Dependence receptor involvement in subtilisin-induced long-term depression and in long-term potentiation

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Abstract

The serine protease subtilisin induces a form of long-term depression (LTD) which is accompanied by a reduced expression of the axo-dendritic guidance molecule Unco-ordinated-5C (Unc-5C). One objective of the present work was to determine whether a loss of Unc-5C function contributed to subtilisin-induced LTD by using Unc-5C antibodies in combination with the pore-forming agents Triton X-100 (0.005%) or streptolysin O in rat hippocampal slices. In addition we have assessed the effect of subtilisin on the related dependence receptor Deleted in Colorectal Cancer (DCC) and used antibodies to this protein for functional studies. Field excitatory postsynaptic potentials (fEPSPs) were analysed in rat hippocampal slices and protein extracts were used for Western blotting. Subtilisin produced a greater loss of DCC than of Unc-5C, but the antibodies had no effect on resting excitability or fEPSPs and did not modify subtilisin-induced LTD. However, antibodies to DCC but not Unc-5C did reduce the amplitude of theta-burst long-term potentiation (LTP). In addition, two inhibitors of endocytosis – dynasore and tat-gluR2(3Y) – were tested and, although the former compound had no effect on neurophysiological responses, tat-gluR2(3Y) did reduce the amplitude of subtilisin-induced LTD without affecting the expression of DCC or Unc-5C but with some loss of PostSynaptic Density Protein-95. The results support the view that the dependence receptor DCC may be involved in LTP and suggest that the endocytotic removal of a membrane protein or proteins may contribute to subtilisin-induced LTD, although it appears that neither Unc-5C nor DCC are involved in this process. (220).

Key-words:-

Deleted in Colorectal Cancer; DCC; plasticity; hippocampus; subtilisin; serine proteases;

INTRODUCTION

Long-term depression (LTD) has been recognised as one of the major forms of neural plasticity in the central nervous system (CNS). Often induced by low frequency electrical stimulation, the phenomenon results in a significant reduction in the amplitude of synaptic potentials recorded *in vitro* (Christie et al., 1994) or in vivo (Goh & Manahan-Vaughan, 2013) with the depressed synaptic transmission correlating with alterations in specific aspects of learning, memory and other cognitive behaviours (Kemp & Manahan-Vaughan, 2004; Dong et al. 2013; Goh & Manahan-Vaughan, 2013, Lemon & Manahan-Vaughan, 2012). LTD can also be induced by chemical stimuli, notably the activaton of N-methyl-D-aspartate (NMDA) (Mulkey & Malenka, 1992) or metabotropic glutamate receptors (Anwyl, 1999, 2006) but can also be generated by some serine proteases, including tissue plasminogen activator (tPA) (Calabresi et al. 2000; Pawlak et al. 2002; Pang and Lu, 2004; Tsirka et al., 1995), neuropsin (Komai et al., 2000) and neurotrypsin (Frischknecht et al., 2008). The enzymes do not necessarily act by the same or similar mechanisms, since tPA is dependent on Brain-Derived Neurotrophic Factor (Pang et al. 2004; Mou et al. 2009; Rodier et al. 2014) but neuropsin modifies the expression or function of adhesion molecules and fibronectin (Matsumoto-Miyai et al. 2003; Tani et al. 2001).

We have demonstrated a similar LTD in hippocampal slices in response to the bacterial serine protease subtilisin (MacGregor et al., 2007) and have reported its association with the loss of specific proteins including Unco-ordinated-5C (Unc-5C, *formerly known as Unc-5H3 in rodents*) (Forrest et al., 2011). Levels of several other proteins remain largely unchanged, including the closely related Unc-5A, the synaptic protein synaptotagmin, the cytoskeletal organisers RhoA and RhoB and the morphogenetic protein sonic hedgehog (Shh) (Forrest et al., 2011). In addition, subtilisin-induced LTD is not a reflection of generalised cellular toxicity as it is not associated with a classical activation of caspase-3 or caspase-9 and it is

not modified by inhibitors of caspase activation (Forrest et al., 2013). These observations indicate that the loss of Unc-5C is not simply the result of a general proteolytic effect of subtilisin and raise the possibility of a specific relationship between Unc-5C and subtilisin induced-LTD.

The family of Unc-5 proteins contains four members with markedly different regional distributions and functional properties. In many cell types they function as receptors for the secreted protein ligand netrin and have become known as 'dependence receptors' since cell survival is dependent on the interaction between netrin and the receptor: a loss of netrin results in the initiation of apoptotic processes leading to cell death (Mehlen and Guenebeaud, 2010; Castets et al., 2012).

However, the same ligand (netrin) and receptors are also involved in axonal guidance, spine development and synapse stabilisation (Masuda et al., 2008; Muramatsu et al., 2010), raising the possibility that the loss of Unc-5C produced by subtilisin could affect these processes and also, therefore, aspects of neural plasticity. The present study was designed to obtain further information on the role of Unc-5C by applying antibodies to Unc-5C receptors to hippocampal slices and assessing their influence on subtilisin-LTD. In addition, the previous finding that subtilisin-LTD was associated with a loss of Unc-5C raised the possibility of some relationship between this protein and other forms of synaptic plasticity.

In their regulation of axon and dendrite formation and guidance Unc-5 receptors operate alone or in conjunction with a second member of the netrin receptor family, Deleted in Colorectal Cancer (DCC). The expression of DCC alone promotes chemoattraction between growing axons and postsynaptic sites (Xu et al., 2010) whereas the expression of Unc-5C, alone or in conjunction with DCC, generates repulsion (Muramatsu et al., 2010). The balance in the expression of these two receptors is, therefore, a critical feature of brain development and anatomical plasticity. On the hypothesis that one or both of these dependence receptors

may also be involved in the LTD response to subtilisin, we have examined the expression of both proteins in response to subtilisin and have tested antibodies to both proteins against electrically-induced LTP and LTD.

Finally, since the removal of proteins from neuronal membranes seems to be largely responsible for several forms of LTD (Kim et al., 2001; Hu et al., 2007) we have used two inhibitors of membrane protein endocytosis: the cell permeant dynamin inhibitor dynasore, and the viral protein derivative tat-GluR2(3Y), to assess whether the internalisation of a membrane protein, possibly including DCC or unc-5C, is relevant to subtilisin-induced LTD.

EXPERIMENTAL PROCEDURES

Electrophysiology

All experiments were performed in accordance with Home Office regulations and the Animals (Scientific Procedures) Act, 1986, and approved by the Glasgow University Ethics Committee. Male Wistar rats weighing 100-150g (approximately postnatal days 28-35) were anaesthetised with urethane (1.5g/kg) and immediately killed by cervical dislocation. The brain was removed into ice-cold artificial cerebrospinal fluid (aCSF) of composition: (in mM) NaCl 115; KH_2PO_4 2.2; KCl 2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 10, gassed with 5%CO_2 in oxygen. The hippocampi were rapidly removed and chopped into 450μm transverse slices using a McIlwain tissue chopper. The slices were pre-incubated at room temperature for at least 1 hour in a water-saturated atmosphere of 5%CO_2 in O_2 before individual slices were transferred to a 1 ml capacity superfusion chamber for recording. Slices were superfused at 28-30°C using ACSF at a flow rate of 3-4 ml/min. A concentric bipolar electrode was used for stimulation of the Schaffer collateral and commissural fibres in stratum radiatum, using stimuli delivered at 0.1 Hz or 0.05 Hz with a pulse width of 50-300

μs, adjusted to evoke a response amplitude approximately 70% of maximum to allow increases or decreases in size to be detected. Extracellular recordings were made via glass microelectrodes containing 1M NaCl (tip diameter approximately 2μm, 2-5MΩ) with the tip positioned under microsopic visualisation in the stratum radiatum of the CA1 region to evoke field excitatory postsynaptic potentials (fEPSPs). Potentials were amplified, digitised and stored on computer via a CED (Cambridge Electronic Design) micro1401 interface. The fEPSPs were routinely quantified by measurement of the early negative-going slope using cursor positions in Signal software (CED, Cambridge, UK). The axonal volley was monitored wherever it was possible to distinguish it clearly from the fEPSP to ensure that no change in synaptic input occurred during experiments.

The fEPSP was allowed to stabilise for a minimum period of 10min before the application of compounds. The degree of LTD and its alteration by the compounds tested was quantified by measuring the size of the evoked potential 40min after terminating the subtilisin application. This allowed standardisation and comparison between slices since the plateau of depression was normally attained approximately 30 min after the end of subtilisin application. One data point per minute (one every six stimuli) is used in the graphical records of fEPSP size for clarity. The fEPSP slope is expressed as a percentage of the potential size obtained immediately prior to the subtilisin perfusion, taken as the mean of the last 10 EPSPs before starting the superfusion of subtilisin.

Long-term potentiation (LTP) was induced by a theta-burst pattern of stimulation as described by Larson et al., (1986), using groups of 4 pulses at 100Hz, delivered 5 times per second for 2 seconds. Electrical LTD was induced using a paradigm modified from that described by Kemp & Bashir (1999). Stimulation was effected by a triplet of pulses, 200 ms apart, delivered at a frequency of 1Hz for 5 min (stim1). Repetition of this sequence (stim2)

increased the magnitude of the induced LTD and in this study two sequences were therefore used routinely, the second being applied 20 min after the end of the first sequence.

Use of antibodies

For the examination of Unc-5C antibodies, exposure of the slices was maximised by pre-incubation with antibodies in the holding chamber for at least 1h before transferring them to the recording bath. After the initial preparation of slices they were placed alternately into aCSF in two petri dishes in the holding chamber. The slices were allowed to rest for 1h to recover from the preparative procedures, after which one batch of slices was designated as the control group while the test antibody was added to the second (experimental) group at a dilution of 1 in 1000. Slices were then transferred alternately from the two dishes into the recording chamber.

In order to increase the penetration of antibodies two approaches were used. Some batches of slices were treated with Triton X-100 at a low concentration of 0.005% for the first 15 minutes of their exposure to antibody. This procedure has previously been used successfully to increase cell permeability to large molecules including antibodies (Tehrani & Barnes, 1991; Chambaut-Guerin et al., 1997; Hirai et al., 2006; Jung et al., 2006) but in order to ensure that cell function was not affected adversely by this non-selective detergent, most slices were treated with the pore-forming molecule streptolysin O. This bacterially derived molecule creates pores up to 50nm in size, large enough for molecules of up to 260 kDa in size to enter cells (Walev et al. 2001; Cassidy and O'Riordan 2013) and has been used previously to promote the entry of antibodies into the cytosol (Bhakdi et al. 1993). In addition, this compound has the advantage that its ability to create pores is greatly enhanced in the absence of calcium, while the subsequent addition of calcium rapidly facilitates re-sealing of the pores (Walev et al., 2001; Gonzalez et al. 2011). This means that the exposure of cells to

the penetrating antibody can be time-limited by removing calcium for 10-15 minutes and can then be protected against any continued leakage of large molecules by the re-addition of calcium (Walev et al., 2001). When streptolysin was used here it was added to the slices for the first 15 minutes of incubation with antibody in calcium-free aCSF and, after 15 minutes, slices were transferred to normal aCSF (with 2.5mM calcium) in the continued presence of antibody at a dilution of 1:1000 until their removal for recording.

Immunoblotting

To obtain further information on the molecular changes underlying subtilisin-induced LTD, the course of the fEPSP depression was followed electrophysiologically as described above to confirm that subtilisin-LTD had been induced. Forty-five minutes after subtilisin application, when a plateau of maximal fEPSP depression had been established, each slice was removed from the recording chamber, transferred into an eppendorf tube, snap frozen in dry ice and stored at -80^{o}C for later analysis. Control slices were also prepared by leaving them in the recording chamber for the same total duration as a subtilisin-treated slice (including the initial stabilisation period) but without the addition of subtilisin to the superfusion medium. The fEPSPs were again recorded to confirm that the slices were maintained in a healthy, functioning state and that subtilisin had produced its typical LTD.

Subsequently, the slices were homogenised in RIPA buffer [50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Triton X-100, 1% IGEPAL, and a Roche complete protease inhibitor tablet]. Protein samples (10µg) were prepared as: 65% protein sample, 25% sample buffer and 10% reducing agent (Invitrogen, Paisley, UK), and heated at 70^{o}C for 10 min to denature the protein. Western blot analysis was carried out using the following primary antibodies raised against target proteins: from BD Pharmingen (Oxford, UK) anti-DCC (mouse monoclonal #554223, 1:5000 dilution); from R&D Systems (Abingdon, UK) - synaptobrevin/VAMP-1

(goat polyclonal, #AF4828, 1:1000 dilution); from Santa Cruz (Insight Biotechnology, Wembley, UK) - unc-5C (goat polyclonal, #sc-54442, 1:500 dilution); Unc-5A (goat polyclonal #sc-67902, 1:1000 dilution); Shh (goat polyclonal, #sc-1194, 1:1000 dilution); RhoA (mouse monoclonal, #sc-418, 1:5000 dilution); actin (goat polyclonal, #sc-1615, 1:10,000 dilution); from Cell Signalling (New England Biolabs, Hitchin, UK) - PSD-95 (rabbit monoclonal, #3450, 1:5000 dilution).

Protein samples were loaded onto NuPAGE Novex 4-12% Bis-Tris (1.0mm) 15 lane gels (Invitrogen, Paisley, UK) and run at 175 volts for 70 min to separate proteins according to their molecular weight. SeeBlue pre-stained standard (Invitrogen, Paisley, UK) was included on each gel as a molecular weight marker. The separated proteins were then blotted onto Invitrolon PVDF membranes (Invitrogen, Paisley, UK) at 35V for 75 min. The membranes were blocked for 1h in 5% non-fat dried milk solution in Tris-buffered saline containing 0.05% Tween (TBST), before overnight incubation at 4^{o}C with the appropriate primary antibody (diluted in 5% milk-TBST). Membranes were then washed 3 times for 15 min with TBST and incubated with the appropriate horse radish peroxidase (HRP) conjugated secondary antibody for 1h at room temperature. All secondary antibodies were prepared in 5% milk-TBST and used at 1:5000 dilution: goat anti-mouse HRP, (#sc-2005), donkey anti-goat HRP (#sc-2313) and donkey anti-goat HRP (#sc-2020), Santa Cruz (Insight Biotechnology, Wembley, UK). Following secondary antibody incubation blots were washed 3 times for 15 min with TBST then visualised using Enhanced Chemiluminescence Plus detection kit (Thermo-Fisher, UK).

Data analysis

A two-tailed *t* test was used to assess the statistical significance of electrophysiological differences between two treatments such as control and experimental slices. For more

complex comparisons, data were analysed using ANOVA followed by Dunnett's multiple comparison test or the Bonferroni test for selected datasets as appropriate. In all cases $P < 0.05$ was adopted as the criterion for significance but the exact P value is shown whenever possible.

Western blots were analysed using 'Image J' densitometric software (http://rsb.info.nih.gov/ij/). Results were expressed as the mean ± s.e.mean optical density and tested for statistical significance using ANOVA followed by Dunnett's multiple comparison test or the Bonferroni test for selected datasets as appropriate.

Sources of materials

In addition to the antibodies listed above, compounds were obtained from the following sources: from Sigma Chemicals (Poole, Dorset, UK) - octylphenol ethoxylate (Triton® X-100); subtilisin catalogue P5380 (subtilisin was dissolved directly in aCSF and fresh solutions were made each day, or dissolved in distilled water and stored at -20°C as aliquots for single use); Streptolysin O catalogue S5265; from Tocris, Bristol, UK - dynasore; from Cambridge Bioscience (Cambridge, UK) - tat-GluR2(3Y).

RESULTS

The Unc-5C and DCC proteins are single pass transmembrane receptors. The anti-Unc-5C antibody corresponds to an epitope on the intracellular domain of the receptor while the anti-DCC antibody (monoclonal ab 16793 [AF5], Abcam, Cambridge, UK) interacts with a sequence in the extracellular domain. The DCC antibody used here has been clearly identified as a function-blocking antibody (Keino-masu et al., 1996; Deiner et al., 1997; Forcet et al., 2002; Powell et al., 2008; Tsai et al., 2003; Nagel et al. 2015; Huang et al. 2015;

Lee et al., 2014; Son et al. 2013; Qu et al. 2013; Manitt et al., 2009). Unc-5 antibodies have been used less often as function-blocking agents, but were successfully used by Koch et al., 2011).

In order to ensure that the antibodies gained access to their intracellular or extracellular binding epitopes some slices were pre-incubated for 15 min in aCSF containing either Triton X-100 at 0.005% or the pore-forming agent streptolysin O at 1μM. Limiting the duration of streptolysin O exposure to 15 min is known to allow pores to re-seal so that intracellular molecules do not leak to the exterior, compromising cell viability (Walev et al., 2001). Preliminary experiments indicated that the exposure of slices to Triton X-100 or streptolysin at the concentrations used had no effect on the excitability of the slices when superfused for 1h:- tests of the stimulus current / fEPSP amplitude relationship indicated no difference between slices pre-incubated in aCSF alone and those which had been exposed to Triton (Fig. 1A) or streptolysin (Fig. 1B).

To quantify the effects of the antibodies on plasticity an initial analysis was performed of slices treated with antibody alone, or antibody plus Triton or streptolysin. That analysis revealed no effects of the antibodies or pore-forming agents on resting fEPSP amplitude or measurements of stimulus current / fEPSP amplitude (Fig. 1C,D), with no significant differences between the groups of experiments. Data on LTD from slices with any of these treatments were therefore pooled.

Western blots were also performed to determine whether the treatment of slices with Triton, streptolysin or antibodies had any direct effect on the expression of the cellular proteins Unc-5C, Unc-5A, DCC, PSD-95, VAMP-1, Shh and actin after the electrophysiological experiments described above. As illustrated in Fig. 1E no changes in protein expression were noted after exposure to any of these agents.

Long-term depression (LTD)

Since we have already reported the down-regulation of Unc-5C protein by subtilisin (Forrest et al. 2011) it was important to determine whether DCC was similarly affected. As shown in Fig. 2A, Unc-5C expression was again reduced by subtilisin but expression of DCC proved to be reduced to a greater extent by the protease, while Unc-5A, the small GTPase enzyme RhoA and the morphogenetic protein sonic hedgehog (Shh) were unaffected at the same concentrations (Fig. 2A).

The superfusion of subtilisin over hippocampal slices invariably generated a depression of fEPSP size. As described previously, this depression was concentration-dependent with the fEPSP amplitude reduced by approximately 90% at 4μM (Forrest et al., 2011, 2013). In this study a concentration of 2 μM was used routinely since it produces LTD which reaches a plateau level of approximately 50% of the initial fEPSP size. The decline in fEPSP size develops slowly after terminating the application of subtilisin, reaching a plateau by approximately 30 min (Fig. 2B).

Preincubation with the Unc-5C or DCC antibodies did not modify the LTD response to subtilisin; the magnitude of the plateau depression of fEPSP slope 50min after ending the subtilisin superfusion was not significantly different in the presence of the Unc-5C or DCC antibodies (Fig. 2B). Analysis of variance on the six data points from 45-50 minutes in antibody-treated slices compared with controls showed no significant difference between any of the curves ($F_{(17,54)} = 0.48$, $p = 0.95$).

LTD induced by electrical stimulation was similarly unaffected by the antibodies (Fig. 2C, D). Analysis of variance on the final six data points showed no significant difference in the amplitude of the fEPSP depression after two periods of low frequency stimulation of control slices and those treated with antibodies to Unc-5C (Fig. 2C; $F_{(11,24)} = 0.059$, $p = 0.99$) or DCC (Fig. 2D; $F_{(11,24)} = 0.077$, $p = 0.99$).

Since it was possible that the antibodies were not able to penetrate into slices sufficiently over the 15min pre-recording exposure or were inactivated during the settling and pre-recording phase after transfer to the recording chamber, a separate series of tests were performed in which slices were again pre-incubated with the antibodies but the antibodies were then also added to the superfusing medium. Following the same pre-incubation protocol as described above using combinations of the antibodies with Triton or streptolysin, individual slices were transferred into the recording chamber and allowed to settle to a constant fEPSP amplitude. Unc-5C or DCC antibodies were then added to the continuously superfused medium for 4h, with no apparent effect themselves on fEPSP amplitude (Fig. 2E). After 6h, LTD was induced by electrical stimulation but this was not significantly different from LTD induced in control slices (Fig. 2D,E; $F_{(17,36)} = 0.223$, $p = 0.99$).

Long-term potentiation (LTP)

To determine whether interference with Unc-5C or DCC would affect different forms of plasticity, slices were subjected to the incubation conditions described above but, after transference to the recording chamber and allowing a stabilisation period to attain a constant fEPSP amplitude, slices were stimulated to induce LTP. A theta-burst pattern was used consisting of 5 pulses at 200Hz, delivered 5 times per second for 2 seconds (Larson et al., 1986). In control slices, stimulation induced LTP was reflected in an increase of fEPSP size to 155.3% ± 5.2 of baseline ($p < 0.0001$, $n = 4$) measured 45 min after stimulation (Fig. 3A). The terminal LTP measured over 40-45 min after stimulation in slices incubated with the Unc-5C antibody was not different between control and test slices ($F_{(11,36)} = 1.96$, $p = 0.064$) (Fig. 3A,B). The magnitude of the early, short-term peak potentiation measured 1min after stimulation was 308.9% ± 10.9 ($n = 4$). Slices that had been exposed to Unc-5C antibody

exhibited a greater initial short-term potentiation of 378.5% ± 22.9 ($p = 0.023$, $n = 4$) (Fig. 3A).

In contrast, slices pre-incubated in antibodies to DCC exhibited a significant change to the LTP. While the peak fEPSP amplitude at 1min was unchanged at 275.4% ± 18.3 of baseline compared with control slices (306.8% ± 16.4) (Fig. 3C; $p = 0.25$), the maximum amplitude of the fEPSP plateau LTP between 40-45min after stimulation was significantly less than in control slices ($F(11,36) = 5.46$, $p < 0.0001$) (Fig.3C,D), with several individual time points showing significantly different values using the Bonferroni multiple comparison test (Fig. 3D).

Paired-pulse interactions

Paired pulse interactions were examined to assess the possible involvement of presynaptic transmitter release in the effects of DCC antibodies on LTP. Exposure of slices to unc-5C antibodies had no effect on paired-pulse interactions at any time point, while DCC antibodies produced significant differences between test and control slices, with less facilitation at the 10 and 20ms interpulse intervals (Fig. 4).

Endocytosis inhibitors

Since the DCC antibodies produced a significant decrease in the amount of electrically-induced LTP, and subtilisin is more potent at removing DCC from cells than Unc-5C (Fig. 2A), we reasoned that subtilisin-induced LTD might involve the endocytotic removal and proteolysis of DCC or Unc-5C, reducing their membrane location for plasticity-related functionality while leaving the total amount of protein unchanged. Slices were therefore treated with two inhibitors of protein endocytosis.

The tat-GluR2(3Y) peptide is produced by the fusion of an inactive form of the GluR2 AMPA receptor subunit with the Human Immunodeficiency Virus (HIV) *tat* peptide, this being an 11-residue peptide that assists the cellular entry of HIV and similarly promotes cell penetration by any protein to which is linked. This construct prevents the endocytotic down-regulation of receptors by low frequency stimulation and reduces electrically-induced LTD (Dalton et al., 2008; Dong et al., 2013) as a result of its blockade of AMPA receptor endocytosis. As illustrated in Fig. 5A, this complex was superfused at a concentration of 2μM for 10min before, during and 30min after subtilisin perfusion. The peptide produced some reduction in the amount of subtilisin-induced LTD for which ANOVA indicated a significant difference across the final six time points and significant differences between several individual data pairs using the Bonferroni tests for selected datasets (Fig. 5A).

Dynasore is an inhibitor of the endocytosis-producing cytoskeletal motor protein dynamin, able to interfere with the internalisation of all membrane proteins (Macia et al. 2006). When superfused for 10min before, during and 30min after subtilisin, dynasore did not modify subtilisin-induced LTD (Fig. 5B).

Immunoblotting

Since tat-gluR2(3Y) had a significant effect on subtilisin-induced LTD it was of interest to determine whether tat-gluR2(3Y) had any effect on the subtilisin-induced removal of DCC or unc-5C. Protein extracts of the hippocampal slices treated with subtilisin and tat-gluR2(3Y) were used for Western blotting but revealed that tat-gluR2(3Y) had no effect on the expression of DCC or Unc-5C and did not prevent the loss of these two proteins produced by subtilisin (Fig. 5C). This result makes it unlikely that down-regulation of these proteins was relevant to subtilisin-induced LTD, supporting the view that the loss of these proteins by endocytosis is not responsible for the LTD. Proteins that were not normally affected by

subtilisin, including Unc-5A, Shh and actin, remained unaffected after treatment with tat-GluR2(3Y), although the presence of tat-GluR2(3Y) did produce a reduced expression of the NMDAR-associated protein PostSynaptic Density protein-95 (PSD-95) (Fig. 5C). This may be related to indications that NMDA receptors are involved in the internalisation of AMPA receptors (Tigaret et al. 2006; Yu et al. 2010) and that specific importance may be attached to the functions of PSD-95 in AMPA receptor endocytosis (Bhattacharyya et al. 2009).

DISCUSSION

The present study was intended to expand our earlier observation that subtilisin-induced LTD was accompanied by a loss of the dependence receptor Unc-5C (Forrest et al. 2011), by extending the work to an examination of a different member of the dependence receptor family, DCC, and by assessing whether interfering with unc-5C or DCC function could underlie, or contribute to, subtilisin-induced LTD or electrically-induced LTD and LTP. Understanding the actions and mechanisms of serine proteases is of great potential pathophysiological relevance since changes in the expression of the proteases or their endogenous inhibitors (serpins) in the CNS have been implicated in abnormal neuronal excitability or viability (Monard, 1988; Hirata et al. 2001). Serine proteases have also been linked to Alzheimer's disease or schizophrenia (Vawter et al., 2004) with evidence for a role in intellectual dysfunction (Molinari et al. 2002). They are also believed to be responsible for brain dysfunction and damage (Gingrich and Traynelis 2000; Siao and Tsirka 2002), including that which follows infection by fungi such as *Aspergillus*, an organism known to produce several serine proteases including cadeprin, which also produces LTD (MacGregor et al. 2007). The subtilase group of serine proteases is especially interesting since these enzymes are involved in a wide range of physiological processes in different species (Seidah & Chretien, 1999) and we have shown that subtilisin itself can induce LTD in parallel with a

reduction in expression of Unc-5C (MacGregor et al. 2007; Forrest et al. 2011, 2013), as can proteinase K (Stone et al. 2012).

Both the DCC and unc-5 receptor proteins have been localised to the neuronal surface, being recruited to the membrane by netrin or depolarisation (Gopal et al., 2016; Bouchard et al., 2004, 2008). The receptors are primarily involved in neurite generation, guidance and contact formation, especially of new synapses (Manitt et al., 2009) and during development the activation of DCC receptors confines growing axons to defined paths, preventing sidewards escape into adjacent axon bundles (Smith et al., 2012; Laumonnerie et al., 2014). Most of the previous work has localised the proteins to axonal and dendritic extensions and growth cones (Matsumoto and Nagashima 2010; Tcherkezian et al. 2010) as might be anticipated of receptors involved in long-term synaptic plasticity. DCC tends to occur primarily on axon branches, terminals and growth cones, while Unc-5 proteins are localised primarily to growth cones and dendritic trees where they determine the extent and direction of local growth (rather than overall neuritic length and branching) (Ren et al. 2008; Norris et al., 2014; Ogura et al. 2012; Purohit et al., 2012; Zarin et al., 2012; Kim and Ackerman 2011; Finger et al., 2002; McKenna et al., 2008). The unc-5 receptors are normally phosphorylated by netrin in order to generate the repulsive activity between neurites (Kruger et al., 2004) and it may be the ratio between unc-5 and DCC receptors which is key to contact formation (Muramatsu et al., 2010). The action of netrin on unc-5 receptors also contributes to regulating the extent to which synaptic machinery is established in dendrites (Poon et al. 2008; Killeen 2009).

While much is known about LTD generated by the activation of NMDAR and metabotropic glutamate receptors there is much less information on mechanisms underlying the LTD induced by proteases. Subtilisin-induced LTD does not seem to involve kinases or phosphatases and is not simply the result of neuronal toxicity or apoptosis since, although

subtilisin does produce a non-canonical proteolysis of caspase-3 and caspase-9, inhibitors of these enzymes do not prevent or modify subtilisin-LTD (Forrest et al. 2013). There may, however, be a significant role for the proteasome in mediating the effects of subtilisin since the eventual plateau level of LTD can be reduced by the proteasome inhibitor MG132 (Forrest et al. 2013).

The discovery that subtilisin can deplete cells of Unc-5C (Forrest et al. 2013) may provide an alternative mechanism by which the generation of LTD could be produced. We report here that the removal of Unc-5C is relatively specific since the expression of several different proteins, including the related molecule Unc-5A, is unaffected by subtilisin. Some of the major actions of Unc-5 members are known to be mediated via the intracellular small GTPase enzyme RhoA (Picard et al. 2009) and the activation of RhoA or RhoB has been linked previously to synaptic plasticity in the hippocampus (Luo et al. 2000; O'Kane et al., 2003a,b, 2004; Zhou et al. 2009; McNair et al. 2010). The absence of changes in the expression of RhoA in the present work, following treatment with subtilisin, suggests that this particular association may not be relevant to the induction of subtilisin-induced LTD. Similarly the morphogenetic protein Shh, which has been implicated in aspects of synaptic plasticity (Mitchell et al., 2012; Yao et al., 2015) was unaffected by subtilisin.

Another molecule which is affected by subtilisin, however, is DCC, a finding which strengthens the possibility that both unc-5C and DCC might be involved in subtilisin-induced LTD since both are receptors for the secreted protein ligand netrin and they function co-operatively in the regulation of axonal growth and guidance. When acting alone, DCC promotes attraction between cells or growth cones and cells, whereas the additional presence of Unc-5 proteins leads to complex formation with DCC and the generation of cell repulsion (Hong et al. 1999; Muramatsu et al., 2010; Kim & Ackerman, 2011). Both proteins are

important in the early development of neurons in the CNS (Dillon et al. 2007). The present results

The relationship between netrin and Unc-5 extends to control of the subcellular localisation of presynaptic and vesicular components that are crucial to the correct orientation and function of synapse formation (Poon et al., 2008) and which are likely to underlie aspects of synaptic plasticity. Both DCC and Unc-5 members are involved in growth cone, dendrite and synapse formation (Norris and Lundquist 2011; Norris et al. 2014; Meriane et al. 2004; Hong et al. 1999). Extrapolating to the role of neurite growth and synaptic rearrangement in plasticity, it might be predicted that the presence of antibodies to these proteins, effectively inhibiting their functional activity, could modify acute changes in plasticity.

The present results reveal that subtilisin reduces the expression of DCC to a greater extent than the expression of Unc-5C (Fig. 2A). However, perfusion of hippocampal slices with antibodies to each of these proteins has no effect on fEPSP size and the antibodies do not modify the amplitude of subtilisin-induced LTD. This is not likely to be due to a failure of the antibodies to gain access to the receptors since the experiments were performed in the presence of either Triton or streptolysin, two agents used to promote cell penetration by antibodies and other large proteins (Bhakdi et al. 1996; Walev et al. 2001; Tehrani and Barnes, 1991). Also it is not likely that these pore-forming agents affected the state of the slices since they did not produce any change in resting excitability or in subtilisin-induced LTD. The results also suggest that Triton or streptolysin had no non-specific toxic effects attributable to the leakage of intracellular proteins from cells influencing the properties of these acute slices.

The loss of the dependence receptors (Unc-5C and DCC) produced by subtilisin is probably not the result of their endocytosis since administration of two different inhibitors of endocytosis, working by different mechanisms, did not prevent their removal by subtilisin.

However, tat-gluR2(3Y) did reduce the degree of LTD generated by subtilisin, implying that the endocytosis of a different molecule could be involved. This might be AMPA receptors or subunits which have been linked previously to the induction of electrical LTD (Beattie et al., 2000; Carroll et al. 2001; Dalton et al. 2008; Waung et al. 2008). It is surprising that dynasore had no comparable activity since its complement of substrates - larger than the substrate pool of tat-gluR2(3Y) and already shown to modify aspects of synaptic plasticity (Hua et al. 2013; Douthitt et al. 2011, Linden 2012) - might have been expected to have some effect on LTD. This result therefore implies a further, substantial, distinction between the mechanisms of LTD induced electrically and that induced by subtilisin.

Long-term potentiation

Of the two antibodies studies, only that for DCC had any significant effect on electrically-induced LTP. The reduction in LTP size is not likely to involve a down-regulation of the target protein as there was no change in the expression of either the DCC (or Unc-5C) proteins. It is more likely, therefore, that the antibodies were binding directly to the targets and interrupting the transduction pathways in which they are involved. It may be that the mechanism by which DCC affects LTP is via interactions with terminal proteins such as syntaxin (Cotrufo et al. 2011). It is also known that DCC can modify neuronal responses to NMDA receptor activation (Kuo et al. 2010) and that depolarisation promotes DCC insertion into cell membranes (Bouchard et al. 2008), consistent with the recent results from Horn et al. (2013) implicating DCC in LTP and supported by our present results. The observation is consistent with the major effect of neuronal DCC being in the mutual attraction between cells, whereas unc-5C (alone or complexed with DCC) generates repulsion (Hong et al. 1999; Muramatsu et al., 2010; Kim & Ackerman, 2011). While the balance between these forces might be expected to determine neuronal connectivity and plasticity, it is the DCC activity

which appears to dominate in the slice preparation (Fig. 6). It should be pointed out that because there is little prior use of an unc-5 function-blocking antibody, the failure to modify LTP or subtilisin-LTD could be due to its binding to an epitope of the protein that does not affect its functional role in plasticity. Future work in this area might include rescue experiments in cell or slice cultures in which transfection with appropriate plasmids were used to restore or over-express DCC or unc-5C function. This would help to confirm the specificity of the results we are reporting here and also address the possibility that synaptic plasticity could be mediated by up- or down-regulation of dependence receptor expression.

Conclusion

In conclusion, the serine protease subtilisin induces a form of LTD which is accompanied by a reduced expression of the axo-dendritic guidance protein Unc-5C and an even greater reduction in expression of the related dependence receptor DCC. However, DCC and Unc-5C are not involved in subtilisin-induced LTD since antibodies to these proteins did not modify the LTD, but the endocytosis inhibitor tat-gluR2(3Y) did reduce the amplitude of subtilisin-induced LTD. Unc-5C is not involved in electrically-induced LTP but DCC antibodies reduce the enhancement of fEPSP amplitude during theta-burst-induced LTP, suggesting the involvement of DCC in this form of neural plasticity. A schematic summary of the proposed events is shown in Fig. 6, which illustrates the balance between DCC and unc-5 in the CNS and the localisation of these receptors. While DCC appears to be involved in the depolarisation-induced enhancement of excitatory transmission, possibly by promoting neurite extension and the formation of new contacts, neither DCC nor unc-5 seem to be involved in electrically-induced or subtilisin-induced LTD. The roles of DCC and unc-5C in attraction and repulsion are not likely, therefore, to be related in any simplistic fashion to structural changes underlying LTD. This conclusion is supported by the marked loss of their

expression induced by subtilisin despite the pronounced LTD produced. It remains possible that there is a differential involvement of the dependence receptors at other types of neuron and synapse combination distinct from the CA3-CA1 connections examined here.

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FIGURE legends.

Figure 1. Excitability of neurons in the CA1 hippocampal region.

Graphs of the changes in fEPSP size at differing stimulus strengths expressed relative to the threshold stimulus. The graphs compare the stimulus-response relationship in control slices (filled circles) and those assessed during superfusion with Triton X-100 (**A:** open circles), streptolysin O (**B:** open circles), the unc-5C antibody (**C:** open circles) and the DCC antibody (**D:** open circles). **E:** Representative Western blots (n=3-4) illustrating the expression of Unc-5C, Unc-5A, DCC, PSD-95, VAMP-1, Shh and actin in control, untreated slices (con) and those superfused with Triton X-100 (0.005%) and antibody (T/Ab) or streptolysin and antibody (Strep/Ab). The presence of antibodies and pore-forming agents did not affect the expression of any of the proteins examined. The graphs indicate the mean ± s.e.m. (n=4-6) of the fEPSP relative to the initial baseline.

Figure 2. Subtilisin-induced LTD in the CA1 region of rat hippocampal slices.

A. Representative Western blots (n=3-4) illustrating the significant and selective reduction in expression of DCC and Unc-5C by subtilisin, with no effect on Unc-5A, RhoA or Shh expression. **B.** Time course and magnitude of the LTD of field EPSPs (fEPSP) produced by subtilisin superfused for 10 min as indicated by the bar above the records from 0 – 10min on the time axis, at a concentration of 2μM (dark circles) or after incubation with the Unc-5C antibody (pale circles) or DCC antibody (filled triangles). There were no significant differences between the different experimental conditions**. C** and **D** summarise the induction of LTD by two periods of low frequency stimulation (indicated as stim 1 and stim 2) in control slices (filled circles) or during superfusion with the Unc-5C antibody (C; open

circles) or the DCC antibody (D; open circles). There were no significant differences between the control and experimental conditions. The graphs shows the mean ± s.e.mean (n = 6 at each condition). **E**. A plot of fEPSP amplitude from experiments in which, after the initial settling period of 10min, the Unc-5C antibody (pale circles) or the DCC antibody (filled triangles) were superfused for 4h as indicated by the bar above the records. After 4h LTD was induced by low frequency stimulation as before but no differences were noted between the control and antibody curves.

For the charts in panel **A:** *P < 0.05; **P < 0.01; ***P < 0.001.

Figure. 3. Effects of antibodies on electrically-induced LTP.

A: summarises a comparison of LTP induced in control slices (filled circles) and those exposed to the Unc-5C antibody (open circles). The main graph and the expansion of the final 15 min (shaded area expanded in **B**) reveal that no significant differences were observed. **C:** summarises a comparison between control slices (filled circles) and those exposed to the DCC antibody (open circles). The main graph and expansion of the final 15 min (shaded area expanded in **D**) indicated a significant difference between the conditions assessed using ANOVA (*** P < 0.001, n = 4) and between several individual time points assessed using the Bonferroni test for post hoc comparisons (+ P < 0.05, n = 4).

Figure 4. Paired pulse interactions in hippocampal slices

Graphs of paired-pulse interactions measured in control slices (dark circles) and those exposed to the Unc-5C antibody (pale circles) or the DCC antibody (triangles). Significant

differences were noted between control and DCC antibody-treated slices at the 10 and 20 ms interpulse intervals (* $P < 0.05$, $n = 6$)

Figure 5. The effects of inhibitors of endocytosis

The graphs summarise the induction of subtilisin-induced LTD in the CA1 region of rat hippocampal slices in control slices (filled circles) and in slices superfused with the endocytosis inhibitors tat-gluR2(3Y) (**A:** open circles) or dynasore (**B:** open circles). Analysis of the final 6 data points revealed a significant difference between control slices and those treated with tat-gluR2(3Y) ($P = 0.0006$) with several individual points being significantly different at *$P < 0.05$. Panel **C:** illustrates representative Western blots (n=3-4) showing the expression of DCC, Unc-5C, Unc-5A, Shh, actin and PSD-95 in control slices, the reduced expression of DCC and Unc-5C induced by subtilisin (sub, 2µM), and the failure of tat-gluR2(3Y) to prevent the loss of Unc-5C or DCC (sub/tat). Note that the presence of tat-gluR2(3Y) reduced the expression of PSD-95.

Figure 6. A schematic of events thought to underlie the results.

A-C:- DCC receptors contribute to LTP: (A) indicates the presence of DCC and unc-5 receptors in a resting neurite and growth cone. (B) Theta-burst stimulation then induces the movement of receptors into an appropriate part of the growth cone for promoting extension towards a nearby neuron, resulting in LTP. (C) Blockade of the DCC receptors, but not unc-5 receptors, by function-blocking antibodies prevents the attraction between cone and neuron, resulting in reduced LTP.

D-F:- The removal of an unknown membrane protein contributes to subtilisin-induced LTD: (D) indicates the presence of an unidentified protein in the neurite membrane which maintains synaptic transmission. (E) Subtilisin induces LTD which involves the loss of the unknown membrane protein. (F) Treatment of slices with tat-GluR2(3Y), which prevents the endocytotic removal of membrane proteins, results in a reduced amount of subtilisin-induced LTD.

Figure 1

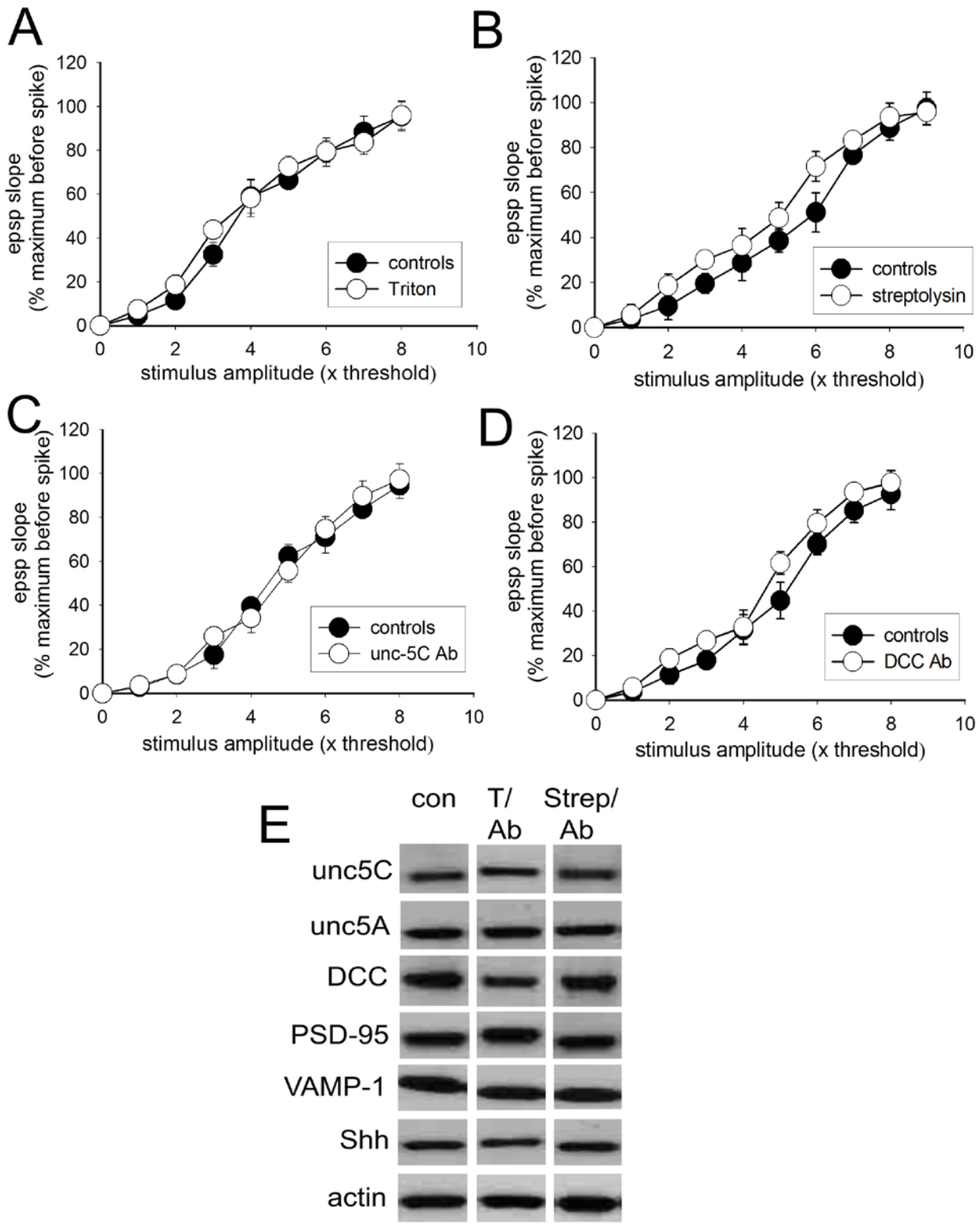

A
epsp slope
(% maximum before spike)
stimulus amplitude (x threshold)
controls
Triton
B
epsp slope
(% maximum before spike)
stimulus amplitude (x threshold)
controls
streptolysin
C
epsp slope
(% maximum before spike)
stimulus amplitude (x threshold)
controls
unc-5C Ab
D
epsp slope
(% maximum before spike)
stimulus amplitude (x threshold)
controls
DCC Ab
E
con
T/
Ab
Strep/
Ab
unc5C
unc5A
DCC
PSD-95
VAMP-1
Shh
actin

Figure 2

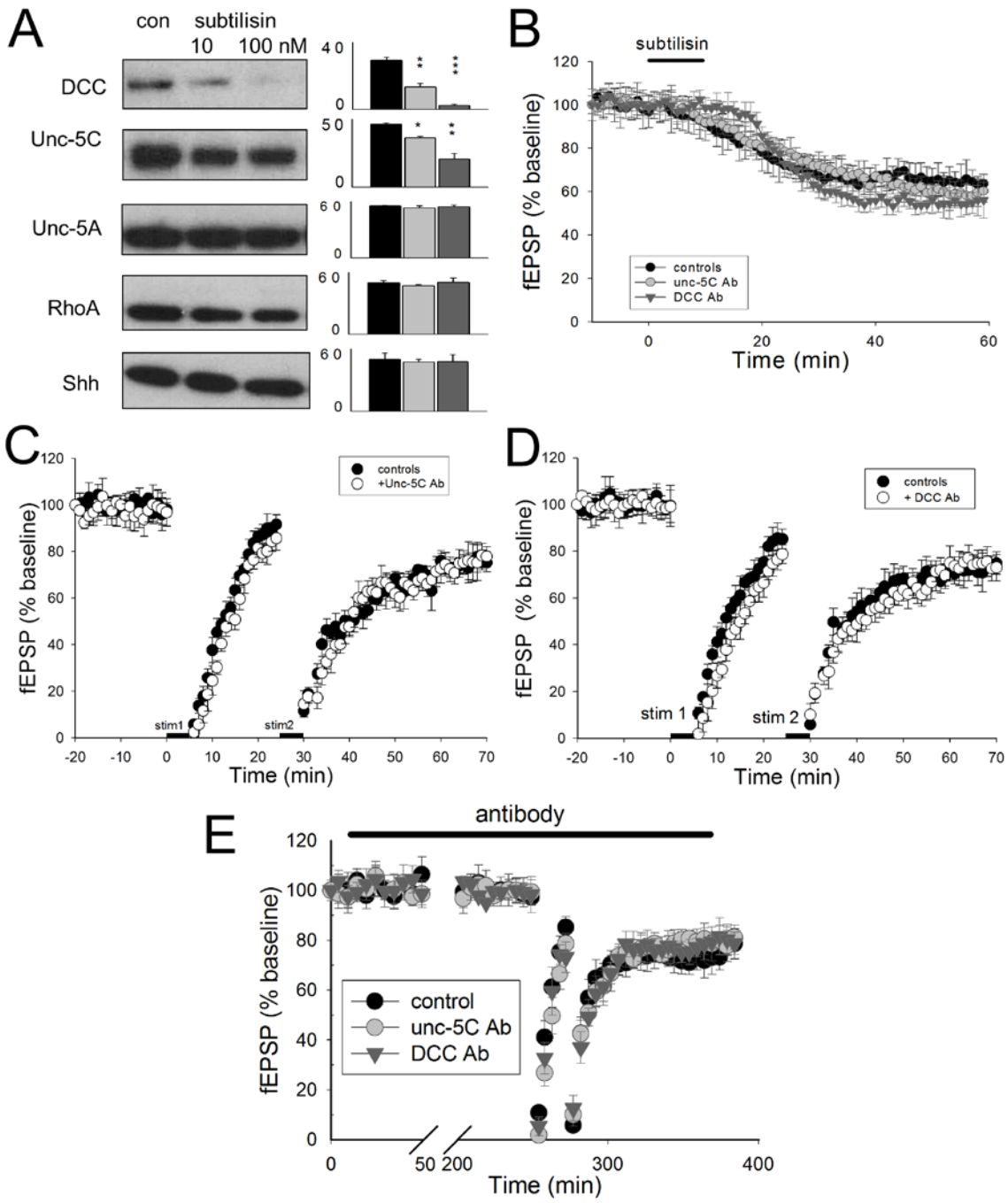

A
con
subtilisin
10
100 nM
DCC
Unc-5C
Unc-5A
RhoA
Shh
B
subtilisin
fEPSP (% baseline)
controls
unc-5C Ab
DCC Ab
Time (min)
C
controls
+Unc-5C Ab
fEPSP (% baseline)
stim1
stim2
Time (min)
D
controls
+ DCC Ab
fEPSP (% baseline)
stim 1
stim 2
Time (min)
E
antibody
fEPSP (% baseline)
control
unc-5C Ab
DCC Ab
Time (min)

Figure 3

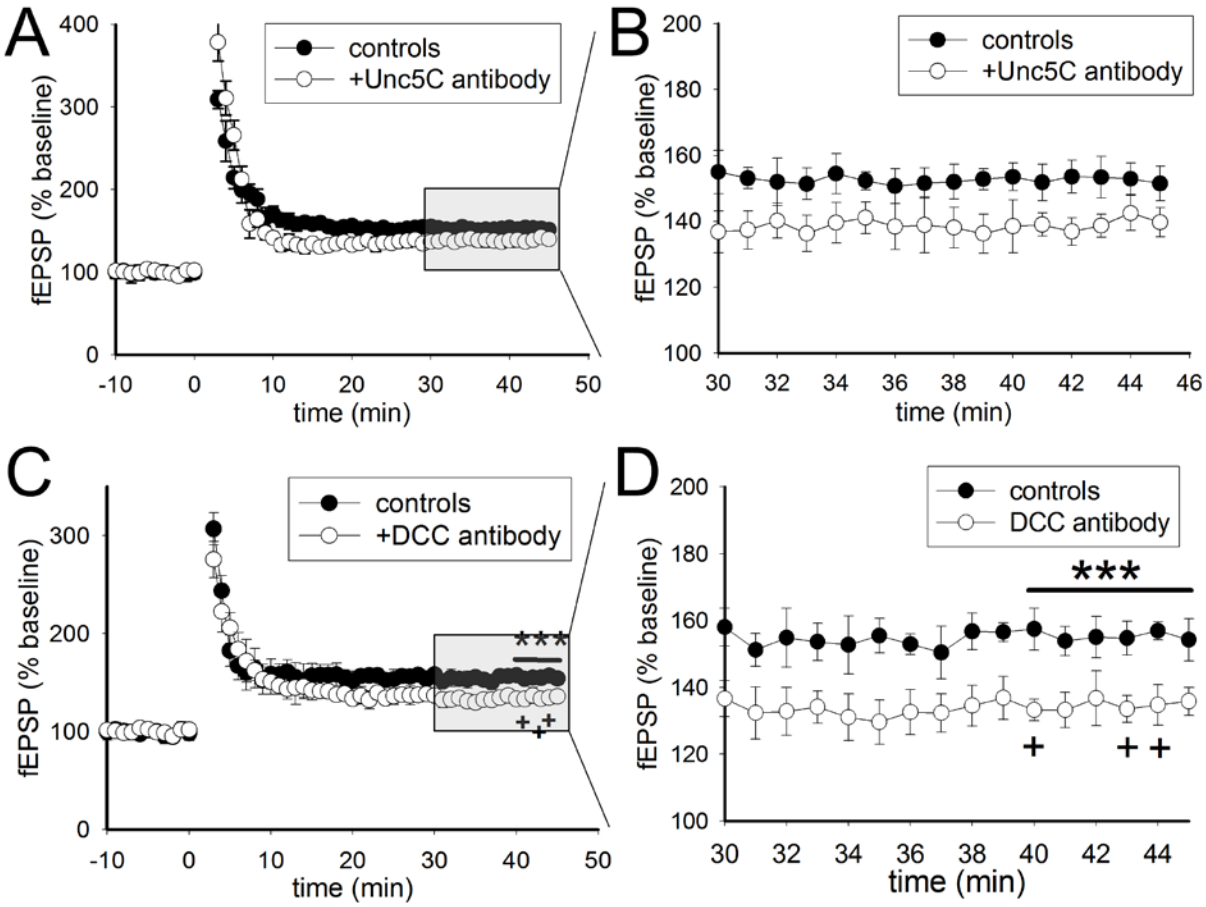

A
controls
+Unc5C antibody
fEPSP (% baseline)
time (min)
B
controls
+Unc5C antibody
fEPSP (% baseline)
time (min)
C
controls
+DCC antibody
***
++
fEPSP (% baseline)
time (min)
D
controls
DCC antibody
***
+
++
fEPSP (% baseline)
time (min)

Figure 4

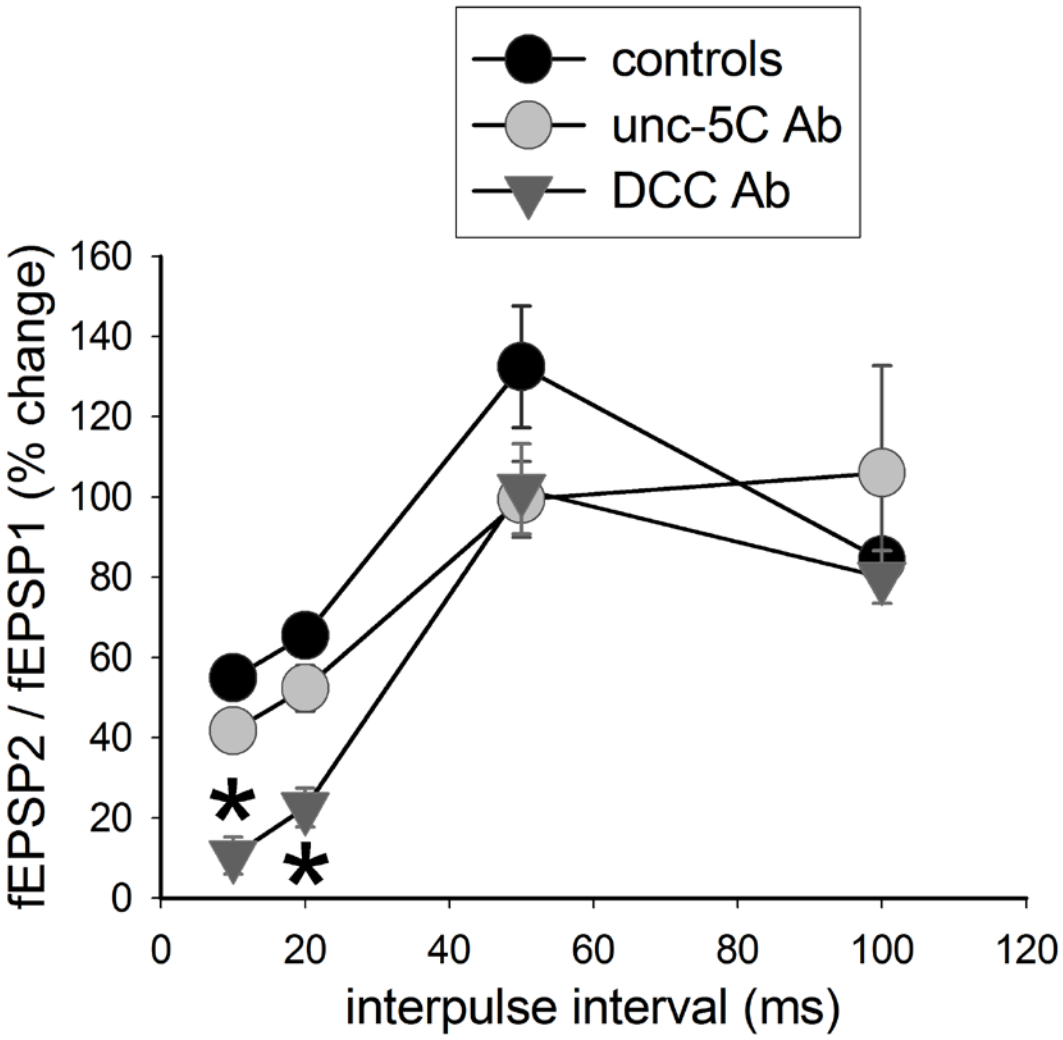

controls
unc-5C Ab
DCC Ab
fEPSP2 / fEPSP1 (% change)
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
interpulse interval (ms)
*
*

Figure 5

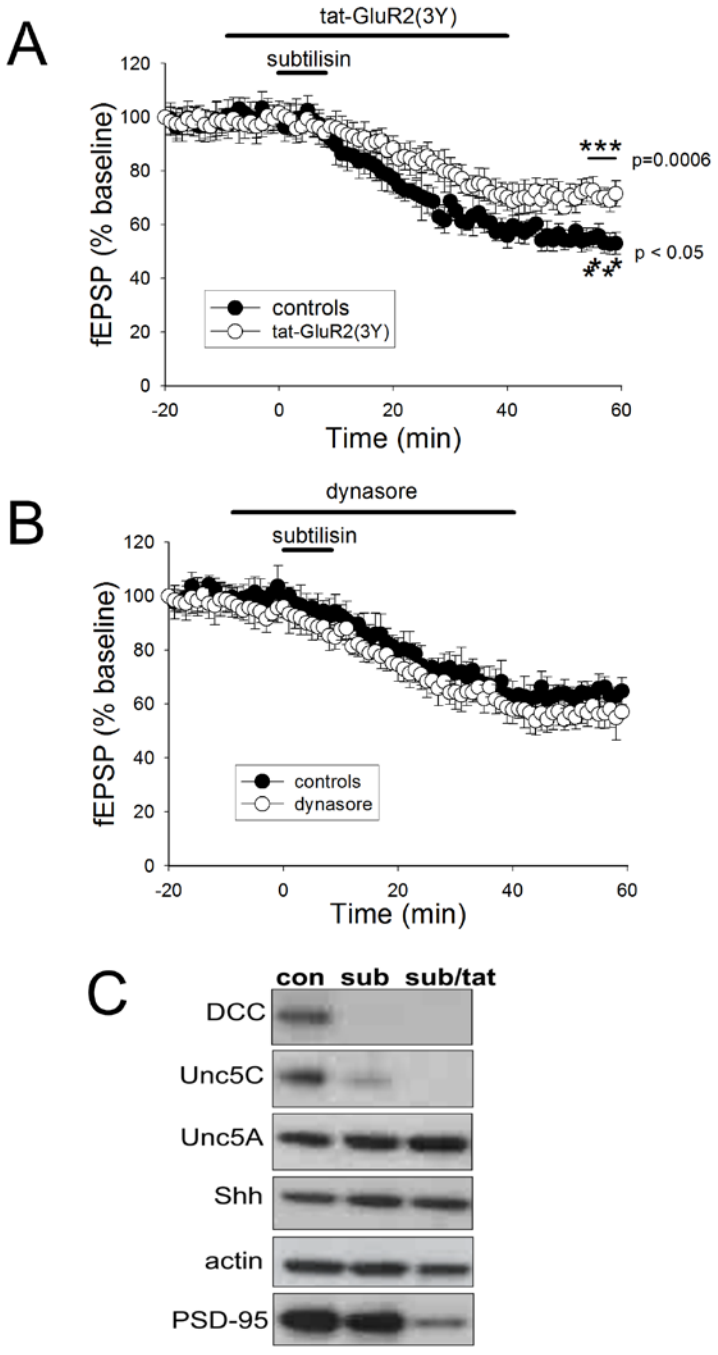
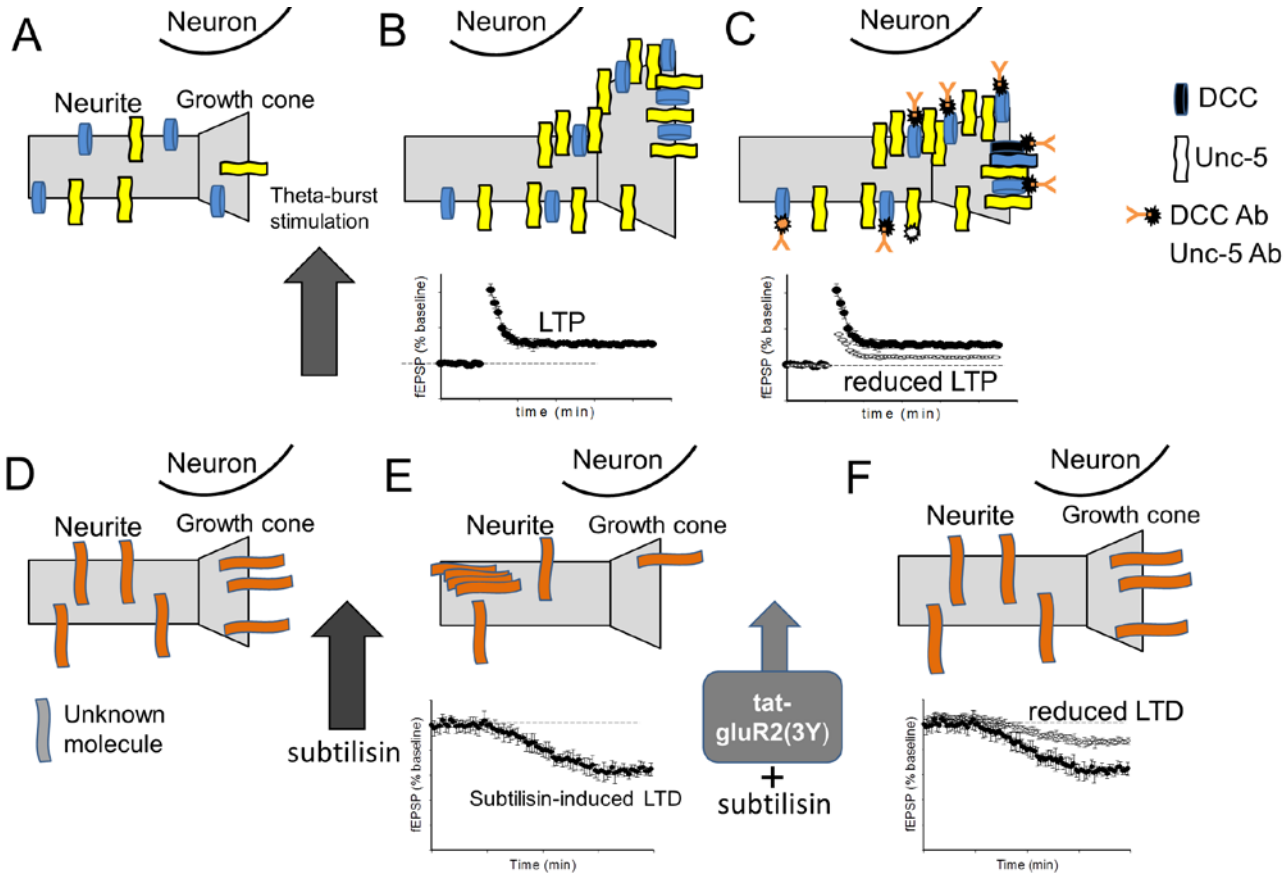

A
tat-GluR2(3Y)
subtilisin
fEPSP (% baseline)
Time (min)
***
p=0.0006
p < 0.05
controls
tat-GluR2(3Y)
B
dynasore
subtilisin
fEPSP (% baseline)
Time (min)
controls
dynasore
C
con sub sub/tat
DCC
Unc5C
Unc5A
Shh
actin
PSD-95

Figure 6


A
Neuron
Neurite
Growth cone
Theta-burst stimulation
B
Neuron
LTP
fEPSP (% baseline)
time (min)
C
Neuron
reduced LTP
fEPSP (% baseline)
time (min)
DCC
Unc-5
DCC Ab
Unc-5 Ab
D
Neuron
Neurite
Growth cone
Unknown molecule
subtilisin
E
Neuron
Neurite
Growth cone
Subtilisin-induced LTD
fEPSP (% baseline)
Time (min)
tat-gluR2(3Y)
+
subtilisin
F
Neuron
Neurite
Growth cone
reduced LTD
fEPSP (% baseline)
Time (min)