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Intracellular mechanisms underlying the Nicotinic Enhancement of LTP in the Rat Dentate Gyrus

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Abstract

We have previously shown that activation of nicotinic acetylcholine receptors enhanced long-term potentiation (LTP) in the rat dentate gyrus *in vitro* via activation of α7 nicotinic acetylcholine receptor (nAChR). In the present studies, mechanisms underlying the acute and chronic nicotinic enhancement of LTP were examined. In particular, the involvement of activation of intracellular kinases was examined using selective kinase antagonists, and the effects of enhancing cholinergic function with a positive allosteric modulators of the α7 nAChR and with acetylcholine inhibitors was also investigated. Activation of extracellular signal-regulated kinase (ERK) and cAMP-dependent protein kinase (PKA) was found to be involved in the induction of the acute nicotinic enhancement of LTP although not control LTP. In contrast, activation of the tyrosine kinase Src, Ca^{2+}-calmodulin-dependent protein kinase II, Janus kinase 2 and p38 mitogen-activated protein kinase were not involved in the acute nicotinic enhancement of LTP although Src activation was necessary for control LTP. Moreover, activation of phosphoinositide 3-kinase was involved in the acute nicotinic enhancement of LTP to a much lesser extent than in control LTP. Chronic nicotine enhancement of LTP was found to be dependent on PKA, ERK and Src kinases. **Acute nicotinic enhancement of LTP was occluded of chronic nicotine treatment.** The positive allosteric modulator PNU-120596 was found to strongly reduce the threshold for nicotinic enhancement of LTP, an affect mediated via the α7 nAChR as it was blocked by the selective antagonist methyllycaconitine. The acetylcholinesterase inhibitors tacrine and physostigmine enhanced control LTP.

Introduction

Cholinergic innervation of the hippocampus modulates electrical activity and other processes that contribute to learning and memory (Decker and McGaugh, 1991; Jones *et al*, 1999; Levin, 2002). This includes modulation of activity dependent synaptic plasticity such as LTP which is the most convincing model of learning and memory (Bliss and Collingridge, 1993; Bear and Malenka, 1994). Loss of high affinity nicotinic receptors and cholinergic neurons is associated with neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease and Lewy body dementia (Whitehouse *et al*, 1982; Arendt *et al*, 1995). Current therapeutic approaches in the treatment of AD utilise AChE inhibition and positive allosteric modulation of cholinergic signalling. Other positive allosteric modulators have been described including ivermectin (Krause *et al*, 1998), 5-hydroxyindole (Zwart *et al*, 2002) and more recently PNU-120596 (Hurst *et al*, 2005).

Neuronal nicotinic receptors are located in both the central and peripheral nervous systems and are members of the superfamily of ligand gated ion channels. Nine alpha (α2-10) and three beta subunits (β2-4) have been identified in the CNS (Picciotto *et al*, 2001), each having unique expression profiles. Within the hippocampus, the single most abundant nicotinic acetylcholine receptor (nAChR) is the homomeric α7 subtype (Seguela *et al*, 1993; Dominguez del Toro *et al*, 1994; Le Novere and Changeux, 1995). It has a pentameric structure forming a channel with high calcium permeability and has consistently been demonstrated to enhance synaptic transmission associated with an increase in intracellular calcium and transmitter release (Gray *et al*, 1996; Radcliffe and Dani, 1998; Ji *et al*, 2001; Sharma and Vijayaraghavan, 2003). Nicotine was found to enhance LTP of excitatory postsynaptic potentials (EPSPs) in the dentate gyrus (Welsby *et al*, 2006, 2007) and to convert weak stimuli evoked short-term potentiation into LTP in the CA1 (Fujii *et al*, 1999; Ji *et al*, 2001; Mann and Greenfield, 2003). The selective

α7 nAChR agonists choline and 2, 4-dimethoxybenzlidine anabaseine have also been found to mimic the facilitative action of nicotine on LTP (Matsuyama *et al*, 2000; Hunter *et al*, 1994).

Stimulation of nAChRs can activate a variety of kinase pathways. Nicotine activation of α7 nAChRs in SH-SY5Y cells and cultured hippocampal neurones leads to PKA and subsequently ERK1/2 activation (Dajas-Bailador *et al*, 2002). In primary cultures of rat cortical neurons the neuroprotective effects of acetylcholinesterase inhibitors are mediated by α7 and α4β2 receptor activation of JAK2 and PI3K (Shaw *et al*, 2002; Takada-Takatori *et al*, 2006). In CA1 interneurons $GABA_A$ receptors are reversibly inhibited following calcium influx via postsynaptic α7 nAChRs and subsequent CaMKII and p38 MAPK activation (Zhang and Berg, 2007). nAChRs themselves are kinase regulated as preventing tyrosine phosphorylation of α7 nAChR's results in increased acetylcholine evoked currents and α7 nAChR cell surface expression (Cho *et al*, 2005; Charpantier *et al*, 2005).

Previously, we described an *in vitro* model where acute or chronic nicotine treatment results in nicotinic enhancement of LTP in hippocampal slices. However, little is known about the mechanisms underlying such nicotinic enhancement. In the present study, we investigated the kinase involvement and effects of AChE inhibitors and positive allosteric modulators on the nicotinic enhanced LTP in the dentate gyrus. We found that acute α7 nAChR stimulated enhancement of high frequency stimulation (HFS) induced LTP required activation of ERK and PKA while chronic nicotine enhancement of LTP was dependent on PKA, ERK and Src kinases. Moreover, LTP could be enhanced in the absence of nicotine by the AChE inhibitors physostigmine and tacrine and the positive allosteric modulator PNU-120596 reduced the threshold for LTP induction.

Materials and Methods

All experiments were carried out on transverse slices of the rat hippocampus (Wistar males, age 3-4 weeks, weight 40-80 g). Animal use was approved by the Bioresources Committee, Trinity College Dublin, and licensed by the Department of Health and Children, Ireland, in accordance with European Communities Council Directive (86/609/EEC) and were designed in such a way as to minimize the number of animals used and their suffering. The brains were rapidly removed after decapitation and placed in cold oxygenated (95% O_2 / 5% CO_2) media. Slices were cut at a thickness of 350μm using an Intracell Plus 1000 and placed in a storage container containing oxygenated medium at room temperature (20 - 22°C) for 1 hr. The slices were then transferred to a recording chamber for submerged slices and continuously superfused at a rate of 5-6ml/min at 30-32°C. The control media contained: (mM) NaCl, 120; KCl 2.5, NaH_2P04, 1.25; $NaHC0_3$ 26; $MgS0_4$, 2.0; $CaCl_2$, 2.0; D-glucose 10. All solutions contained 100μM picrotoxin (Sigma) to block $GABA_A$-mediated activity.

Drugs used were from Sigma unless otherwise stated; (-)-Nicotine, U0126, H-89, Rp-8-CPT-cAMPs, wortmannin, genistein, SU-6656, KN-62, tyrphostin AG-490, SB-203580, MLA, physostigmine, tacrine, PP2 (Calbiochem) and PNU-120596 (Tocris). In chronic studies, nicotine (1mg/kg), its vehicle (sterile 0.9% NaCl), PNU-120596 (1mg/kg) and its vehicle (5% DMSO and 5% Solutol in PBS) was administered to male rats (age 3-4 weeks, weight 40-80 g) twice daily by subcutaneous injection for 14 days prior to *in vitro* slice preparation as described above.

Standard electrophysiological techniques were used to record field potentials. Presynaptic stimulation was applied to the medial perforant pathway of the dentate gyrus using a bipolar insulated tungsten wire electrode, and field EPSPs were recorded at a control test frequency of 0.033 Hz from the middle one-third of the molecular layer

of the dentate gyrus with a glass microelectrode. The inner blade of the dentate gyrus was used in all studies. In each experiment, an input-output curve (afferent stimulus intensity versus EPSP amplitude) was plotted at the test frequency. For all experiments, the amplitude of the test EPSP was adjusted to one-third of maximum (~1.0 mV). LTP was evoked by HFS consisting of 8 trains, each of 8 stimuli at 200Hz, intertrain interval 2s, with the stimulation voltage increased during the HFS so as to elicit an initial EPSP of the train of double the normal test EPSP amplitude. Measurements of LTP are given at 60 min post-HFS. Recordings were analysed using p-CLAMP (Axon Instruments, CA, USA). Values are the means ± S.E.M. for n slices. Two-tailed Student's t-test was used for statistical comparison.

Results

ERK and PKA are required for the acute nicotinic enhancement of LTP but not control LTP

In the medial perforant pathway of the dentate gyrus HFS induced control LTP measuring 154±8%, n=7 (60 min post HFS) (Fig. 1A). As in our previous studies, perfusion of nicotine (5μM) for ten minutes prior to HFS significantly enhanced the induction of control LTP, which measured 187±9% ($p < 0.05$; n=6; 60 min post HFS) (Fig. 1A).

U0126 is a selective inhibitor of both the active and inactive forms of MAP kinase kinase (MEK1/2), the immediate upstream activator of ERK1/2**, and has been previously shown to inhibit ERK1/2 activation in acute slices (Roberson *et al*, 1999; Giovannini *et al*, 2003).** U0126 (5μM) did not significantly affect the induction of control LTP (139±9%, $p > 0.1$; n=7) but completely prevented the induction of the nicotinic enhancement of LTP, which was not significantly different from control in the presence of U0126 (138±8%, $p > 0.8$; n=6) (Fig. 1B). Control LTP was also unaffected

by PKA inhibition. Thus induction of LTP was not blocked by **effective concentrations of** the PKA inhibitor H-89 (2μM) **(Yang *et al*, 2008)** (147±3%, $p > 0.9$; n=7) (Fig. 1C) or the non hydrolysable cAMP analogue Rp-8-CPT-cAMPS (150±5%, $p > 0.8$; n=7) (100μM) (Fig. 1D). However, both H-89 and Rp-8-CPT-cAMPs completely inhibited nicotinic enhancement of LTP, with LTP induction in the presence of either H-89 or Rp-8-CPT-cAMPs plus nicotine not significantly different from control LTP (144±7%, $p > 0.5$; n=9 and 145±5%, $p > 0.8$; n=6 respectively) (Fig. 1C, D).

Thus nicotinic-enhanced LTP, unlike control LTP, is dependent on activation of ERK and PKA.

Src is required for control LTP but not acute nicotinic enhancement of LTP, while PI3K is involved in acute nicotinic-enhancement of LTP to a lesser extent than in control LTP.

The isoflavone genistein is a potent competitive inhibitor of tyrosine kinase phosphorylation, both receptor and non-receptor mediated **(Kawakami *et al*, 2001).** As in previously published studies (Casey *et al*, 2002; Huang and Hsu 1999; O'Dell *et al*, 1991) control HFS induced LTP was strongly inhibited in the presence of genistein (10μM) with LTP not being significantly different from baseline (113±12%, $p > 0.6$; n=11) (Fig. 2A)**.** In contrast, substantial LTP was induced in the presence of nicotine plus genistein (154±10%, $p < 0.04$; n=10), significantly increased LTP compared to that in genistein alone ($p < 0.005$).

The potential involvement of the tyrosine kinase Src in the induction of LTP was further investigated by examining the effects of two potent and selective Src kinase inhibitors, PP2 and SU-6656 **(Sanna *et al*, 2000; Meriane *et al*, 2004)**. Src kinases have previously been demonstrated to alter NMDAR activation (Yu *et al*, 1997; Lu *et al*, 1998) and are required in mGluR5 activation of the MAPK signalling cascade (Peavy *et*

al, 2001). PP2 (1μM) completely blocked control HFS induced LTP (101±5%, $p > 0.5$; n=5, 60min post HFS) (Fig. 2B). In contrast, substantial LTP was induced in the presence of nicotine plus PP2 (137±2%, $p < 0.001$; n=5), with significantly increased LTP compared to that in PP2 alone ($p < 0.005$). A second selective Src kinase inhibitor SU-6656 (10μM) produced similar results (Fig. 2C). Thus the induction of control LTP was strongly blocked by SU-6656, with LTP not significantly different from baseline (110±3%, $p > 0.1$; n=6). In contrast, substantial LTP was induced in the presence of nicotine plus SU-6656 (137±4%, $p < 0.001$; n=7, 60 min post HFS), with significantly increased LTP compared to that in SU-6656 alone ($p < 0.005$).

Inhibition of PI3K has previously been reported to disrupt LTP in the hippocampus (Kelly and Lynch, 2000; Opazo *et al*, 2003; Man *et al* 2003; Sanna *et al*, 2002**), with wortmannin blocking LTP associated PI3K phosphorylation (Kelly and Lynch, 2000).** Moreover, stimulation of α7 nAChR in cultured cortical neurons was shown to activate PI3K (Kihara *et al*, 2001). Using the fungal metabolite wortmannin which is a potent and highly selective inhibitor of PI3K activation with an IC50 for PI3K of 5-10nM (Davies *et al*, 2000), we investigated the role of PI3K in nicotinic enhanced LTP. The induction of control HFS-LTP was strongly inhibited by wortmannin (100 nM or 1μM) with LTP measuring 115±9% ($p > 0.1$; n=1) and 102±6% ($p > 0.8$; n=7) respectively (60 min post HFS) (Fig. 2D, E). The nicotinic enhancement of LTP was less sensitive to inhibition by wortmannin. Thus the induction of nicotinic enhanced LTP was unaffected by the low concentration of wortmannin (100 nM) (177±16%, $p > 0.1$; n=9) (Fig. 2D), but was inhibited by the higher concentration (1μM), LTP measuring 127±3%, $p < 0.001$; n=7) (Fig. 2E).

Thus nicotinic-enhancement of LTP, unlike control LTP, is not dependent on activation of the tyrosine kinase Src. Moreover, nicotinic-enhancement of LTP is less dependent on activation of PI3K than control LTP.

Acute nicotinic enhancement of LTP does not require CaMKII, JAK2 or p38 MAPK activity

A number of kinases previously reported to couple to nicotinic receptor activation were also investigated. Nicotinic activation of the α7 nAChR was previously shown to activate CaMKII (Klein and Yakel, 2005). The selective cell permeable CaMKII inhibitor KN-62 **(Dhavan *et al*, 2002)** (3μM) did not significantly alter control HFS induced LTP (148±6%, $p > 0.9$; n=9) (Fig. 3A), confirming a recent study from the present laboratory that LTP induction in the dentate gyrus could not be blocked by inhibition of CaMKII alone, unlike CA1 (Wu *et al*, 2006). We also found that KN-62 also did not inhibit LTP induced in the presence of nicotine (202±8%, $p > 0.4$; n=5) (Fig. 3A).

Previous reports have found that nicotine activation of JAK2 *via* α7 nAChR's has a neuroprotective role against the Alzheimer's associated protein Aβ1-42 (Shaw *et al*, 2002; Takada-Takatori *et al*, 2006). However, in the present study, inhibition of JAK2 kinase with Tyrphostin AG-490 (10μM) **(Marrero *et al*, 2003; Shaw *et al*, 2003)** did not inhibit control HFS-LTP (136±11%, $p > 0.5$; n=5) (Fig. 3B) or LTP induced in the presence of nicotine (174±23%, $p > 0.5$; n=5).

Finally, p38 MAPK is known to be involved in mGluR dependent LTD in the dentate gyrus (Rush *et al*, 2002; Wang *et al*, 2007) but not HFS - LTP (Wang *et al*, 2004). However, nicotine has been shown to activate p38 MAPK (Wada *et al*, 2007; Li *et al*, 2004). The involvement of p38 MAPK in nicotinic enhanced LTP was investigated using the selective inhibitor SB-203580 (1μM **(**Fig. 3C**) and 10μM) at pharmacologically relevant concentrations (Bain *et al*, 2007; Blanquet, 2000).** SB-203580 did not inhibit either the induction of control LTP (159±11%, $p > 0.4$; n=10) or

LTP induced in the presence of nicotine (190±6%, p > 0.8; n=5 **@ 1μM and 188±14%, p > 0.7; n=4).**

Thus nicotinic-enhancement of LTP is not dependent on activation of CaMKII, JAK or p38 MAPK activity.

Chronic nicotine enhancement of LTP is dependent on activation of PKA, ERK and Src kinases

Previous results from our laboratory have demonstrated that the chronic administration of nicotine (1mg/kg twice daily for two weeks) significantly enhanced HFS induced LTP in the medial perforant pathway of the dentate gyrus in the absence of acute nicotinic receptor activation (Welsby *et al*, 2006). This result was confirmed in the present studies in which HFS-LTP in nicotine treated rats was significantly enhanced when compared to vehicle controls (192±10%, p < 0.01; n=7, 60min post HFS) (Fig. 4A). The kinase dependence of chronic nicotine enhanced LTP was subsequently investigated. The MEK1/2 inhibitor U0126 completely blocked the enhancement of HFS-LTP by chronic nicotine, LTP induced in the presence of U0126 not being different from the saline control (148±10%, p > 0.7; n=5, 60min post HFS) (Fig. 4B). In addition, LTP induced in the presence of the PKA inhibitor Rp-8-CPT-cAMPs was also not significantly different from saline control LTP (139±12%, p > 0.8; n=5, 60min post HFS) (Fig. 4C). Thus the enhancement of induction of LTP caused by chronic nicotine application, although not induction of control LTP, was dependent on activation of ERK and PKA.

The Src kinase inhibitor SU-6656 completely blocked control LTP (Fig. 2A, 4D) and the enhanced LTP following chronic nicotine application, LTP measuring 101±3%, p > 0.5; n=5 (Fig. 4D). Thus Src is necessary for both control LTP and the chronic nicotine enhancement of LTP.

The effect of acute nicotine on chronic nicotine enhanced LTP was also examined (Fig. 4E). Chronic nicotine treatment increased the magnitude of LTP significantly ($p < 0.05$) over saline treated controls (189±8%, n=5 vs. 159±9%, n=5) (Fig. 4E). Additional acute nicotine treatment of hippocampal slices from chronic nicotine treated animals did not further increase the magnitude of LTP (201±5%, $p > 0.5$; n=5).

A positive allosteric modulator of the α7 nAChR reduces the threshold concentration of nicotine for enhancement of LTP

PNU-120596 is a positive allosteric modulator at the α7 nAChR and has been demonstrated to enhance isolated acetylcholine elicited α7 receptor currents (Hurst *et al*, 2005). In the present studies, PNU-120596 (1μM) did not significantly enhance control LTP (148±9%, $p > 0.5$; n=9, 60min post HFS) (Fig. 5A) or the maximally enhanced LTP induced by a high concentration of nicotine (5μM) (Welsby *et al*, 2006) (192±9%, $p > 0.5$; n=5, 60min post HFS) (Fig. 5B). However, PNU-120596 reduced the threshold concentration of nicotine required to enhance LTP. Thus while 500 nM nicotine alone did not enhance LTP (157±12%, $p > 0.6$; n=8) (Fig. 5C), the same concentration of nicotine did significantly enhance LTP in the presence of PNU-120596 (191±5%, $p < 0.01$; n=5) (Fig. 5D). In order to confirm that nicotine was solely activating the α7 nAChR in the presence of PNU-120596, we found that while MLA alone (50nM) had no significant effect on LTP compared with control (149±14%, $p > 0.5$; n=5) (Fig. 5E) (see also Welsby *et al*, 2006), MLA completely blocked the enhancement of LTP in the presence of nicotine (500nM) and PNU-120596 (162±11%, $p > 0.1$; n=7) (Fig. 5F).

Acetylcholinesterase inhibitors enhance HFS-LTP

In order to determine if an increase in endogenous acetylcholine levels could enhance HFS-LTP in a manner similar to nicotine the effects of two different AChE

inhibitors, physostigmine and tacrine, were examined. Physostigmine applied at a concentration of 10μM, although not 1μM, enhanced HFS-LTP (179±11%, $p > 0.7$; n=5, and 138±13%, $p > 0.5$; n=5 respectively) (Fig. 6A). Tacrine (10μM) also produced a strongly enhanced LTP (181±8%, $p > 0.9$; n=9, 60min post HFS) (Fig. 6B). The enhanced LTP in physostigmine and tacrine was identical to that produced by nicotine.

Discussion

We previously described both acute and chronic nicotinic enhancement of LTP in the perforant pathway of the rat dentate gyrus (Welsby *et al*, 2006, 2007). In the current studies we have utilized this model to identify a number of kinases critical for the effects of nicotine and also assessed the effectiveness of a number of modulators of cholinergic signalling in mimicking or further augmenting LTP enhancement. We find that activation of ERK and PKA is required for the acute nicotinic enhancement of LTP but not control LTP, while activation of PI3K and Src is required for control LTP but not acute nicotinic-enhancement of LTP. Moreover, chronic nicotine enhancement of LTP is dependent on PKA, ERK and Src kinases.

The involvement of the kinases PKA and ERK1/2 MAPK in LTP and memory processes has been demonstrated in a number of previous studies. Transgenic mice over-expressing a dominant negative form of the PKA regulatory subunit showed significantly reduced LTP in the CA1 region of the hippocampus (Abel *et al*, 1997; Woo *et al*, 2000) while inhibitors of PKA prevent the induction of early and late phase LTP *in vitro* (Blitzer *et al*, 1995, 1998; Huang and Kandel 1994; Matthies and Reymann 1993). Many *in vivo* studies have demonstrated a role for the cAMP/PKA pathway in memory. Acute infusion of modulators of PKA activity into the CA1 region altered spatial memory in the Morris water maze task in male rats (Sharifzadeh *et al*, 2005, 2007); infusion of the PKA inhibitor H-89 impaired spatial memory retention in trained animals (Sharifzadeh *et al*, 2005); and conversely the membrane permeable PKA

activator dibutyryl cyclic AMP caused a significant improvement in special memory (Sharifzadeh *et al*, 2007). ERK1/2 MAPK has also been implicated in synaptic plasticity and memory. The formation of contextual and spatial memories in mammals has requires activation of ERK/MAPK (Sweatt, 2001) and various inhibitors of the ERK1/2 pathway also attenuate LTP induction in CA1 (English and Sweatt, 1997; Davis *et al*, 2000; Ying *et al*, 2002). However, in the dentate gyrus, control LTP is mediated by the induction of one of two parallel pathways (Wu *et al*, 2006), the first requiring CaMKII activation and the second PKA or MAPK. The involvement in nicotine activated PKA in synaptic plasticity is less well described. *In vitro* studies using SH-SY5Y cells and cultured hippocampal neurones have previously shown that acute activation of the α7 nAChR stimulates ERK1/2 *via* PKA activation (Dajas-Bailador *et al*, 2002). An interaction between PKA and ERK1/2 following nicotine stimulation may also occur during LTP enhancement. Our studies found that the acute inhibition of PKA and MEK, the kinase immediately upstream from ERK1/2, did not alter induction of control LTP but completely prevented nicotinic augmentation of LTP. Thus activation of the α7 nAChR links to stimulation of the PKA and MEK intracellular signalling pathways and thence enhances LTP induction.

The enhanced HFS-LTP following chronic nicotine treatment was also dependent on PKA and ERK activation. The chronic enhancement of LTP may be due to alterations in the expression of proteins in the cAMP-PKA signalling pathway following chronic nicotine treatment. It has been shown that in the brains of human smokers both PKA and adenylate cyclase levels are increased (Hope *et al*, 2007). Also, in rats chronically exposed to nicotine, phosphodiesterase levels are decreased (Polesskaya *et al*, 2007a, b). It is also known that both PKA and adenylate cyclase demonstrate basal activity in the absence of agonist activation (Bogan and Niswender, 2007; Salazar and Gonzalez, 2002). Under control conditions inhibition of this basal activity does not impact on LTP induction. However, nicotine induced upregulation of

the pathway at least in part may be responsible for enhanced HFS-LTP in the absence of acute nAChR activation. This hypothesis is supported by a study showing that repetitive activation of PKA induces a persistent potentiation in fEPSP amplitude recorded from the CA1 region in cultured hippocampal slices (Tominaga-Yoshino *et al*, 2002).

Src kinases have been implicated previously in LTP induction and α7 nAChR functioning. Thus tyrosine phosphorylation of the NMDAR and Src mediated insertion of AMPAR's are required for LTP induction *in vitro* (Casey *et al*, 2002; Huang and Hsu 1999; O'Dell *et al*, 1991). Conversely, phosphorylation of the α7 nAChR by Src kinase reduces acetylcholine evoked currents and decreases receptor expression at the cell surface (Charpantier *et al*, 2005; Cho *et al*, 2005). Block of Src kinase activation prevented the induction of control LTP in our present studies but did not prevent the acute nicotine enhancement of LTP component. In contrast, the enhancement of LTP by chronic nicotine treatment was found to be dependent on Src activation, further evidence for different mechanisms underlying acute and chronic enhancement of LTP as well as control LTP and nicotinic-enhanced LTP.

HFS induced LTP in the dentate gyrus has previously been shown to require PI3K activation (Kelly and Lynch, 2000; Opazo *et al*, 2003; Man *et al* 2003; Sanna *et al*, 2002), a finding that our studies confirm and additionally establish that control and nicotinic enhancement of LTP display differential sensitivities to PI3K inhibition. In the present studies, concentrations of wortmanin sufficient to block control plasticity did not block nicotinic-enhanced LTP, although such nicotinic-enhanced LTP was inhibited using a higher although still selective concentration of the PI3K antagonist (Davies *et al*, 2000). This result shows that nicotinic-enhanced LTP is less dependent on activation of PI3K than control LTP. The activation of PI3K following α7 nAChR stimulation reported by Kihara *et al* and others (Shaw *et al*, 2002) established a neuroprotective role for nicotine in AD. This neuroprotective pathway and the nicotinic enhancement of LTP

which we describe appear to diverge following receptor activation but both involve PI3K adding further complexity to the many reported effects of nicotine treatment.

Hippocampal cholinergic deficits associated with AD have been treated with partial success through modulation of endogenous signalling with drugs such as galantamine which acts as both an inhibitor of AChE and as a positive allosteric modulator at nAChR's (Santos *et al*, 2002). Modulation of endogenous signalling may avoid both over stimulation and desensitisation associated with direct agonist stimulation and also problems with agonist selectivity due to the high sequence homology of nAChR orthosteric binding sites and also other members of the cys-loop family. To date a number of positive allosteric modulators of the α7 nAChR have been described including PNU-120596 which was demonstrated to have therapeutic potential by improving auditory gating deficits (Hurst *et al*, 2005). Our current studies found a lack of effect of acute PNU-120596 on LTP in the absence of exogenous agonist activation. However, acute PNU-120596 did produce an approximate tenfold reduction in the threshold for nicotinic enhancement of LTP. We also showed that control LTP is increased by tacrine in a manner consistent with its primary activity as an AChE inhibitor and thus enhancement of HFS released acetylcholine (Liston *et al*, 2004). A similar enhancement was produced by physostigmine which has activity both as an AChE inhibitor (Atack *et al*, 1989) and as a positive allosteric modulator of α7 nAChRs (Faghih *et al*, 2008). However given the lack of affect of PNU-120596, a drug reported not to inhibit AChE activity but to modulate positively the activity of α7 nAChRs through an allosteric site (Hurst *et al*, 2005), it is reasonable to conclude that the enhancement of control LTP by physostigmine and tacrine is as a result of AChE inhibition and not allosteric modulation.

In summary, this study shows that the control and nicotine enhanced components of HFS-LTP in the perforant pathway of the rat dentate gyrus display diverse and

differential kinase sensitivities. Allosteric modulation of α7 nAChR signalling reduces the threshold for nicotinic enhancement of HFS-LTP and inhibition of AChE activity enhances HFS-LTP in a manner consistent with α7 nAChR activation. This information and further characterisation of nicotine enhanced LTP will aid in the development of novel cholinergic treatments for AD.

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Abbreviations: AD, Alzheimer's disease; LTP, long-term potentiation; nAChR, nicotinic acetylcholine receptor; ERK, extracellular signal-regulated kinase; PKA, cAMP-dependent protein kinase; CaMKII, Ca^{2+}-calmodulin-regulated kinase II; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; AChE, acetylcholinesterase; EPSP, excitatory-postsynaptic potential; HFS, high frequency stimulation; MEK, mitogen activated protein kinase kinase; MLA, methyllycaconitine

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Legends

Fig 1. Kinase dependence of control and nicotinic enhanced HFS-LTP in rat dentate gyrus. A, The graph shows the induction of control LTP (filled circles) and the enhancement of LTP induction by pre-application of nicotine (5μM) (open circles). **B**, Acute application of the MEK1/2 inhibitor U0126 (5μM) prevents nicotine enhancement of HFS-LTP (closed circles). **C**, The PKA inhibitor H-89 (2μM) blocks nicotine enhancement of HFS-LTP (closed circles). **D**, A non-hydrolysable analogue of cAMP, Rp-8-CPT-cAMPs (100μM), prevents nicotinic enhancement of HFS-LTP (closed circles). Traces illustrate EPSP's before and 60min after HFS.

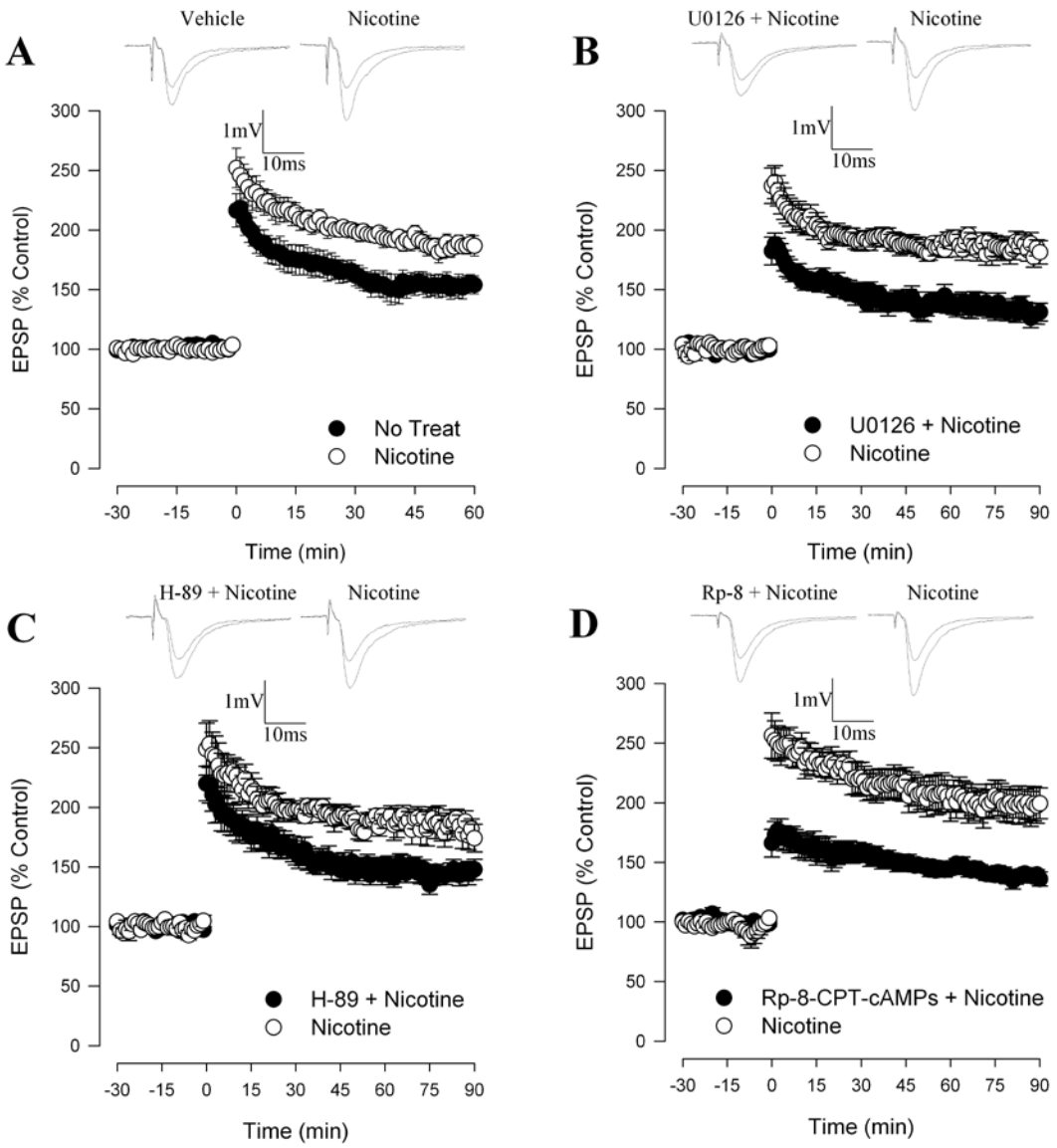

A
Vehicle
Nicotine
1mV
10ms
EPSP (% Control)
No Treat
Nicotine
Time (min)
B
U0126 + Nicotine
Nicotine
1mV
10ms
EPSP (% Control)
U0126 + Nicotine
Nicotine
Time (min)
C
H-89 + Nicotine
Nicotine
1mV
10ms
EPSP (% Control)
H-89 + Nicotine
Nicotine
Time (min)
D
Rp-8 + Nicotine
Nicotine
1mV
10ms
EPSP (% Control)
Rp-8-CPT-cAMPs + Nicotine
Nicotine
Time (min)

Fig 2. Kinase dependence of control and nicotine enhanced HFS-LTP in the rat dentate gyrus. A, The graph shows the induction of LTP in control (filled circles) and nicotine treated (open circles) slices in the presence of the tyrosine kinase inhibitor genistein (10μM). **B**, The Src kinase inhibitor PP2 (1μM) blocks control LTP (closed circles) and reduces the magnitude of nicotinic enhanced LTP (open circles). **C**, SU-6656 (10μM) also blocks control HFS-LTP (closed circles) but HFS-LTP in the presence of nicotine remains significantly above baseline (open circles). **D**, A concentration of wortmannin (100nM) sufficient to completely block control HFS-LTP (closed circles) does not significantly affect nicotinic enhancement of LTP (open circles). **E**, A higher concentration of wortmannin (1μM) inhibits nicotinic LTP (open circles) however, LTP remains significantly above baseline. Traces illustrate EPSP's before and 60min after HFS.

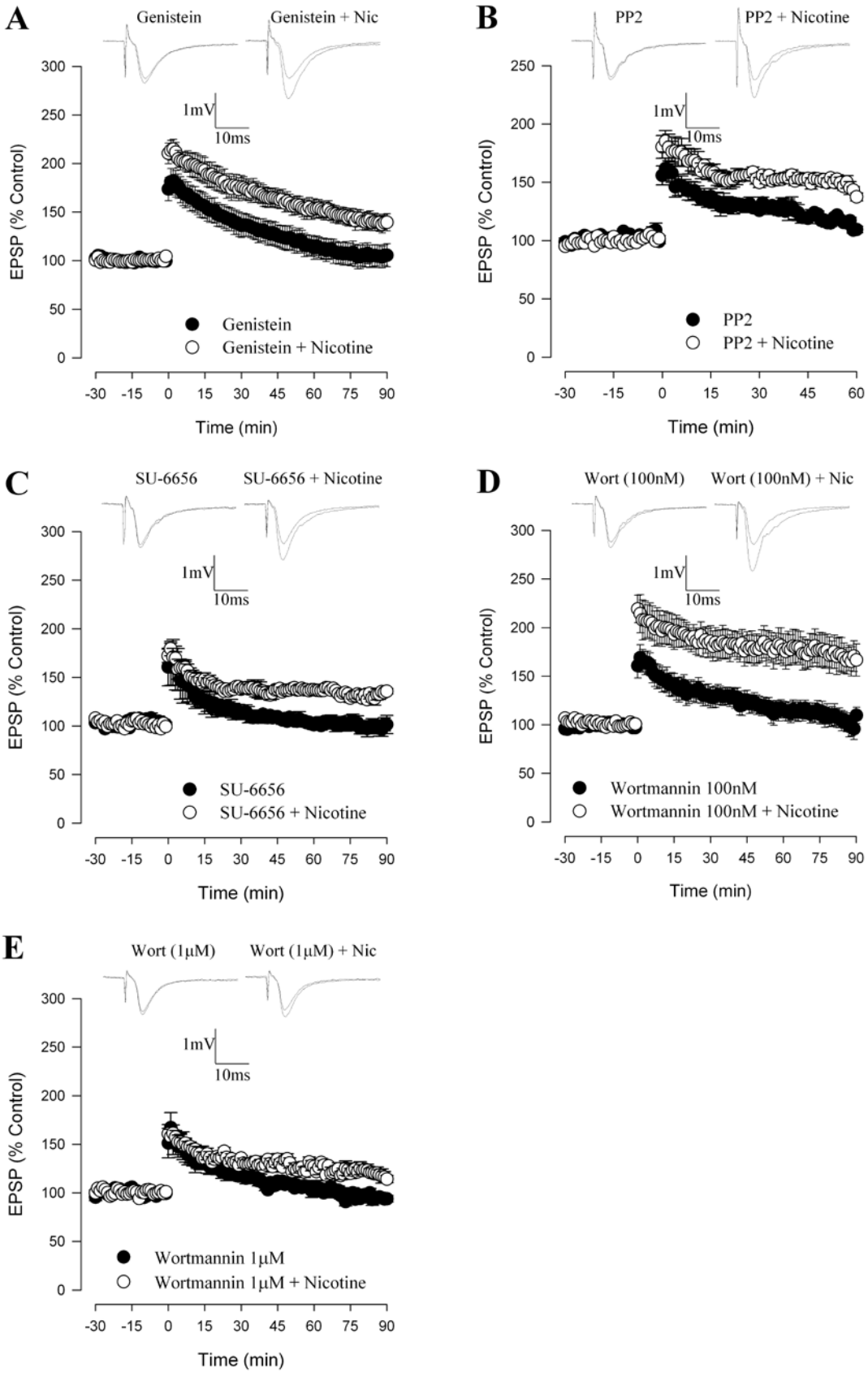

A
Genistein
Genistein + Nic
1mV
10ms
EPSP (% Control)
Genistein
Genistein + Nicotine
Time (min)
B
PP2
PP2 + Nicotine
1mV
10ms
EPSP (% Control)
PP2
PP2 + Nicotine
Time (min)
C
SU-6656
SU-6656 + Nicotine
1mV
10ms
EPSP (% Control)
SU-6656
SU-6656 + Nicotine
Time (min)
D
Wort (100nM)
Wort (100nM) + Nic
1mV
10ms
EPSP (% Control)
Wortmannin 100nM
Wortmannin 100nM + Nicotine
Time (min)
E
Wort (1μM)
Wort (1μM) + Nic
1mV
10ms
EPSP (% Control)
Wortmannin 1μM
Wortmannin 1μM + Nicotine
Time (min)

Fig 3. Acute nicotinic enhancement of LTP is not dependent on CaMKII, JAK or p38 MAPK activation. A, Induction of control LTP is unaffected by the selective CaMKII inhibitor KN-62 (3μM) (closed circles), as is nicotinic enhancement of HFS induced LTP (open circles). **B**, Acute application of the JAK inhibitor tyrphostin AG-490 (10μM) affects neither control (closed circles) nor nicotine enhanced (open circles) **C**, The selective p38 MAPK inhibitor SB-205380 (1μM) also does not block control (closed circles) or nicotine enhanced LTP (open circles) in the rat dentate gyrus. Traces illustrate EPSP's before and 60min after HFS.

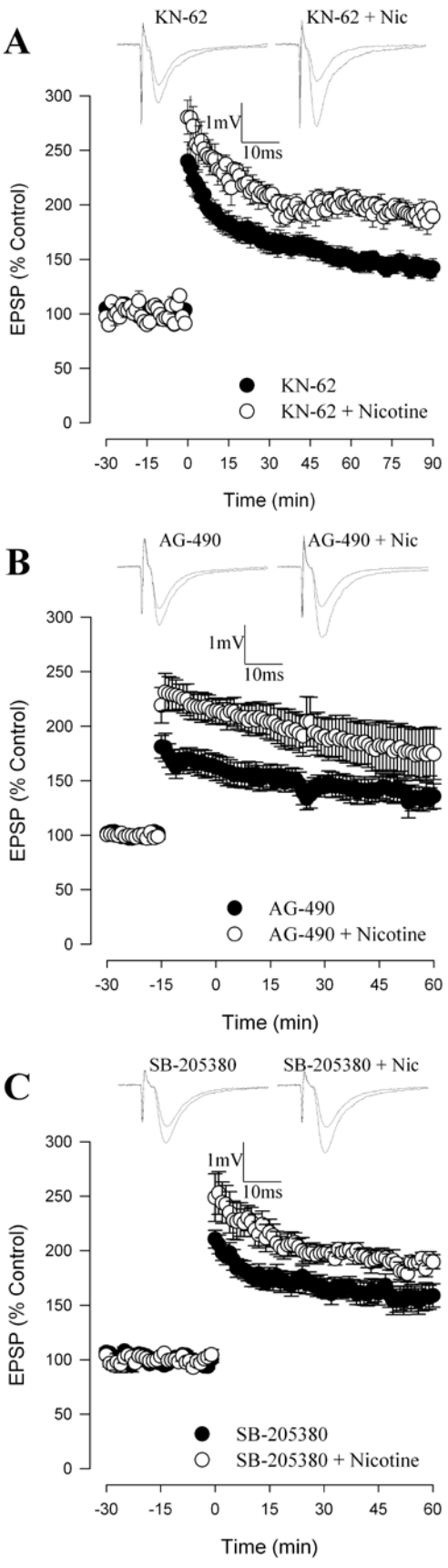

A
KN-62
KN-62 + Nic
1mV
10ms
EPSP (% Control)
KN-62
KN-62 + Nicotine
Time (min)
B
AG-490
AG-490 + Nic
1mV
10ms
EPSP (% Control)
AG-490
AG-490 + Nicotine
Time (min)
C
SB-205380
SB-205380 + Nic
1mV
10ms
EPSP (% Control)
SB-205380
SB-205380 + Nicotine
Time (min)

Fig 4. Chronic nicotine enhanced HFS-LTP is dependent on ERK1/2, PKA and Src family kinase activation in the rat dentate gyrus. A, Chronic treatment with nicotine (1mg/kg twice daily for two weeks) significantly enhances HFS-LTP (open circles) in the absence of acute nicotine when compared with saline control (closed circles). **B**, The MEK1/2 inhibitor U0126 (5μM) significantly inhibits chronic nicotinic enhancement of HFS-LTP (open circles). **C**, The PKA inhibitor Rp-8-CPT-cAMPs (100μM) also inhibits chronic nicotine enhanced LTP (open circles). **D**, SU-6656 (10μM), the Src family kinase inhibitor completely blocks LTP following chronic nicotine administration (open circles). **E, Acute nicotine treatment (5μM) (open circles) does not enhance LTP significantly over chronic nicotine treatment.** Traces illustrate EPSP's before and 60min after HFS.

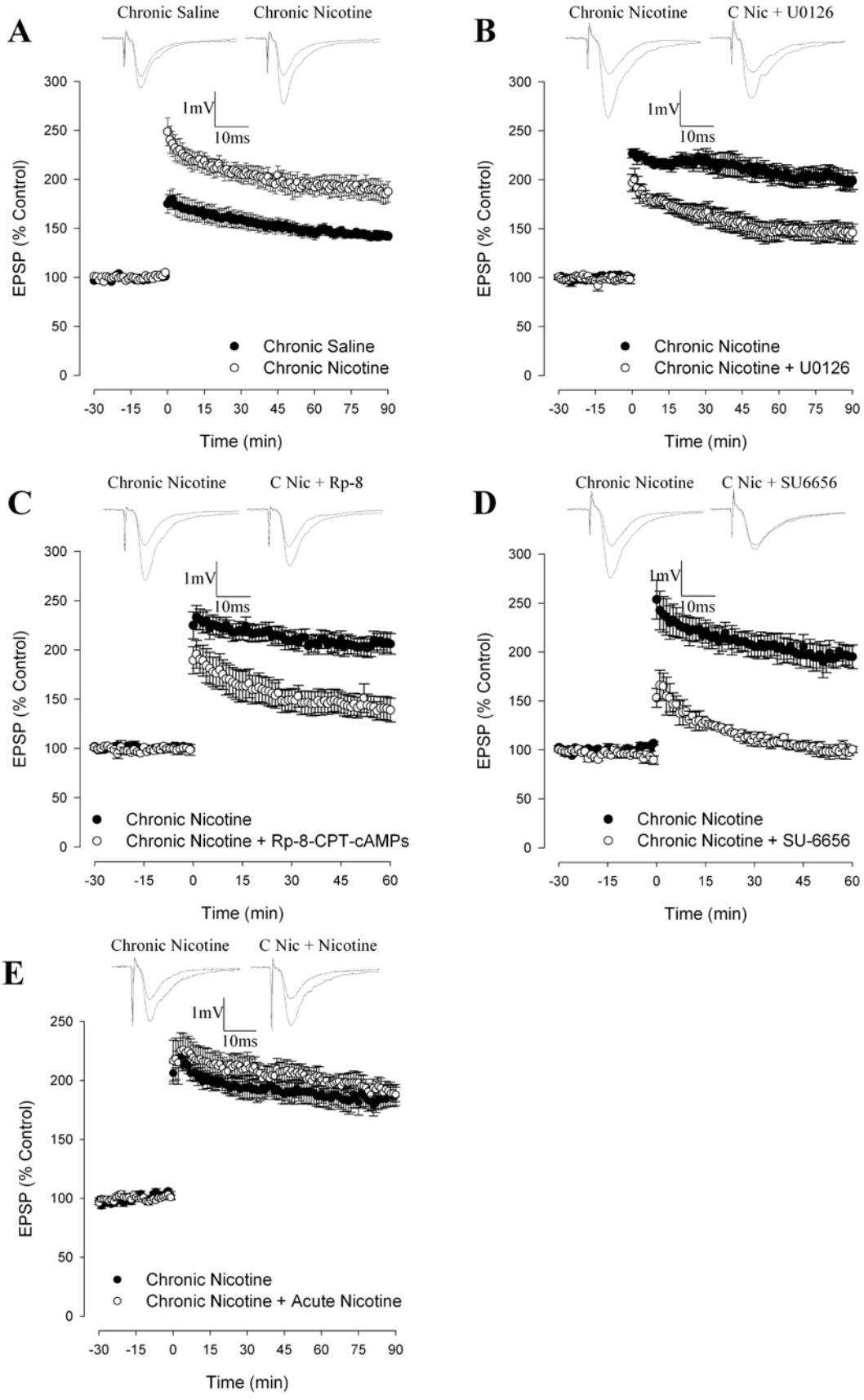

A
Chronic Saline
Chronic Nicotine
1mV
10ms
EPSP (% Control)
Chronic Saline
Chronic Nicotine
Time (min)
B
Chronic Nicotine
C Nic + U0126
1mV
10ms
EPSP (% Control)
Chronic Nicotine
Chronic Nicotine + U0126
Time (min)
C
Chronic Nicotine
C Nic + Rp-8
1mV
10ms
EPSP (% Control)
Chronic Nicotine
Chronic Nicotine + Rp-8-CPT-cAMPs
Time (min)
D
Chronic Nicotine
C Nic + SU6656
1mV
10ms
EPSP (% Control)
Chronic Nicotine
Chronic Nicotine + SU-6656
Time (min)
E
Chronic Nicotine
C Nic + Nicotine
1mV
10ms
EPSP (% Control)
Chronic Nicotine
Chronic Nicotine + Acute Nicotine
Time (min)

Fig 5. PNU modulation of HFS induced LTP in the presence of nicotine in rat dentate gyrus. A, Acute application of the α7 nAChR positive allosteric modulator PNU-120596 (1μM) did not significantly effect LTP (open circles) when compared with control (closed circles). **B**, PNU-120596 also did not significantly affect nicotine (5μM) enhanced HFS-LTP (open circles) when compared with nicotine treatment alone (closed circles). **C**, The bar graph summarises the affects on HFS-LTP of a range of nicotine concentrations. Only 5μM nicotine significantly enhanced LTP when compared with control. 500nM and 100nM were sub enhancing concentrations of nicotine. **D**, LTP in the presence of both acute PNU-120596 and 500nM nicotine (open circles) is significantly enhanced when compared with 500nM nicotine alone (closed circles). **E**, The α7 nAChR selective antagonist MLA (50nM) (open circles) did not alter LTP when compared with control (closed circles). **F**, MLA does not significantly affect LTP in the presence of nicotine (500nM) (closed circles) when compared with control (not shown). In the presence of MLA (50nM), the combination of PNU and 500nM nicotine (open circles) no longer significantly enhanced LTP when compared with control (not shown). Traces illustrate EPSP's before and 60min after HFS.

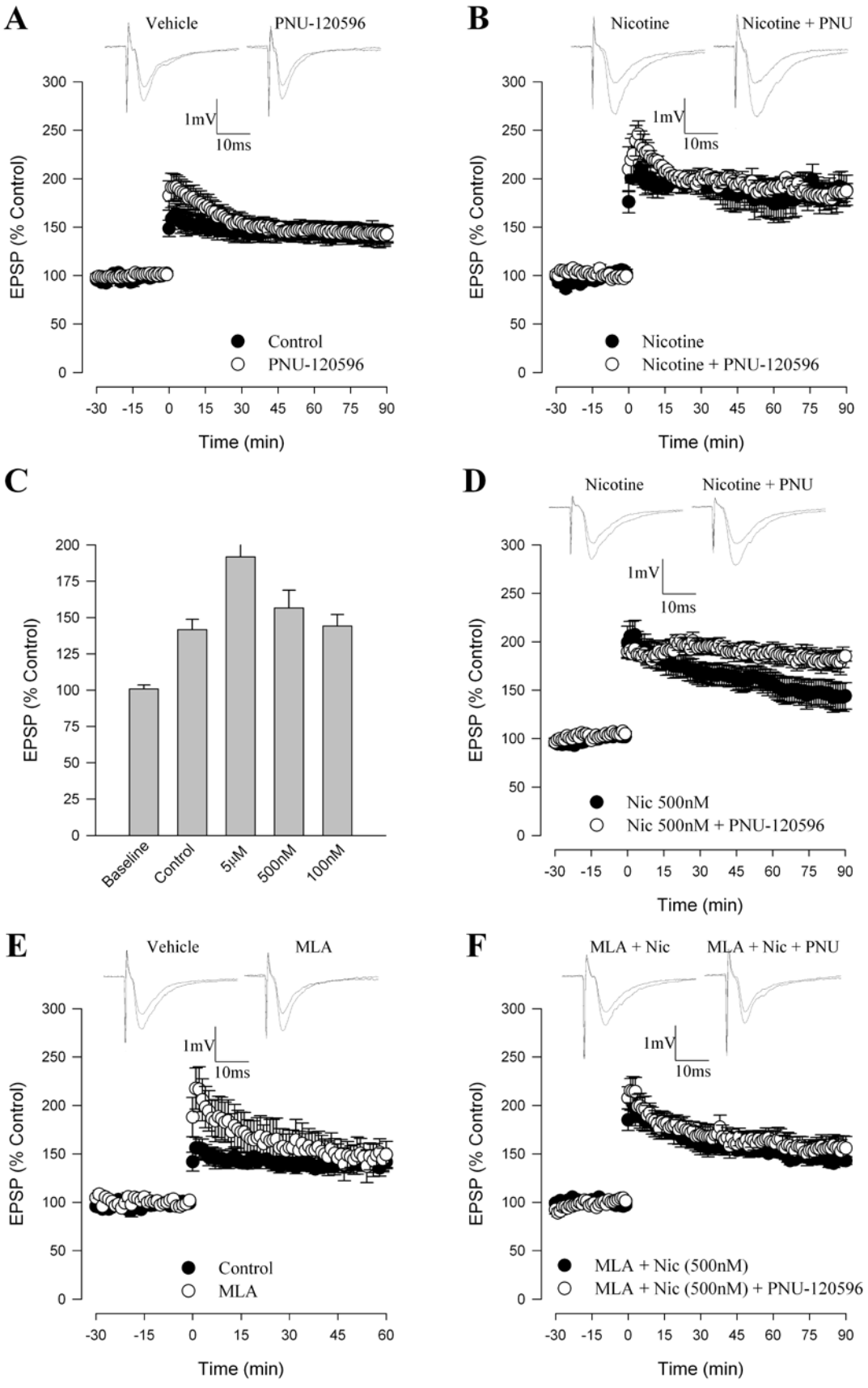
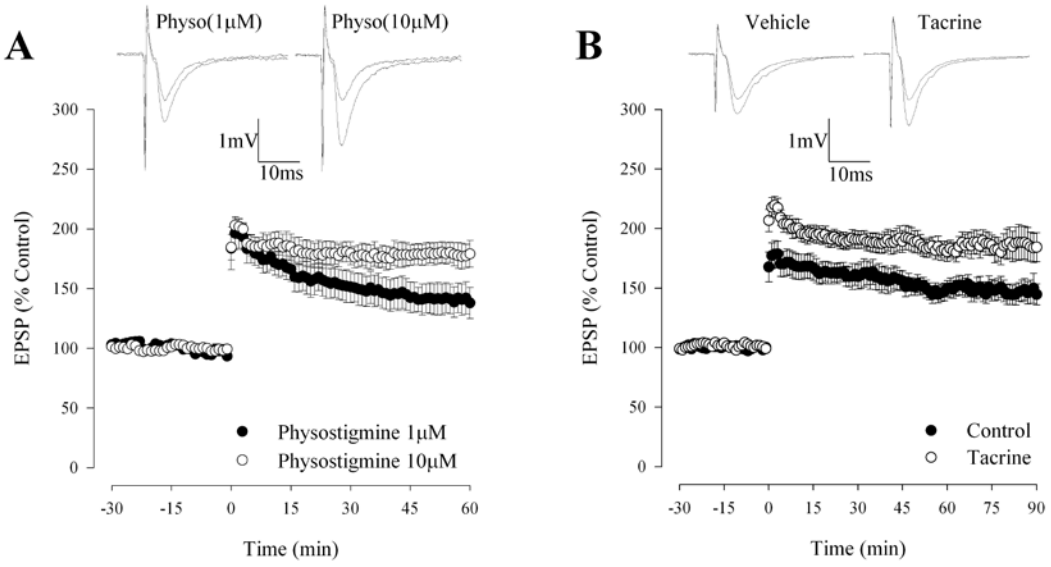

A
Vehicle
PNU-120596
1mV
10ms
EPSP (% Control)
Control
PNU-120596
Time (min)
B
Nicotine
Nicotine + PNU
1mV
10ms
EPSP (% Control)
Nicotine
Nicotine + PNU-120596
Time (min)
C
EPSP (% Control)
Baseline
Control
5μM
500nM
100nM
D
Nicotine
Nicotine + PNU
1mV
10ms
EPSP (% Control)
Nic 500nM
Nic 500nM + PNU-120596
Time (min)
E
Vehicle
MLA
1mV
10ms
EPSP (% Control)
Control
MLA
Time (min)
F
MLA + Nic
MLA + Nic + PNU
1mV
10ms
EPSP (% Control)
MLA + Nic (500nM)
MLA + Nic (500nM) + PNU-120596
Time (min)

Fig 6. Inhibition of AChE results in enhanced HFS-LTP in the rat dentate gyrus. A, LTP is unaffected by 1μM physostigmine (closed circles), but is significantly enhanced in the presence of 10μM physostigmine (open circles). **B**, The AChE inhibitor, tacrine (10μM), enhanced HFS-LTP (open circles) when compared to control (closed circles). Traces illustrate EPSP's before and 60min after HFS.


A
Physo(1μM)
Physo(10μM)
1mV
10ms
EPSP (% Control)
Physostigmine 1μM
Physostigmine 10μM
Time (min)
B
Vehicle
Tacrine
1mV
10ms
EPSP (% Control)
Control
Tacrine
Time (min)