Involvement of the proteasome and caspase activation in hippocampal long-term depression induced by the serine protease subtilisin

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Abstract

The serine protease subtilisin A produces a long-lasting depression (LTD) of synaptic potentials in hippocampal slices which differs mechanistically from classical LTD. Since caspases have been implicated in hippocampal plasticity, this study examined a possible role for these enzymes in subtilisin-induced LTD. Subtilisin produced a concentration-dependent decrease in the size of field excitatory synaptic potentials (fEPSPs), which was not prevented or modified by the caspase inhibitors Z-VAD(OMe)-fmk or Z-DEVD-fmk. Similarly Z-VAD(OMe)-fmk did not modify the selective loss of protein expression produced by subtilisin. Subtilisin reduced the expression of procaspase-3 and caspase-9 but, while caspase-9 was converted to its conventionally activated form (39kDa), caspase-3 was metabolised along a non-canonical pathway to a 29/30kDa protein rather than the classical 17/19kDa fragments. Both Z-VAD(OMe)-fmk and were unable to prevent the reduced expression of PSD-95, VAMP-1 and Unc5H3 proteins produced by subtilisin, although MG132 did produce partial recovery from subtilisin-induced depression of fEPSPs. When tested on long-term potentiation (LTP) induced by theta stimulation in the stratum radiatum, MG132 inhibited the immediate increase in fEPSP size but generated a higher plateau LTP. Twin LTP stimulation generated a further increase in LTP amplitude in control slices but not in slices exposed to MG132. The results indicate that subtilisin does produce caspase activation but that this does not contribute to its induction of LTD. However, activation of the proteasome does contribute to subtilisin-induced LTD and may also play a modulatory role in electrically-induced LTP.

Key-words:- Long-term depression; LTD, synaptic plasticity; subtilisin; caspase-3; proteasome

1. Introduction

The two most widely studied forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) probably underlie many aspects of cognition. LTD has become associated with specific aspects of learning and memory processing such as the exploration of novel environments (Kemp & Manahan-Vaughan, 2004). Previously, there appeared to be two distinct forms of LTD, depending on whether they are triggered by or involve the activation of N-methyl-D-aspartate (NMDA) receptors (Mulkey & Malenka, 1992) or metabotropic glutamate receptors (mGluR) (Anwyl, 1999, 2006) respectively. While both are generated by much lower frequencies of electrical stimulation than LTP – typically around 1Hz – the nature of the LTD is partly dependent on species, age and the precise frequency and pattern of stimulation (Anwyl, 2006; Kemp et al. 2000).

However, we recently reported a novel form of LTD that can be induced by the group 8A serine protease subtilisin A (MacGregor et al., 2007). This subA-LTD appears to be mediated by transduction systems distinct from those reported for electrical or glutamate-induced LTD and is not dependent on either NMDA or metabotropic glutamate receptors (Forrest et al., 2011). In a recent study, Jo et al. (2011) have reported that β-amyloid fragments inhibit the generation of LTP in the hippocampus, and that this effect was dependent on caspase activation. In view of caspase involvement in that modulation of plasticity, and the lack of any clear transduction process mediating subA-LTD, the possibility was considered that caspases might also be involved in the latter phenomenon. We have therefore sought to determine whether caspase activity might contribute to subA-LTD and in addition we have examined any involvement of proteasomal activation by using the proteasome inhibitor MG132.

2. Experimental Procedures

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.

2.1 Electrophysiology

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 preincubated 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 with a pulse width of 200 μs and amplitude adjusted to evoke field excitatory postsynaptic potentials (fEPSPs) approximately 70% of the maximum obtainable before the appearance of a population spike, 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 microscopic visualisation in the stratum radiatum of the CA1 region. 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 of the EPSP,

using cursors 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 of 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 alterations 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. The fEPSP slope was expressed as a percentage of the potential size obtained immediately prior to the subtilisin-A perfusion, taken as the mean of the last 10 EPSPs before starting the superfusion of subtilisin-A.

When long-term potentiation (LTP) was examined, it 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 controlled by a Digitimer 4030 multichannel timing unit.

2.2 Immunoblotting

In order to examine the molecular changes underlying the electrophysiology, each slice was removed from the recording chamber immediately following electrophysiological recording, transferred into an eppendorf tube, snap frozen in dry ice and stored at -80^{o}C until analysis. 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) and centrifuged at 18000 *g* for 5 min at 4^{o}C. Supernatants were collected for protein concentration determination using the Bio-Rad Coomassie Blue protein assay (Bio-Rad, Hemel Hempstead, UK). Samples were then normalised to 10μg

and prepared as; 65% protein sample, 25% sample buffer and 10% reducing agent (Life Technologies, Paisley, UK), and heated at 70^{o}C for 10 min. The protein samples were loaded onto NuPAGE Novex 4-12% Bis-Tris (1.0mm) 15 lane gels (Life Technologies, Paisley, UK) and run at 150 volts for 80 min to separate proteins according to their molecular weight. SeeBlue pre-stained standard (Life Technologies, Paisley, UK) was included on each gel as a molecular weight marker. The separated proteins were then blotted onto Invitrolon poly(vinylidene difluoride) membranes (Life Technologies, Paisley, UK) at 35V for 70 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). The following day, membranes were then washed 3 times for 15 min with TBST and incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (prepared in 5% milk-TBST) for 1h at room temperature. Following secondary antibody incubation, blots were washed 3 times for 15 min with TBST then visualised using Enhanced Chemiluminescence Plus detection kit (GE Healthcare, Chalfont St Giles, UK). The blots were analysed using Image J densitometric software (http://rsb.info.nih.gov/ij/). In order to control for variations in the total amount of protein loaded onto gels, all membranes were stained with Ponceau S solution (Sigma, Poole, UK). Results were expressed as the mean ± s.e.mean optical density and tested for statistical significance.

2.3 Antibodies

Western blot analysis was carried out using the following primary antibodies raised against target proteins:

PSD-95 (rabbit monoclonal, 3450, 1 : 10000 dilution), caspase-3 (detects full length 35kDa and cleaved 19/17kDa caspase-3 fragments, rabbit polyclonal, 9662, 1 : 1000 dilution),

caspase-9 (detects full length 51kDa and cleaved 39/37kDa fragments, mouse monoclonal, 9508, 1 : 1000 dilution) (Cell Signalling, New England Biolabs, Hitchin, UK); VAMP-1/synaptobrevin (goat polyclonal, AF4828, 1 : 10000 dilution) (R&D Systems, Abingdon, UK); SHH (goat polyclonal, sc-1194, 1 : 1000 dilution), unc5H1 (goat polyclonal, sc-67902, 1 : 1000 dilution), unc5H3 (goat polyclonal, sc-54442, 1 : 500 dilution) (Santa Cruz, Insight Biotechnology, Wembley, UK).

The following secondary HRP-conjugated antibodies were used at 1 : 5000 dilution: goat anti-rabbit HRP (12-348) (Millipore, Watford, UK); donkey anti-goat HRP (sc-2020) and goat anti-mouse (sc-2005) (Santa Cruz, Insight Biotechnology, Wembley, UK).

2.4 Analysis

The statistical significance of differences between two treatments such as control and experimental slices was determined using a *t* test. For more complex comparisons, data were analysed using analysis of variance followed by Dunnett's multiple comparison test relative to control values or the Bonferroni test for selected datasets. In all cases $P < 0.05$ was adopted as the guiding criterion for significance but the exact P value is shown whenever possible.

2.5 Sources of materials

Compounds were obtained from the following sources: NMDA, subtilisin-A (catalogue P5380), from Sigma Chemicals (Poole, Dorset, UK); Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (Z-DEVD-fmk), Benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (Z-VAD(OMe)-fmk) and N-[(phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide (MG132) from Tocris bioscience (Bristol, UK).

Subtilisin was dissolved directly in aCSF and fresh solutions were made each day. Caspase inhibitors and MG132 were dissolved in DMSO and subsequently diluted into aCSF at the desired concentration such that the final level of DMSO did not exceed 0.01% v/v.

3. Results.

3.1 Caspase inhibitors - fEPSPs

Superfusion of subtilisin invariably generated a depression of fEPSP size as described previously. Concentrations of 1, 2 or 4 μM were used in most experiments since they produce LTD which reaches a plateau level of approximately 80%, 45% and 20% respectively of the initial fEPSP size. The decline in fEPSP size develops slowly after the application of subtilisin, reaching a plateau by approximately 30min. Control responses to subtilisin together with samples of the fEPSPs recorded are illustrated in Fig. 1A-E.

For the examination of caspase inhibitors, exposure of the slices to inhibitor compounds was maximised by pre-incubation with concentrations shown by previous workers to be appropriate for inhibition of the relevant enzymes. After their initial preparation, alternate slices were transferred into aCSF in two petri dishes in the holding chamber. These were allowed to rest for 1h for slices to recover from the preparative procedures, after which one batch of slices was designated as the control group, and the enzyme inhibitors were added to the second, experimental dish. Slices were subsequently transferred into the recording chamber (submerged mode) alternately from the two dishes. In addition, each inhibitor was superfused over slices for 20min before the application of subtilisin and superfusion was continued during and for 30 mins after ending the subtilisin application.

To examine whether any of the caspases were implicated in subtilisin-LTD, the non-selective but cell-permeant and irreversible caspase inhibitor Z-VAD(OMe)-fmk was included in the pre-incubation medium at a concentration of 5μM, and was superfused over slices at 1μM. These concentrations were selected on the basis of values for IC50 that have been reported (Kunstle et al., 1997; Dunstl et al., 2007). A second group of slices was incubated in Z-VAD(OMe)-FMK at 100μM for 2-6h before transferring to the recording chamber. These higher concentrations have been found necessary by some authors to produce complete caspase inhibition (Perez-de-la-Cruz et al., 2010; Gerace et al. 2012; Ortega and Moran, 2011) but the results were similar to those seen with the lower concentrations.

Exposure of the slices to Z-VAD(OMe)-fmk did not itself affect excitability of the slices since an initial test of fEPSP size at various stimulus currents indicated no difference between slices pre-incubated in aCSF alone and those pre-incubated with Z-VAD(OMe)-fmk (Fig. 1F-H)

Preincubation and superfusion with Z-VAD(OMe)-fmk did not modify the LTD response to subtilisin when quantified as the magnitude of the fEPSP depression at its plateau. Analysis of variance of the six data points from 45-50 minutes and from 75-80 minutes in Z-VAD(OMe)-fmk-treated slices compared with controls showed no significant difference between the two sets of curves (Fig. 1A-E).

The selective caspase-3 inhibitor Z-DEVD-fmk was also examined in case Z-VAD(OMe)-fmk had interfered with any effect of subtilisin, or had introduced a confounding element of toxicity. Z-DEVD-fmk is also an irreversible but cell-permeant compound and was included in the pre-incubation medium at a concentration of 5μM, and in the superfusion medium at 1μM. These concentrations were based on the 2μM incubation levels used by Li et al. (2010) to fully block caspase-3 and the IC50 values obtained by Valanne et al. (2008) and

Zhang et al., (2007). The inhibitor did not itself affect excitability of the slices since an initial test of stimulus current – fEPSP amplitude indicated no difference between slices pre-incubated in aCSF alone and those pre-incubated with Z-DEVD-fmk (data not shown). Again, the preincubation and superfusion with Z-DEVD-fmk did not modify the LTD response to subtilisin. Analysis of variance on the six data points from 45-50 minutes in Z-DEVD-fmk-treated slices compared with controls showed no significant difference between the two curves for LTD induced by subtilisin : subtilisin 4μM alone produced an end-of-plateau fEPSP size of 22.4% ± 5.8 (percent baseline, n = 3) and after treatment with Z-DEVD-fmk a plateau of 16.6% ± 3.5 (n = 3, P = 0.33; data not shown).

3.2 Immunoblotting

One objective of this project was to obtain further information on the relationship between the effects of subtilisin on protein expression and LTD. Our previous study found that subtilisin decreased the expression of several proteins but with substantial differences in potency, while others remained unaffected by the protease (Forrest et al., 2011). The question therefore arose as to whether the effects of subtilisin on LTD and protein expression were related or not, a question which could be addressed by examining whether caspase inhibition prevented the protein loss, even though it did not affect subA-LTD. To answer this question, some slices were retained for the examination of protein expression by western blotting. The proteins examined included those which had previously been shown to be sensitive to subtilisin, including Vesicle Associated Membrane Protein-1 (VAMP-1; synaptobrevin-1), Postsynaptic Density Protein-95 (PSD-95) and Unco-ordinated 5H3 (Unc5H3), as well as several which appear to be relatively resistant such as Unc5H1 and sonic hedgehog (SHH), a protein found primarily in the earliest phases of CNS development. SHH continues to be expressed in adult tissue

where it is found especially in areas exhibiting neurogenesis and is associated with the maturation and differentiation of progenitors into cells with a neuronal phenotype.

In order to compare the relative sensitivity of these proteins to subtilisin, a concentration of 2μM was selected as one which produced approximately 50% reduction in the size of the electrical fEPSP. Slices were taken for analysis 10min after ending the subtilisin superfusion at a time when the rate of development of the LTD was maximal in order to assess whether caspase inhibitors were able to prevent the early development of protein loss. In a second series of experiments slices were retained in the incubating chamber until 40min after ending the subtilisin perfusion to assess whether any recovery occurred compared with the 10min time point and whether caspase inhibition altered the development of protein expression or recovery.

The results revealed that for those proteins examined in our previous study, the same pattern was apparent at the end of the 10min washing period after subtilisin (Fig. 2A), with levels of PSD-95 and VAMP-1 being very significantly reduced by subtilisin while SHH protein expression was not significantly affected (Fig. 2A). Expression of Unc5H3 was profoundly reduced while Unc5H1 was relatively resistant and not significantly affected (Fig. 3A). After washing the slices for 40min after ending the superfusion with subtilisin, all these proteins showed a degree of recovery, with no significant reduction remaining in the expression of PSD-95 or VAMP-1 (Fig. 2B). Expression of Unc5H1 remained not significantly different, with mean levels being restored to control values (Fig. 3B). Levels of Unc5H3 showed some recovery such that the levels were no longer significantly different from controls (Fig. 3B).

In slices which had been treated with Z-VAD(OMe)-fmk, the early losses of Unc5H3, VAMP-1 and PSD-95 were not affected (Fig. 2A and Fig. 3A). Indeed, there was a

suggestion that Z-VAD(OMe)-fmk increased the loss of Unc5H1 since the reduction in its expression was now statistically significant (Fig. 3A).

3.3 Caspase activation

An examination of the expression of caspase-3 and caspase-9 was also performed to determine whether subtilisin-induced LTD was associated with activation of these enzymes independently of any role in the LTD itself. In concentrations at which subtilisin produced LTD of fEPSPs, it did not produce a classical activation of the 'effector' caspase-3. As illustrated in Fig. 4A the concentrations of subtilisin that produced LTD were associated with a non-canonical protein pattern for caspase-3. There was a trend towards a reduction in the full-length caspase at 500nM and 1μM subtilisin which became a significant decrease at 4μM. This was paralleled by significant increases in the presence of the cleaved, active form of the enzyme (30kDa) at 1μM subtilisin, with the result that the ratio of activated to normal protein showed a concentration-related trend to increase, with a highly significant change at 4μM subtilisin (Fig.4A).

In contrast, caspase-9 showed a classical response to subtilisin, with a reduction of the full-length protein (51kDa) at 500nM, 1μM and 4μM subtilisin, and the appearance of a band corresponding to the activated form of the enzyme at 37/39kDa such that the ratio of cleaved to native forms revealed a concentration-related trend becoming significant at 4μM subtilisin (Fig. 4B).

To determine whether any of these effects were shared by more conventional inducers of LTD, the effects of electrically-induced LTD, and the metabotropic receptor agonist dihydroxyphenylglycine (DHPG) were examined. The former was induced by triple pulse stimulation (3 stimuli at 5Hz) delivered every second for 5 min, while chemical induction was achieved using 100μM DHPG superfused for 10 min. Both of these procedures have

previously been shown to induce LTD in our laboratory (Forrest et al. 2011) but they had no effect on the activation of caspase-3 or caspase-9 (Fig. 4A,B).

Recovery experiments similar to those outlined above showed that, after treatment with subtilisin at 2μM, the early loss of full-length caspase-3 was no longer statistically significant after 40min (Fig. 5A,B). Nevertheless, caspase-3 remained activated at this time as revealed by the ratio of the activated protein relative to the native molecule (Fig. 5B). Examination of slices that had been exposed to Z-VAD(OMe)-fmk and then used for immunoblotting of the caspase proteins 10min after the subtilisin superfusion indicated that the inhibitor did not change the effects of subtilisin on the patterns of caspase-3. Subtilisin still produced a loss of the native procaspase-3 which was not prevented by Z-VAD(OMe)-fmk (Fig. 5A). Even after 40min, when the levels of caspase-3 were showing clear signs of recovery, Z-VAD(OMe)-fmk was not able to reduce the loss of full-length protein and did not prevent the activation of caspase-3 expressed as the ratio of activated to native protein (Fig. 5B).

The loss of full length caspase-9 by subtilisin at 2μM was also not prevented by Z-VAD(OMe)-fmk after 10min (Fig. 5A). Some recovery of caspase-9 protein was noted after 40min, but again Z-VAD(OMe)-fmk showed no ability to prevent the loss of protein (Fig. 5B).

As a positive control to confirm that Z-VAD(OMe)-FMK was able to penetrate into the tissue slices and inhibit caspase activation, a group of slices were incubated for 1h either with staurosporine alone (2μM) or staurosporine with Z-VAD(OMe)-FMK (100μM) followed by 8h recovery. Western blots generated from these slices showed that staurosporine did activate the initiator caspase-9, demonstrated by the appearance of the activated protein band at 39kDa (Fig. 6). Inclusion of Z-VAD(OMe)-fmk, however, prevented the activation of the caspase (Fig. 6). In contract to subtilisin, it was noted that staurosporine had no

significant effect on those proteins sensitive to elimination by subtilisin such as SHH, PSD-95, Unc5H1 and Unc5H3 (Fig. 6) providing further supportive evidence that caspase activation is not related to the protein loss induced by subtilsin.

3.4 Proteasomal inhibition

In view of the non-classical effects of subtilisin on caspase expression, without any alteration in the fEPSP LTD by caspase inhibitors, an alternative mechanism for the effects of subtilisin was considered. The possible involvement of proteasomal activation was examined, especially since the proteasome is known to be activated in some forms of plasticity. The proteasome activation might then contribute to either the subA-LTD or the loss of cellular proteins, or both.

The cell-permeable compound Cbz-leu-leu-leucinal (MG132) is a widely used inhibitor of proteasomal activity and was included in the slice holding chamber at a concentration of 10μM, with subsequent superfusion over the slices at 2μM for 20 min before, during and for 30mins following applications of subtilisin. The concentrations chosen were based on the original reports of the potency and mechanisms of proteasome inhibition by MG132 (Lee and Goldberg, 1998).

MG132 did not itself affect excitability of the slices since an initial test of stimulus current – fEPSP amplitude indicated no difference between slices pre-incubated in aCSF alone and those pre-incubated with MG132 (Fig. 7A-C). Similarly there was no overall significant difference in the maximum amplitude of the subtilisin-LTD (Fig. 7D-H). A comparison between the size of the fEPSPs 40 minutes after the application of subtilisin in the presence and absence of MG132 showed no significant difference (for subtilisin 4μM: 20.5 ± 4.5 in controls, 30.5 ± 4.4 in MG132, $P = 0.18$, n.s; for subtilisin 1μM: 83.0 ± 5.2 in controls, 77.6 ± 4.1 in MG132, $P = 0.44$, n.s.). However, there was a clear tendency for the

potentials to recover partially after treatment with MG132 such that the final size of the plateau LTD, 90 mins after beginning superfusion with subtilisin, was significantly less than with subtilisin alone (Fig. 7G). Analysis of variance of the final 5 data points at 85-90 mins after subtilisin 4μM revealed a highly significant difference between the curves ($P < 0.001$; Fig. 7G), with 4 of the individual data points being significantly different (Bonferroni post hoc test).

3.5 Long-term potentiation (LTP)

The ability of MG132 to effect a partial recovery from subtilisin suggested an ability to modify the excitability of neurons participating in plastic changes. The effect of MG132 was therefore examined upon LTP. LTP was induced by theta-burst stimulation and quantified as the increase in fEPSP amplitude measured 40min after the induction. As illustrated in Fig. 8, there was a smaller maximum degree of potentiation immediately following the stimulation bursts in the presence of MG132, although the rate of decline in fEPSP size was slower with MG132 treatment than in control slices, with the result that the initial increase in fEPSP size and the final degree of LTP attained after 40min was greater than that seen in controls. Analysis of variance of the six data points immediately following the stimulation period in MG132-treated slices compared with controls showed a highly significant difference between the two curves ($P < 0.001$), with several of the individual points being highly significant as indicated in Fig. 8 (Bonferroni post hoc test). The final degree of LTP measured at 40min was also very significantly different between control and MG132-treated slices. Analysis of variance on the six data points from 40-45 minutes in MG132-treated slices compared with controls showed a highly significant difference between the two curves ($P < 0.001$), with several of the individual points being highly significant (Bonferroni test) (Fig. 8).

To examine this change further, some slices were subjected to two bursts of theta stimulation delivered 20 min apart. The control slices usually exhibited a second, additional degree of LTP which attained a mean value of 180.0% of baseline fEPSP size over the last 5 time points of the experiment compared with 130.4% over the five time points immediately preceding the second period of stimulation ($P < 0.001$) (Fig. 9). Slices treated with MG132, however, showed a much smaller difference over the same time periods (154.3% after stim2 compared with 150.8% immediately preceding stim2 ($P = 0.05$) (Fig. 9), suggesting a limit to their ability to respond to the dual stimuli.

3.6 Immunoblotting

In view of the implication from these results that proteasomal activation might be involved in the electrophysiological actions of subtilisin, slices were used for the examination of protein expression after exposure to normal aCSF or MG132. The blots revealed that MG132 did not modify the effects of subtilisin on the proteins described above, with the protease still able to suppress the expression of PSD-95, VAMP-1 and Unc5H3 (Figs. 2, 3). MG132 appears to have increased the loss of Unc5H1 at the 40min time point (Fig. 3B).

This pattern, therefore, of failure to prevent the reduced protein expression by subtilisin with possible enhancement in the loss of Unc5H1, resembled that described above after exposure to Z-VAD(OMe)-fmk. A further group of slices were collected 100min after ending the subtilisin application to determine whether the partial recovery in fEPSP size was accompanied by any restoration of proteins at that time. However, expression of the proteins remained depressed at this point, despite the partial electrical recovery (data not shown).

MG132 did not prevent the loss of caspase-9 by subtilisin (Fig. 5A) but may have reduced the loss of full-length caspase-3 after 10 min and the ratio of cleaved to native procaspase protein after 40 min (Fig. 5B).

In view of the intriguing effect of MG132 on LTP, the effects of MG132 perfusion and LTP on protein expression were examined by immunoblotting. Hippocampal slices were collected for western blotting following incubation with MG132. Another group of slices were collected 30min after the induction of LTP, and slices were also examined that had been pre-incubated and superfused with MG132 before stimulation to induce LTP. Treatment with MG132 and the induction of LTP had no significant effect on the expression of PSD-95, Unc-5H1, Unc-5H3 and VAMP-1 proteins. In addition, MG132 and LTP failed to modify the expression of full-length caspase-3 and caspase-9: it did not induce cleavage of either of the caspases and no cleaved products were detected following the various treatments (data not shown).

4. Discussion

The caspase family of proteases has been studied primarily in relation to cell death cascades such as apoptosis. The most widely studied enzymes are caspase-3, which is one of the final effector enzymes in the caspase cascade leading to DNA fragmentation and apoptosis, and caspase-9 which is normally activated by cytochrome-C release from mitochondria in response to insults such as hypoxia (Stefanis et al.,1999). These enzymes are, however, also involved in a range of other cell functions including aspects of synaptic and dendritic modelling which probably underlie the growing evidence for roles in synaptic transmission and neuronal plasticity (see Chan and Mattson 1999).

Indeed, members of the caspase family have a number of actions that may compromise cellular function independently of any induction of apoptosis (Maelfait and Beyaert 2008; Li

and Yuan, 2008; Feinstein-Rotkopf and Arama, 2009; Kuranaga, 2011; Crawford et al. 2011; Chowdhury et al., 2008) such as the modulation of immune function (Yi and Yuan, 2009) and the modification of neurogenesis and neurotransmission (D'Amelio et al., 2010). In some cases these alternative functions of caspases involve actions on a variety of substrates other than interleukins, such as poly(ADP-ribose)polymerase (PARP) (Gunn-Moore and Tavare, 1998).

The work of Li et al. (2010) clearly implicates caspases in the LTD produced by electrical stimulation in the hippocampus, an observation that is consistent with evidence for a role of caspases in the internalisation of AMPA receptors that is required for NMDA receptor-dependent LTD (Collingridge et al., 2010; Shepherd and Huganir 2007). It has also been reported that caspase activation is required for the reduction of LTP by β-amyloid fragments (Jo et al., 2011).

These various considerations prompted the present investigation to examine whether the caspases were involved in LTD produced by the serine protease subtilisin (MacGregor et al. 2007; Forrest et al., 2011) and whether the loss of cellular proteins that we have described previously (Forrest et al., 2011) was a factor in this. The study was given additional impetus by previous studies which have shown that caspase involvement in neuronal damage and plasticity can be dissociated (Gillardon et al 1999). Following cerebral ischaemia Z-DEVD-fmk reduced cell death but did not prevent the reduction of LTP, implying that the two phenomena are not directly related and that the loss of LTP can be dissociated from caspase activity and cell death. Z-VAD(OMe)-fmk is an irreversible, cell-permeable but non-selective inhibitor of most members of the caspase group.The failure of this compound to modify subtilisin-induced LTD, therefore, provides a marked contrast from electrically-induced LTD. Of particular interest is the finding that caspase

inhibition does not prevent either the LTD or the selective loss of cellular proteins, leaving open the question of whether the protein loss is a necessary feature of the LTD.

Despite the lack of caspase involvement in the LTD, subtilisin does produce an activation of the enzymes. Caspase-9 is activated in a classical fashion with the conversion of full-length native enzyme into a 39kDa protein. This cleavage has been shown to occur in response to mitochondrial damage by stimuli such as oxidative stress, which leads to the release of cytochrome C, its interaction with full size native 51kDa procaspase-9 and generation of the 39KDa fragment.

Caspase-3 was affected by subtilisin in an atypical manner resembling that described by Csordas et al. (2006). The induction of oxidative stress by peroxide in that study led to a novel form of caspase cleavage in which a fragment of approximately 29kDa was generated. Its production was not prevented by Z-VAD-fmk, although it was not seen in the presence of general protease inhibitors, inviting comparison with an earlier report which suggested that the activation of an endogenous serine protease might mediate a non-apoptotic (and non-caspase-dependent) pathway of cell death (O'Connell et al. 2006). At lower concentrations, peroxide induced the expression of two smaller fragments of caspase-3 with molecular weights of approximately 17 and 19kDa, which would correspond to the p20 subunit of the caspase. Prolonged applications of peroxide resulted in the loss of these and their replacement by the larger 29kDa band, although it remains unclear whether this might be the result of a re-combination of the two smaller fragments.

Alternative, non-canonical forms of caspase cleavage such as this have been previously studied in relation to cell death processes, especially in the clarification of whether cell death sequences are primarily of a classically apoptotic type or involve necrotic features. The atypical cleavage of caspases has also been reported in relation to specific aspects of cellular function (Shultz et al., 2010, 2011; Hoffman et al., 2009)

although there has been less examination of non-canonical cleavage in neuronal plasticity. This report is among the first to observe this non-canonical catabolism associated with a form of LTD.

The differences between LTD induced by classical methods and that produced by subtilisin suggest that different transduction pathways are involved. It is clear that subtilisin-LTD is not simply the result of a non-selective global destruction of proteins produced by this serine protease since, of several proteins that have been examined to date only some, including PSD-95, VAMP-1 and Unc5H3 are lost after subtilisin perfusion of hippocampal slices at concentrations that produced significant levels of LTD. Several other proteins, including synaptophysin, synaptotagmin, RhoA and RhoB remain much less affected at the same concentrations of subtilisin (Forrest et al., 2011). The present report adds further to this selectivity by showing that the morphogenetic protein sonic hedgehog is relatively little affected by subtilisin.

The failure of caspase inhibitors to affect subtilisin-induced LTD led to the testing of MG132, a potent, cell-permeable inhibitor of the cellular proteasome. This compound did induce a degree of recovery of subtilisin-LTD, suggesting that proteasomal activation might contribute to the establishment and maintenance of subtilisin-induced LTD. Indeed, it is now recognised that the removal of AMPA receptors is a major factor in LTD, and such internalisation can be inhibited by MG132 (Collingridge et al. 2010; Bingol and Schuman, 2004).

The reduction of subtilisin-induced LTD by MG132 emphasises the difference between this form of synaptic inhibition and that which is produced by the activation of metabotropic glutamate receptors, since the latter is increased by proteasomal inhibition (Citri et al., 2009). LTD which involves NMDA receptors, on the other hand appears not to be affected by MG132 (Citri et al., 2009). These results are consistent with our earlier reports that

subtilisin-LTD is not dependent on the activation of NMDA receptor (MacGregor et al., 2007) or metabotropic glutamate receptors (Forrest et al., 2011).

The ability of MG132 to increase the size of the subtilisin-inhibited fEPSPs (although having no effect itself on potential size), led to an examination of its effects in electrically-induced LTP. Intriguingly, this revealed a lower short-term potentiation but a significantly higher LTP after 30min. This result is partly similar to that reported by Karpova et al., (2006) in which they observed a similar decrease by MG132 in the immediate short-term potentiation of synaptic potentials. The comparable depression of short-term potentiation seen here and by Karpova et al., (2006) suggests a possible role for proteasomal activity in regulating new protein generation or destruction in relation to the immediate potentiation of fEPSPs. A role for proteins in short-term as well as long-term changes of fEPSP size has been demonstrated previously (Behnisch et al., 2004; Tsokas et al., 2005) and is consistent with the present data.

In addition, we have noted that MG132 produces a larger degree of LTP than seen in control slices after 30min, an effect not seen by Karpova et al., (2006). The precise reasons for this difference are not clear, although the earlier study employed a higher concentration of MG132 (10μM) to superfuse the slices and LTP was only quantified after applying the third of a series of three bursts of 100Hz stimuli. Since theta stimulation, as used here, was originally noted as a mechanism which induced clearly separate short and long-term aspects of LTP (Larson et al., 1986), it is possible that the differences of stimulation pattern determine the relative contributions of these LTP components and their sensitivity to MG132. A similar explanation may account for the conclusion by Cai et al. (2010) that proteasome activity was required for the induction, although not the maintenance of late LTP induced by 100Hz tetanic stimulation. The present data may suggest that proteasomal activation normally limits the degree of potentiation generated in

slices, or may facilitate its decay to a significant extent. The use of two periods of stimulation supports this proposal since, in contrast to control slices in which a second stimulation usually generates further potentiation, the presence of MG132 promotes saturation of LTP after only a single stimulation, with no further increase developing thereafter. Despite possible differences in the role of the proteasome in different experimental protocols, an involvement in aspects of synaptic plasticity is strongly supported by evidence that it regulates activity-dependent structural changes in spine morphology (Hamilton et al., 2012).

Overall, these results strengthen the view that the serine protease subtilisin induces a novel form of LTD, but demonstrate that it does not involve caspase activity and therefore cannot be attributed to caspase-mediated cellular damage or indiscriminate proteolysis. This is despite the ability of subtilisin to produce a classical activation of caspase-9 and an atypical cleavage of caspase-3. The results also raise the possibility that activation of the proteasome could contribute to the mechanisms underlying subtilisin-induced LTD and may also modulate electrically-induced LTP in the rat hippocampus.

Acknowledgements

We are grateful to Epsom Medical Research and the Haddon Family Trust for funding support, and to Dr. Alexandra Ferguson for performing some of the experiments in mice.

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Figure legends

Figure 1. Effects of Z-VAD(OMe)-fmk on subtilisin-A-induced LTD in the CA1 region of rat hippocampal slices.

A: Sample fEPSPs before (a) and 20min after (b) the superfusion of subtilisin 1μM; **B:** Comparable sample EPSPs in the presence of Z-VAD(OMe)-fmk; **C:** Time course and magnitude of the LTD of field EPSPs produced by subtilisin-A superfused for 10 min as indicated by the bar, at concentrations of 1μM (filled circles), or 4μM (filled squares) alone or in the presence of Z-VAD(OMe)-fmk (open diamonds and open triangles respectively). The graph shows the mean ± s.e.mean (*n* = 4 at each concentration).

D: Sample fEPSPs before (a) and 20min after (b) the superfusion of subtilisin 4μM; **E:** Comparable sample EPSPs in the presence of Z-VAD(OMe)-fmk.

Calibrations 1mV and 5ms.

F: sample fEPSPs at (increasing amplitude) 90, 100, 110, 120 and 130nA stimulating current, normalised to the amplitude obtained at 130nA above which slices exhibited indentations due to population spike generation; **G:** Comparable fEPSPs recorded after preincubation and superfusion with Z-VAD(OMe)-FMK; **H:** Graph showing mean ± s.e.mean values for control slices (filled circles) and slices preincubated and superfused with Z-VAD(OMe)-FMK (open circles).

Figure 2. Effects of Z-VAD(OMe)-fmk and MG132 on subtilisin-A-induced loss of protein expression in rat hippocampal slices.

Representative western blots are illustrated from control slices, slices treated with subtilisin 2μM, subtilisin 2μM with MG132 2μM, and subtilisin 2μM with Z-VAD(OMe)-fmk 1μM. The

corresponding bar charts show mean ± s.e.mean (n=3) optical density (O.D.) for PSD-95 (95kDa), VAMP-1 (synaptobrevin; 18kDa) and SHH (45kDa; n=2).

A. blots and quantified protein expression in slices taken after 10min washing following superfusion of subtilisin.

B. Comparable blots and quantified levels in slices taken after 40min washing following superfusion of subtilisin.

*P< 0.05, ***P< 0.001 ANOVA followed by Bonferroni post hoc test compared with control slices.

Figure 3. Effects of Z-VAD(OMe)-fmk and MG132 on subtilisin-A-induced loss of protein expression in rat hippocampal slices.

Representative western blots are illustrated from control slices, slices treated with subtilisin 2μM, subtilisin 2μM with MG132 2μM, and subtilisin 2μM with Z-VAD(OMe)-fmk 1μM. The corresponding bar charts show mean ± s.e.mean (n=3) optical density (O.D.) for Unc-5H3 (130kDa) and Unc-5H1 (98kDa).

A. blots and quantified protein expression in slices taken after 10min washing following superfusion of subtilisin.

B. Comparable blots and quantified levels in slices taken after 40min washing following superfusion of subtilisin.

*P< 0.05, ***P< 0.001 ANOVA followed by Bonferroni post hoc test compared with control slices.

Figure 4. Effects of LTD induction on caspase expression.

Representative western blots are illustrated from control slices, slices with electrically-induced LTD, slices treated with DHPG (100μM) and slices treated with subA (500nM, 1μM and 4μM). The corresponding bar charts show mean ± s.e.mean (n=3) optical density (O.D.) for

A. caspase-3 (a) full length (35kDa) (b) ratio of cleaved (30kDa) to full length (35kDa)

B. caspase-9 (a) full length (51kDa) (b) ratio of cleaved (39kDa) to full length (51kDa)

*P< 0.05, **P< 0.01 ANOVA followed by Dunnett's post hoc test compared with control slices.

Figure 5. Effects of Z-VAD(OMe)-fmk and MG132 on subtilisin-A-induced loss of caspase expression in rat hippocampal slices.

Representative western blots are illustrated from control slices, slices treated with subtilisin 2μM, subtilisin 2μM with MG132 2μM, and subtilisin 2μM with Z-VAD(OMe)-fmk 1μM. The corresponding bar charts show mean ± s.e.mean (n=3) optical density (O.D.) for caspase-3 (full length 35kDa; cleaved 30kDa) and caspase-9 (full length 51kDa) expression.

A. blots and quantified protein expression in slices taken after 10min washing following superfusion of subtilisin.

B. Comparable blots and quantified levels in slices taken after 40min washing following superfusion of subtilisin.

*P< 0.05, **P<0.01, ***P< 0.001 ANOVA followed by Bonferroni post hoc test compared with control slices.

Figure 6. Effects of staurosporine and Z-VAD(OMe)-fmk on caspase activation.

Representative western blots are illustrated from control slices, slices treated with staurosporine 2μM for 1h followed by 8h recovery or a combination of staurosporine with Z-VAD(OMe)-fmk 100μM. **A.** Activation of caspase-9 (detected as the appearance of the 39kDa band shown) in response to staurosporine and the complete inhibition of activation in the presence of Z-VAD(OMe)-fmk. **B.** Lack of effect of staurosporine on the expression of four proteins sensitive to subtilisin.

The corresponding bar charts show mean ± s.e.mean (n=3) of the optical density (OD) of the proteins.

***P< 0.001 ANOVA followed by Bonferroni *post hoc* test compared with control slices.

Figure 7. Effects of MG132 on neuronal excitability and subtilisin-induced LTD.

A: sample fEPSPs at (increasing amplitude) 90, 100, 110, 120 and 130nA stimulating current, normalised to the amplitude obtained at 130nA above which slices exhibited indentations due to population spike generation. **B:** Comparable fEPSPs recorded after preincubation and superfusion with MG132. **C:** Graph showing mean ± s.e.mean values for control slices (filled circles) and slices preincubated and superfused with MG132 (open circles).

D: Sample fEPSPs recorded at the times indicated by the broken lines at ***a:*** before, ***b:*** 40min after and ***c:*** 80 min after the superfusion of subtilisin 1μM.

E: Comparable sample EPSPs in the presence of MG132.

F: Time course and magnitude of the LTD of field EPSPs produced by subtilisin-A superfused for 10 min as indicated by the bar, at concentrations of 1μM (filled circles), or

4μM (filled triangles) alone or in the presence of MG132 (open circles and open triangles respectively). The graph shows the mean ± s.e.mean (*n* = 4 at each concentration).

G: Sample fEPSPs at ***a:*** before, ***b:*** 40min after and ***c:*** 80min after the superfusion of subtilisin 4μM.

H: Comparable sample EPSPs in the presence of MG132.

Calibrations 1mV and 5ms.

Figure 8. Long-term potentiation.

A: Sample fEPSPs recorded (a) immediately before and (b) 45 mins after the induction of LTP using a theta-stimulation protocol. **B**: Comparable potentials recorded in the presence of MG132. C: Time course of the development of LTP. Data are shown as mean ± s.e.mean.

***P < 0.001 using ANOVA for the 5 consecutive time points immediately after stimulation and the final 5 points at 41-45min.

+P < 0.05, ++P < 0.01, +++P < 0.001 for individual pairs of data points within the 5 minute periods immediately after stimulation and at 41-45min.

Figure 9. Long-term potentiation.

A: Sample fEPSPs recorded (a) immediately before the first LTP stimulation (stim1) (b) immediately before the second stimulation (stim2) and (c) 30-35min after stim2. LTP was induced using a theta-stimulation protocol. **B**: Comparable potentials recorded in the presence of MG132. C: Time course of the development of LTP. Data are shown as mean ± s.e.mean. For control slices (filled circles) the second stimulation generates LTP at (bar 4) which is significantly greater than that seen after stim1(bar 3) (ANOVA P < 0.001, each point is significantly different at P < 0.001 using Bonferroni multiple comparison test). For

slices in MG132, (open circles) the second stimulation generates LTP at (bar 2) which is not significantly greater than that seen after stim1(bar 1) (none of the points is significantly different using Bonferroni multiple comparison test).

Figure 1

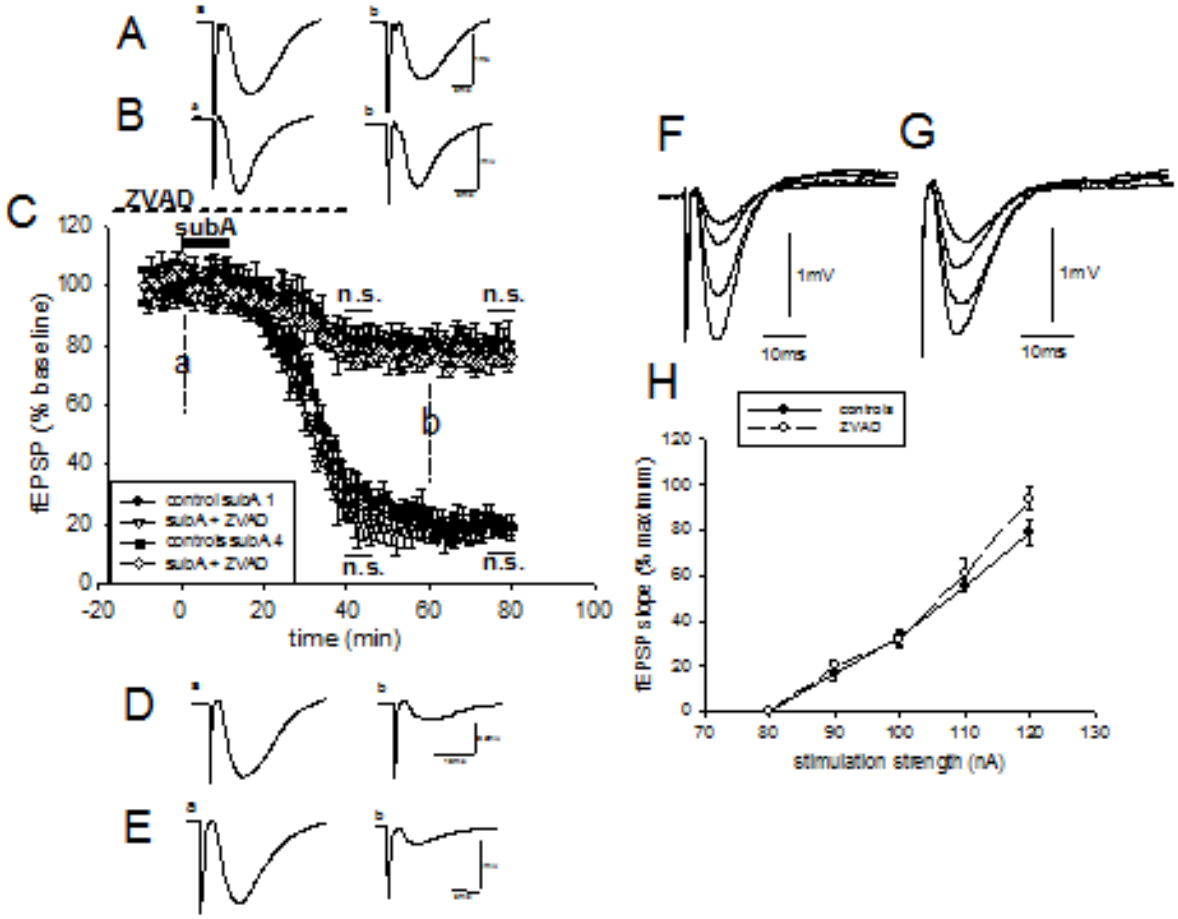

A
B
C
ZVAD
subA
n.s.
n.s.
a
b
n.s.
n.s.
fEPSP (% baseline)
time (min)
control subA 1
subA + ZVAD
control subA 4
subA + ZVAD
D
E
F
1mV
10ms
G
1mV
10ms
H
control
ZVAD
fEPSP slope (% maximum)
stimulation strength (nA)

Figure 2

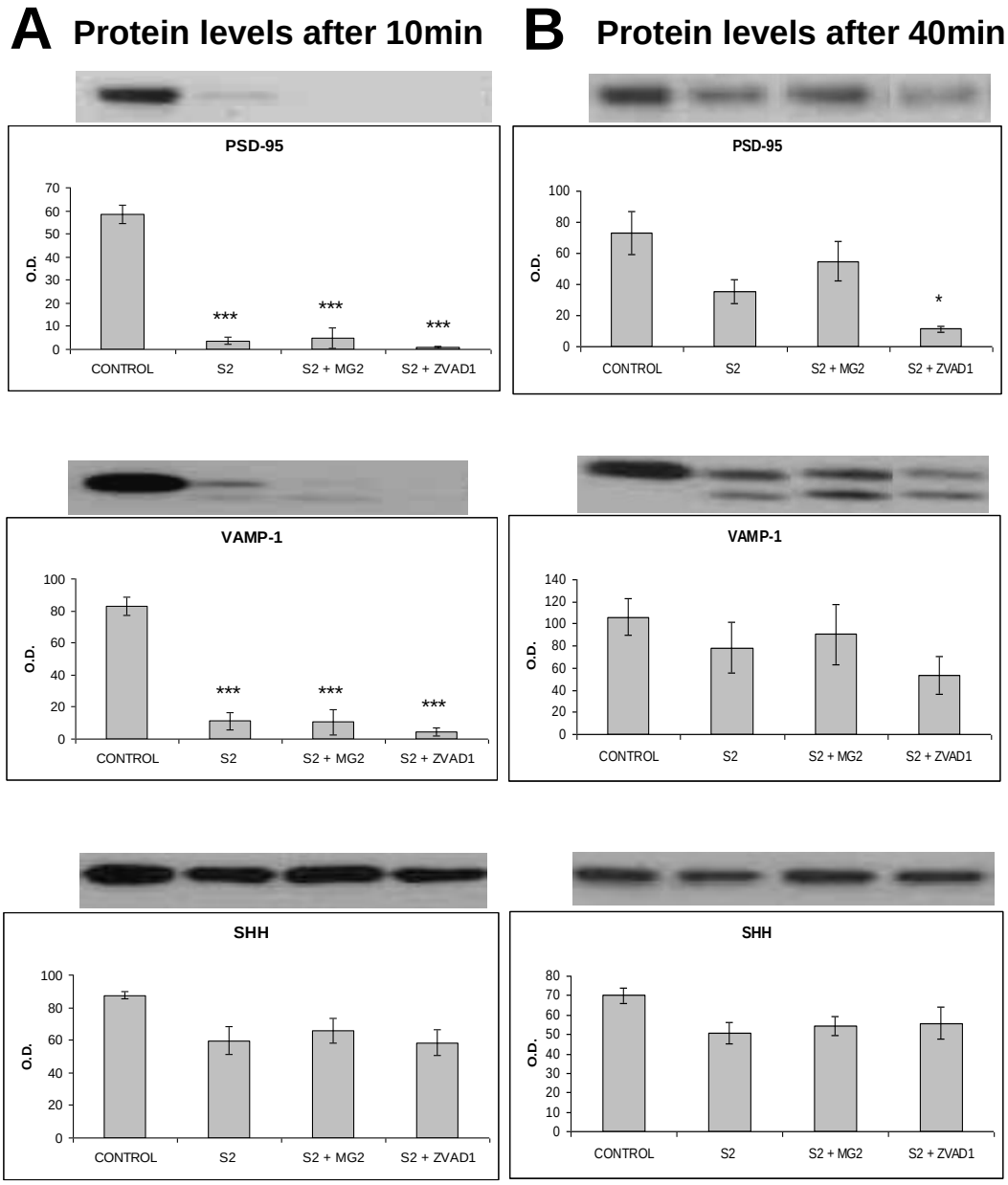

A Protein levels after 10min
B Protein levels after 40min
PSD-95
O.D.
CONTROL
S2
S2 + MG2
S2 + ZVAD1
***
*
VAMP-1
SHH

Figure 3

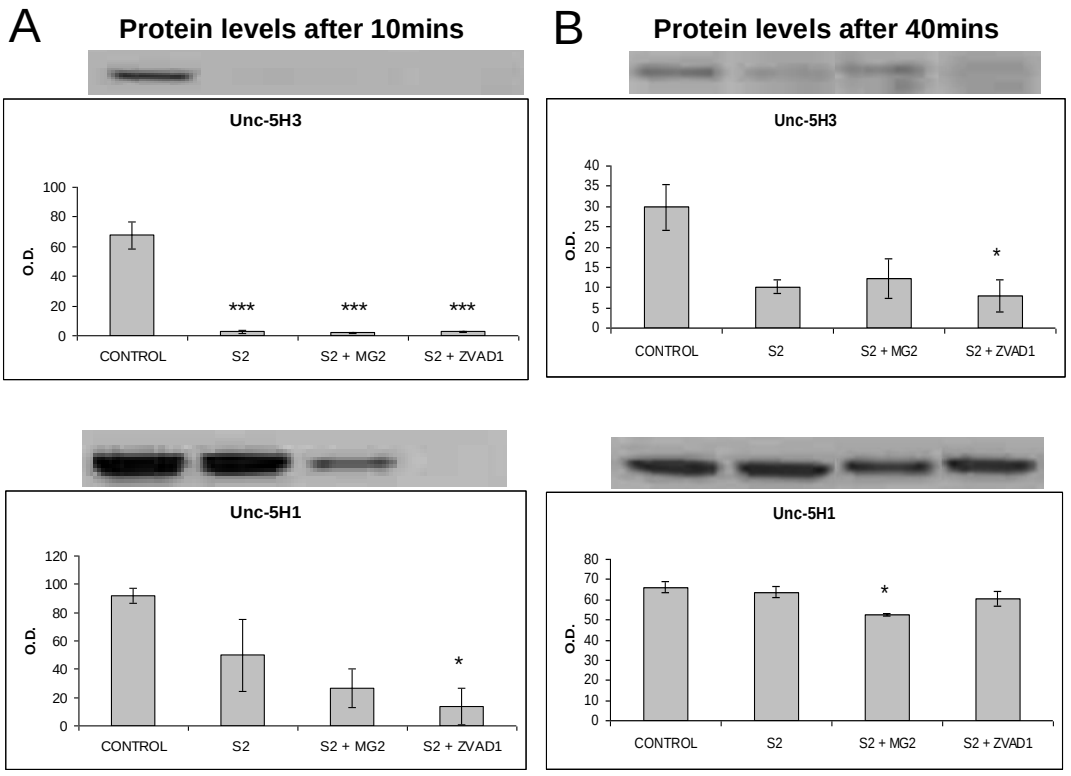

A
Protein levels after 10mins
Unc-5H3
O.D.
100
80
60
40
20
0
***
***
***
CONTROL
S2
S2 + MG2
S2 + ZVAD1
B
Protein levels after 40mins
Unc-5H3
O.D.
40
35
30
25
20
15
10
5
0
*
CONTROL
S2
S2 + MG2
S2 + ZVAD1
Unc-5H1
O.D.
120
100
80
60
40
20
0
*
CONTROL
S2
S2 + MG2
S2 + ZVAD1
Unc-5H1
O.D.
80
70
60
50
40
30
20
10
0
*
CONTROL
S2
S2 + MG2
S2 + ZVAD1

Figure 4

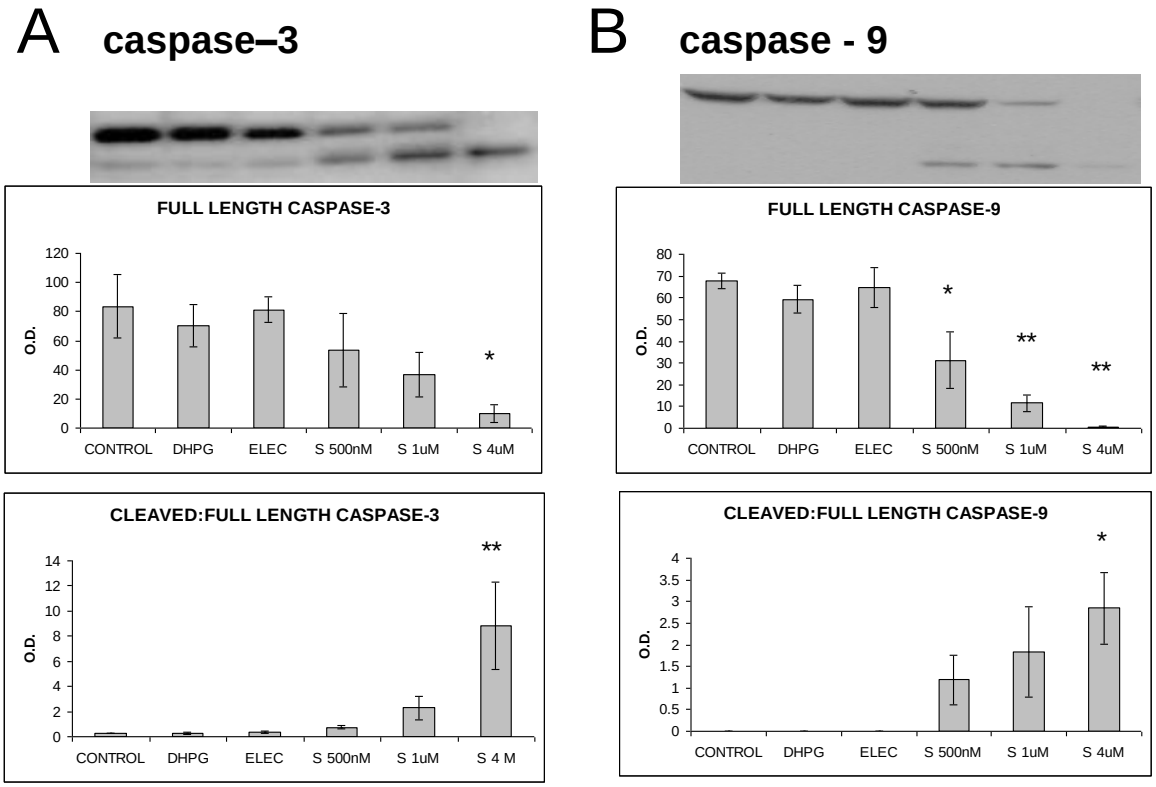

A caspase–3
B caspase - 9
FULL LENGTH CASPASE-3
FULL LENGTH CASPASE-9
O.D.
CONTROL
DHPG
ELEC
S 500nM
S 1uM
S 4uM
CLEAVED:FULL LENGTH CASPASE-3
CLEAVED:FULL LENGTH CASPASE-9
S 4 M

Figure 5

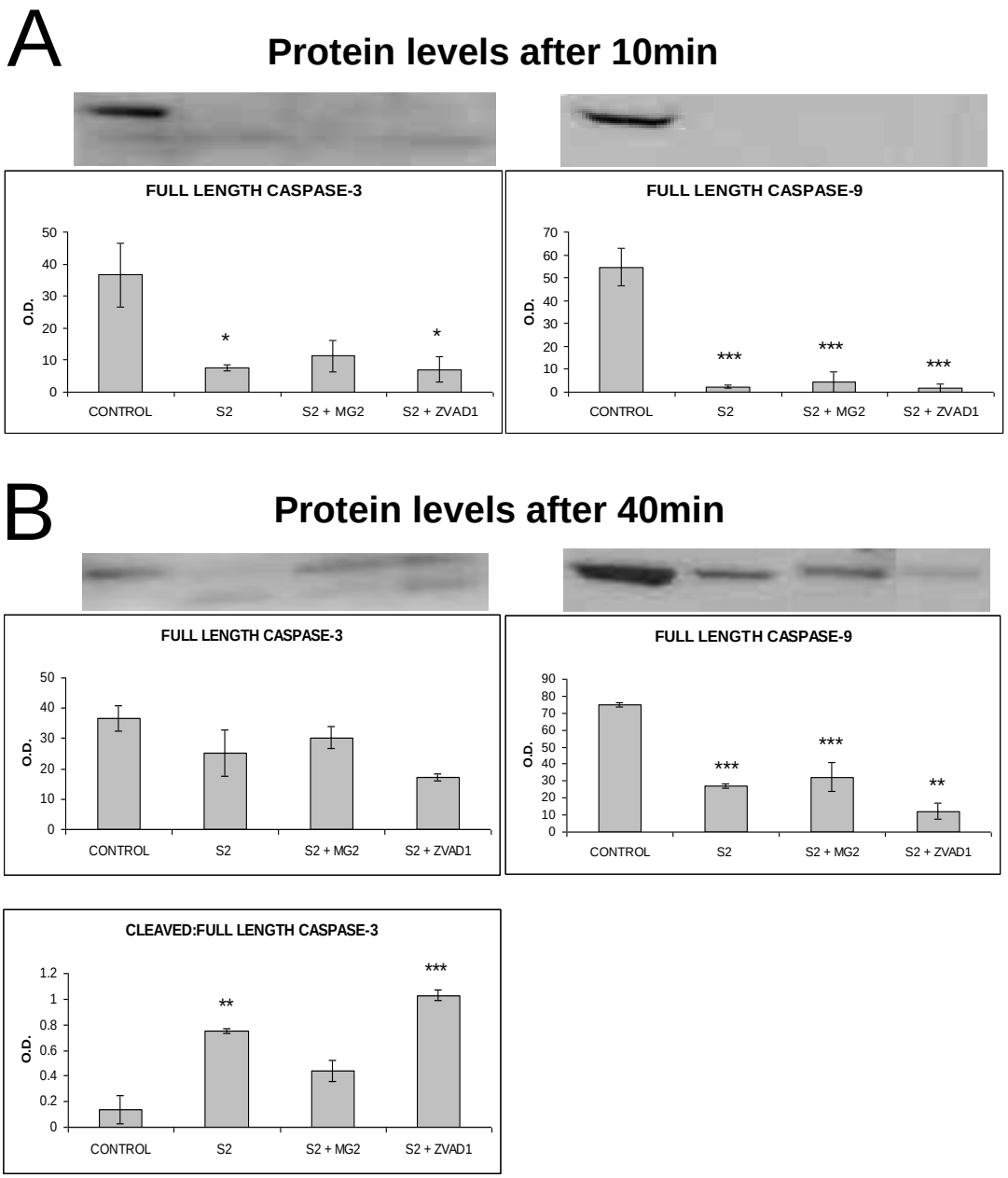

A
Protein levels after 10min
FULL LENGTH CASPASE-3
O.D.
CONTROL
S2
S2 + MG2
S2 + ZVAD1
FULL LENGTH CASPASE-9
B
Protein levels after 40min
FULL LENGTH CASPASE-3
FULL LENGTH CASPASE-9
CLEAVED:FULL LENGTH CASPASE-3

Figure 6

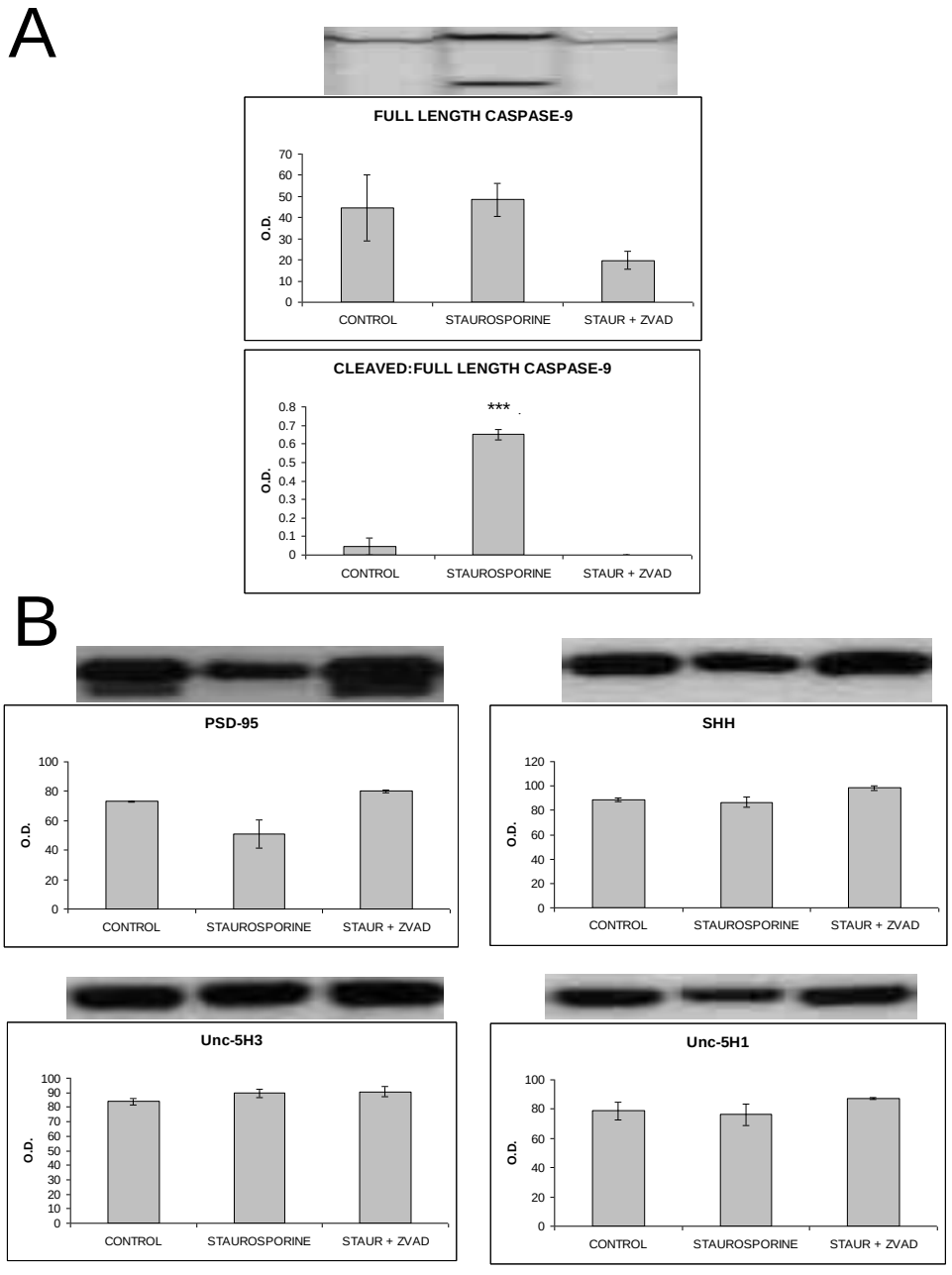

A
FULL LENGTH CASPASE-9
O.D.
CONTROL
STAUROSPORINE
STAUR + ZVAD
CLEAVED:FULL LENGTH CASPASE-9
***
B
PSD-95
SHH
Unc-5H3
Unc-5H1

Figure 7

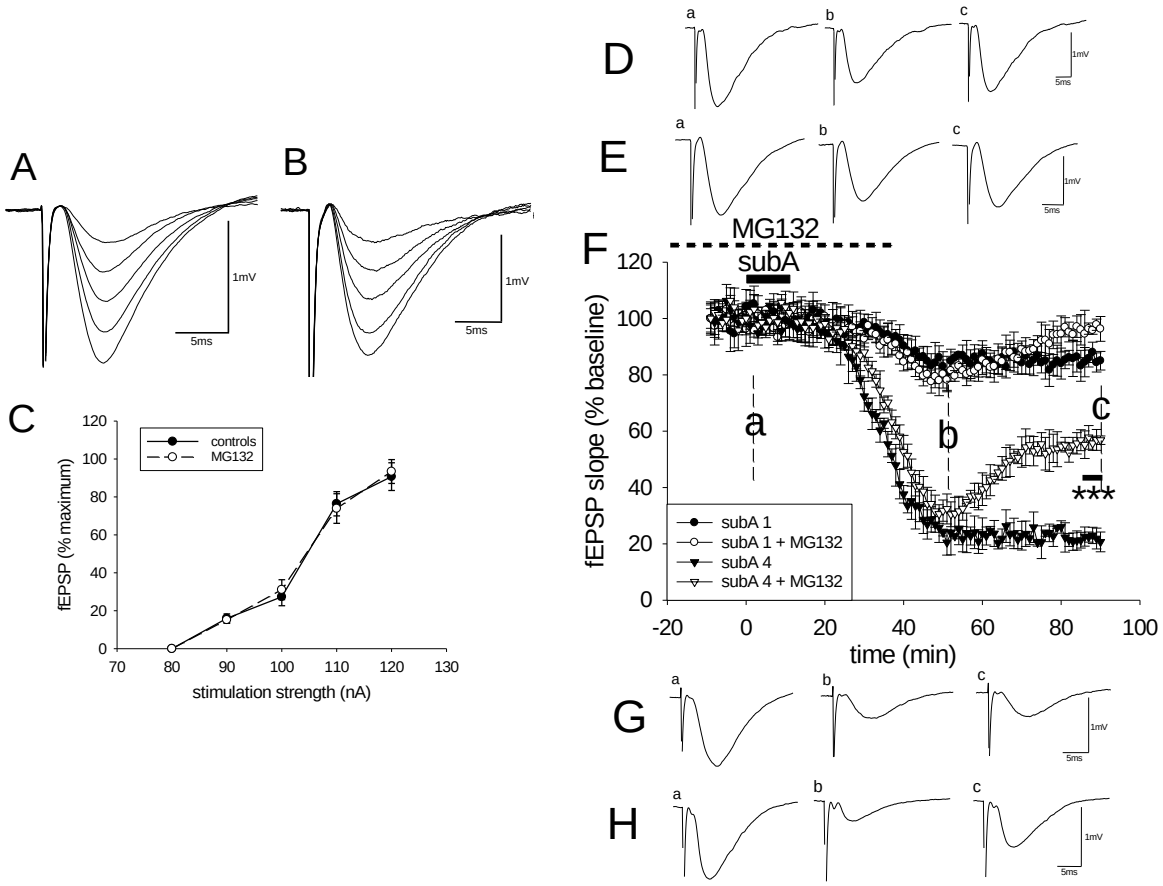

A
B
1mV
5ms
C
controls
MG132
fEPSP (% maximum)
stimulation strength (nA)
D
E
a
b
c
F
MG132
subA
fEPSP slope (% baseline)
time (min)
subA 1
subA 1 + MG132
subA 4
subA 4 + MG132
***
G
H

Figure 8

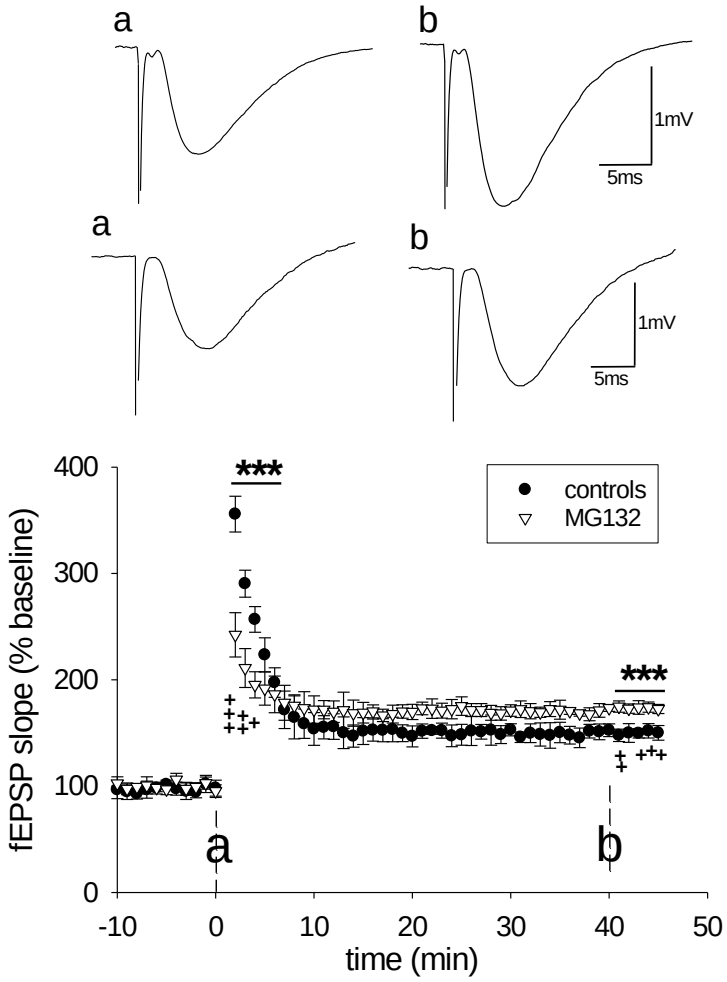
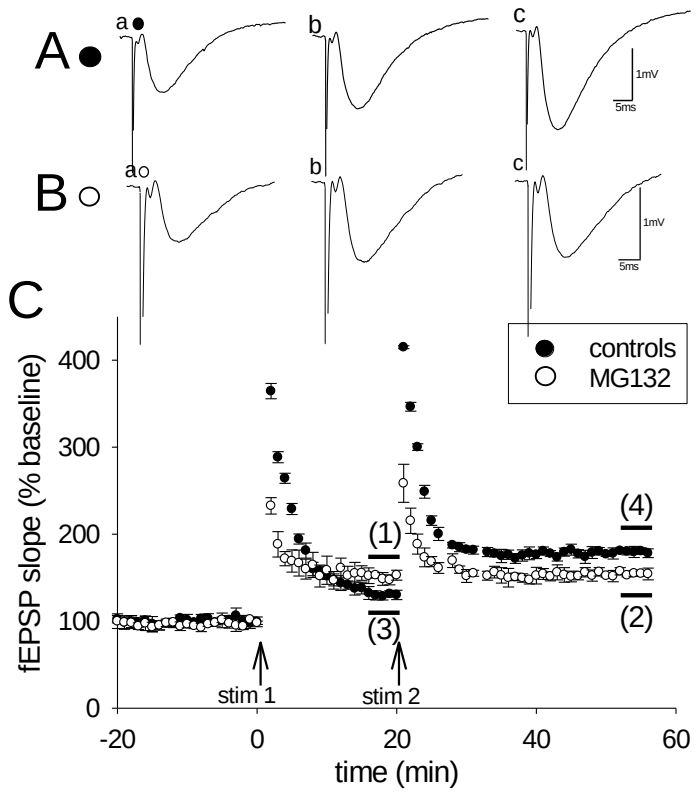

a
b
1mV
5ms
a
b
1mV
5ms
400
300
200
100
0
fEPSP slope (% baseline)
***
***
controls
MG132
a
b
-10
0
10
20
30
40
50
time (min)

Figure 9


A
a
b
c
1mV
5ms
B
a
b
c
1mV
5ms
C
controls
MG132
fEPSP slope (% baseline)
400
300
200
100
0
(1)
(2)
(3)
(4)
stim 1
stim 2
-20
0
20
40
60
time (min)