of 1 – 2 weeks post-transfection.

In dissociated striatal MSN cultures from both the YAC128 mouse model and from MSN cultures
transfected with N-terminal mutant (148Q) huntingtin for 48 – 92 hrs, increased extrasynaptic
NMDA receptor-mediated currents and toxicity have been shown (Okamoto et al., 2009,
Milnerwood et al., 2012). Our results indicate that changes in synaptic and extrasynaptic
glutamatergic transmission do not occur in hippocampal neurons transfected with mutant (97Q)
huntingtin. This lack of a significant change may be due to the polyQ (97Q) expansion not being
severe enough as compared to the 148Q huntingtin used in striatal cultures (Okamoto et al., 2009).
However it is also likely that the pathophysiological phenotype observed in striatal MSNs is not the
same in hippocampal neurons in HD. This is interesting as mechanisms other than changes in NMDA
receptor-mediated currents could underlie the pathophysiology of the hippocampus in HD.

In HD the striatum is the primary site of severe cells loss and volume changes (Vonsattel et al., 1985)
whereas cell loss occurs to a lesser extent in the human hippocampus (Spargo et al., 1993, Rosas et
al., 2003) or is spared in animal models of HD (Slow et al., 2003, Van Raamsdonk et al., 2005a). The
increased extrasynaptic NMDA receptor signalling that occurs in the HD striatum (Milnerwood et al.,
2010, Milnerwood et al., 2012) is thought to underlie the MSN death (Hardingham et al., 2002,
Okamoto et al., 2009). On the other hand, the lack of a significant change in extrasynaptic NMDA
receptor signalling as we have shown here in mutant huntingtin-expressing hippocampal neurons
correlates with the relative sparing of hippocampal neurons in HD. However, cognitive deficits and
memory impairment related to hippocampal function occurs early in the HD process in humans and
animal models (Foroud et al., 1995, Lawrence et al., 1998, Van Raamsdonk et al., 2005b, Paulsen et
al., 2008). Therefore mechanisms such as altered MAGUK-dependent trafficking of glutamate
receptors during LTP and LTD (thought to underlie memory and learning) may be the origin of
cognitive deficits in HD. We have not yet measured LTP or LTD in the HD culture model system used
here but regulators of LTP and LTD are likely to underlie cognitive pathophysiology rather than
altered extrasynaptic signalling.

Other mechanisms that could underlie hippocampal pathophysiology in HD may be related to
changes in presynaptic function. We did not measure and compare presynaptic glutamate release
probability in mutant huntingtin transfected neurons with the use of PPF or spontaneous mEPSCs,
which are routinely used to assess presynaptic function (reviewed in (Thomson, 2000)). However,
although not measured in cultured dissociated hippocampal neurons transfected with mutant huntingtin, acute hippocampal slices from HD animal models have shown no change in presynaptic function (our results in Chapter 3, (Hodgson et al., 1999, Murphy et al., 2000, Milnerwood et al., 2006)).

4.5.2 DISASSOCIATED HIPPOCAMPAL NEURONS TRANSFECTED WITH PBWN-97Q AS A MODEL FOR HD

One of the advantages of using dissociated hippocampal cultures is that they can be easily transfected with DNA of interest where both the pBWN plasmid and another protein of interest can be transfected into a single hippocampal neuron (Goetze et al., 2004). Therefore primary hippocampal neuronal cultures used as a cellular model for disease is very flexible and rapid investigations can be performed to study synaptic function in HD. Here in our dissociated hippocampal cell model of HD, the 97Q mutant huntingtin produced aggregates of the mutant protein in both the nucleus and cytoplasm as found in HD in the human brain where aggregate number corresponds to CAG repeat length and increase in size in advanced grades of the disease (DiFiglia et al., 1997, Becher et al., 1998, Gutekunst et al., 1999).

4.5.2.1 Glutamate Uptake in Dissociated Cultures

In dissociated hippocampal cultures the cells are not as densely packed as in hippocampal slices, therefore the important effect of glial cells on glutamate uptake will be limited in dissociated cultures. As reviewed in Chapter 1, glutamate uptake transporters such as GLT-1 are present on glial and neuronal cells in the hippocampus and remove glutamate quickly from the synaptic cleft after release (Rothstein et al., 1994, Chen et al., 2004). Glia are abundant in number and tightly packed in close proximity to neurons in hippocampal slices but less so in our dissociated neuronal cultures. The use of TBOA to block the GLT-1 transporter was found to be less efficient in our dissociated hippocampal neurons as compared to hippocampal slices. This reduced efficiency was observed as a lack of change in NMDA receptor-mediated charge with the application of TBOA in some paired recordings. This is most likely due to the lack of abundant numbers of closely packed astroglia in the dissociated cultures. The low number of recordings in TBOA may also contribute to the lack of a significant change in total NMDA receptor-mediated current that we report here. Since glutamate uptake is important in the context of our study of synaptic vs. extrasynaptic NMDA receptor activation, future experiments must investigate this with methods other than blocking GLT-1. For example, extrasynaptic NMDA receptor-mediated currents could be measured by applying exogenous NMDA via a Picospritzer to the dendrites of the postsynaptic cell to measure total NMDA
receptor-mediated current (e.g. (Li et al., 2011)). This can then be compared to the synaptic NMDA receptor-mediated currents that we have measured here.

4.5.2.2 Glutamate spillover and the effect of temperature in dissociated cultures

Glutamate spillover during our synaptic NMDA receptor-mediated current recording will cause extrasynaptically located receptors to be already activated during the synaptic recording and may lead to an underestimation or a lack of change in the total NMDA receptor-mediated current in the presence of TBOA. Spillover in acute slices have been shown to occur only when there is intense tetanic stimulation of the presynaptic fibres or when glutamate uptake is blocked (see Chapter 3) (Diamond, 2001, Arnth-Jensen et al., 2002). Presynaptic stimulation frequency was minimal (0.1 Hz) in our recordings and only one presynaptic cell was stimulated to fire a single action potential (unlike in the slice where many presynaptic fibres are stimulated at once), hence our presynaptic stimulation protocol did not cause any spillover during our synaptic recording.

In hippocampal dissociated cell cultures it has been shown that glutamate transporters buffer free glutamate in the cleft slower at room temperature as compared to 34 °C (Tong and Jahr, 1994, Diamond and Jahr, 1997). These experiments were performed with the use of a rapidly dissociating AMPA receptor competitive antagonist kynurenate (KYN) and glutamate transporter blocker D,L-threo-bhydroxyaspartic acid (THA) while measuring AMPA receptor-mediated mEPSCs. KYN binds and unbinds from AMPA receptors during the rising phase of the mEPSC as it is replaced by glutamate in a competitive manner. The addition of THA produces a prolonged glutamate transient. At room temperature blocking transporters slows the mEPSC rise time but does not affect its amplitude (Diamond and Jahr, 1997). This is due to the transporters buffering glutamate during the first few hundred microseconds after release and thereby slowing the decay phase of the glutamate transient but not affecting its peak (which occurs within 60 µs of vesicle fusion (Bruns and Jahn, 1995)). On the other hand, at 34 °C transporter blockers increase the mEPSC amplitude (Tong and Jahr, 1994). This indicates that the transporters bind glutamate fast enough at higher temperatures to affect the peak glutamate concentration in the cleft but at room temperature the buffers act over a slower time course without affecting peak glutamate concentration in dissociated cultured neurons.

The effect of temperature has been shown in both dissociated cultures and slices where glutamate transport is more efficacious at physiological temperatures in removing cleft glutamate and preventing spillover (Tong and Jahr, 1994, Asztely et al., 1997, Diamond and Jahr, 1997). Increased temperature is reported to increase transporter turnover rate (Schwartz and Tachibana, 1990), which would result in faster removal of free glutamate from the synaptic cleft. Even if transporter
turnover rate is very fast, these transporters still need to be located very near the synaptic cleft close to release sites to produce adequate glutamate buffering capacity. Thus, glutamate transporters in dissociated cultures still remove free glutamate from the cleft but less effectively at room temperature and less effectively as compared to slices. Therefore possible glutamate spillover to extrasynaptic sites may be present in our cultures during synaptic stimulation in the absence of TBOA and thereby render our TBOA application less efficient at affecting total NMDA receptor-mediated current.

4.5.2.3 Receptor Saturation and the effect of temperature in dissociated cultures

Furthermore, the interpretation of our TBOA results is affected by whether there is synaptic glutamate receptor saturation during our synaptic stimulation before TBOA application. If synaptic receptors are not saturated in the absence of TBOA then applying TBOA will increase both the synaptic pool of receptors as well as activating the extrasynaptic pool. As discussed in Chapter 3, AMPA and NMDA receptors are not saturated by a single transmitter release event in brain slices at physiological temperature (Mainen et al., 1999). However, if glutamate transporters are not as effective at removing cleft glutamate in dissociated cultures especially at room temperature, then AMPA and NMDA receptors may be saturated during a single release event.

In dissociated hippocampal cultures at room temperature activation of a single presynaptic terminal (by local application of a high Ca²⁺ solution), produces large variation in EPSC size between local applications (Liu et al., 1999). If postsynaptic receptors were saturated, the peak amplitude should remain relatively constant but instead a great variation in amplitude is observed. This group also compared the glutamate-evoked current, produced by applying a saturating dose of glutamate by focal iontophoretic application at a single synaptic site, with the mEPSC current in the same hippocampal neuron. Their results showed that the median mEPSC size was only 40% that of the maximal glutamate-evoked current. This indicates that under normal conditions endogenously released glutamate is insufficient to saturate AMPA receptors at a single synapse at room temperature in dissociated hippocampal cultures. Furthermore, NMDA receptors have a much greater affinity for glutamate (Patneau and Mayer, 1990) and as a result it can be expected that even if AMPA receptors are not 100% saturated, NMDA receptors may be saturated. However, later it was shown that NMDA receptors are also not saturated during a single release event at room temperature in cortical dissociated cultures (Umemiya et al., 1999). Therefore, our experiments at room temperature indicate that TBOA may increase synaptic NMDA receptor-mediated currents as well as extrasynaptic pools of NMDA receptors.
4.5.2.4 Cell types recorded from in dissociated hippocampal neurons

Dissociated hippocampal cultures are no longer part of an intact hippocampal circuit as it is in hippocampal slices and it is difficult to discern between pyramidal neurons and interneurons by cell morphology using DIC optics (see Experimental Procedures). Interneurons can be identified electrophysiologically by their lack of spike frequency adaptation (Schwartzkroin and Mathers, 1978, Kawaguchi and Hama, 1987), which is a phenomena that occurs in pyramidal neurons and is due to Ca²⁺-activated K⁺ channels (BK channels) (Lancaster and Nicoll, 1987, Storm, 1990). However, the use of cesium (Cs⁺) in our internal solution in the postsynaptic neuron precludes the identification of interneurons electrophysiologically as Cs⁺ blocks voltage-gated K⁺ channels and thereby affects action potential firing. Cs⁺ depolarises resting membrane potential by blocking delayed outward K⁺ currents (Chandler and Meves, 1965, Adelman and Senft, 1966) as it binds to the inner mouth of the pore of the K⁺ channel that is relatively non-selective for certain cations (unlike the rest of the channel) (Bezanilla and Armstrong, 1972). Cs⁺ prolongs the action potential duration from the normal 2 ms to approximately 45 ms and causes it to have a long plateau phase (Sjodin, 1966), hence spike frequency adaptation cannot be measured.

Hippocampal interneurons also contain AMPA and NMDA receptors which mediate EPSCs (Baude et al., 1995, He et al., 1998). AMPA receptors on inhibitory interneurons are kinetically different from those on excitatory neurons. For example, faster spontaneous EPSC decay times and faster desensitisation rates in response to glutamate occur in interneurons (Hestrin, 1993, Livsey et al., 1993). However, such kinetic measurements are time consuming to perform before commencing the experiments done here in our study. Therefore the postsynaptic neuron that we recorded from may have been a transfected interneuron or a hippocampal pyramidal neuron. In intact slices (Chapter 3) this is largely avoided as interneurons are located in specific layers in the hippocampus (see Chapter 1). Future experiments could inject current immediately once the whole cell mode is obtained as the cell will not be dialysed by the internal Cs⁺ solution at this point and action potential firing properties could be recorded. However, this time frame must be determined experimentally.

4.6 FUTURE DIRECTIONS

To measure total NMDA receptor-mediated currents more efficiently in dissociated cultures, future experiments must make use of exogenous NMDA applied with the use of a Picospritzer to the dendrites of a transfected neuron and compared between GFP, wild type 25Q and mutant 97Q transfected neurons. This should also be done at physiological temperatures as glutamate transporters are more efficient at removing free glutamate at these temperatures (see Section 4.6.1).
(Tong and Jahr, 1994, Asztely et al., 1997, Diamond and Jahr, 1997). This will add to our knowledge obtained from this study where synaptic transmission did not appear to be altered by mutant huntingtin in hippocampal neurons. Alongside these experiments, LTP and LTD should be measured in WT and mutant huntingtin transfected neurons to determine whether the presence of mutant huntingtin alters synaptic plasticity in this cell model of HD. It would also be of interest to use our transient cell model of HD to study the effect of important hippocampal inputs (e.g. cortical input) on excitatory transmission. When NMDA receptor-mediated currents and GluN2 expression was assessed in MSNs cultured from YAC128 animals, no increase in NMDA receptor-mediated current or GluN2 expression was found (Fernandes et al., 2007). However, when co-cultures of cortical as well as striatal neurons were made from YAC128 animals, a unique phenotype emerged where extrasynaptic NMDA receptor-mediated current and GluN2 surface expression was significantly increased (Milnerwood et al., 2012). Therefore, cortico-hippocampal cultures may be interesting to study with regard to their excitatory transmission when mutant huntingtin is present after transfection or when cultures are made from YAC128 animals. Cortical input to the hippocampus CA1 area has been shown to be important for the consolidation of long term memory, plasticity and spiking in CA1 pyramidal neurons (Remondes and Schuman, 2002, 2004). The cultures from YAC128 animals can be made either from P0 pups as we have done here and in other studies (Fernandes et al., 2007, Milnerwood et al., 2012) or from adult animals, which have been done successfully is control animals (Brewer, 1997).

Molecular mechanisms underlying changes in synaptic vs. extrasynaptic glutamate receptor currents in HD has not been explored. This should be studied in the striatum where altered synaptic vs. extrasynaptic NMDA receptor-mediated currents have been shown, in contrast to our lack of a significant change in mutant huntingtin hippocampal neurons. Due to the role of SAP97 in trafficking glutamate receptors to synaptic and extrasynaptic sites in an isoform-dependent manner (Waites et al., 2009, Li et al., 2011), studies involving α and βSAP97 and their role in HD will further our understanding of synaptic dysfunction in HD. These experiments will make use of a transient HD cell model using striatal cultures or cortico-striatal co-cultures and involve co-transfections of the pBWN vector and either α or β SAP97.

It will also be important to assess the expression levels of GLT-1 in our transient HD cell model and to determine if GLT-1 transporters function normally or abnormally in the mutant huntingtin neurons. The effect of temperature on glutamate transport should be included in this study. GLT-1 function will have important consequences for extrasynaptic NMDA receptor-mediated currents, glutamate spillover and EPSC kinetics. Altogether the suggested future experiments will further our
understanding of synaptic and extrasynaptic excitatory transmission in the hippocampus and striatum in HD and the possible mechanisms underlying HD pathophysiology.
4.7 CONCLUSION

Here we have produced a transient cell model of HD by expressing wild type 25Q and mutant 97Q huntingtin protein in primary neuronal cultures of the hippocampus. Only mutant 97Q-expressing neurons formed diffuse and nuclear aggregates of the mutant huntingtin protein. Using this model system of HD we have found no significant changes in synaptic transmission (through AMPA and NMDA receptors) or in total NMDA receptor-mediated current (involving synaptic plus extrasynaptic NMDA receptors) in mutant 97Q cells. Further experiments with exogenous application of NMDA will more efficiently measure total NMDA receptor-mediated currents in this HD model system. Our results in hippocampal neurons are in contrast to increased extrasynaptic NMDA receptor signalling in MSNs of the striatum in a model of HD (Milnerwood et al., 2010, Milnerwood et al., 2012). Hence, our results suggest that hippocampal extrasynaptic NMDA receptor-mediated currents are not affected by mutant huntingtin and that other mechanisms, such as presynaptic function, could underlie hippocampal pathophysiology in HD.
CHAPTER FIVE: GENERAL DISCUSSION

5.1 SYNAPTOPATHY IN THE HIPPOCAMPUS: INSIGHTS INTO BRAIN DISEASE

The work in this thesis examined the mechanisms underlying synaptic dysfunction in neurodegenerative disease by studying the expression of important glutamatergic synaptic proteins as well as synaptic function. The synapse is the elementary unit of the brain and is the anatomical locus for cell communication and synaptic plasticity. The significant results found here in the human brain and the electrophysiological results found in the hippocampus in a cell model and animal model of HD has furthered our understanding of synaptopathy in neurodegenerative disease. Both cognitive and motor symptoms appear before overt neuronal cell loss in the brain in HD (Vonsattel et al., 1985, Levine et al., 2004, Cepeda et al., 2007), which suggests that cognitive and motor deficits are caused by cellular dysfunction rather than overt neuronal death. Studying synaptic function therefore may reveal early changes that occur in the brain in response to disease. We show here that significant and specific changes occur in synaptic protein expression and synaptic plasticity in response to neurodegenerative disease.

The medium spiny neurons (MSNs) of the striatum are particularly vulnerable and eventually die in the HD human brain (Vonsattel et al., 1985, Vonsattel and DiFiglia, 1998) and in HD animal models such as the YAC128 (Slow et al., 2003, Van Raamsdonk et al., 2005a). However the principal cells of the hippocampus are relatively spared in animal models of HD (Mangiarini et al., 1996, Hodgson et al., 1999, Van Raamsdonk et al., 2005a) although cell loss in the CA1 region of the hippocampus is known to occur in the human brain in HD (Spargo et al., 1993). Although the hippocampus is relatively spared with regard to cells loss in HD animal models, we have shown reduced synaptic plasticity (LTP) in YAC128 hippocampal slices similar to that reported in the YAC72 (Hodgson et al., 1999) and other animal models of HD (Usdin et al., 1999, Murphy et al., 2000). Huntingtin aggregate formation in the hippocampus has been shown to correlate with this deficit in synaptic plasticity and cognitive decline (Murphy et al., 2000). Therefore regardless of cell loss, important changes are occurring at the synaptic level in the hippocampus that could underlie the cognitive deficits in both human HD and animal models of HD. Synaptic dysfunction in the hippocampus is therefore of particular interest as cognitive deficits related to the hippocampus in HD (see Chapter 1) precede motor deficits both in human cases (Foroud et al., 1995, Lawrence et al., 1998, Paulsen et al., 2008) and in the YAC128 model of HD (Van Raamsdonk et al., 2005b) and as stated before, occurs before
overt neuronal loss in the human brain. Therefore changes in synaptic function are evident early in
the disease process and are of great significance to therapeutic strategies. The hippocampus is also
an area less studied than the striatum in PD and HD, which is involved in the classical motor
symptoms present in these diseases. Pathophysiological changes found in the hippocampus will be
greatly useful for future therapeutic strategies that are focussed on early intervention and future
studies of the mechanisms underlying the disease process.

5.2 THE SYNAPTIC FOOTPRINT IN HD AND PD IS DIFFERENT IN THE
HIPPOCAMPUS VS. THE STRIATUM

5.2.1 MAGUK AND GLUTAMATE RECEPTOR SUBUNIT EXPRESSION

In the human brain, using immunohistochemistry, we show that differential and in some cases
opposite changes occur in the hippocampus vs. the striatum in both HD and PD. The changes in
expression of synaptic scaffold proteins is expected to alter glutamate receptor trafficking and
localisation as both SAP97 and PSD95 are known to bind glutamate receptors (Kornau et al., 1995,
Leonard et al., 1998, Chen et al., 2000, Schnell et al., 2002, Gardoni et al., 2003). In human HD we
report significant increases in the hippocampus in SAP97, PSD95 and GluN1 with no change in GluA2
levels. In the striatum on the other hand we report no change in SAP97 and GluN1 and a decrease in
PSD95 and GluA2. This indicates that a unique “synaptic footprint” exists in HD in the hippocampus
vs. the striatum, which will have important consequences for therapeutic strategies targeting
cognitive vs. motor deficits. We have shown here that in the human hippocampus these synaptic
changes involve a dysregulation of the expression of glutamatergic receptor subunits and synaptic
scaffolding proteins. While these proteins are mostly upregulated in the hippocampus, they show no
change or a downregulation in the human striatum in our study.

It is intriguing that the synaptic footprint with regard to MAGUK and glutamate receptor subunit
expression in HD is different in the hippocampus vs. the striatum. One reason for this could be that
mutant huntingtin alters striatal vs. hippocampal neurons differently. The function of the huntingtin
protein is largely unknown although it binds to and regulates various proteins including transcription
factors such as NF-KB (Takano and Gusella, 2002), the synaptic scaffold protein PSD95 (Sun et al.,
2001) and signalling molecules such as calmodulin (a Ca\(^2+\) binding protein) (Bao et al., 1996).
Aggregated mutant huntingtin is present in both the human striatum and hippocampus (Gutekunst
et al., 1999). In HD it is known that excitotoxicity and MSN loss occurs extensively in the striatum
(Vonsattel et al., 1985, Zeron et al., 2002, Fan and Raymond, 2007). Interestingly, BDNF expression is
specifically reduced in the striatum as the presence of mutant huntingtin causes a deficit in vesicular transport of BDNF (Gauthier et al., 2004). This reduced BDNF expression along with defects in mitochondrial energy metabolism that also occur specifically in the human HD striatum (Gu et al., 1996) causes MSNs to be sensitised to excitotoxicity. This increased sensitivity may lead to the reduced MAGUK expression and AMPA receptor subunit expression in the human HD striatum that we have reported. This may occur to reduce signalling through glutamate receptors and thereby attempt to reduce excitotoxicity. On the other hand, in the hippocampus cognitive deficits and memory impairment are related to reduced LTP (Hebb, 1949, Bliss and Lømo, 1973, Murphy et al., 2000). This may trigger the increase in MAGUK and glutamate receptor subunit expression that we report in the human hippocampus in an attempt to increase trafficking and anchoring of glutamate receptors at the synapse during LTP. In contrast it may also have a causative effect and lead to the hippocampal cell loss reported in human HD (Spargo et al., 1993, Rosas et al., 2003) by causing excitotoxicity through increased glutamate receptor activity. It is difficult to know if our results in the human brain are a cause or consequence of the disease. Altogether, the increased sensitivity of MSNs to excitotoxicity in HD vs. altered synaptic plasticity in hippocampal neurons render the synaptic footprint in these two neuronal populations very different.

Furthermore, in the YAC128 animal model of HD we show no changes in total synaptic protein expression in the hippocampus at 12 months of age (symptomatic) while in the striatum most other animal models of HD at symptomatic ages show decreased total striatal levels of SAP97 (Torres-Peraza et al., 2008), decreased total striatal levels of PSD95 (Jarabek et al., 2004, Torres-Peraza et al., 2008), no change in synaptic GluA2 (Benn et al., 2007, Torres-Peraza et al., 2008) and either an increase in total GluN1 (Ariano et al., 2005) (Cepeda et al., 2001) or no change in total striatal GluN1 (Jarabek et al., 2004, Fan et al., 2007, Torres-Peraza et al., 2008). This difference may reflect the severe cell loss in the striatum at 12 months of age in the YAC128 while the hippocampus is relatively spared in this model (Slow et al., 2003, Van Raamsdonk et al., 2005a). Therefore, with regard to total expression levels of synaptic proteins, our results here report a different synaptic footprint in the hippocampus vs. the striatum in both human HD and an animal model of HD.

Moreover, while we show no change in total expression levels of synaptic proteins in the YAC128 hippocampus, redistribution of MAGUKs and/or glutamate receptors may occur. However, our electrophysiological data in YAC128 hippocampal slices does not support this redistribution in the hippocampus (at 1 month of age) as synaptic AMPA and NMDA receptor currents and extrasynaptic NMDA receptor currents are normal as compared to WT slices. On the other hand, a redistribution of MAGUKs and their bound glutamate receptors has been shown to occur in the striatum of HD
animal models and thereby alter synaptic vs. extrasynaptic receptor activity. In the YAC128 striatum PSD95-NMDA receptor complexes are redistributed to non-PSD sites where they mediated increased NMDA receptor-mediated currents, which are linked to cell death pathways (Hardingham et al., 2002, Fan et al., 2009, Milnerwood et al., 2010, Fan et al., 2012, Milnerwood et al., 2012).

Similarly, in PD we show differential changes to occur in the hippocampus vs. striatum in the human brain. While SAP97 and PSD95 are upregulated in the hippocampus alongside no changes in glutamate receptor subunits, there is a decrease in GluA2 in the striatum alongside no changes in other synaptic proteins. Interestingly, in the striatum NMDA receptors are trafficked between subcellular compartments in a dopamine dependent manner. Specifically, dopamine D1 receptor agonists have been shown to redistribute NMDA receptor subunits from intracellular compartments to synaptosomal membranes (Dunah et al., 2000, Dunah and Standaert, 2001). Therefore the loss of dopaminergic input to the striatum in PD may cause a redistribution of synaptic proteins rather than a change in total protein levels as we have measured here in the human striatum. Certainly in an animal model of PD it has been shown that both PSD95 and SAP97 are redistributed away from the synapse into vesicular compartments in the striatum (Nash et al., 2005). On the other hand, our results indicate that upregulation of SAP97 and PSD95 occurs specifically in the hippocampus, possibly in an attempt to restore cognitive and memory deficits (Emre, 2003, Whittington et al., 2006, Ziemssen and Reichmann, 2007). Memory deficits in PD have been shown to be related to reduced CA1 hippocampal LTP as well as reduced CaMKII phosphorylation (Moriguchi et al., 2012). The upregulation of MAGUKs that we found in our study may occur in an attempt to increase glutamate receptor trafficking to the synapse to restore LTP.

5.2.2 SYNAPTIC VS. EXTRASYNAPTIC NMDA RECEPTOR ACTIVITY IN HD

The work in this thesis was the first to investigate the functional changes in NMDA receptors in the hippocampus in both an animal and cell model of HD, specifically investigating synaptic vs. extrasynaptic NMDA receptor function. In the striatum of YAC128 animals at 1 month of age, increased extrasynaptic NMDA receptor signalling occurs (Milnerwood et al., 2010) however we have shown no change in synaptic or extrasynaptic NMDA receptor signalling in the hippocampus at this age. Therefore, again the synaptic footprint is different in the hippocampus vs. the striatum. The striatal MSNs abundantly express GluN2B subunits in both rat and human brain (Landwehrmeyer et al., 1995, Rigby et al., 1996). GluN2B subunits are thought to be mostly located at extrasynaptic sites (Williams, 1993, Tovar and Westbrook, 1999, Hardingham et al., 2002) (but see (Petralia et al., 2010)). The increased extrasynaptic NMDA receptor signalling in the striatum has been attributed to a redistribution of NMDA receptor subunits from the PSD to non-PSD sites (Milnerwood et al., 2010).
and has been linked to apoptotic pathways (Hardingham et al., 2002). This renders MSNs particularly vulnerable in HD. While increased extrasynaptic NMDA receptor signalling is thought to underlie cell death in the striatum as well as the classic motor symptoms in HD we find that this is not the mechanism that underlies cognitive decline and synaptic plasticity deficits in HD in the hippocampus. The unaltered extrasynaptic NMDA receptor activity that we report in the hippocampus may underlie the relatively sparing of hippocampal neurons reported in the YAC128 and other animal models of HD (Mangiarini et al., 1996, Hodgson et al., 1999, Van Raamsdonk et al., 2005a) which is not the case in the striatum. It will be very important to determine if this phenotype persists in the hippocampus in older YAC128 animals where the disease has progressed.

Furthermore, memantine (an open channel NMDA receptor blocker, see Chapter 3) has been shown to reverse the motor deficits in the YAC128 model of HD, thought to be due to the preferential blocking of extrasynaptic NMDA receptors (Milnerwood et al., 2010). It is currently used for the treatment of moderate to severe Alzheimer’s disease (AD) and significantly improves cognition (as assessed with neuropsychological tests for semantic memory involving the medial temporal lobe) (Reisberg et al., 2003, Tariot Pn and et al., 2004, de Jonghe et al., 2009) and delays time to death (Zhu et al., 2013). However, it has also been shown that memantine does not improve the rate of total brain or hippocampal atrophy in AD (Wilkinson et al., 2012). When used for the treatment of HD, memantine significantly improves motor symptoms but fails to improve cognition (Ondo et al., 2007, Milnerwood et al., 2010). This indicates that the synaptic pathology underlying cognitive deficits in AD but not in HD involves altered extrasynaptic NMDA receptor activity. The use of memantine in HD to preferentially block extrasynaptic NMDA receptors seems to be only effective in the striatum (involved in motor deficits) but is not effective in the hippocampus (involved in cognitive and memory decline). The results in this thesis provide further support for this as we find no change in extrasynaptic NMDA receptor activity in the hippocampus in an animal or hippocampal cell model of HD, which could render blocking of extrasynaptic receptors ineffective as a treatment.

5.2.3 PRESYNAPTIC GLUTAMATE RELEASE

In this thesis we have shown no change in presynaptic release probability in the YAC128 hippocampus at 1 month of age (Chapter 3). Similarly, other studies in hippocampal slices from HD animal models show no alterations in presynaptic release as assessed by PPF (Hodgson et al., 1999, Murphy et al., 2000, Milnerwood et al., 2006). In the cortex there is also no change in PPF in an animal model of HD (Dallérac et al., 2011). However in contrast to this, in the striatum of the YAC128 animal model of HD significantly increased PPF (indicating reduced release probability) has been shown at corticostriatal synapses (Milnerwood and Raymond, 2007). This increased PPF in the
YAC128 striatum occurs at 1 month of age, the same age at which we show no change in PPF in the hippocampus. Various presynaptic mechanisms may underlie the reduced striatal release probability including decreased vesicle exocytosis, which has been related to synaptic vesicle release protein complexin II (CPLXII) expression (Reim et al., 2001, Edwardson et al., 2003). CPLXII is specifically depleted in striatal cells in an animal model of HD (Morton and Edwardson, 2001) and also in the human HD brain (DiProspero et al., 2004). Therefore our data shows that presynaptic release probability is differentially affected in HD at glutamatergic synapses in the hippocampus vs. the striatum.

5.3 The Synaptic Footprint in HD is Different in the Human Brain vs. Disease Models

As well as presenting results in this thesis that indicate a different synaptic footprint in the hippocampus vs. striatum, we also show that the footprint within the hippocampus is different between the human brain and animal models. We show that total synaptic protein expression in the human brain is altered but there are no changes in the symptomatic YAC128 animal model (at 12 months of age). Similarly we also highlight important differences with regard to synaptic protein expression in the PD human brain and animal models used in other studies (see Chapter 2). This highlights important differences in synaptopathy in human vs. animal models and shows that future studies in the human brain are important. However, for obvious reasons only the end stage of the human disease can be studied in post-mortem brain tissue and animal models are therefore still greatly valuable to study progressive changes that occur in neurodegenerative disease.

While we show differences in total synaptic protein expression in the HD human brain vs. HD animal model, other studies also show differential results in the human brain vs. animal models regarding neuronal cell loss and volume changes in the hippocampus. Using stereology, the YAC128 hippocampus at 12 months of age has been shown to have a normal volume as compared to WT animals (Van Raamsdonk et al., 2005a). However, it is known that volume analysis using stereology-based techniques are not as sensitive in detecting subtle changes in volume as magnetic resonance imaging (MRI) (Lerch et al., 2008). When using MRI techniques, the YAC128 hippocampal volume has been shown to be normal at 1 month of age with a trend towards a decrease from 3 to 12 months but there was no change in hippocampal volume when it was normalised to total brain volume (Carroll et al., 2011). Therefore, the hippocampus is relatively spared at all ages in the YAC128 mouse model despite the cognitive deficit that is present at a presymptomatic age and continues to decline with disease progression (Van Raamsdonk et al., 2005b).
On the other hand human studies have shown a significant loss (35%) of neuronal density in the CA1 area of the HD hippocampus as compared to control subjects as assessed by stereological techniques (Spargo et al., 1993). Even when MRI based techniques are used there is a significant reduction (9%) in hippocampal volume in human HD (Rosas et al., 2003). Our study shows that the synaptic proteins SAP97, PSD95 and GluN1 are increased in the human HD hippocampus. This may lead to increased glutamatergic signalling, leading to excitotoxicity and cell death. This may underlie the neuron loss reported in HD in the hippocampus. On the other hand, in the YAC128 animal model we report no such changes in synaptic proteins, which may correlate with the relative sparing of the hippocampus in this model (Van Raamsdonk et al., 2005a, Carroll et al., 2011).

5.4 THE ROLE OF MAGUKS IN HUNTINGTON’S DISEASE

While great focus has been placed on excitotoxicity and NMDA receptor function in HD (Fan and Raymond, 2007), studies investigating the link between altered MAGUK expression and glutamate ion channel function in HD are limited. MAGUKs are the downstream organisers of glutamate receptor location and function and are critical for functions such as synaptic plasticity, which is thought to underlie learning and memory in the brain (Hebb, 1949, Bliss and Lømo, 1973). Various groups have investigated MAGUK expression changes with the use of western blot analysis in animal models of HD and shown significant changes to occur in the striatum especially (Luthi-Carter et al., 2003, Benn et al., 2007, Fan et al., 2007, Torres-Peraza et al., 2008). However, these changes in expression have not been directly related to altered glutamate receptor function or trafficking.

On the other hand electrophysiological studies in the striatum of HD animal models have shown increased extrasynaptic NMDA receptor activity, leading to cell death pathways (Hardingham et al., 2002, Milnerwood et al., 2010, Milnerwood et al., 2012). The role of MAGUKs in this mislocalisation of receptors to extrasynaptic sites has only recently been studied in the striatum of YAC128 animals. One group showed that there is an increased association between PSD95 and GluN2B receptors (Fan et al., 2009) and this occurs specifically at non-PSD sites (Fan et al., 2012). This is thought to underlie or contribute to the NMDA receptor toxicity seen in the YAC128 striatum. While we show significant increases in MAGUK expression in the human hippocampus, we do not know if there is also an increase in MAGUK association with glutamate receptors and if this translates into altered glutamate receptor function and trafficking in the human brain. Coimmunoprecipitation studies in the human brain will reveal if there is an increased association but the use of human tissue precludes the use of electrophysiology to study functional changes in glutamate receptor function due to long post-mortem delays.
However, with the use of electrophysiological studies in an animal model of HD we measured glutamate receptor function and synaptic plasticity in the hippocampus in response to HD. Various acute knock down and knock-out mice studies have investigated the effects of the loss of a particular MAGUK on synaptic plasticity and synaptic transmission (Migaud et al., 1998, Stein et al., 2003, Nakagawa et al., 2004, Ehrlich et al., 2007, Howard et al., 2010, Li et al., 2011). Although these studies have greatly contributed to our understanding of the role of MAGUKs, our data indicate that such complete loss of a particular MAGUK in HD does not occur. We have suggested a potential role for SAP97 in the pathophysiology underlying a deficit in hippocampal LTP in HD for various reasons discussed in Chapter 3. While our results show that glutamate receptor surface expression is normal during baseline synaptic transmission, it is likely that the activity-dependent trafficking of AMPA receptors to the synapse during high frequency synaptic activity (such as during LTP induction) is deficient in YAC128 animals. It will therefore be important to continue this study to directly investigate MAGUK-dependent receptor trafficking in response to LTP in HD with a combination of electrophysiological and live cell imaging approaches.

Furthermore, it is not known if the increased PSD95-GluN2B complexes at extrasynaptic sites shown in the HD striatum (Fan et al., 2009, Fan et al., 2012), the increased hippocampal expression of SAP97 and PSD95 in the human brain (Chapter 2) or the potential role of SAP97 in hippocampal synaptic plasticity in HD (Chapter 3) are isoform specific. MAGUK isoform-dependent receptor localisation has been shown for SAP97 where αSAP97 regulates the synaptic location of AMPA receptors and βSAP97 regulates the extrasynaptic location of AMPA and NMDA receptors (Li et al., 2011). It is not known if α and β isoforms of SAP97 and PSD95 are altered in the diseased human brain or in animal models of disease and will be important to determine. NMDA receptors are important for many processes and generally blocking NMDA receptors as a therapeutic target may have serious side effects (Chen and Lipton, 2006, Hardingham and Bading, 2010). However, a novel and more specific therapeutic target may involve regulating MAGUK expression levels in an isoform specific manner to reduce extrasynaptic NMDA receptor signalling that has been reported in the striatum while maintaining adequate levels of synaptic NMDA receptor activity for cell survival. Similarly, targeting a specific isoform of SAP97 may be of therapeutic benefit for rescuing LTP deficits in the hippocampus in HD.

5.5 FUTURE DIRECTIONS

Specific future directions have been discussed in the previous chapters. However, important questions regarding neurodegenerative disease and synaptic function remain. In the human brain
future studies need to determine if the increased expression of MAGUKs and glutamate receptor subunits that we report in the hippocampus also lead to an increased association between MAGUKs and glutamate receptors. Coimmunoprecipitation and/or double fluorescence labelling to determine colocalisation could be used to answer this question. Due to the inherent variability in human tissue, more cases are required for our synaptosome study to determine the subcellular location of the changes in MAGUK and glutamate receptor subunit expression. Together these data will reveal in more detail any changes that occur in MAGUK-dependent glutamate receptor trafficking and the localisation of altered MAGUK and glutamate receptor expression.

Furthermore, we have shown that the synaptic footprint in the YAC128 hippocampus at 1 month of age is different in the hippocampus (Chapter 3) than what is reported in the striatum (Milnerwood et al., 2010). It will be very important to determine if the lack of any significant change in synaptic and extrasynaptic transmission in the YAC128 hippocampus persists in older animals. This will determine if the synaptic footprint in the HD hippocampus remains different to the striatum or if it becomes similar once the disease and the related symptoms have progressed.

Moreover, determining the underlying mechanisms that cause increased extrasynaptic NMDA receptor signalling in the striatum (Milnerwood et al., 2010, Milnerwood et al., 2012) will be of great interest. We suggest that important changes in α vs. βSAP97 may be occurring that underlie the disruption of the balance between synaptic vs. extrasynaptic NMDA receptor activity in the striatum (Li et al., 2011). An HD striatal cell culture model (similar to that described in Chapter 4) will be greatly useful to overexpress or knock down MAGUKs in an isoform specific manner in mutant huntingtin expressing neurons. These experiments will potentially identify new therapeutic targets in the treatment of HD.

5.6 SUMMARY

In summary, the work in this thesis has found altered total levels of MAGUK protein and glutamate receptor subunit expression in the human hippocampus and striatum of HD and PD cases. However, this synaptic footprint was different in the striatum vs. the hippocampus. We also show that these changes are not evident in the hippocampus of the YAC128 animal model of HD at 12 months of age. We found no changes in synaptic vs. extrasynaptic NMDA receptor signalling in the hippocampus of YAC128 animals at 1 month of age or in a hippocampal cell model of HD, unlike that reported in the striatum (Milnerwood et al., 2010, Milnerwood et al., 2012). However, we report reduced synaptic plasticity (LTP) in YAC128 hippocampal slices at 1 month of age and suggest a potential role for
SAP97 in this deficit. Manipulating MAGUK expression in an isoform specific manner may be a novel and more specific therapeutic target for HD in the future.


Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin I (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the
substantia nigra by N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine. Proceedings of the National Academy of Sciences 80:4546-4550.


Disease mouse models: rescue by D1 dopamine receptor activation. Neurodegenerative Diseases 8:230-239.


Johnson KA, Conn PJ, Niswender CM (2009) Glutamate Receptors as Therapeutic Targets for Parkinsons Disease. CNS & Neurological Disorders - Drug Targets (Formerly Current Drug Targets 8:475-491.


Mayer ML, Vylicky L, Clements J (1989) Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine.


Patterson MA, Szatmari EM, Yasuda R (2010) AMPA receptors are exocytosed in stimulated spines and adjacent dendrites in a Ras-ERK-dependent manner during long-term potentiation. Proceedings of the National Academy of Sciences 107:15951-15956.


