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Running Title: A role for GSK3β in synaptic plasticity

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Summary

Glycogen synthase kinase-3 (GSK3) has been implicated in major neurological disorders but its role in normal neuronal function is largely unknown. Here we show that GSK3β mediates a novel interaction between two major forms of synaptic plasticity in the brain, N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) and NMDA receptor-dependent long-term depression (LTD). In rat hippocampal slices, GSK3β inhibitors block the induction of LTD. Furthermore, the activity of GSK3β is enhanced during LTD, via activation of PP1. Conversely, following the induction of LTP there is inhibition of GSK3β activity. This regulation of GSK3β during LTP involves activation of NMDA receptors and the PI3K-Akt pathway and disrupts the ability of synapses to undergo LTD for up to one hour. We conclude that the regulation of GSK3β activity provides a powerful mechanism to preserve information encoded during LTP from erasure by subsequent LTD, perhaps thereby permitting the initial consolidation of learnt information.

Introduction

GSK3 is a multifunctional serine / threonine (ser/thr) kinase that was originally identified as a regulator of glycogen metabolism (Embi et al., 1980). Ubiquitously expressed in eukaryotes (see Ali et al., 2001), GSK3 plays a fundamental role in a wide variety of functions, including the division, proliferation, differentiation and adhesion of cells (Frame and Cohen, 2001; Grimes and Jope, 2001). GSK3 dysfunction is implicated in major diseases including cancer and diabetes (Frame and Cohen, 2001). There are two known isoforms of GSK3 in mammals that are encoded by different genes (GSK3α and GSK3β) (Woodgett, 1990). Only GSK3β, however, is highly enriched in the brain (Leroy and Brion, 1999; Woodgett, 1990; Takahashi et al., 1994), where it has been implicated in several central nervous system (CNS) dysfunctions, such as Alzheimer's disease (Alvarez et al., 2002; Anderton, 1999; Bhat et al., 2004; Eldar-Finkelman, 2002; Grimes and Jope, 2001), schizophrenia (Beasley et al., 2001; Eldar-Finkelman, 2002; Kozlovsky et al., 2002) and bipolar disorders (Eldar-Finkelman, 2002; Grimes and Jope, 2001; Klein and Melton, 1996). Therefore, GSK3β is a prime drug target for a variety of CNS therapies. Nonetheless, its normal functions in the nervous system have remained largely unknown.

The GSK3β isoform is an unusual enzyme in that it has high basal activity, which is primarily determined by the phosphorylation status of ser9. The dephosphorylation of this residue by ser/thr protein phosphatases leads to further activation of GSK3β. Conversely, phosphorylation of ser9 by a variety of kinases results in inhibition of its activity. For example, in glycogen metabolism, insulin stimulates PI3K which leads to activation of Akt (also known as protein kinase B). This then results in

phosphorylation of GSK3β to inhibit its activity allowing for dephosphorylation of glycogen synthase and the stimulation of glycogen synthesis (Doble and Woodgett, 2003; Frame and Cohen, 2001). Other upstream signalling pathways can also regulate GSK3β and, in the case of Wnt signalling, this involves direct protein:protein interactions with GBP/FRAT1 (Doble and Woodgett, 2003). Numerous potential substrates for GSK3β have been identified, including several different transcription factors, metabolic enzymes, proteins that bind to microtubules, and components of the machinery involved in cell division and cell adhesion (Doble and Woodgett, 2003; Frame and Cohen, 2001). Of particular relevance to neurological disorders, GSK3β has been shown to bind to and phosphorylate both presenilin-1 and tau; proteins implicated in the aetiology of Alzheimer's disease (Avila et al., 2004; Hanger et al., 1992; Kirschenbaum et al., 2001). There are a variety of inhibitors available to address the function of GSK3β in the brain. Lithium is of particular interest, because inhibition of GSK3β may account for its ability to act as a mood stabilising drug (Klein and Melton, 1996).

A major function of the brain is to store information. It is widely believed that most information is stored at synapses in the form of alterations in synaptic efficiency. In particular, two forms of synaptic plasticity, LTP and LTD, have been extensively investigated in the pursuit of understanding the molecular and cellular basis of learning and memory (Bliss and Collingridge, 1993; Bear and Abraham, 1996). The dominant forms of both LTP and LTD are triggered by the synaptic activation of one class of glutamate receptor, the NMDA receptor, and are expressed as alterations in synaptic transmission mediated by another class of glutamate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Collingridge et al.,

1983; Dudek and Bear, 1992; Mulkey and Malenka, 1992). Most information has been derived from studies in the hippocampus, a brain region that is critically involved in learning and memory. It is known, for example, that during LTD the transient activation of NMDA receptors leads to internalisation of AMPA receptors from the surface of the neuron (Beattie et al., 2000; Collingridge et al., 2004). Synapses are able to undergo bidirectional plasticity, such that different patterns of synaptic activation result in NMDA receptor signals that can induce either LTP or LTD. This property is likely to be critically important to enable the brain to store vast amounts of information (Bliss and Collingridge, 1993; Malenka and Bear, 2004). However, it is not known whether mechanisms exist to prevent interference between LTP and LTD at synapses.

To address the normal function of GSK3β in the CNS we have made recordings from hippocampal neurons and have discovered that various inhibitors of GSK3β completely prevent the induction of LTD. Furthermore, we describe a physiological function for the inhibition of GSK3β. Thus, we show that LTP inhibits the induction of LTD for up to an hour, via activation of the PI3K-Akt-GSK3β pathway.

Results

GSK3 inhibitors block the induction of LTD

To determine whether GSK3β is involved in synaptic plasticity in the brain, we tested the effects of the highly selective GSK3 inhibitor 3-[(3-chloro-4-

hydroxyphenyl)amino]-4-(2-nitrophenyl)-1H-pyrrole-2,5-dione (SB415286) (Coghlan et al., 2000) in rat hippocampal slices. We applied SB415286 (10 μM) to the perfusing medium and used extracellular recording to investigate its effects on LTD, LTP and on the reversal of LTP by depotentiation (Fig. 1). SB415286 had no effect on baseline synaptic transmission (data not shown) but it blocked the induction of LTD induced by low frequency stimulation (LFS; 900 stimuli delivered at 1 Hz; n = 9). Thus, 60 min following LFS the slope of the field excitatory postsynaptic potential (fEPSP) of SB415286 treated slices was 103 ± 2% of baseline (n = 9) compared with 65 ± 5% (n = 3) of baseline, for untreated slices ($p < 0.001$). In contrast, in an independent input of SB415286 treated slices, LTP was readily induced by tetanic stimulation (either one tetanus (n = 5) or four tetani delivered at 30 s intervals (n = 4); each tetanus comprising 100 Hz stimulation for 1s) and was reversed by depotentiation (900 stimuli delivered at 1 Hz) in each of the seven slices tested. Therefore, of the three major forms of synaptic plasticity studied, SB415286 specifically blocks the induction of LTD.

Since LTD is induced and expressed postsynaptically (Collingridge et al., 2004; Kemp and Bashir, 2001), we infused individual CA1 pyramidal neurons in rat hippocampal slices with SB415286 via a whole-cell patch electrode to selectively block only postsynaptic GSK3β. Consistent with no effect on basal synaptic transmission, excitatory postsynaptic currents (EPSCs), evoked by stimulating Schaffer collateral-commissural fibres, were similar in the presence or absence of intracellular SB415286 (10 μM). However, SB415286 blocked the induction of LTD (Fig. 2B and D), which was routinely induced in interleaved control neurons by delivering 300 pulses at -40 mV (Luthi et al., 1999; Fig. 2A and C). Thus, 20 min

following this stimulation the EPSC amplitude of the test pathway was 100 ± 3% (n = 7) and 63 ± 4% (n = 16) of baseline, for the SB415286 treated and untreated neurons, respectively ($p < 0.001$). We next sought to firmly establish a role for GSK3β in LTD by further exploring the pharmacological block of LTD. We first tested SB415286 at a lower concentration and found that this inhibitor exhibited a concentration dependent inhibition of LTD; with 3 μM producing ~50% inhibition (Fig. 2H). Although SB415286 has previously been found to be a highly selective inhibitor of GSK3β (Coghlan et al., 2000) we wanted to rule out the possibility that it was acting through inhibition of other kinases over this concentration range. We therefore, tested the effects of two other structurally unrelated GSK3β inhibitors, lithium (Klein and Melton, 1996) and kenpaullone (Leost et al., 2000). Lithium also produced a concentration-dependent inhibition of LTD, with a partial inhibition at 2 mM and a complete block at 20 mM (Fig. 2E and H) while kenpaullone fully blocked LTD at a concentration of 10 μM (Fig. 2F and H). GSK3β is most closely related to the cyclin-dependent protein kinases (CDKs). We therefore, tested the potent CDK inhibitor roscovitine (Meijer et al., 1997), which does not affect GSK3β (Bain et al., 2003). This compound had no effect on LTD when applied at 10 μM (Fig. 2G and H), a concentration 40 times the IC_{50} for inhibition of CDK2 (Bain et al., 2003). The ability of all three GSK3β inhibitors to prevent LTD over the concentration range at which they are inhibitors of GSK3β (Bain et al., 2003), considered together with the insensitivity to the CDK2 inhibitor, strongly implicates a role for this enzyme in LTD.

LTD is associated with an increase in GSK3β activity

These pharmacological experiments demonstrate the requirement for GSK3β activity for the induction of LTD. To determine whether the activity of GSK3β is altered

during LTD, we delivered LFS and investigated the phosphorylation status of GSK3β in the CA1 area of hippocampal slices. LTD induction was associated with a reduction in the phosphorylation of ser9 of GSK3β, but no change in total GSK3β (Fig. 3A), which corresponds to activation of the enzyme. Since Akt can phosphorylate ser9 of GSK3β, we also measured the effects of LFS on this enzyme. LFS caused a decrease in phosphorylation of thr308 of Akt, which corresponds to inhibition of this enzyme (Fig. 3A). To confirm that LTD actually involves an alteration in GSK3β enzymatic activity and to quantify this change, we measured the GSK3β activity of the homogenates directly using ^{32}P labelling of a synthetic GSK3β substrate. After LTD induction, there was a significant increase in kinase activity above basal levels (32 ± 4%; n = 3; $p < 0.05$; Fig. 3B). Consistent with a role in LTD, the alteration in ser9 phosphorylation of GSK3β was prevented when LFS was delivered in the presence of the NMDA receptor antagonist, 50 μM D-2-amino-5-phosphonopentanoate (D-AP5; data not shown) or 1 μM okadaic acid (n = 5, Fig. 3C), a compound that also blocks LTD via inhibition of PP1 (Mulkey et al., 1994). Interestingly, okadaic acid also caused an increase in basal phosphorylation of GSK3β, which suggests that protein phosphatases maintain the constitutive activity of GSK3β in neurons. Okadaic acid also prevented the LFS induced decrease in phosphorylation of Akt, but had no effect on basal levels (Fig. 3D). Therefore, during LTD the activation of GSK3β may be due to the phosphatase inhibiting Akt and dephosphorylating ser9 of GSK3β.

To determine whether the activation of GSK3β is transient we measured its phosphorylation immediately and 20 min following the delivery of LFS. We also examined the effects of a tetanus (100 Hz, 1 s). LFS and tetanic stimulation caused

opposite effects on the phosphorylation status of ser9 of GSK3β that were similar in magnitude at both time points (Fig. 3E, F). The finding that the change in GSK3β phosphorylation persisted beyond the delivery of LFS suggests that its increase in activity could be involved in the maintenance of LTD, though alternatively it may be an epiphenomenon. To directly examine the possibility that GSK3β activity may be required beyond the delivery of LFS we investigated the effects of its inhibition after LTD had been induced. For these experiments we used lithium, since it is rapidly membrane permeant. Lithium (20 mM) had no effect on baseline transmission but fully blocked the induction of LTD (Fig. 3G). Interestingly, lithium was also fully effective when applied after the delivery of LFS (Fig. 3H).

GSK3β is present in dendritic spines and forms a complex with AMPA receptors

GSK3β is mainly found in the cytosol. However, our experiments predict that a fraction of GSK3β should be present in the vicinity of synapses. As shown in Fig. 4A, GSK3β is distributed throughout hippocampal neurons and to a lesser extent in glial cells too. Importantly, GSK3β is present in dendritic spines. Consistent with the immunocytochemistry, western blotting revealed the presence of GSK3β in both cytosolic and synaptosomal fractions prepared from both hippocampal (Fig. 4B) and neocortical slices (data not shown).

NMDA receptor-dependent LTD is due to the internalisation of AMPA receptors and involves protein interactions directly associated with the AMPA receptor subunits, in particular GluR2 (Collingridge et al., 2004; Malinow and Malenka, 2002). We reasoned that GSK3β might form a complex with AMPA receptors, and thus sought to investigate this by probing for an association of native GSK3β with AMPA

receptors in the CA1 area of hippocampal slices. A specific antibody against GSK3β was able to co-immunoprecipitate the GluR1 and GluR2 AMPA receptor subunits (Fig. 4C) and conversely immunoprecipitation of AMPA receptors produced coimmunoprecipitation of GSK3β (Fig. 4D). To determine the functional status of AMPA receptor associated GSK3β we immunoprecipitated AMPA receptors, using antibodies against either GluR1 or GluR2, and then assayed for kinase activity. GSK3β activity was readily detected in both GluR1 and GluR2 immunoprecipitates relative to the background IgG control (Fig. 4E), demonstrating that endogenous GSK3β associates with native AMPA receptors in the brain, and that the bound GSK3β is functionally active. This association of GSK3β with AMPA receptors suggests a compartmentalization of this enzyme for the efficient regulation of AMPA receptors during LTD.

We wondered whether the GSK3β activity that is associated with AMPA receptors could be regulated. Previous work has shown that transient exposure of cultured neurons to a solution containing sucrose plus glycine leads to an NMDA receptor-dependent insertion of AMPA receptors into the plasma membrane (Man et al., 2003). Interestingly, this effect is associated with an increase in the activity of PI3K that is associated with AMPA receptors. Since PI3K is an upstream regulator of GSK3β, we explored whether this treatment also affected the AMPA receptor-associated GSK3β enzymic activity. Neurons were treated with sucrose (200 mM) plus glycine (100 μM) for two min and this led to the insertion of AMPA receptors into the plasma membrane, as determined approximately 15 min later using surface biotinylation assays (30 ± 7% increase, $n = 6$; $p < 0.01$ for GluR1and 35 ± 8% increase, $n = 11$; $p < 0.05$ for GluR2; Fig. 4F). This chemically-induced AMPA receptor insertion was

associated with a decrease in AMPA receptor associated GSK3β activity (31 ± 4% inhibition; $n = 4$; $p < 0.05$; Fig. 4G).

LTP inhibits the induction of LTD

The finding that LTP stimuli can inhibit GSK3β and that GSK3β activity is required for LTD suggests that LTP may inhibit subsequent LTD at the same set of synapses. To test this idea we developed a protocol whereby the effects of an LTP stimulus, which normally leads to a long-lasting increase in synaptic transmission, could be examined on the subsequent ability to induce LTD in the same input, without the complication of LTP-induced alterations in synaptic strength. For this we delivered an LTP induction protocol of 60 pulses at 0.5 Hz, while the neuron was depolarised to 0 mV. This well established "pairing" protocol produced a robust LTP when applied soon after commencement of a whole-cell recording (Fig. 5A). However when this pairing protocol was applied after more than 10 minutes of whole-cell dialysis, LTP did not occur (Fig. 5A), due to the phenomenon of 'washout' of LTP (Malinow and Tsien, 1990). We reasoned that pairing would still drive the PI3K-Akt-GSK3β pathway following "washout". This pathway is resistant to dialysis since both PI3K inhibitors (Daw et al., 2002) and GSK3β inhibitors (Fig. 2) still affect LTD after an extended period of whole-cell recording. All our subsequent whole-cell experiments were, therefore, conducted after at least 10 min of recording to enable washout to occur. Consistent with our prediction, when pairing was delivered immediately prior to the LTD induction protocol it completely prevented the generation of LTD (Fig. 5B). The conditioning effect was fully reversible since the full level of LTD could be obtained if the induction protocol was delivered one hour following pairing (Fig. 5C). The effect of conditioning was, however, fairly long-lasting. For example, when

pairing was delivered 20 min beforehand it still largely blocked the generation of LTD (Fig. 5D, F). Conditioning involved the activation of NMDA receptors, since it was prevented by the NMDA receptor antagonist D-AP5 (Fig. 5E, F).

Although it is unlikely that washout affected the signalling mechanisms involved in the NMDA receptor-mediated inhibition of LTD, we went on to investigate whether LTD was blocked following the production of LTP under more physiological conditions. So as not to perturb the neurons, we performed extracellular recordings and induced LTP with tetanic stimuli. An inherent complication of these experiments is that the stimulus used to induce LTD can also induce an mGluR-dependent form of depotentiation (Bortolotto et al., 1994). To remove this additional factor, we performed these experiments in the presence of a broad spectrum metabotropic glutamate (mGlu) receptor antagonist LY341495 (100 μM), which blocks this type of depotentiation (Fitzjohn et al., 1998). In the presence of LY341495, LFS consistently induced LTD of baseline responses (control (input 1): 64 ± 4 % of baseline; LY341495 (input 2): 68 ± 2% of baseline; n = 3; data not illustrated). In contrast, if the same stimulation was delivered shortly after inducing LTP, then long-term synaptic depression was fully blocked (Fig. 5G). Consistent with the reversibility of the conditioning effect, the same stimulation delivered 60 min following the induction of LTP was able to induce long-term synaptic depression (Fig. 5H) that was dependent on the synaptic activation of NMDA receptors (Fig. 5I). Therefore, the synaptic activation of NMDA receptors during LTP inhibits the induction of LTD for approximately one hour.

A role for PI3K and Akt in the conditioned block of LTD

We next investigated whether this block of LTD, by prior NMDA receptor activation, was due to activation of the PI3K-Akt pathway using whole-cell recordings. First we tested the role of PI3K using the selective inhibitors wortmannin (500 nM) or LY294002 (10 μM). In the presence of wortmannin, EPSCs were readily evoked and pairing alone, after LTP washout, had no effect on transmission (Fig. 6A). Treatment with wortmannin also had no effect on the amount of LTD induced (Fig. 6B). However, wortmannin completely prevented the block of LTD by the LTP pairing stimulus (Fig. 6C). LY294002 had the same effect; also completely preventing the effect of the prior conditioning on LTD (Fig. 6D). Thus, using two structurally unrelated inhibitors, our data indicate that PI3K is required for the NMDAR-dependent inhibition of LTD. An alternative mechanism for inhibition of GSK3β may be via the mammalian target of rapamycin (mTOR) pathway (Frame and Cohen, 2001). However, inhibition of mTOR using rapamycin did not affect conditioning (Fig. 6E). A possible alternative explanation for this result is that rapamycin directly blocks LTD. However, LTD was readily induced in the presence of rapamycin if the conditioning pairing stimulus was not delivered (Fig. 6F). Thus, NMDA receptor dependent inhibition of LTD involves the PI3K, but not the mTOR, pathway.

As discussed above, our data suggest that the PI3K-Akt-GSK3β pathway operates normally in whole-cell recording experiments regardless of washout of LTP. However, we further confirmed the involvement of PI3K in the synaptic inhibition of long-term synaptic depression under circumstances in which LTP was induced. To do this, we again performed extracellular recording experiments in the presence of the broad spectrum mGluR antagonist to block mGluR-dependent depotentiation. We performed two-input experiments and, firstly, induced LTP and then tested for long-

term synaptic depression in the presence of a reversible PI3K inhibitor and, secondly, applied these same protocols to the second input after washout of the PI3K inhibitor. The results after washout of LY294002 were the same as the control experiments (illustrated in Fig. 5G) and show that LTP blocks long-term synaptic depression (Fig. 6G). In contrast, long-term synaptic depression is readily induced in the presence of LY294002 (Fig. 6G). This is due to block of conditioning rather than an effect of LY294002 on LTP *per se* since, in interleaved experiments, LY294002 did not affect LTP induced by four tetani (Fig. 6H) (see also Horwood et al, 2006).

We next investigated whether Akt is involved in this process using three complementary strategies, using whole-cell recording (Fig. 7A). First, we used a peptide (TCS 183) that mimics the Akt phosphorylation site on GSK3β. Inclusion of this false substrate at a concentration of 300 μM in the patch electrode solution prevented the effect of conditioning (Fig. 7B). Next we used a construct containing just the pleckstrin homology (PH) domain of Akt, since this acts as a highly specific inhibitor of Akt within this signalling pathway (Wang et al., 2003). In interleaved blind experiments, GST-PH-Akt (Fig. 7D), but not GST alone (Fig. 7C), also blocked conditioning. Finally, we examined the effects of an antibody that inhibits the function of Akt (Hill et al., 1999). This antibody also inhibited the conditioning effect (Fig. 7F). In contrast, the same antibody when heat-inactivated proved ineffective at blocking conditioning (Fig. 7E) as was an antibody against mTOR (99 ± 3 % of control; n = 4; data not illustrated). These experiments demonstrate the essential role of Akt in the conditioning blockade of LTD. Collectively, these experiments demonstrate that the transient activation of NMDA receptors during LTP inhibits subsequent LTD via the PI3K-Akt-GSK3β pathway.

Discussion

In the present study we have identified a form of regulation of synaptic plasticity, in which the transient synaptic activation of NMDA receptors, as occurs during LTP, leads to inhibition of LTD. This regulation is very powerful since LTD is fully inhibited immediately following the conditioning stimulus and the effect lasts for approximately one hour. We have also identified some of the signalling pathways responsible for this potent regulation of synaptic plasticity. We have found that GSK3β activity is an absolute requirement for the induction of LTD and that the conditioning stimulus inhibits its activity via activation of the PI3K-Akt pathway. Finally, we show that there is a correlation between the phosphorylation state of GSK3β ser9 and whether NMDA receptor activation leads to the induction or inhibition of LTD. This new regulatory mechanism is shown schematically in Fig. 8.

A function for GSK3β in the CNS

GSK3β is an unusual kinase that has been implicated in many diseases. However, very little is known about its normal function in the nervous system. It is important during early development and it has been shown to play a key role in cell polarity (Etienne-Manneville and Hall, 2003) and in the growth of neuromuscular junctions (Franco et al., 2004). Recently, it has been shown that GSK3β is important for determining neuronal polarity during the development of hippocampal neurons (Jiang et al., 2005; Yoshimura et al., 2005). However, though GSK3β is also highly expressed in the mature brain (Woodgett, 1990) its function in the nervous system has, hitherto, been largely unexplored. One intriguing function has been in the nucleus

where it is involved in the regulation of gene transcription, such as promoting the nuclear export of the transcription factor NF-ATc4 in hippocampal neurons (Graef et al., 1999). In addition, it has been shown that over-expression of GSK3β impairs spatial learning (Hernandez et al., 2002), though the mechanism underlying this effect is unknown. Here we show that in two-week-old rats, a time when the expression of GSK3β is near its peak (Takahashi et al., 2000), GSK3β activity is essential for NMDA receptor-dependent LTD in the hippocampus. This form of LTD is widespread throughout the brain and has been strongly implicated in development and learning and memory (Bear and Abraham, 1996; Kemp and Bashir, 2001). Therefore, this novel GSK3β-dependent mechanism may be of general significance in regulating the interaction between LTP and LTD throughout the brain.

Activation of GSK3β during LTD

GSK3β, unlike most enzymes, possesses high basal level constitutive activity and can be bi-directionally regulated to either further increase or decrease its activity. Our experiments show that during LTD there is additional activation of GSK3β activity, probably via dephosphorylation of ser9. We show further that this effect is prevented by an inhibitor of PP1/PP2A. This suggests that the activation of PP1, which is known to occur during LTD (Mulkey et al., 1993), is responsible for the activation of GSK3β, via its dephosphorylation of ser9 (Lee et al., 2005; Morfini et al., 2004; Szatmari et al., 2005). We also show that LTD is associated with inhibition of Akt, probably also via the activation of PP1. These data suggest that GSK3β activity is increased during LTD because the phosphatase concomitantly inhibits Akt and directly dephosphorylates ser9 of GSK3β.

Interestingly, the alteration in the phosphorylation status of GSK3β persisted beyond the delivery of LFS and lithium completely blocked LTD when applied after the delivery of LFS. These data suggest that GSK3β is required for the LTD process beyond the initial induction phase. Further studies are required to determine the full time-course of the involvement of GSK3β in LTD.

Signalling pathways involved in the actions of GSK3β

GSK3β has several upstream regulators and numerous downstream targets. In the present study, we have identified two of its upstream regulators. During LTD, GSK3β is activated via an okadaic acid-sensitive protein phosphatase, which is probably PP1 (Mulkey et al., 1993; Szatmari et al., 2005). During LTP, GSK3β is inhibited via the PI3K-Akt pathway. Since GSK3β is such a ubiquitous kinase it needs mechanisms to localise its access to its substrates. This is achieved in part via direct interactions with other proteins to form complexes. For example, in the canonical wnt pathway GSK3β-binding proteins control access of β-catenin (Jope and Johnson, 2004). It seems likely that the association between GSK3β and AMPA receptors serves to localise the kinase close to substrates that are involved in the trafficking of these receptors during synaptic plasticity. Further studies are required to establish the mechanism of this interaction as well as the downstream pathways mediated by GSK3β in the regulation of LTD.

The finding that the synaptic activation of NMDA receptors during LTP inhibits NMDA receptor-dependent LTD raises an intriguing issue; what determines whether the synaptic activation of NMDA receptors leads to the induction or to the inhibition

of LTD? We present evidence that the phosphorylation state of ser9 of GSK3β is a critical determinant. Thus, during LTP, activation of the PI3K-Akt pathway results in phosphorylation of GSK3β and hence inhibition of its activity. In contrast, during LTD, activation of PP1 results in inhibition of Akt and the dephosphorylation of GSK3β at ser9, and this leads to an increase in the enzyme's activity. The activation of PI3K-Akt (Man et al., 2003) and inhibition of PP1 (Atkins et al., 2005) during LTP, but inhibition of Akt during LTD (the present study) as well as the selective activation of PP1 during LTD (Mulkey et al., 1993) can be explained by the differences in the magnitude and spatiotemporal properties of the Ca^{2+} rise associated with the synaptic activation of NMDA receptors during these two forms of synaptic plasticity.

Implications of GSK3β-mediated metaplasticity

Previous work (e.g., Huang et al., 1992; Montgomery and Madison, 2002) has described other ways in which synaptic plasticity can be powerfully influenced by the prior history of synaptic activity. However, the mechanisms involved in these forms of metaplasticity are not known. Why synapses need such regulatory mechanisms is a matter of conjecture. One intriguing possible role for the regulation described here is to stabilise a synaptic modification over the short-term, by protecting synapses from the effects of additional NMDA receptor-dependent plasticity, until the information can be either consolidated or erased by NMDA receptor-independent mechanisms.

The regulation of synaptic plasticity is further complicated by the involvement of mGluRs, which are involved in depotentiation (Bortolotto et al., 1994), LTD of baseline transmission (Bolshakov and Siegelbaum, 1994), heterosynaptic LTD (Daw et al., 2002) and metaplasticity (Abraham and Bear, 1996). So that we could focus on

interactions between the NMDA receptor-dependent forms of synaptic plasticity we eliminated the additional complication of mGluR-dependent synaptic plasticity by using the broad spectrum mGluR antagonist LY341495 and by employing stimulus protocols optimised for NMDA receptor-dependent synaptic plasticity. However, given that PI3K has been implicated in a chemically-induced form of mGluR-dependent LTD (Hou and Klann, 2004) and heterosynaptic LTD (Daw et al., 2002) it will be interesting to determine whether GSK3β is also involved in these forms of synaptic plasticity. One possibility is the PI3K-Akt-GSK3β pathway serves to inhibit NMDA receptor-dependent LTD both homosynaptically following the induction of LTP and heterosynaptically following the induction of LTD.

Clinical implications

Our finding that in the normal brain activation of GSK3β is essential for NMDA receptor dependent LTD, and that its activity can be regulated by LTP, may offer clues to the pathological role of this enzyme in neurological disorders. For example, the primary therapeutic action of lithium in bipolar disorders may be via inhibition of GSK3β (Grimes and Jope, 2001; Klein and Melton, 1996). Indeed, specific inhibition of GSK3β has recently been shown to produce antidepressive-like activity *in vivo* (Kaidanovich-Beilin et al., 2004). Over-activity of GSK3β may, therefore, lead to this mood disorder by affecting the balance and interplay between NMDA receptor-dependent LTP and LTD.

Experimental Procedures

General antibodies and reagents – Rabbit anti GSK3β was obtained from Santa Cruz. Rabbit phospho-GSK3β (ser9), mouse monoclonal antibody (mAb) Akt1 and rabbit mAb phospho-Akt (thr308) were obtained from Cell Signaling Technology (Beverly, MA). Mouse mAb MAP2 was obtained from BD Pharmagen (San Diego, CA). ProLong Gold mounting medium and secondary fluorophore bound IgG's (Alexafluor series) for immunocytochemistry were obtained from Invitrogen-Molecular Probes (Portland, OR). Rabbit IgG, recombinant rat GSK3β and phospho-glycogen synthase peptide-2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). [γ-^{32}P]ATP was purchased from Amersham Biosciences (Quebec, Canada). Leupeptin protease inhibitor was obtained from Peptides International (Louisville, Kentucky) and all other lysate buffer components, including phosphatase and protease inhibitors were obtained from Sigma Aldritch (St Louise, MO). Kenpaullone was obtained from Calbiochem, okadaic acid was obtained from LC laboratories (Woburn, MA) and the other chemicals from Tocris Cookson (Avonmouth, U.K.). TCS 183 (MSGRPRTTSFAES) was custom synthesized by Tocris Cookson. The Akt antibody (Akt 1/2 (N-19), sc-1619) and mTOR antibody (FRAP (C-19):msc-1550) were obtained from Santa Cruz. GST-PH Akt was constructed as follows. The DNA fragment for bovine Akt N terminus (amino acids 1-148) containing the PH domain (1-106) (GST-PH-Akt) was generated by PCR using the following primers: 5'-cgcggattcatgaacgacgtggccatcgtg-'3' and 5'-ccggaattctcaaaactcattcatggtcac-3'. The PCR generated DNA fragment was ligated into the BamHI and EcoRI sites of the pGEX-4T-2 vector (Amersham Biosciences biotech) cloned, and then verified by sequencing. The GST-PH-Akt (amino acids 1-148) and GST alone proteins were prepared from bacterial lysates and purified according to the protocol provided by Amersham Biosciences.

Electrophysiology – Experiments were performed on 400 µm thick transverse hippocampal slices obtained from juvenile (13 - 15 day old) rats, which were perfused with artificial cerebrospinal fluid (ACSF) which comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; Glucose, 15; Ascorbate, 2; (-)-bicuculline methochloride, 0.01. In experiments in which LiCl was added to the perfusuate and the equivalent amount of NaCl was omitted from the ACSF. Visually-guided, whole-cell recordings were obtained at room temperature from the soma of CA1 neurons using patch electrodes that contained (mM): $CsMeSO_4$, 130; HEPES, 10; NaCl, 8; EGTA, 0.5; Mg-ATP, 4; Na-GTP, 0.3; QX-314, 5. In experiments in which LiCl was included in the whole-cell solution, the equivalent concentration of CsMeSO4 was removed. Some control experiments were performed in this way but by substituting CsCl for LiCl. When antibodies were added to the whole-cell solution the NaCl concentration was adjusted appropriately. Two independent sets of Schaffer collateral-commissural fibres were stimulated alternately, each at a frequency of 0.1 Hz and EPSC amplitude and access resistance were recorded on-line at a holding potential of -70 mV. LTD was induced by delivering 300 pulses (at 0.75 Hz) at -40 mV to one of the two inputs. The induction of this form of LTD was completely inhibited by the NMDA receptor antagonist, D-AP5 (50 µM). Kinase inhibitors were included in the patch solution. For experiments using kenpaullone or roscovitine these were also added to the perfusate (at 10 µM). Extracellular recordings were obtained as described previously (Fitzjohn et al., 1998). All inhibitors were added to the perfusing medium. Data were stored and analysed using the LTP Program (Anderson and Collingridge, 2001).

Western blotting for alterations in GSK3β phosphorylation state – For western blotting, 40 - 50 µg of the hippocampal slice lysate from each treatment condition was

separated with SDS-PAGE, transferred onto a PVDF membrane and probed with the relevant antibodies. For sequential reprobing of the same blots, the membranes were stripped of the initial primary and secondary antibodies and subjected to immunoblotting with another target antibody. Blots were developed using enhanced chemiluminescence detection (Amersham). Band intensities were quantified using NIH ImageJ software and normalized to the quantity of β-tubulin in each sample lane. LFS comprised delivering 900 stimuli at 1 or 3 Hz. A small region of the slice (approximately 1.25 mm^2) surrounding the stimulating electrode was then dissected and homogenised. Tetanic stimulation comprised delivering 100 stimuli at 100 Hz to three separate locations.

GSK3β kinase assay after LTD – To measure GSK3β activity in hippocampal slices following electrode induced LTD (900 stimuli delivered at 3 Hz) microdissected areas of slices were solubilized and 30 μg of the lysate was used for each assay as described (Li et al., 2003) with some modifications. Briefly, the kinase reaction occurred in a 50 μl total volume containing 20 mM MOPS pH 7.4, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM NA_3VO_4, 1 mM DTT, 15 mM $MgCl_2$, 100 μM cold ATP, 7.5 μl phosphoglycogen synthase peptide-2 (1mg/ml stock solution, Upstate, USA), 30 μg whole slice lysate (10 μl in volume) or 10 μl of water as negative control, and 2.5 μCi γ-^{32}P-ATP. In order to take into account the contribution of the other kinases in the consumption of γ-^{32}P-ATP, 20 mM LiCl was added to a second set of the same samples. The reactions were incubated for 30 min at 30°C, then stopped with the addition of 50 μl of 10% H_3PO_4, mixed, and 30 μl spotted on filter paper for subsequent analysis. Finally, CPM for GSK3β activity was calculated by deducting total counts from the CPM of the duplicate samples which contained LiCl.

Coimmunoprecipitation of GSKβ and AMPA receptor complexes –

Coimmunoprecipitation and Western blotting were carried out as previously reported (Taghibiglou et al., 2002). The CA1 areas of hippocampal slices from 20 days old SD rats were microdissected and then homogenized on ice in lyses buffer (150 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 1% Nonidet P 40) containing an inhibitor cocktail (2mM PMSF, 10 µg/ml aprotinin, 10µg/ml leupeptin, 100 mM NaF, 10 mM Na pyrophosphate, and 2 mM NA_3VO_4) for blocking phosphatase and protease activity. Lysates were subjected to coimmunoprecipitation for 16-18 hrs at 4°C with a rabbit polyclonal antibody against GSK3β (2-10 µg of antibody/1-1.5 mg of total lysate), or rabbit polyclonal antibodies against the C-terminus of either GluR1 or GluR2 (both raised in house), or control IgG antibodies bound to 50 µl of a 50 % (w/v) protein A-sepharose bead slurry per sample. Precipitates were washed 3-4 times on ice with wash buffer; phosphate-buffered saline (PBS) containing 0.1% Nonidet P 40, 0.1% Triton X-100 with the inhibitor cocktail, and boiled in 60 µl sample buffer for 5-10 min. Precipitates were loaded for SDS-PAGE separation and subjected to western blotting for the appropriate targets as described below.

GSK3β kinase assay for GSK3β- AMPA receptor associated activity – In order to assess any GSK3β kinase activity associated with GluR1 and GluR2 subunits of AMPA receptor, we subjected 1 mg of slice lysate to coimmunoprecipitation with either rabbit IgG (background control) or antibodies against GluR1 and GluR2. These bead bound coimmunoprecipitates were subsequently used for GSK3β kinase assays according to the supplier's protocol (Upstate Biotechnology, Inc. Lake Placid, NY) with some modifications. Briefly, kinase activity was assayed in a total volume of 50

µL of kinase buffer containing 8 mM MOPS (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 250 µM ATP, 2 µCi of [γ-^{32}P]ATP, and 100 µM phosphoglycogen synthase peptide-2 (YRRAAVPPSPSLSRHSSPHQSEDEEE) and incubated at 30°C for 30-60 min. Recombinant rat GSK3β (1-3 µg active kinase per assay) was used as a positive control. After pelleting the beads, 25 µL of reaction supernatant was spotted onto filter paper (Millipore, Bedford, MA) and the proteins were briefly allowed to bind the filter paper for 30 seconds. The filter papers were washed three times in 60-100 ml 0.75% phosphoric acid for a total time of 15 min, once more in acetone for 5 min, dried and then counted in a liquid scintillation counter. Background subtraction was done using counts from IgG controls for each independent experiment.

GSK3β kinase assay associated with AMPA receptor insertion – GSK3β activity assays were performed on GluR2-GSK3β coimmunopreciptated lysates from cortical neurons (12-14 DIV), cultured as previously described (Man et al., 2003). AMPA receptor insertion was induced using 200 mM sucrose plus 100 µM glycine, applied for 2 min at 37 °C as previously described (Lu et al., 2001). Cells were lysed as before and coimmunoprecipitated for 2-4 hrs at 4°C with a mouse GluR2 N-terminal monocolonal antibody (10 µg/mL; Chemicon) bound to Protein G-sepharose beads. Kinase activity was assessed on coimmunoprecipitates as described above and background subtracted using counts from IgG controls.

Immunocytochemistry – Coverslip cultured hippocampal neurons (DIV 14-17) were fixed in 4% PFA, 4% sucrose/ PBS solution for 10 min, permeabilized in 0.1% Triton X-100 for 5 min and blocked with 2% goat serum diluted in PBS for 30 min with extensive PBS washings between each step. Blocking buffer diluted antibodies against GSK3β (1:250) and MAP2 (1:2,000) were incubated for either 1 hr at room

temperature (RT) or overnight at 4ºC, respectively. Goat raised secondary antibodies (1:1,000), rabbit Alexa555 (for GSK3β) and mouse Alexa488 (for MAP2), were incubated for 1 hour at RT and washed extensively in 0.5M NaCl supplemented PBS prior to mounting on slides in ProLong Gold medium. Captured images were obtained from a Leica DMIRE2 deconvolution microscope using OpenLab software. Representative images have been adjusted to maximize the signal:noise ratio. Relative intensities from each fluorophore channel were adjusted for a 50:50 contribution of signal intensities prior to merging the two channel images and subsequent analysis of the cellular location of neuronal GSK3β.

Preparation of Synaptosomes – Slices of cerebral cortex or hippocampus were homogenized in 10 volumes of solution containing 20 mM HEPES pH 7.4, 0.32 M sucrose, 1 mM EDTA, 5mM DTT, and protease/phosphatase inhibitor cocktail. Homogenates were subjected to centrifugation at 1000 X g_{max}, 4^{o}C, for 10 min. The supernatant was centrifuged at 17000 gmax at 4^{o}C, for 15 min using a SW55 Beckman rotor. The resultant pellet was resuspended in 4 ml of the above mentioned homogenization buffer and layered top to bottom with 4 ml of 0.8 M sucrose overlaid with 4 ml of 1.2 M sucrose. The gradient was centrifuged at 54000 g_{max}, 4^{o}C, for 90 min, using a SWTi41 Beckman rotor. The synaptosome fraction was removed from the 0.8 M and 1.2 M sucrose interface. The collected fraction (~ 0.5 ml) was diluted with 10 volumes of ice-cold 0.32 M sucrose and centrifuged at 20000 g_{max}, 4^{o}C, for 15 min, using a SW55 Beckman rotor. The resulting synaptosomal pellet was dissolved in a solubilizing buffer.

Biotin labeling of neuronal surface proteins – Cultured cortical neurons subjected to chemical LTP induction, were cooled to 4 °C after treatment, and labelled for 30 min

with sulfo-NHS-LC-Biotin (1 mg/ ml). Cells were washed three times in cold phosphate buffered saline and then harvested in lysis buffer. Lysates were subjected to overnight avidin precipitation (280 μg of total protein/ 60 μl of avidin suspension), washed four to five times, loaded on a gel for SDS-PAGE, and western blotted for surface AMPA receptor expression.

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Figure 1. A GSK3 inhibitor blocks the induction of LTD but not LTP or depotentiation.

(A) A single example of an extracellular recording experiment to show that 10 μM SB415286 (bath applied for 4 h) blocked the induction of LTD (input 1) but had no effect on LTP and depotentiation (input 2). Each point is the mean of 4 successive responses normalised to baseline. In this and subsequent figures, the LTD induction protocol is marked by a horizontal line and the LTP induction protocol by an arrowhead. Representative recordings (averages of 4 successive responses) are shown at the times indicated. The calibration bars depict 1 mV and 20 ms.

(B) Pooled data (mean ± SE. mean) from 9 experiments performed as in (A), except that in 4 experiments LTP was induced using 4 tetani.

(C) The LTD portion of the experiment shown in (B) is re-plotted.

(D) Interleaved control experiments (n = 3) illustrating LTD.

Figure 2. Pharmacological evidence that GSK3 activity is required for LTD.

(A) A single experiment illustrating homosynaptic LTD. Each point plots 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 0.75 Hz to the test input for the duration indicated by the bar. EPSCs obtained before and following the induction of LTD are illustrated for the control and test inputs at the times indicated (1, 2). In this and Fig. 5 the calibration bars for the traces depict 40 pA and 50 ms.

(B) A single example from an experiment in which 10 μM SB415286 was contained within the patch pipette.

(C) Pooled data (mean ± SE) from 16 control experiments.

(D) Pooled data from 7 experiments performed as in (B).

(E) Effects of 20 mM lithium (n = 5).

(F) Effects of 10 μM kenpaullone (n = 5).

(G) Effects of 10 μM roscovitine (n = 5).

(H) Summary graphs illustrating the effects of various inhibitors on homosynaptic LTD quantified 20 min following induction. * $p < 0.05$, *** $p < 0.001$.

Figure 3. GSK3β activity is increased during LTD.

(A) Representative western blots from three separate experiments showing a reduction in phosphorylated GSK3β, but no change in expression of total GSK3β, following LFS. There is also a reduction in phosphothreonine Akt but no change in Akt mass. β-tubulin represents equal loading of lysates for each condition.

(B) LFS-induced increase in GSK3β activity relative to controls (n = 3).

(C) Pooled data to show LFS-induced changes in phosphorylated GSK3β under control conditions (n = 10) and following treatment with 1 μM okadaic acid (OA; n = 5).

(D) Pooled data to show LFS-induced changes in phosphorylated Akt under control conditions (n = 4) and following treatment with okadaic acid (n = 4).

(E) Representative western blots (upper) and pooled data from 4 experiments (lower) showing a decrease in phosphorylated GSK3β immediately and 20 min following LFS.

(F) Representative western blots (upper) and pooled data from 5 experiments (lower) showing an increase in phosphorylated GSK3β immediately and 20 min following tetanic stimulation. All quantified data are plotted as mean ± SE; * $p < 0.05$. ** $p < 0.01$. NS, not significant.

(G) Pooled data from 6 experiments showing that lithium blocks the induction of LTD.

(H) Pooled data from 4 experiments showing that lithium reverses LTD when applied following the induction stimulus.

Figure 4. Localisation of GSK3β.

(A) Immunocytochemical localisation of GSK3β in hippocampal cultures. The images show (from left to right) GSK3β, MAP2 and their co-localisation. Note that GSK3β is present throughout neurons and that there is a faint staining of glial cells. GSK3β is present in dendritic spines, as most clearly visualised in the high magnification co-localisation image.

(B) GSK3β is present in both cytosolic and synaptosomal fractions. PSD95 is localised only in synaptosomes, which is consistent with its synaptic localisation.

(C) Representative western blots showing that immunoprecipitation with GSK3β coimmunoprecipitates GluR1 or GluR2 (n = 3). (IP = Immunoprecipitate, IB = Immunoblot).

(D) Immunoprecipitation for either GluR1 or GluR2 coimmunoprecipitates GSK3β (n = 3).

(E) Co-immunoprecipitation of GSK3β complexed with GluR1 and GluR2 shows associated functional activity (n = 3; * $p < 0.05$).

(F) Insertion of AMPA receptors in cultured neurons as determined using cell surface biotinylation. Neurons were treated with sucrose (200 mM) plus glycine (100 μM) (SG) for 2 min and maintained for a further 10-15 min before being placed on ice. Top: representative western blots. Bottom: quantification of surface GluR1 (n = 6)

and GluR2 (n = 11) containing AMPA receptors, normalized to the surface expression of the transferrin receptor.

(G) The insertion of AMPA receptors is accompanied with a decrease in GluR2 associated GSK3β activity. Top: Representative western blot showing equal immunoprecipitation of GluR2 and co-immunoprecipitated GSK3β in unstimulated controls and LTP induced lysates used in the subsequent kinase reactions. Bottom: quantification of GSK3β kinase activity after LTP induction and AMPA receptor immunoprecipitation (n = 4).

All quantified data are plotted as mean ± SE; * $p < 0.05$.

Figure 5. The synaptic activation of NMDA receptors inhibits subsequent LTD.

(A) Shows that a conditioning stimulus (60 pulses, 0.5 Hz, 0 mV; arrowhead) induces LTP when delivered within 5 min of whole-cell recording (filled symbols; n = 8) but not when baseline recording was extended for at least 10 min (open symbols; n = 18).

(B) Shows that a conditioning stimulation completely blocks the induction of LTD (n = 8). (Note that interleaved control experiments invariably induced LTD and these experiments are included in the grand pool illustrated in Fig. 2C).

(C) Shows full recovery from the effects of conditioning when the LTD induction protocol was delivered 1 h after the pairing (n = 5).

(D) LTD is largely blocked when the LTD induction protocol was delivered 20 min following pairing (n = 4).

(E) The conditioning effect of pairing is prevented by an NMDA receptor antagonist (D-2-amino-5-phosphonopentanoate; AP5, 50 μM; n = 4).

(F) Time-course of conditioning. Each point plots the mean ± SE of at least 4 experiments (* $p < 0.05$, *** $p < 0.001$).

(G) The induction of LTP (4 bursts of 100 pulses at 100 Hz, delivered at 30 s intervals) blocks the induction of long-term synaptic depression. LY341495 was present to block mGlu receptor-dependent synaptic plasticity (n = 5)

(H) Long-term synaptic depression can be induced 1 h after the induction of LTP (n = 4).

(I) The long-term synaptic depression induced 1 h after LTP is dependent of activation of NMDA receptors (n = 4).

Figure 6. Conditioning of LTD requires activation of PI3K.

(A) Wortmannin (500 nM) has no effect on the conditioning train (n = 3).

(B) Wortmannin has no effect on LTD (n = 5).

(C) Wortmannin completely prevents the conditioning of LTD (n = 9).

(D) LY294002 (10 μM) completely prevents the conditioning of LTD (n = 6).

(E) Rapamycin (0.1 μM) has no effect on conditioning (n = 4).

(F) Rapamycin has no effect on LTD (n = 5).

(G) Prior induction of LTP did not inhibit long-term synaptic depression, when the experiment was performed in the presence of LY294002 (10 μM). Following washout of LY294002 the block of long-term synaptic depression by LTP was invariably observed (tested on an independent input; n = 5).

(H) LY294002 (10 μM) did not affect the generation of LTP (n = 4).

Figure 7. Conditioning of LTD requires activation of Akt.

(A) Strategies for inhibiting Akt.

(B) A peptide that mimics the Akt phosphorylation site on GSK3β prevents conditioning of LTD (n = 4).

(C) GST has no effect on conditioning (n = 4).

(D) GST-PH-Akt inhibits conditioning of LTD (n = 7).

(E) The heat-inactivated antibody has no effect on conditioning (n = 5).

(F) An Akt neutralising antibody prevents conditioning of LTD (n = 6).

Figure 8. The regulation of GSK3β in the control of NMDA receptor-dependent synaptic plasticity.

The activity of GSK3β determines whether NMDA receptor activation induces LTD or inhibits LTD. During LTD activation of PP1 leads to dephosphorylation of GSK3β at ser9 to further activate GSK3β and enable LTD to occur. PP1 also inhibits Akt. During LTP activation of NMDA receptors leads to stimulation of the PI3K / Akt pathway, which phosphorylates GSK3β at ser9 to inhibit its activity and prevent the induction of LTD. Thus GSK3β, under the control of Akt and PP1, is a critical determinant of the direction of NMDA receptor-dependent plasticity.

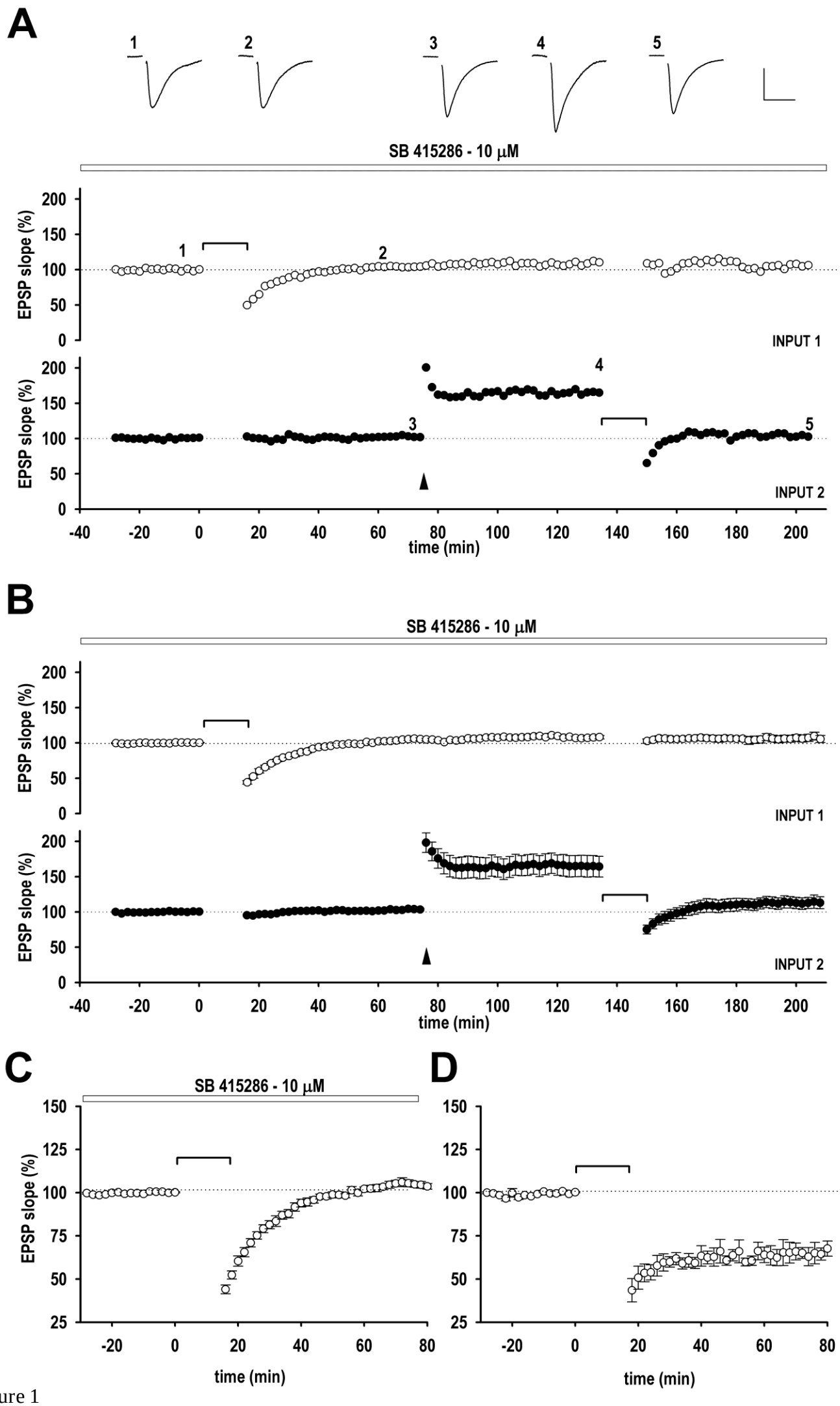
A
1
2
3
4
5
SB 415286 - 10 μM
EPSP slope (%)
INPUT 1
INPUT 2
time (min)
B
SB 415286 - 10 μM
EPSP slope (%)
INPUT 1
INPUT 2
time (min)
C
SB 415286 - 10 μM
EPSP slope (%)
time (min)
D
time (min)

Figure 1

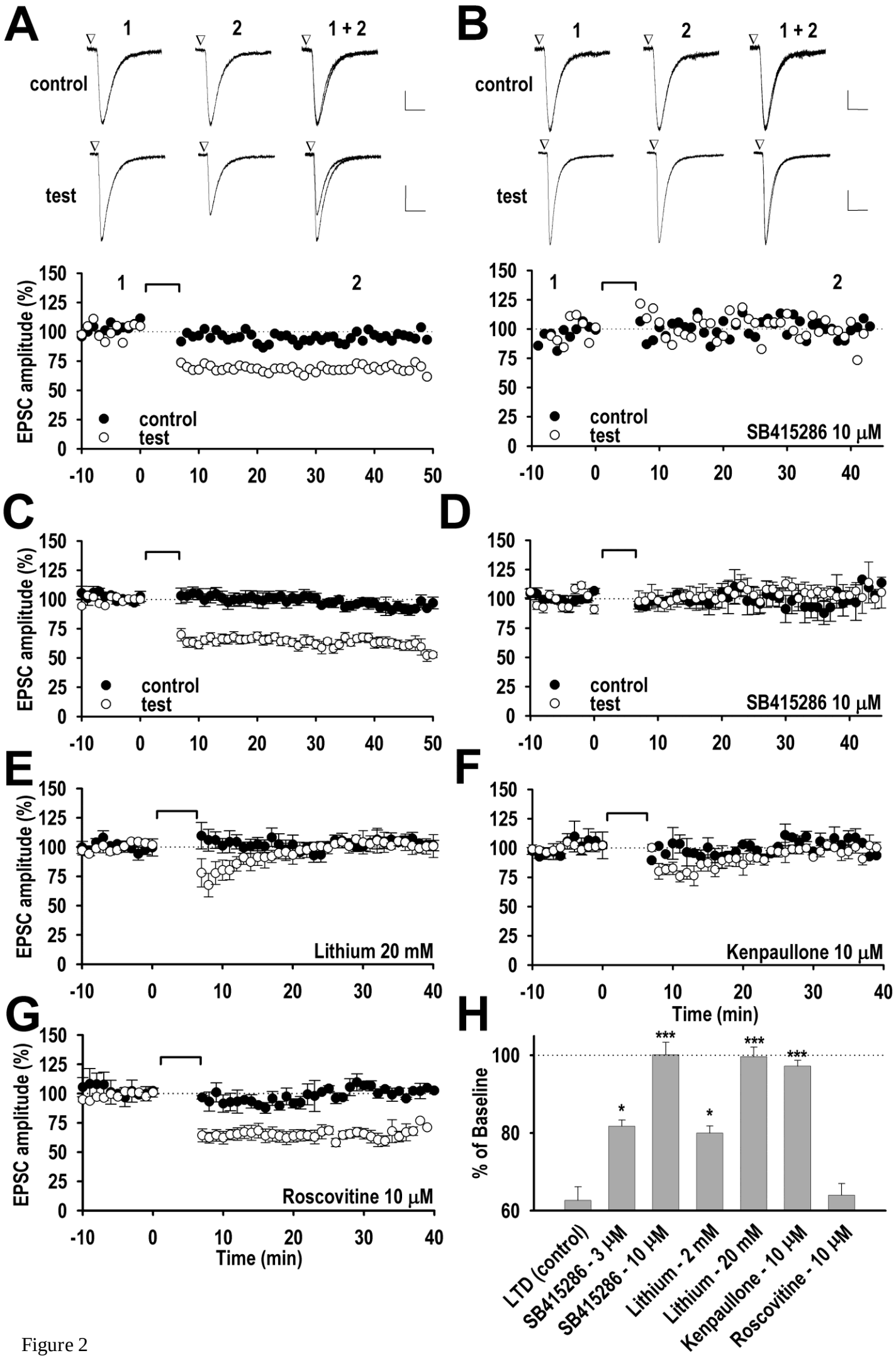
A
1
2
1 + 2
control
test
B
1
2
1 + 2
control
test
EPSC amplitude (%)
1
2
control
test
SB415286 10 μM
C
D
E
Lithium 20 mM
F
Kenpaullone 10 μM
Time (min)
G
Roscovitine 10 μM
H
% of Baseline
LTD (control)
SB415286 - 3 μM
SB415286 - 10 μM
Lithium - 2 mM
Lithium - 20 mM
Kenpaullone - 10 μM
Roscovitine - 10 μM

Figure 2

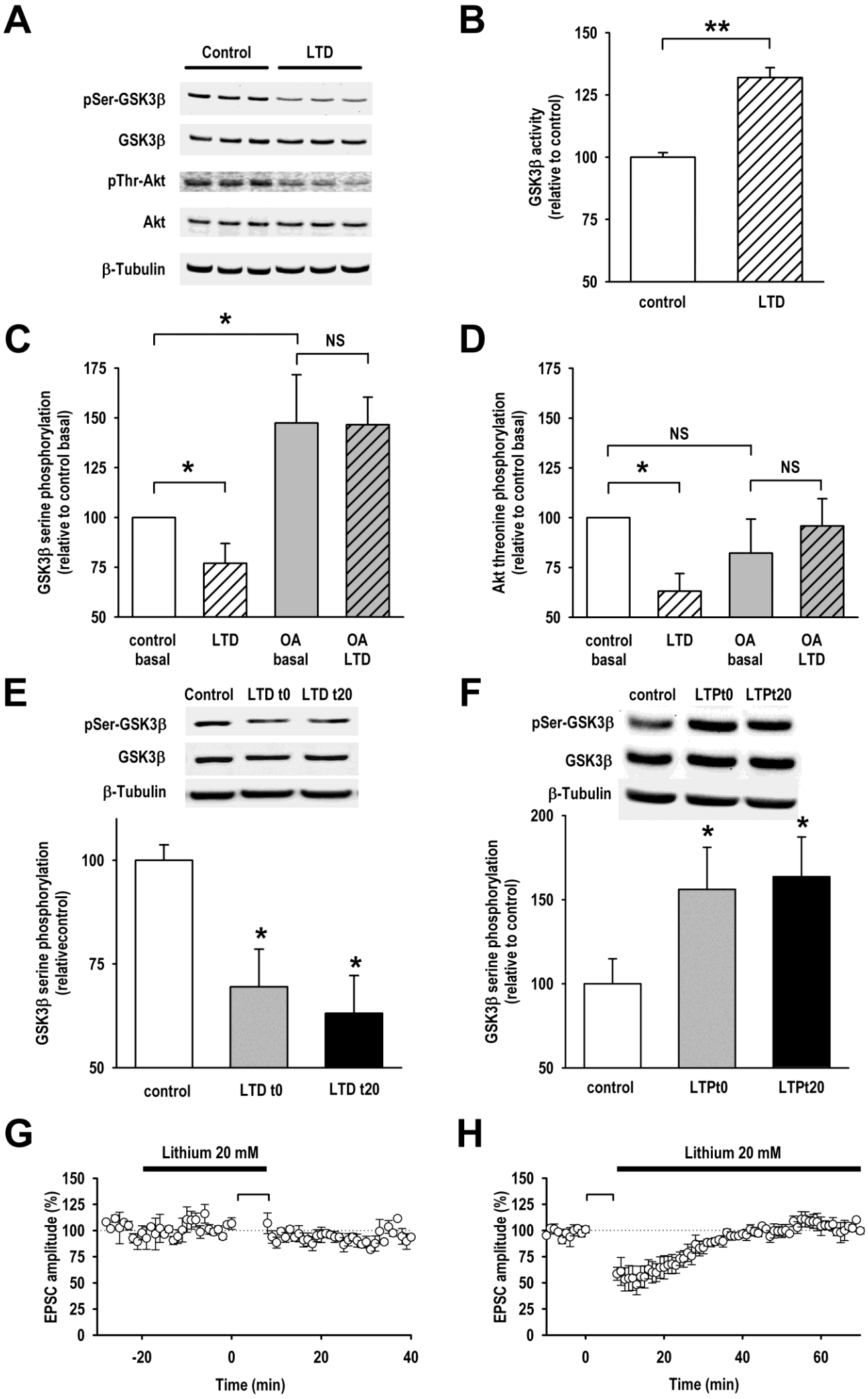
A
Control
LTD
pSer-GSK3β
GSK3β
pThr-Akt
Akt
β-Tubulin
B
**
GSK3β activity (relative to control)
150
125
100
75
50
control
LTD
C
*
NS
*
GSK3β serine phosphorylation (relative to control basal)
175
150
125
100
75
50
control basal
LTD
OA basal
OA LTD
D
NS
*
NS
Akt threonine phosphorylation (relative to control basal)
175
150
125
100
75
50
control basal
LTD
OA basal
OA LTD
E
Control
LTD t0
LTD t20
pSer-GSK3β
GSK3β
β-Tubulin
GSK3β serine phosphorylation (relativecontrol)
100
75
50
*
*
control
LTD t0
LTD t20
F
control
LTPt0
LTPt20
pSer-GSK3β
GSK3β
β-Tubulin
GSK3β serine phosphorylation (relative to control)
200
150
100
50
*
*
control
LTPt0
LTPt20
G
Lithium 20 mM
EPSC amplitude (%)
150
125
100
75
50
25
0
-20
0
20
40
Time (min)
H
Lithium 20 mM
EPSC amplitude (%)
150
125
100
75
50
25
0
0
20
40
60
Time (min)

Figure 3

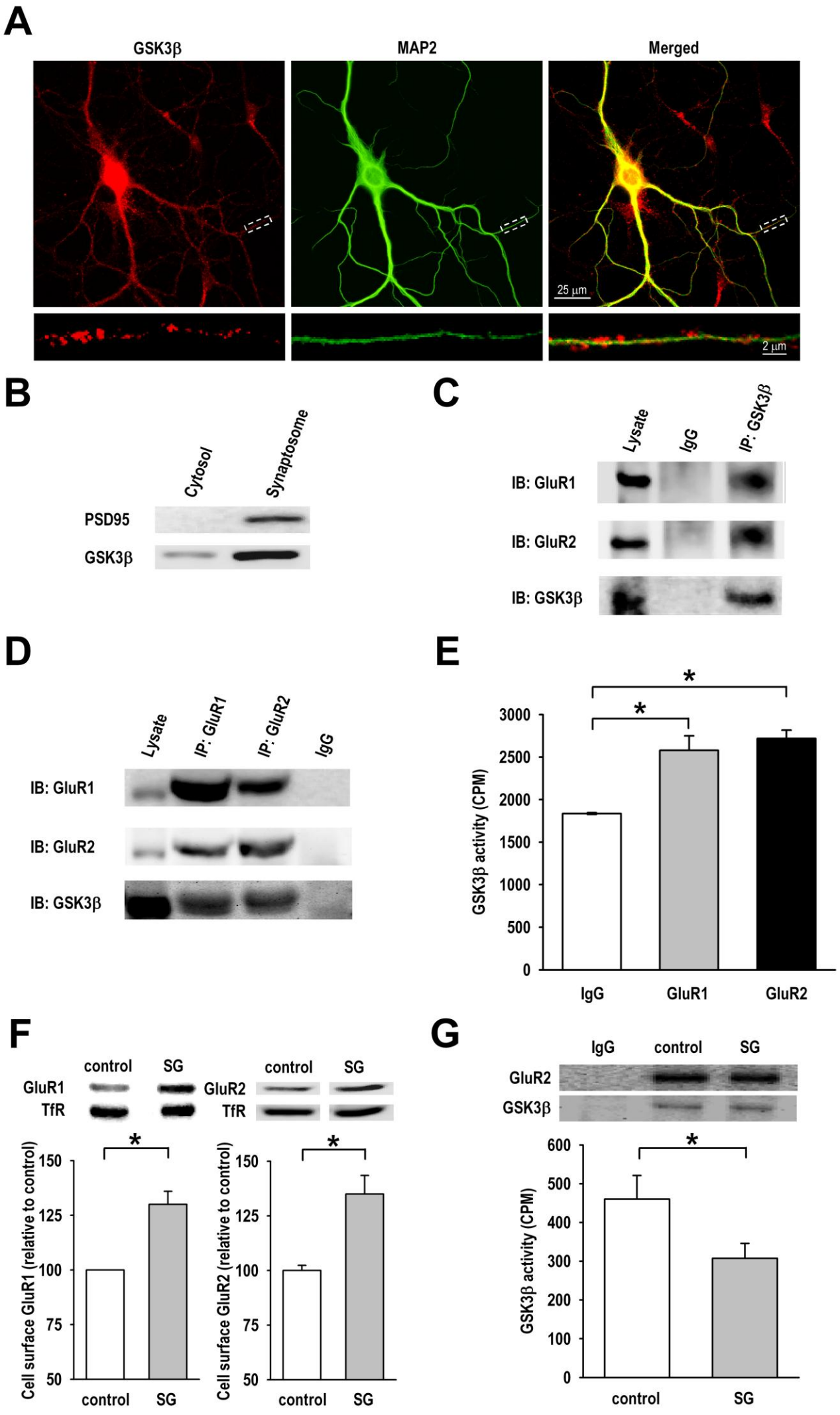
A
GSK3β
MAP2
Merged
25 μm
2 μm
B
Cytosol
Synaptosome
PSD95
GSK3β
C
Lysate
IgG
IP: GSK3β
IB: GluR1
IB: GluR2
IB: GSK3β
D
Lysate
IP: GluR1
IP: GluR2
IgG
IB: GluR1
IB: GluR2
IB: GSK3β
E
GSK3β activity (CPM)
3000
2500
2000
1500
1000
500
0
IgG
GluR1
GluR2
F
control
SG
GluR1
TfR
GluR2
Cell surface GluR1 (relative to control)
Cell surface GluR2 (relative to control)
150
125
100
75
50
G
IgG
control
SG
GluR2
GSK3β
GSK3β activity (CPM)
600
500
400
300
200
100
0

Figure 4

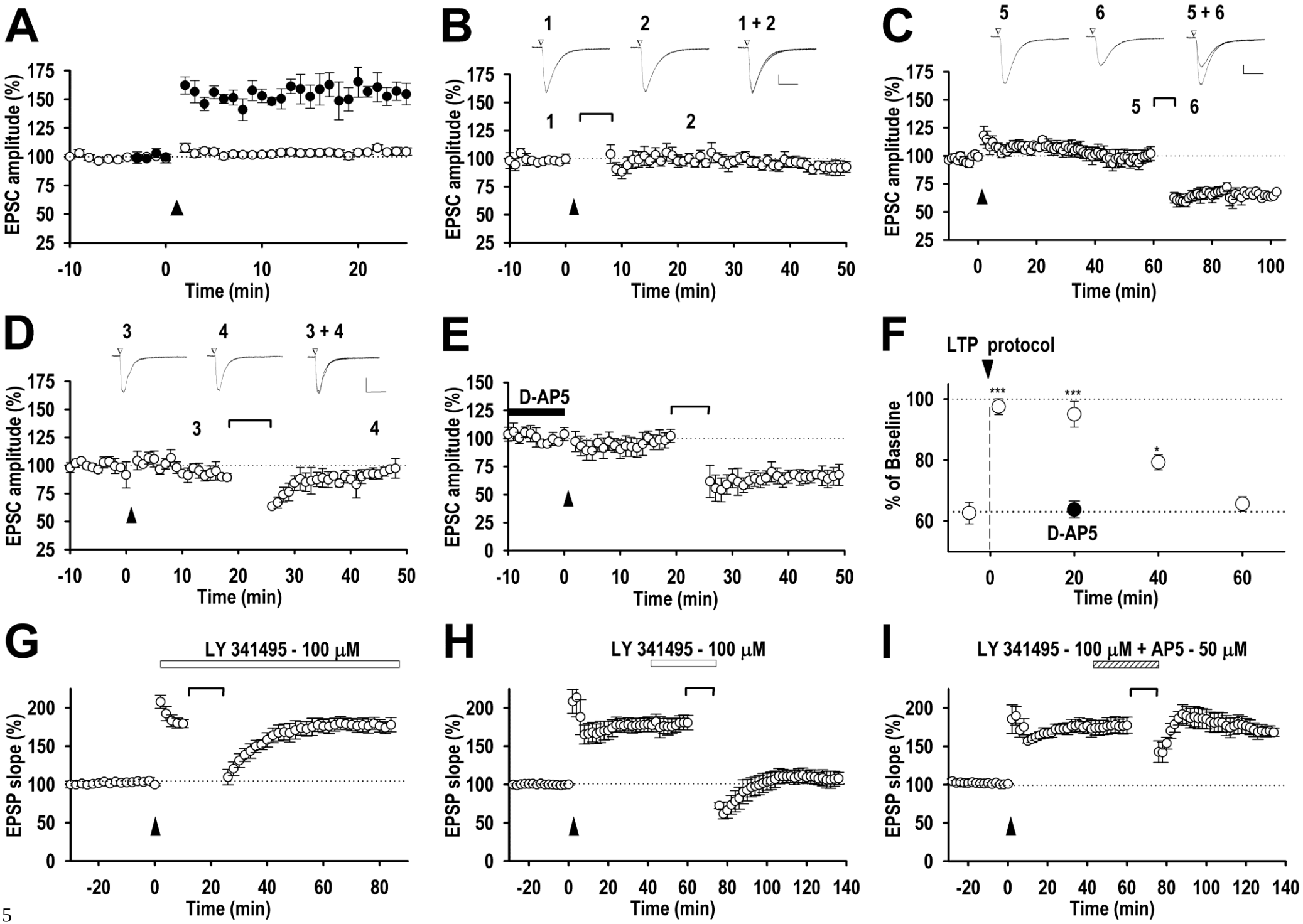
A
EPSC amplitude (%)
Time (min)
B
1
2
1 + 2
C
5
6
5 + 6
D
3
4
3 + 4
E
D-AP5
F
LTP protocol
% of Baseline
D-AP5
G
LY 341495 - 100 µM
EPSP slope (%)
H
LY 341495 - 100 µM
I
LY 341495 - 100 µM + AP5 - 50 µM

Figure 5

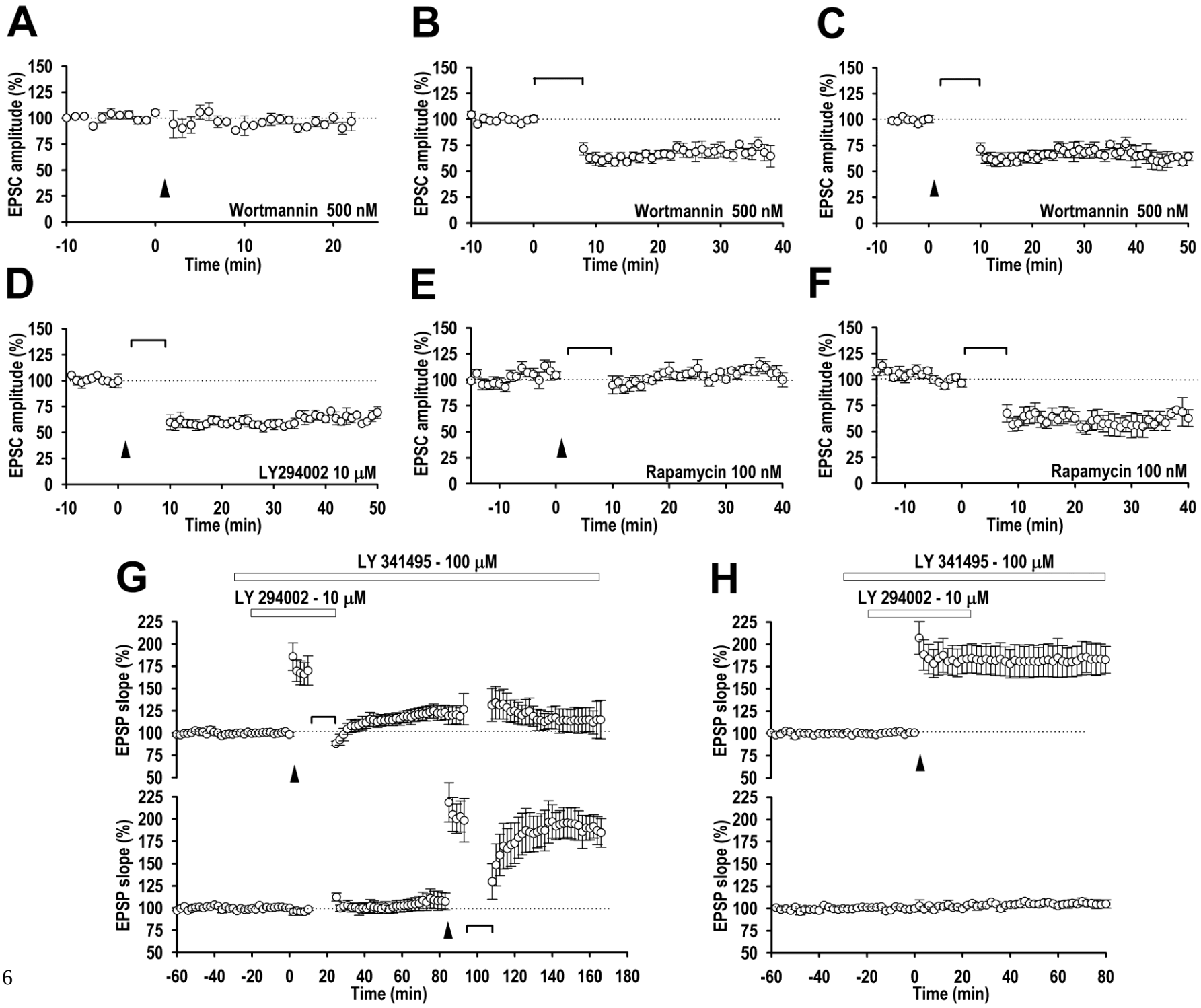
A
B
C
D
E
F
G
H
EPSC amplitude (%)
EPSP slope (%)
Time (min)
Wortmannin 500 nM
LY294002 10 μM
Rapamycin 100 nM
LY 341495 - 100 μM
LY 294002 - 10 μM

Figure 6

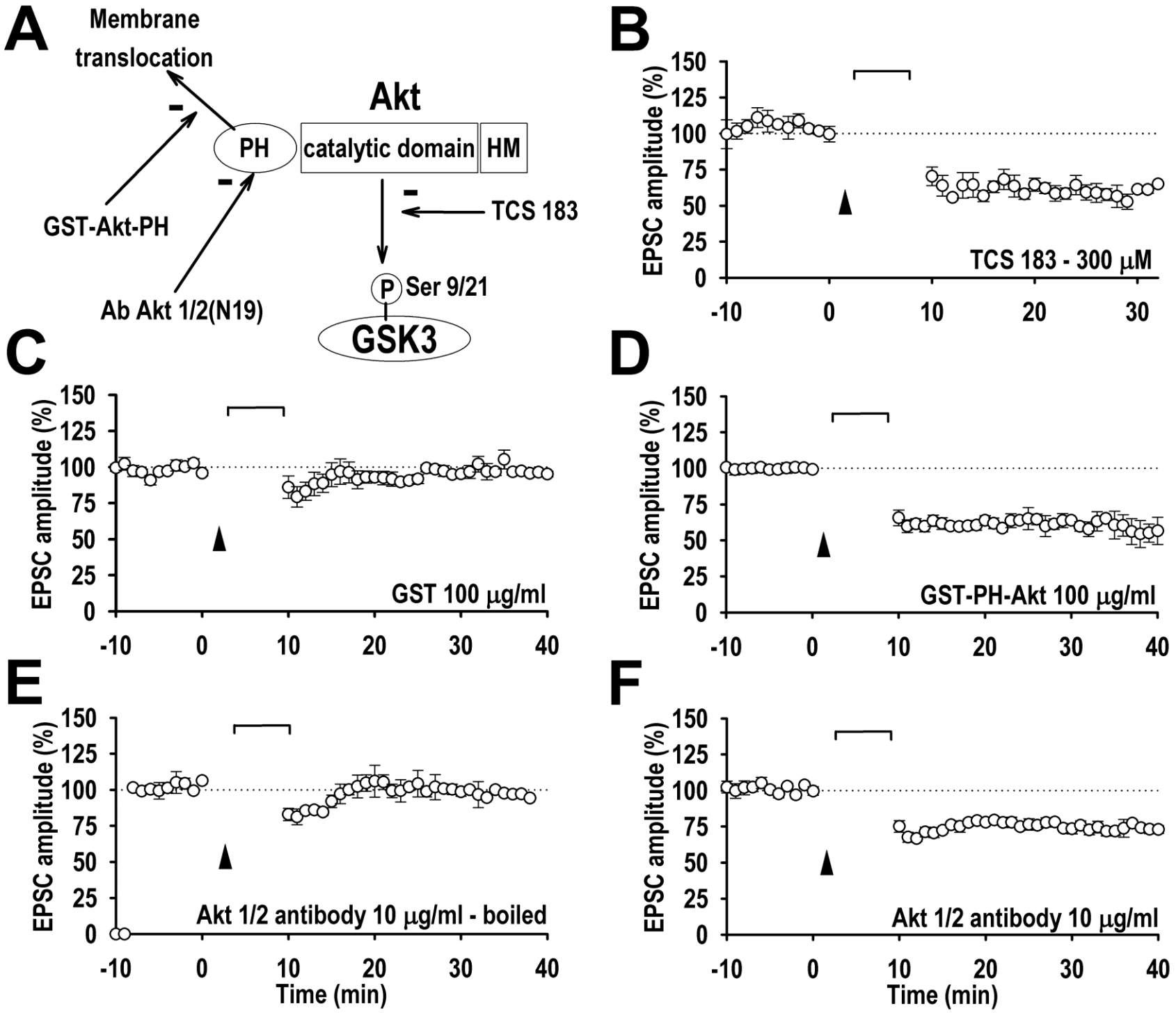
A
Membrane translocation
Akt
PH
catalytic domain
HM
GST-Akt-PH
TCS 183
Ab Akt 1/2(N19)
P
Ser 9/21
GSK3
B
EPSC amplitude (%)
TCS 183 - 300 μM
C
GST 100 μg/ml
D
GST-PH-Akt 100 μg/ml
E
Akt 1/2 antibody 10 μg/ml - boiled
F
Akt 1/2 antibody 10 μg/ml
Time (min)

Figure 7

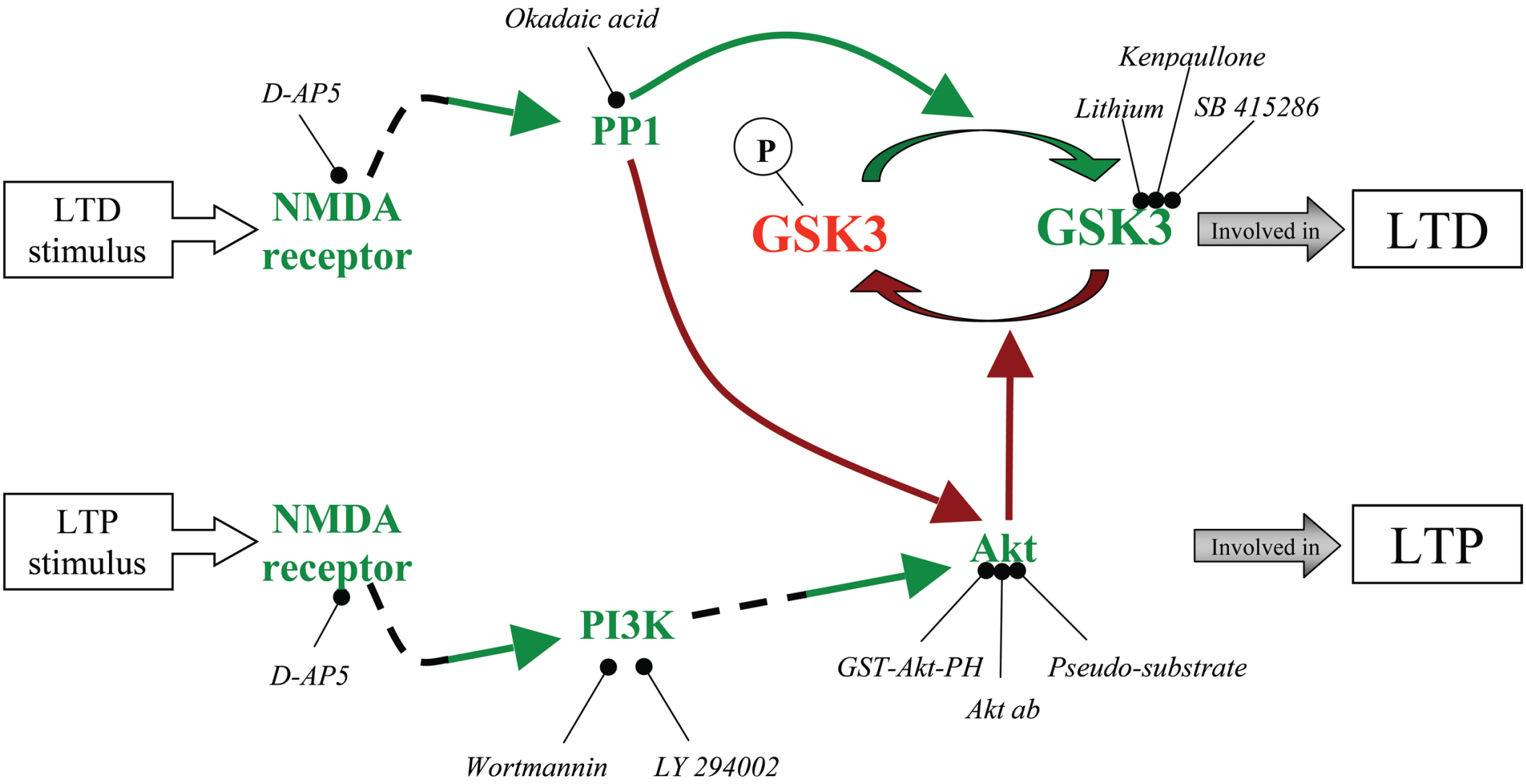
Okadaic acid
D-AP5
PP1
Kenpaullone
Lithium
SB 415286
P
LTD stimulus
NMDA receptor
GSK3
GSK3
Involved in
LTD
LTP stimulus
NMDA receptor
Akt
Involved in
LTP
PI3K
D-AP5
GST-Akt-PH
Pseudo-substrate
Akt ab
Wortmannin
LY 294002

Figure 8