Part 2: DESCRIPTION OF THE PROPOSED RESEARCH
Project title: **The signalling pathways involved in NMDAR-dependent LTD**

BACKGROUND

**Synaptic plasticity**

Information storage in the brain depends on changes in the efficiency of synaptic transmission. Synaptic plasticity is the process by which synapses can alter their efficiency of transmission; there are two main long-lasting forms of synaptic plasticity termed long-term potentiation (LTP) and long-term depression (LTD). The principal excitatory neurotransmitter in the brain, L-glutamate, exerts its physiological actions via three types of ionotropic receptors, which are named after their various agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate, as well as a family of G-protein coupled metabotropic glutamate receptors.
Since many synapses in the brain utilise L-glutamate as their neurotransmitter, it comes as no surprise that all classes of glutamate receptors are critically involved in LTP and LTD. Most forms of LTP, and many forms of LTD, are triggered by the synaptic activation of NMDA receptors (NMDARs) and subsequently involve alterations in the efficiency of transmission that is mediated by AMPA receptors (AMPARs)[1]. It has become clear, through the work of many groups, that NMDAR-mediated LTP (hereafter referred to as LTP) and NMDAR-mediated LTD (hereafter referred to as LTD) are general mechanisms for inducing synaptic change and occur at many different excitatory synapses throughout the brain. In all cases they are involved in plasticity events that relate to that particular brain region. In the hippocampus, for example, they are involved in spatial learning and memory[2,3,4], in the perirhinal cortex in the visual recognition memory[5], in the amygdala in plasticity underpinning fear conditioning[6], in the basal ganglia in motor learning[7], in the spinal cord in neuropathic pain[8] and in the neocortex in higher level cognitive functioning.

Most of the studies of LTP and LTD are performed at the Schaffer-collateral commissural pathway, which is the monosynaptic glutamatergic connection between hippocampal CA3 and CA1 pyramidal neurons (CA3-CA1 synapses). Many general principles of synaptic plasticity have been first observed at these synapses. For example, it was at these synapses that NMDARs were discovered to be the primary receptors that trigger the induction of LTP[9] and LTD[10]. Therefore, the study of plasticity at these synapses offers the best hope for a detailed molecular understanding of the synaptic basis of learning and memory.

**Signalling mechanisms involved in LTD**

Most evidence suggests that LTD is due to a reduction in the number of AMPARs at the post synaptic density (PSD)[11]. One likely scenario for LTD expression starts by dissociation of the AMPAR from their anchoring proteins, followed by their lateral movement to the dendritic shaft out of the PSD where they are then internalised[12]. AMPARs comprise four subunits (GluA1-4)[13] arranged in various combinations. The GluA2 subunit is the site of several key interacting proteins, including N-ethylmaleimide sensitive factor (NSF), AMPAR binding protein (ABP), glutamate receptor interacting protein (GRIP) and protein interacting with C-kinase (PICK1)[14]. There is good evidence that LTD involves these proteins[15,16]. For example, it has been shown that the binding of NSF to the GluA2 subunit stabilises AMPARs at synapses[17,18] and that the NSF-sensitive component of synaptic transmission is specifically involved in LTD[15]. Based on the finding that NSF and AP2 (a protein initiating clathrin-dependent endocytosis) bind to overlapping regions of GluA2[19], the current proposed model for LTD induction involves the $Ca^{2+}$-dependent exchange of AP2 for NSF. In support of this, hippocalcin was identified as a $Ca^{2+}$ sensor that likely regulates this exchange[20].

Alterations in protein phosphorylation are known to be crucial in LTD induction. In particular, it is established that LTD involves a protein phosphatase (PP) cascade activation in which $Ca^{2+}$/calmodulin activates PP2B (calcineurin), which then inhibits inhibitor-1 to activate PP1[21,22,23]. More recently, tyrosine phosphorylation of the C-terminal tail of GluA2 has been shown to cause internalisation of AMPARs and is associated with NMDAR-LTD[24,25], leading to the suggestion that a tyr kinase may play a role in this process. Unfortunately, the identity of this kinase is not known. However, several ser/thr protein kinases have been implicated in LTD, including PKA[26,27] (but see ref 28), cyclin-dependent kinase 5 (CDK5)[29], calcium/calmodulin-dependent protein kinase II (CaMKII)[30,31] and p38 MAPK[32].

**The involvement of GSK-3β in synaptic plasticity**

Our own previous work has shown that glycogen synthase kinase-3 β (GSK-3β) is required for LTD[33]. We found that three structurally-distinct inhibitors of GSK-3 blocked the induction of LTD. Furthermore, we found that LTD was associated with the activation of GSK-3β (caused by PP1 induced dephosphorylation of ser9). In addition, we identified a second upstream regulatory pathway, involving PI3K-Akt, by which LTP can directly regulate the induction of LTD. However, the downstream effectors of GSK-3β that mediate its effects on LTD are unknown. GSK-3β is involved in many cellular processes and has numerous substrates comprising metabolic and signalling proteins such as PKA and PP1, structural proteins such as tau and neurofilaments, and transcription factors like CREB and c-Myc[34]. Interestingly, GSK-3β can associate with GluA1 and GluA2[33]. Thus, there are many potential mechanisms by which GSK-3β could be involved in LTD.

**Discovery of a role for JAKs in synaptic plasticity**

We have recently performed a systematic investigation of the role of protein kinases in LTD Twenty-eight inhibitors were applied individually and directly into the cell under investigation via the patch-pipette, to avoid potential problems of access and to minimise the possibility of presynaptic effects. The involvement of over 60 PKs could then be determined in hippocampal LTD. Of these, we found no evidence for PKA, PKC, CaMKII, p38MAPK, cdk5 or src family tyr kinases. Indeed, we found evidence for only two protein kinases, GSK-3 and Janus kinase (JAK): we confirmed the role of GSK-3 in LTD, using three additional inhibitors (including CT99021, the most specific known inhibitor of this kinase[3]). In addition, we discovered a role for a tyr kinase, a member of the JAK family. Some of these unpublished data are shown in Figure 1.

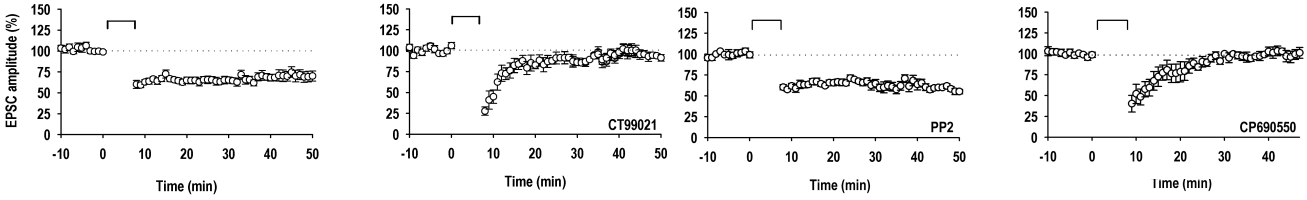



***Figure 1**: GSK-3β and JAK inhibitors block induction of LTD. Pooled data illustrating LTD under control conditions (left, n = 28) or when a GSK-3β inhibitor (CT99021, 1 μM, n = 6), src family tyrosine kinase (PP2, 10 μM, n = 7) or JAK inhibitor (CP690550, 1 μM, n = 5) is applied via the patch pipette. In each panel, the points are the average amplitude of 6 successive EPSCs normalised with respect to the baseline. At t = 0, the neuron was depolarised to -40 mV and stimuli delivered at 0.66 Hz for the duration indicated by the bar.*

Whilst JAK has not been previously implicated in LTD, there is evidence that one or more JAK isoforms are expressed in the hippocampus[36] where they may play a role in synaptic regulation and learning and memory[37,38]. However, nothing is known about the molecular mechanisms involved.

PROGRAMME AND METHODOLOGY

**Overall aims and individual objectives**

The overall aim of this proposal is to determine the molecular mechanism that lead from NMDAR activation to AMPAR internalisation during hippocampal LTD. The basic mechanisms and questions that we wish to address are represented schematically in Figure 2.

In particular, this study will investigate the role of two protein kinases, GSK-3 and JAK, in this process. Our specific objectives are to establish:

1. Which isoform(s) of the JAK family are involved in LTD
2. Whether the activity of JAK is altered during LTD
3. Whether JAK is part of the AMPAR or NMDAR complex
4. What are the upstream regulators of JAK
5. What are the downstream effectors of JAK
6. What are the downstream effectors of GSK-3

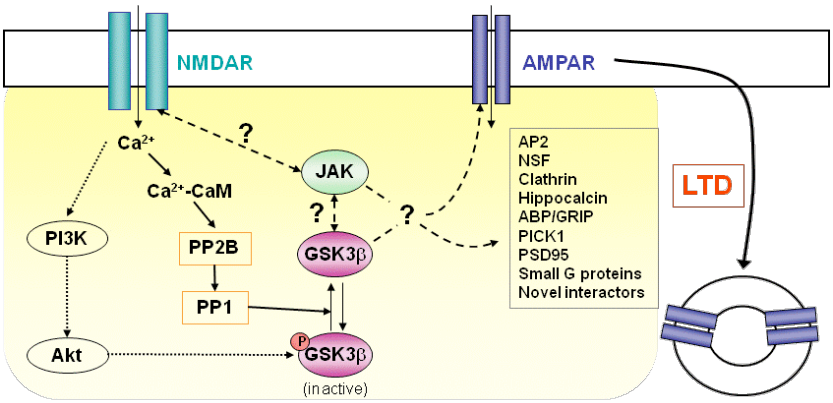
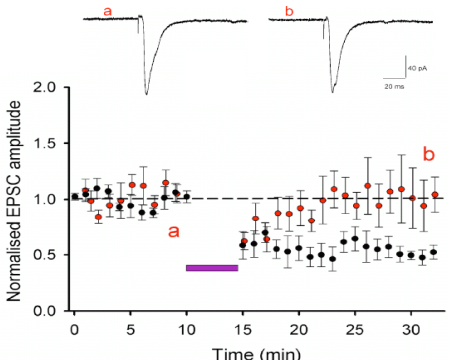



***Figure 2**: Scheme representing some of the main signalling molecules involved, and the questions we wish to address (?) in LTD.*

**1. Which isoform(s) of the JAK family are involved in LTD ?**

We have found that three structurally distinct inhibitors of JAK (CP690550, AG490 and JAK inhibitor I) block the induction of LTD. Whilst this provides strong evidence that a member of the JAK family is required, it provides little information on the isoform, since the inhibitors are not isoform specific. There are four JAK isoforms (JAK1, JAK2, JAK3 and TYK2), of which JAK2 is, by far, the most highly expressed in the brain[39] and the only isoform found in the postsynaptic density (PSD) fraction[36]. It is therefore the strongest candidate. We will therefore complement the pharmacological investigations with experiments using RNA interference (RNAi) to knockdown JAK2, and other isoforms as required (as a control / if we find that JAK2 is not the isoform involved). JAKs are widely studied in other systems and the different short-hairpin RNA (shRNA) required for these experiments are commercialy available. Furthermore, we have established the necessary transfection techniques in house[40] (Figure 3). For additional controls we will use scrambled shRNA. These experiments will be complemented with the use of subtype selective JAK inhibitors, should such compounds become available.

***Figure 3**: Knocking down the expression of hippocalcin blocks the induction of LTD in organotypic hippocampal slices. LTD was blocked in hippocalcin RNAi expressing cells (red circles, n = 5) but reliably induced in interleaved control cells (black circles, n = 5). Insets are representative traces from a hippocalcin RNAi transfected cell at the indicated time* points.




**2. Is the activity of JAK altered during LTD?**

We found that LTD is associated with an increase in the activity of GSK-3β, as assessed by determining the phosphorylation status of the enzyme or by measuring enzymatic activity in microdissected regions of hipocampal slices[33]. We will use similar techniques to determine whether JAK is also activated during LTD. Assuming there is an alteration in the tyrosine phosphorylation status of one or more of the isoforms (revealing their activity[41]), we will determine the time course of the effect. This will inform as to whether JAK is required just for the induction or, like GSK-3, also the expression of LTD.

**3. Is JAK part of the AMPAR or NMDAR complex ?**

The next question that we wish to address pertains to where JAK is located. In the case of GSK-3β we found that it was part of the AMPAR complex. We will perform similar experiments in which we will immuno-precipitate (IP) AMPARs and look for the co-IP of JAK isoforms. We will also perform the reverse co-IPs. Similar experiments will then be conducted with NMDARs. All the antibodies for IP of AMPAR and NMDAR subunits and the different JAK isoforms are available in-house / commercially available and have been widely used. To identify the binding site, a GST-pull down assay will also be performed with mutated or truncated forms of the receptors[40]. If JAK is found to

be part of one or other of these receptor complexes then we can test whether this pool of JAK is regulated during LTD.

**4. What are the upstream regulators of JAK?**

In the case of GSK-3β we were able to identify both activators (PP1) and inhibitors (Akt) of the enzyme and showed that these pathways were operational during LTD and LTP, respectively[33]. For JAK, we have no idea how it might be activated during LTD and whether it is also inhibited during LTP. JAK is usually associated with a receptor by a binding site located in its N-terminal domain[42], and activated following the receptor activation[43]. If JAK is found in the same complex as NMDAR and AMPAR, we will then verify if its activation depends on its association with the receptor / complex. We will, for example, use peptides directed against the interacting domain (identified with the GST-pulldown assay), to selectively disrupt this interaction.

**5. What are the downstream effectors of JAK?**

Similarly we have no information on what the downstream effector(s) of JAK are during LTD. We shall address this in two ways; (i) by investigating logical targets based on the knowledge of JAK from other systems or from what is currently known about LTD, and (ii) by performing a proteomics screen for novel targets.

(i) It has been shown that the GluA2 AMPAR subunit is tyrosine phosphorylated during LTD on residue(s) 869, 873 and/or 876. It was assumed (but not shown) that the kinase responsible is src, or a family member, but our finding that PP2 (Figure 1) and other src PTK inhibitors (unpublished observations) do not block LTD raises the possibility that the PTK responsible might be JAK. We will test this by determining (i) whether the corresponding region of GluA2 is phosphorylated by JAK in a cell free assay and (ii) whether JAK inhibitors block the tyrosine phosphorylation of the GluA2 c-terminal tail that is observed during LTD.

In many other systems, JAK interacts with signal transducer and activators of transcription (STAT). Interestingly, STAT3 is found in the PSD[36] and GSK-3β is required for STAT3 (as well as STAT5) activation by JAK[44]. We will therefore examine whether the activity of STAT3, and other isoforms, are modulated during LTD and whether their inhibition, either by pharmacological means or by RNAi directed against STAT isoforms, has any effect on LTD.

NMDARs are highly tyrosine phosphorylated in the PSD[45] and their function and insertion into membranes are regulated by tyrosine phosphorylation[46,47]. However, little is known concerning the role of NMDAR tyrosine phosphorylation in hippocampal LTD. NMDARs can be regulated by JAK[38] and some JAK2 consensus phosphorylation sites are present in both GluN2A (NR2A) and GluN2B (NR2B) C-terminal tails. Therefore, a modulation of NMDAR function by JAKs could affect the induction of LTD. Initially, we will assess the tyrosine phosphorylation state of NMDARs during the induction of LTD and the effects of JAK inhibitors.

(ii) To look for novel targets for JAK during the induction of LTD, we will use mass spectrometry (MS) on lysates from LTD-induced hippocampal slices, treated with or without JAK inhibitors. For interleaved controls, some slices will be treated with an NMDAR antagonist during the induction of LTD. The phosphorylated peptides will be selected and those containing the JAK phosphorylation sites analysed by MS. We will then confirm that the potential substrate is phosphorylated during LTD by performing western blots of micro-dissected slice tissue. This part of the project will be performed with the expertise and advice of Sir Philip Cohen and Dr. Nick Morrice, MRC Protein Phosphorylation Unit, Dundee, UK.

**6. What are the downstream effectors of GSK-3β**

To investigate the downstream effectors of GSK-3β we will adopt a similar two-pronged strategy as that described in section 5.

The possible targets could be GluA1 or GluA2 since they are in the same complex as GSK-3β[33] and possess GSK-3β consensus phosphorylation sites. Also, β-catenin, a protein linked to the AMPAR complex via its interaction with N-cadherin, is a target of GSK-3β[48]. Phosphorylation of β-catenin by GSK-3β is a signal for ubiquitination which could lead to internalisation of the β-catenin-AMPAR complex.

As the GSK-3β phosphorylation consensus motif is well known, an analysis of the phosphopeptides by MS/MS will also be performed.

Once potential downstream effectors have been identified for GSK-3β, their role in LTD will be established in similar ways to that described above for JAK. In brief, the downstream substrates

will be inhibited using pharmacological agents, if available, and/or using RNAi (specifically designed if not commercially available). Biochemical experiments will also be performed in parallel to assess, for example, the activity of the proteins.

The experiments described above will help us to determine how GSK-3 and JAK interact during the induction of LTD. In other systems, GSK-3β can be upstream of JAK or *vice versa*. Alternatively, these kinases may act in parallel, potentially phosphorylating the same substrate(s)[49,50].

**Methods to be used**

*Electrophysiology:* Conventional whole-cell patch-clamp recording and LTD induction will be used as described previously on acute slices from two-week old rats[33] or organotypic slices[40]. Stimulating electrodes will be placed in the Schaffer collateral-commissural pathway. EPSC amplitude, series resistance and input resistance will be monitored on-line and re-analyzed off-line, using the WinLTP program[51].

*Organotypic slice culture & transfection:* Hippocampal slice cultures will be prepared from 6-8 day old rats and cultured for 7-10 days, as described previously[52]. Neurons will be transfected with various RNAi constructs, using a biolistic gene gun (Helios Gene-gun system, Bio Rad, U.S.A.) at DIV 2–3. Electrophysiological recordings will be performed 3-5 days after transfection.

*Western Blot*: Standard electrophoretic transfer of proteins (sodium dodecyl sulphate polyacrylamide gel electrophoresis; SDS-PAGE) and immunoblot analysis of samples will be used[53]. Briefly, whole cell extracts will be collected from hippocampal slices. The immunoreaction of, for example, anti-GSK-3β, JAK2, GluA2, GluN2B or phospho-tyrosine, will be quantified by optical density, and will be normalized to the levels of, for example, β-actin[19].

*Co-immunoprecipitations (co-IP):* Antibodies will be conjugated to protein G-Sepharose beads by rotating for 2 hr at 4°C. The protein G-antibody-bound beads will be washed, added to 500 µg of lysates and incubated for 4 hr at 4°C. The immunoprecipitates will be washed four times, eluted with sample buffer and western blotting will be carried out[54].

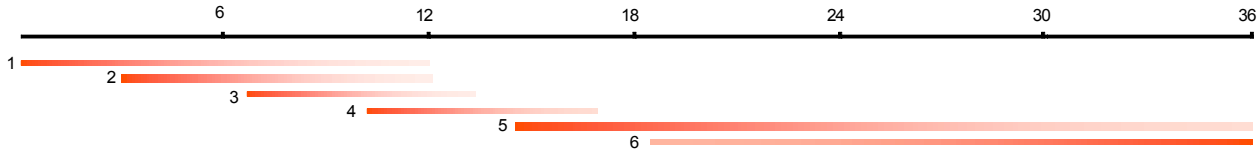
*Mass spectrometry (MS):* Control and treated samples will be lysed and trypsinated to obtain small peptides. The peptides will then be separated by liquid chromatography and globally analysed by MS. The phosphorylated peptides will be selected by using a precursor ion scan of m/z –79 ($PO_3^-$) on a triple quadrupole instrument which permits a selective detection of serine, threonine and tyrosine phosphorylation[55,56]. If needed, prior to the MS analysis, a phosphopeptide enrichment could be performed using hydrophilic interaction chromatography technique (HILIC/IMAC)[57]. The phosphopeptides containing the appropriate sequence (GSK-3β and JAK consensus motif) will be analysed by MS/MS. The use of techniques such as iTRAQ (isobaric tag for relative and absolute quantitation) will allow a relative quantification of the peptides in digest as well as the proteins from where they originate to compare the different conditions (before and after LTD induction, treated and not treated with inhibitors)[58]. Bioinformatics will be used to analyse the several thousand phosphopeptides that will be obtained and map them to the primary sequences of the proteins.

**Timeliness and novelty**: The role of GSK-3β in LTD was recently discovered by the principal applicant's team[33]. Whilst the signalling pathway leading to GSK-3β activation (and inhibition by LTP) is established in this model, nothing is known about how GSK-3β induces the expression of LTD. Furthermore, a very recent investigation in this lab, conducted in part by Celine Nicolas, also showed that from over 60 kinases tested, only GSK-3β and JAK are involved in LTD. These new findings are of great relevance since they constitute the first evidence for a role of JAK in synaptic plasticity and opens up the way to establish a molecular mechanism for a major form of synaptic plasticity. Thus this multidisciplinary project proposed here will establish how JAK and GSK-3β are activated and linked and what are the downstream pathways leading to AMPAR internalisation during LTD. As JAK and GSK-3β have been extensively studied in other fundamental systems, all the materials required to study their role in LTD (inhibitors, antibodies, RNAi) are available. Furthermore, this project will for the first time establish a formal collaboration between the Bristol group and Professor Philip Cohen, an expert in protein phosphorylation and signalling pathways.

**Programme of work and milestones**:

The project will be supervised by the principal applicant (Graham Collingridge) and the co-applicant (Kei Cho). Celine Nicolas (Research co-investigator) who discovered the role of JAK in

LTD and provided pilot data for this proposal will be the main research assistant associated with this project and will realise or manage all the experiments. A half-time technician will also be associated to the project to maintain the organotypic cell cultures and help prepare the constructs and other materials required for the experiments.




1: JAK isoforms implicated in LTD (RNAi in organotypic cultured slices)
2: JAK activity during LTD (phosphorylation state by WB)
3: JAK localisation (co-IP with AMPAR and NMDAR and GST-pull down assay)
4: Upstream regulators of JAK (e.g., inhibitory peptides)
5: Targeted downstream regulators of JAK and GSK3 (biochemistry and electrophysiology)
6: Identification of novel JAK and GSK3 targets and validation of their role in LTD (MS, biochemistry and electrophysiology)

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