Glutamate-induced depression of E-S coupling in hippocampal CA1 neurons and modulation by adenosine receptors

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Abstract

The presence of high concentrations of glutamate in the extracellular fluid following brain trauma or ischaemia may contribute substantially to subsequent impairments of neuronal function. In this study, glutamate was applied to hippocampal slices for several minutes, producing over-depolarisation which was reflected in an initial loss of evoked population potential size in the CA1 region. Orthodromic population spikes recovered only partially over the following 60 minutes, whereas antidromic spikes and excitatory postsynaptic potentials (epsps) showed greater recovery, implying a change in epsp – spike coupling (E-S coupling) which was confirmed by intracellular recording from CA1 pyramidal cells. The recovery of epsps was enhanced further by dizocilpine suggesting that the long-lasting glutamate-induced change in E-S coupling involves NMDA receptors. This was supported by experiments showing that when isolated NMDA-receptor mediated epsps were studied in isolation, there was only partial recovery following glutamate, unlike the composite epsps. The recovery of orthodromic population spikes and NMDA receptor-mediated epsps following glutamate was enhanced by the adenosine A_1 receptor blocker DPCPX, the A2A receptor antagonist SCH58261, or adenosine deaminase, associated with a loss of restoration to normal of the glutamate-induced E-S depression. The results indicate that the long-lasting depression of neuronal excitability following recovery from glutamate is associated with a depression of E-S coupling, This effect is partly dependent on activation of NMDA receptors which modify adenosine release or the sensitivity of adenosine receptors. The results may have implications for the use of A1 and A2A receptor ligands as cognitive enhancers or neuroprotectants.

Key-words:

Glutamate; adenosine; purines; hippocampus; epsps; population spikes; E-S coupling;

Introduction

It is well established that the activation of adenosine A1 or A2A receptors has major influences on neuronal activity including the ability to modulate plastic phenomena such as long-term potentiation (LTP) and long-term depression (LTD) (Costenla et al., 1999; de Mendonca & Ribeiro, 2001; Fontinha et al., 2009; Kukley et al. 2005; Moore et al. 2003) In addition, both A1 and A2A receptors are known to influence neuronal recovery following injurious stimuli or events such as ischaemia or CNS trauma (von Lubitz et al., 1996; Jones et al., 1998; Kitagawa et al., 2002; Pugliese et al., 2003; Stone et al., 2009), the subsequent neurodegeneration in these cases being considered attributable to locally elevated concentrations of glutamate or a related agonist such as quinolinic acid (Benveniste et al., 1984; Baratte et al., 1998; Nishizawa, 2001; Szatkowski and Attwell, 1994; Stone, 2001). Many attempts have been made to devise electrophysiological techniques in acute slice preparations which might represent an appropriate model for such events (Schurr et al., 2001; Latini et al., 1999a,b; Pugliese et al. 2003), facilitating an understanding of their electrophysiological and molecular basis.

Here we describe the effects on synaptic transmission of a relatively intense exposure to glutamate (5mM applied for 10min) which generates a depression of evoked field potentials lasting at least 60 min. We have examined the associated electrophysiological changes in this situation and then assessed the effects of A1 and A2A receptor blockade on those changes. The results suggest that the procedures used might form the basis for further investigation into the basis of interactions between glutamate and adenosine receptor function in relation to long-term changes of neuronal function. In particular, the results suggest that adenosine receptors are able to modulate long-lasting alterations of synaptic transmission and neuronal excitability caused by glutamate, supporting the view that adenosine receptors have therapeutic potential as targets for preventing glutamate-induced dysfunction.

Methods

Ethical information

All procedures performed in this work were in accordance with the regulations and recommendations of the Animals (Scientific Procedures) Act, 1986 of the United Kingdom Home Office.

Hippocampal slices were prepared as described previously (Ferguson and Stone, 2008). Briefly, male Wistar rats (100-150g) were killed by an overdose of urethane (1ml/100g body weight, administered i.p. as a 25% solution) and 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 removed and sliced into 450μm transverse slices using a McIlwain tissue chopper. Slices were selected from approximately the middle one third of each hippocampus, a restriction which provided approximately 6 slices from each. 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. For extracellular recording, slices were superfused at 28-30°C with aCSF at a flow rate of 3-4 ml/min. A concentric bipolar electrode (Harvard Apparatus, Edenbridge, UK) was used for stimulation in stratum radiatum, using stimuli delivered at 0.1 Hz with a pulse width of 50-300μs. The stimulating electrode tip was located immediately internal to the stratum pyramidale at the border between the CA1 and CA2 regions. For antidromically-evoked potentials, the stimulating electrode was placed in contact with the alvear white matter containing the axons of the pyramidal neurons. Recording electrodes were constructed from fibre-containing borosilicate glass capillary tubing (Harvard Apparatus, Edenbridge,

Kent, UK), with the tips broken back to 2-4μM, DC resistance approximately 5MΩ. Electrodes were filled with a solution of 1M NaCl. The recording electrode tip was placed either in the stratum pyramidale (for population spike recordings) or internal to the CA1 pyramidal cell layer at the point of maximum epsp amplitude. The signal was amplified and captured on a micro1401 interface (CED, Cambridge Electronic Design, Cambridge, UK) for storage on computer and subsequent analysis.

Intracellular recordings were made as described previously (Stone, 2007), using sharp electrodes pulled from borosilicate fibre-containing glass tubing (Clark Electromedical, Reading, UK, and Harvard Apparatus, Edenbridge, UK) using a vertical Narashige puller. The electrodes were filled with 1M potassium acetate, and had tip resistances of 90-120 MΩ. Potentials were amplified via an Axoclamp-2 system (Axon Instruments, Molecular Devices, New Orleans, USA)) or a Neurolog NL102 DC amplifier (Digitimer Ltd., Welwyn Garden City, UK) in bridge balance mode, with a filter bandwidth between DC and 500Hz to reduce higher frequency components of epsp recordings. A modification of the amplifier allowed 1-10 ms periods of increased capacitance compensation (buzz) to be applied remotely to facilitate penetration. Following penetration, cells were allowed to reach a maximum resting potential and then left for 30 minutes before the addition of compounds. Cells were only used if they exhibited stable resting potentials of at least –60mV. Neuronal excitability was tested by applying intracellular depolarising current pulses between 0.05 and 1.0nA amplitude for 300, 500 or 800ms. Input resistance was monitored using 0.05 to 1.0nA hyperpolarizing pulses lasting 300 or 500ms. Responses were digitised at 20kHz via a CED (Cambridge Electronic Design, Cambridge, UK) micro1401 interface and stored on computer for later analysis using the Signal programme. Stable cells were maintained for between 1 and 6 hours after penetration. Only one cell was studied in each slice, to preclude the possibility that drug superfusion might alter the responsiveness of subsequent neurons

in the same slice. Stimulation and recording electrode locations were as for the extracellular recordings noted above.

Sources

L-glutamate, N-methyl-D-aspartate (NMDA), adenosine and adenosine deaminase (type VII) were obtained from Sigma-Aldrich Chemicals (Poole, Dorset, UK), while dizocilpine, 6,7 – dinitroquinoxaline-2,3-dione (DNQX),1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5-c]-pyrimidine (SCH58261) were obtained from Tocris Bioscience (Bristol, UK).

Measurements and statistics

The amplitude of population spikes was taken as the potential difference between the negative and positive peak. The negative slope of the epsp was taken as the gradient of the line of best fit of all sampled points between approximately 25% and 75% after the start of the negative slope. The epsp amplitude was taken as the potential difference between the negative peak and the return to baseline.

Data are presented as mean ± standard error. Baseline values were obtained from a stable 10min period of evoked potential size prior to the addition of any drugs, and which was then taken as 100%. Linear and non-linear regression analyses were performed using GraphPad Instat and Prism software.

For statistical comparisons between groups, unpaired two-tailed *t*-tests were used except where two or more data sets were compared with a common control, in which case a one-way analysis of variance (ANOVA) was normally performed, followed by Dunnett's multiple comparisons test or Bonferroni's multiple comparison test when sample groups were of different sizes.

Results

Glutamate and orthodromic population spikes

In preliminary investigations, glutamate was applied at various concentrations to assess the effects upon the orthodromic population spikes evoked from CA1 neurons. While micromolar concentrations could induce a partial and fully reversible loss of response size, higher millimolar concentrations produced a complete loss of the potential with only partial recovery. A concentration of 5mM was selected for routine use as it produced an initial over-depolarisation leading to a complete loss of response which persisted for 10-20min following glutamate perfusion. The response then partially recovered until it reached a stable plateau. A higher concentration of 10mM produced similar effects but with a slower time course and a poorer and more variable plateau recovery (Fig. 1Aa, b).

Antidromic population spikes

In contrast, when applications of 5mM glutamate were examined on antidromically-evoked population spikes, the potentials were seen to decrease only partially in response to 5mM glutamate, but were never suppressed completely. Another distinction from orthodromic spikes was that, on removing the glutamate, antidromic spikes recovered to a level which was not significantly different from the initial baseline (90.3% $\pm$ 8.5, $n = 4$, $P = 0.34$, one sample t-test) and with no long-lasting decrease in potential size (Fig. 1Ba,b).

Composite field epsps

As in the case of orthodromic population spikes, 5mM glutamate perfusion for 10min resulted in a complete loss of the field epsps. This again persisted for approximately 10-20min after glutamate was removed. The epsps then recovered but, unlike orthodromic spikes, they recovered to the initial pre-glutamate levels and did not normally show pronounced long-lasting changes in slope or amplitude compared with pre-glutamate treatment (Fig. 1Ca,b). Taken together with the partial reversibility of population spikes, these results suggest a reduction of epsp-spike coupling (E-S coupling).

NMDA receptor involvement

The use-dependent NMDA channel blocker, dizocilpine (10μM) was perfused prior to, during and after 5mM glutamate exposure. The overdepolarisation produced by glutamate still decreased the epsp size to zero for approximately 10-20min post-glutamate, as in the experiments described above (Fig. 2Aa,b). However, in slices superfused with dizocilpine, post-glutamate recovery significantly exceeded not only the post-glutamate recoveries of control responses without the antagonist but also exceeded even the initial baseline size with respect to both epsp slope and amplitude (for slope, $P = 0.011$ relative to treatment with glutamate alone, $P = 0.004$ relative to baseline; for amplitude, $P = 0.0003$ relative to treatment with glutamate alone; $P = 0.002$ relative to baseline).

A five minute perfusion of 25μM NMDA produced an initial loss of the orthodromic population spike. After a washout period of several minutes it was observed that, as with glutamate superfusion, the population spike returned to a plateau level that was lower than the pre-NMDA control potential size (Fig. 2Ba,b).

Isolated NMDA receptor-mediated epsps

In order to probe further the involvement of NMDA receptors in the long-lasting effects of glutamate, NMDA receptor-mediated epsps were isolated by superfusing slices with magnesium-free aCSF containing 20μM DNQX. Superfusion with glutamate at 5mM then induced a change in epsp slope and amplitude similar to that observed in the examination of composite epsps, with a period of overdepolarisation and complete loss of the epsp but followed by only a partial recovery to around 50% of the baseline (Fig. 2Ca,b).

From these results, it was hypothesized that the use of 5mM glutamate may induce an NMDA receptor-mediated dissociation between the field epsp and the orthodromic population spike which could be further investigated with E-S coupling experiments.

Intracellular experiments.

To obtain precise information on the E-S coupling in the CA1 region, individual neurons were examined using intracellular recordings rather than the more traditional, but cruder, analysis of population field potentials. The intracellular correlate of extracellular E-S coupling is the probability of action potential initiation in individual cells in response to constant epsp inputs, this probability in turn being associated with changes in firing threshold (Andersen et al., 1980; Pugliese et al., 1994; Jester et al., 1995). A total of 11 neurons were examined in which the probability of epsp-induced spike initiation was measured under control conditions and 50min after the superfusion of the slice with glutamate. The number of successful epsp-induced spikes was recorded at 5μA stimulus intervals and these were plotted to allow interpolation of the stimulus current able to generate a theoretical spike success of 50%. Responses from a typical neuron are illustrated in Fig. 3A, together with a summary graph which shows the mean ± s.e.m. spike failure rates at different stimulus intensities in the control period

and during recovery 50min after glutamate superfusion (Fig. 3B). From plots of the stimulus – probability relationship for individual neurons, the stimulus strength required to elicit a failure rate of 50% spike initiation was increased from 76.1 μA ± 1.64, to 80.5 μA ± 1.44 (n = 11, P = 0.019, paired t test) (Fig. 3B). In addition, the membrane potential threshold for the action potential, measured as the point of discontinuity between the epsp and the spike potential, was increased by an average of 2.48 mV ± 0.25 (n = 11, P < 0.001; one-sample t test).

Adenosine A_1 receptors

Since adenosine is known to be present in the extracellular space of tissue slices and its release can be increased by the activation of glutamate receptors (Manzoni et al., 1994; Hoehn & White, 1990), it was hypothesised that it could contribute to the long-lasting effects of glutamate on the evoked potentials. The adenosine antagonist DPCPX, at an A_1-receptor-selective concentration of 30nM, was perfused for 10min prior to and during the application of 5mM glutamate. The blockade of A_1 receptors did not produce any change in the immediate response to glutamate, but it did result in a significant improvement in recovery of the orthodromic population spike amplitude compared with controls, with a larger potential size recorded at the 60min recovery plateau following glutamate with DPCPX (n = 11) compared with glutamate alone (64.5% ± 3.1; n = 9; P = 0.01)(Fig. 4A,B). DMSO, the solvent used to dissolve DPCPX was tested at its final superfusion concentration of 0.05%, as it is a free radical scavenger known to alter electrophysiological responses in hippocampal slices (Hülsmann et al., 1999; Greiner et al., 2000). However, DMSO did not significantly affect the post-glutamate recovery of orthodromic population spikes (76.0% ± 8.9, n = 5) compared to those treated with glutamate alone.

The same experimental protocol was then tested using antidromic population spikes and field epsps. When 30nM DPCPX was perfused 10min prior to and during 5mM glutamate, there was no difference in the observed changes in antidromic spike amplitude (Fig. 4C) or composite field epsps (Fig. 4D). As the recovery for the orthodromic field epsps may have been influenced by differences in the epsp response sizes between groups (see section on relationships below), *t*-tests were performed which revealed no significant differences in this parameter (epsp slope: glutamate: -0.56 ± 0.15mV/ms, glutamate + DPCPX: -0.92 ± 0.29mV/ms, $P = 0.25$; epsp amplitude: glutamate 1.3 ± 1.0mV, glutamate + DPCPX: 1.8 ± 0.5mV, $P = 0.25$).

Since the effect of A_1 receptor blockade upon the response to glutamate of orthodromic population spikes was not observed using antidromic population spikes or field epsps, the results imply that A1 receptor blockade prevents the NMDA receptor-induced depression of E-S coupling. Therefore, the experiments were repeated using NMDA receptor-mediated epsps isolated by superfusing the slices with DNQX in nominally magnesium-free aCSF. DPCPX was perfused for 10min prior to and during 5mM glutamate as before. Control responses that were over 0.8mV in amplitude were selected to match the range of orthodromic composite epsp sizes used above. It was noted that DPCPX significantly enhanced the recovery from glutamate of NMDA receptor-mediated epsps (82.0% ± 7.7, $n = 6$) compared with the use of glutamate alone (53.3% ± 9.7, $n = 7$; $P = 0.045$, unpaired *t* test), supporting the possibility that the effects of A_1 receptor blockade upon the response to glutamate of orthodromic spikes may involve the modulation of NMDA receptors (Fig. 4E,F).

Intracellular measurements of neuronal firing probability, as outlined above, were made in slices subjected to the DPCPX and glutamate perfusion protocol. A total of 8 neurons were tested, in which the stimulus strength required to elicit a failure rate of 50% spike initiation was unchanged between baseline conditions and measurements

made 50 min after DPCPX and glutamate perfusion (baseline 74.33 μA ± 1.5; after glutamate and DPCPX 75.45 μA ± 1.48 (*P* = 0.16, *n* = 8, paired *t* test) (Fig. 5). The difference in action potential thresholds between the control and test conditions was 0.72 mV ± 0.45, which was also not statistically significantly different (*P* = 0.15, one-sample *t* test, *n* = 8), indicating that DPCPX prevented the alterations of E-S coupling produced by glutamate application.

Adenosine A_{2A} receptors

Since the effects of DPCPX pointed to a role for endogenous adenosine in the degree of recovery after glutamate, it was felt appropriate to assess whether there might also be any influence on recovery of A_{2A} receptor activation, especially since A_{2A} receptors are known to facilitate glutamate release and could therefore increase the degree of glutamate receptor activation. The A_{2A} receptor antagonist, SCH 58261 (1μM), was used in a similar manner to DPCPX. SCH 58261 was perfused prior to, during and after 5mM glutamate during the recording of orthodromic population spikes. Following perfusion with SCH58261, there was a significantly greater recovery of orthodromic spike amplitude after the glutamate application, with the potentials recovering to 101.7% ± 8.5 of the initial baseline after SCH58261 (*n* = 9) compared with recovery to only 71.7% ± 9.3 of the baseline in slices not treated with SCH58261 (*n* = 7, *P* = 0.033, unpaired t test). Interestingly, it was also observed that perfusion of SCH 58261 alone for 60 min resulted in a significant depression of the orthodromic population spike amplitude (85.0% ± 2.2; *n* = 5; *P* = 0.003 one sample *t* test).

Adenosine deaminase

The use of antagonist compounds is inevitably complicated by questions of selectivity, especially when differences between pre- and post-synaptic receptors for the same agonist are under discussion. Therefore, it was decided to repeat the same experimental protocol as above but using adenosine deaminase to metabolise endogenous adenosine and thus remove its activation of receptors. Adenosine deaminase (ADA, 0.2U/ml) was superfused from 10 minutes prior to glutamate application until 60 minutes after ending the application. There was no change in the size of the population spikes during perfusion with ADA in the control period (mean potential size at baseline 100.25% $\pm$ 1.73; size in ADA 100.5% $\pm$ 1.78, n = 8, n.s.). However, there was a significantly greater degree of recovery following the glutamate, control slices recovering to 68.4% $\pm$ 4.07 of the baseline potential size, while those treated with ADA showed recovery to 80.1% $\pm$ 3.33 of baseline (n = 8, P = 0.042).

Relationships between neuronal recovery and initial response size

During the course of this work, a number of observations were made which indicated a relationship between the initial evoked potential size and the degree of recovery observed following glutamate. These relationships suggest a differential role of NMDA and non-NMDA receptor-mediated components in the recovery which would be entirely consistent with the main body of results reported here but which, to our knowledge, have not been reported before.

The extent of post-glutamate recovery of the orthodromic population spike was dependent on the initial spike amplitude. Smaller initial spikes were associated with a lesser recovery after glutamate applications. This was supported by scatter plots of the post-glutamate recovery against the initial population spike size, which revealed significant positive correlations between these parameters using both 5mM and 10mM glutamate (Fig. 1A,c). In subsequent experiments, the average size of population spike

amplitudes between treatment groups were compared to ensure that the outcomes were not influenced by this covariate (see section on *Adenosine A1 Receptors*, above). In contrast, there was no correlation between initial antidromic spike size and percentage recovery following glutamate (n = 4).

Recovery of epsps in different slices could occur to less than, or more than, the initial potential size. This was reflected in scatter-plots of epsp slope or amplitude and percentage recovery of either parameter which suggested that smaller initial epsps generally recovered to a greater extent than larger epsps. This relationship was opposite to that seen for the population spikes and was non-linear. The data were fitted to a one phase exponential decay curve (slope R^2 = 0.74, amplitude R^2 = 0.57) (Fig. 1Cc,d).

NMDA receptors

Davies & Collingridge (1989) have previously reported that smaller epsp amplitudes were associated with a smaller NMDA receptor-mediated contribution: small epsps were insensitive to NMDA receptor antagonists until stimulation was increased sufficiently to obtain a slowly-emerging NMDA receptor component. Since, in the present work, smaller epsps showed a tendency to recover better than large epsps following glutamate treatment, it was hypothesized that this might be due to a smaller NMDA receptor-mediated component of the smaller epsp. The effects of glutamate upon field epsps were therefore studied further by testing the effects of glutamate in the presence of dizocilpine, and by examining isolated NMDA receptor-mediated epsps.

Scatter-plots were created of the range of initial epsp size versus the percentage recoveries of epsp slope and epsp amplitude following glutamate perfusion. In those experiments involving dizocilpine and glutamate, the recovery of field epsp amplitude depended significantly upon the initial epsp slope and amplitude (R^2 = 0.83, P < 0.001)

whereas no such significant relationship existed in the absence of dizocilpine. These results suggested that it is the non-NMDA sensitive component of the epsp which determines the degree of recovery following glutamate. The extent of recovery of population spikes after slice superfusion with NMDA was also affected by the initial spike amplitude, as it had been after glutamate application. However, in contrast to the effect of glutamate, a contrary, negative relationship was obtained, in which smaller initial population spikes were significantly correlated with greater post-NMDA recoveries whilst larger potentials had smaller recoveries (Fig. 2B, c).

Discussion

The effects of glutamate observed here are broadly consistent with those reported in the literature with a complete loss of evoked responses upon application of millimolar concentrations of glutamate for several minutes followed by return to a reduced potential size during washout (Wallis et al., 1994; Alici et al., 1996). Others have noted a varying degree and time course of recovery, depending on factors such as calcium levels (Alici et al. 1996; Limbrick et al., 2003). Coulter et al. (1992) and Limbrick et al. (2003) have observed what is probably a related phenomenon, of prolonged changes in membrane potential in hippocampal neuronal cultures using 500μM glutamate in the presence of 10μM glycine, which they termed “extended neuronal depolarization”.

The facts that orthodromic spikes behave in a manner opposite to antidromic spikes and epsps, and that, overall, the latter potentials recover to a much greater extent than orthodromic spikes, suggest that a major determinant of recovery lies in events at the level of the synapse. The most likely event would be an action of glutamate receptors on E-S coupling. This possibility has been confirmed by our intracellular recordings

which show that the post-glutamate recovery period is associated with a significant decrease in neuronal firing probability, often (but not always) associated with an increased action potential threshold in response to synaptic stimulation. Indeed, decreased E-S coupling has previously been described after electrical stimulation leading to LTD (Daoudal et al., 2002), consistent with the possibility that common mechanisms underlie both electrical LTD and the long-lasting depression described here. The converse relationship, with enhanced E-S coupling after high-frequency stimulation or tetra-ethylammonium, has also been described (Bernard and Wheal, 1995; Bernard et al., 1998; Taube and Schwartzkroin, 1988).

Whereas long-term potentiation (LTP) is known to involve a degree of epsp-spike (E-S) potentiation, the role of E-S coupling in LTD, has received less experimental attention. Increased E-S potentiation implies an increased efficiency of transmission resulting in larger population spikes being evoked by a constant epsp amplitude (Andersen et al., 1980; Jester et al. 1995). There are a few studies describing stimulation-induced or high K+-induced E-S depression but few have considered the effects of glutamate itself.

Adenosine

The possible role of adenosine receptors in the phenomena described here was examined as it is known that adenosine is released at high concentrations during ischaemia (Latini et al., 1999b) and is a known preconditioning agent (Liu et al., 1991; Heurteaux et al., 1995; Perez-Pinzon et al., 1996; Kitagawa et al., 2002; Ferguson & Stone 2008). It was found that blockade of adenosine A_1 receptors increased post-glutamate recovery, an observation that would be consistent with previous evidence that glutamate or NMDA can induce adenosine release (Hoehn & White, 1990; Pedata et al.,

1991; Manzoni et al., 1994). There is ample evidence from several groups studying evoked potential size or the levels of adenosine present in the bathing medium that adenosine release from slices in response to glutamate or other depolarising stimuli can reach concentrations sufficient to inhibit spike potential size or recovery (Manzoni et al., 1994; Brunedge and Dunwiddie, 1996; Cunha et al., 1996; 1998; Sebastiao et al., 2001; Frenguelli et al., 2003, 2007; Wall and Dale, 2008)

However, adenosine release could not account on its own for the relatively poor recovery of orthodromic spikes compared with the full recovery of antidromic spikes and epsps. In addition, the adenosine release induced by glutamate or NMDA has been shown to be relatively brief : release was normalised within minutes of the washout period, with no lasting increase which might readily account for the present results (Hoehn & White, 1990; Manzoni et al., 1994). The fact that adenosine deaminase improved recovery in a similar fashion to DPCPX supports the view that the presence of adenosine – at some stage in the experiment – is largely responsible for the phenomena described here, but there must be an additional mechanism to account for the different responses of population spikes and epsps.

One such possibility to account for the delayed modulatory effects on recovery is that a change occurs in the number, distribution or sensitivity of adenosine receptors in response to high concentrations of glutamate, especially involving receptors at synaptic regions where, as noted above, the results would be consistent with a change of E-S coupling (O'Kane & Stone, 1998, 2004). In particular, the results imply that there may be an increased sensitivity of postsynaptic adenosine receptors associated with E-S coupling, but not presynaptic adenosine receptors regulating transmitter release and epsp generation. This is a particularly important distinction to make since the presynaptic A1 receptors have been regarded as high affinity sites (Vizi, 2000) which

could conceivably respond to very low residual concentrations of adenosine persisting beyond the usual detection limits.

The present work does not provide any information on the origin of the adenosine released by glutamate, whether as a direct result of cellular depolarisation or caused by the increased cellular metabolism which is induced by prolonged depolarisation. Certainly under conditions such as ischaemia, in which endogenous glutamate release is thought to play a significant role in the subsequent cell dysfunction, adenosine release can arise either from neurones or from astrocytes. The latter can release large quantities of adenosine directly or indirectly (from the metabolism of ATP) under such conditions (Sperlagh and Vizi, 1996; Cunha, 2008),

The efficacy of DPCPX may involve interactions between A_1 receptors and NMDA receptors in the hippocampus (de Mendonça & Ribeiro, 1993; Schubert and Kreutzberg, 1993; Canhão et al., 1994; de Mendonça et al., 1995; Klishin et al., 1995; Nikbakht & Stone 2001). The effects of glutamate and A_1 receptor blockade were therefore examined on NMDA receptor-mediated epsps. When DPCPX was applied in the presence of glutamate during the recordings of pharmacologically isolated NMDA receptor-mediated epsps, post-glutamate recovery was increased. Previous work on NMDA receptor currents in the hippocampus has shown that A_1 receptor antagonism enhances responses to NMDA receptors. In particular, DPCPX both facilitates the NMDA receptor-mediated component of epsps in the rat hippocampal slice (Canhão et al., 1994) and 8-cyclopentyl-theophylline (8-CPT) increases the NMDA receptor component of hippocampal synaptic currents in hippocampal neurons (Klishin et al., 1995). Conversely the agonist, 2-chloroadenosine, suppresses NMDA receptor-mediated epsps in slices (de Mendonça & Ribeiro, 1993) and NMDA-induced currents in isolated hippocampal neurons (de Mendonça et al., 1995). Thus, the increase in post-

glutamate recovery of orthodromic population spikes and NMDA receptor-mediated epsps when DPCPX is present may be due to its removal of A_1 receptor inhibition of NMDA receptors.

The A1 adenosine receptors do not seem to be involved in the glutamate-induced depression of antidromic potentials, since DPCPX did not prevent the effect. Since it has recently been noted that agonists at group I metabotropic glutamate receptors are able to produce a prominent depression of antidromic spikes (Clement et al., 2009), an effect that might be related to the ability of those agonists to inhibit sodium currents in CA1 pyramidal neurons (Carlier et al., 2006), it is possible that a similar effect is responsible for the depression seen here.

The A_{2A} adenosine receptors enjoy a rather paradoxical pharmacology. Their activation tends to facilitate activity in neuronal networks, by a combination of pre- and post-synaptic actions on excitability and transmitter release (Ribeiro, 1999; Lopes et al., 2002), reflected in the enhancement of hippocampal epsps (Cunha et al., 1994) and population spikes (Sebastião & Ribeiro, 1992) observed upon exposure to 2-[4-(2-carboxyethyl)-phenylethylamino]-5'N-ethyl-carboxamido-adenosine (CGS21680), an A_{2A} receptor agonist. This activity probably accounts for the significant depression induced by SCH 58261 alone.

However, activation of A_{2A} receptors can inhibit NMDA receptor-mediated depolarisation (Wirkner et al., 2000, 2004) and can regulate synaptic plasticity differentially on population spikes and epsps. The induction of LTP and depotentiation of epsps are increased and decreased respectively when A2 receptors are activated, whereas these forms of plasticity in population spikes are unaffected by A2 receptors (Sekino et al., 1991; Fujii et al., 1992, 1999; Forghani & Krnjević, 1995). Antagonists at A_{2A} receptors are also neuroprotective, reducing cell death in response to a range of insults including glutamate, NMDA, quinolinic acid, oxidative stressors and the selective

dopaminergic toxin MPP+ (Lau & Mouradian, 1993; Jones et al., 1998; Connick & Stone, 1989, Behan & Stone, 2002; von Lubitz et al., 1996). Indeed, both agonists and antagonists seem able to protect neurones (Macgregor & Stone, 1994; Jones et al., 1998; see Stone et al., 2009).

Response sizes and post-glutamate/NMDA recovery

The relationship between initial potential size and recovery appears to be a novel observation, although a fuller explanation of the phenomenon will require a separate study to explore changes of pre-synaptic and post-synaptic function, the relative contributions of different glutamate receptors subtypes, and the roles of the major voltage-dependent ion channels.

A positive correlation was discovered between the initial amplitude of orthodromic population spikes and the degree of post-glutamate recovery. A similar correlation between resting membrane potential and total voltage change has been noted for compounds such as cyanide (Englund et al., 2001). This mitochondrial poison tended to return membrane potential to a fixed point, so that the induced voltage change depended on the initial resting potential. However, such a simple relationship is not likely to apply to the present results since no correlation was found for antidromic spike size and subsequent recovery, implying that the correlation of orthodromic potentials and recovery involves a change in synaptic transmission.

On the other hand, field epsps showed a negative trend with respect to post-glutamate recovery. There was also a negative correlation between orthodromic population spike amplitude and post-NMDA recovery: small initial spike amplitudes produced larger post-NMDA recoveries whilst larger starting potentials did not recover well. An explanation of these observations may lie in the proposal that smaller epsp

amplitudes were often associated with a smaller NMDA receptor-mediated component compared with larger epsps (Davies & Collingridge, 1989). Thus, in view of the key role of NMDA receptors in neuronal function, especially in relation to calcium influx, synaptic plasticity and toxicity, it is possible that recovery from glutamate applications depends largely on the extent to which activation of NMDA receptors is involved. This conclusion is supported by two further results. Firstly the application of dizocilpine, to block NMDA receptors during perfusion with glutamate, induced a greater recovery of epsp responses. Secondly, isolated NMDA receptor-mediated epsps showed a negative trend between epsp size and post-glutamate recovery, as did the normal composite epsps.

It is possible that the size of the NMDA receptor component of an evoked potential will have a major influence on the overall behaviour of that potential in the presence of high concentrations of glutamate, and its recovery. For example, it has been shown that a form of inhibitory interaction exists between NMDA receptors and AMPA receptors at CA1 dendrites (Bazhenov & Kleshchevnikov, 1999) whereby antagonism of one receptor-mediated component of the epsp will induce a potentiation of the other. These studies are consistent with NMDA receptor stimulation causing a depression of the AMPA receptor component of the epsp, and with the effects of dizocilpine in potentiating the post-glutamate recovery of epsps. This explanation of our results is supported by previous studies of epsp composition. The NMDA receptor is known to increase the linearity of spatial summation of epsps (Cash & Yuste, 1999), which may be relevant to the fact that the relationships we have observed with NMDA receptor-mediated epsps were more clearly seen in relation to amplitude than slope. In addition, since the NMDA component of composite epsps arises after the AMPA / kainate component (as channel blockade by magnesium is relieved), NMDA receptor blockade

induces a decrease in amplitude but not the initial, AMPA/kainate-mediated negative slope of epsps in the hippocampal slice (Dahl & Sarvey, 1990).

Overall, therefore, the presence of a high concentration of glutamate for 10 minutes is sufficient to induce long-lasting changes of E-S coupling, but those changes depend on the initial size of the recorded potentials. The degree of recovery can be increased by apparent adenosine A_1 or A_{2A} receptor blockade, further justifying the possible value of such compounds in the prophylaxis or treatment of cerebral ischaemia and trauma. The protocol employed here may be a useful *in vitro* model of long-term impaired neuronal function which follows exposure to elevated or pathological concentrations of glutamate or agonists such as quinolinic acid.

Abbreviations

ACSF : - artificial cerebrospinal fluid

ADA : - adenosine deaminase

AMPA : - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANCOVA : - analysis of covariance

ANOVA : - analysis of variance

DMSO : - dimethylsulphoxide

DNQX : - 6,7-dinitroquinoxaline-2,3-dione (disodium salt)

DPCPX : - 1,3-dipropyl-8-cyclopentylxanthine

Epsps: - excitatory postsynaptic potentials

E-S coupling : - epsp-spike coupling

LTD : - long-term depression

LTP : - long-term potentiation

NMDA : - N-methyl-D-aspartate.

SCH58261 : - 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5-c]-pyrimidine

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Figure 1

The effects of glutamate on CA1 evoked responses

A) **a** Time course graph showing the average changes in orthodromic population spike amplitude in response to 5mM (*n* = 3) or 10mM (*n* = 3) glutamate

b Sample traces of orthodromic population spikes taken from the time points as indicated in **a**.

Calibrations: 2mV, 5ms

c Scatterplot showing the relationship between % recovery of the population spike following treatment with 5mM or 10mM glutamate and the initial orthodromic spike size. Recovery is taken as the average % spike size of a stable 10min plateau period following glutamate treatment. There is a significant difference in recovery following applications of glutamate at 5mM and 10mM ($P<0.0001$, ANCOVA).

B) **a** Time course graph showing the percentage changes in antidromic population spike amplitude produced by superfusion with 5mM glutamate. The population spikes show a recovery following glutamate that does not differ significantly from the baseline (*n* = 4)

b Sample waveforms showing an antidromic population spike before, during and after perfusion of 5mM glutamate. Calibrations: 2mV, 5ms.

C) **a** Time course graphs showing the percentage changes in slope and amplitude for field epsps before, during and after superfusion with 5mM glutamate (*n* = 12). The arrows indicate time points from which the sample waveforms in **b** are taken

b Sample waveforms of a single field epsp taken from the time points indicated in **a**. Calibrations: 2mV, 5ms. **c,d** Scatterplots showing the relationship between epsp amplitude **c** or slope **d** and the % response recovery after glutamate perfusion.

Figure 2

The role of NMDA receptors in mediating responses to glutamate

A) Time course graphs showing the average changes in field epsp slope and amplitude in response to 5mM glutamate alone or in the presence of 10μM dizocilpine. **a** The presence of dizocilpine significantly increases the recovery of the epsp slope following glutamate perfusion compared with the initial pre-glutamate baseline (n = 9, P = 0.0044, one-sample t-test) and also compared to controls treated with glutamate alone (n = 13, P = 0.011, unpaired t- test). **b** The presence of dizocilpine significantly increases the recovery of the epsp amplitude following glutamate perfusion compared with the initial pre-glutamate baseline (n = 9; P = 0.0018, one-sample t test), and also compared to controls treated with glutamate alone (n = 13, P = 0.0003, unpaired t test).

B) **a** Time course graph showing the average percentage changes in amplitude of orthodromic population spikes before, during and after perfusion of 25μM NMDA. The arrows indicate the time points from which the sample waveforms in **b** are taken.

b Sample waveforms showing an orthodromic population spike before and after perfusion of 25μM NMDA. Calibrations: 2mV, 5ms.

c Scatterplot showing the inverse relationship between orthodromic spike size and % recovery of spike size following treatment with 25μM NMDA for 5min. There is a significant linear relationship (R^2 = 0.69, P = 0.0005).

C) **a** Time course graph showing the percentage changes in epsp amplitudes of the isolated NMDA receptor-mediated component (obtained in perfusion medium containing no magnesium and 20μM DNQX) before, during and after 5mM glutamate perfusion (solid circles, n = 8). Changes in pre-synaptic volley amplitudes are also shown (open circles). **b** Sample waveforms of a composite field epsp and its corresponding isolated NMDA receptor-mediated component taken before and after glutamate application. Calibrations: 1mV, 10ms.

Figure 3

Intracellular recording of epsps and spike probability

A) Panel (a) illustrates recordings from a pyramidal neurone under control conditions. The stimulus used here to activate the Schaffer collateral and commissural axons was 80μA, which gave approximately 50% probability of epsp-induced spike firing (7 successes, 7 failures). Fifty minutes after the application of glutamate, the same stimulus failed to evoke any spikes, and increasing the stimulation to 85μA also failed to do so (panel b). In panel (c) the stimulation was increased further to 90μA, when a probability of approximately 50% was restored (6 successes, 8 failures).

B) summarises all the intracellular data, plotting the probability of spike firing at different stimulus intensities. Data are shown as mean ± s.e.m (*n* = 11 neurons). At several stimulus intensities there was a highly significant difference between the experimental points.

Calibrations: 10mV and 10ms. The amplitudes of the spikes are restricted by the recording bandwidth.

****P* < 0.001 between baseline and post-glutamate probabilities

Figure 4

Modulation by adenosine A1 receptor blockade of the effects of glutamate

A) Bar chart summarising the % orthodromic spike recovery following 5mM glutamate with or without DPCPX. There is a significant difference between the recovery of potentials affected by glutamate (*n* = 9) and those recorded after glutamate + DPCPX (*n* = 11; * *P* < 0.01 Bonferroni Multiple Comparisons test against 5mM glutamate control).

B) Sample waveforms of orthodromic population spikes before and after glutamate alone or DPCPX + glutamate. Calibrations: 1mV, 5ms.

C) Bar chart summarising the % antidromic spike recovery following 5mM glutamate (*n* = 4) or 30nM DPCPX + 5mM glutamate (*n* = 4).

D) Bar chart summarising the % field epsp slope and amplitude recovery following 5mM glutamate (*n* = 8) or 30nM DPCPX + 5mM glutamate (*n* = 12).

E) Bar chart summarising the % NMDA receptor-mediated epsp slope and amplitude recovery following 5mM glutamate with or without DPCPX. There is a significant difference between NMDA receptor-mediated epsp amplitudes affected by glutamate (*n* = 7) and those obtained after perfusion by glutamate + DPCPX (*n* = 6)(**P* = 0.045, unpaired t test). However, there was no significant difference between the epsp slopes for each treatment group (*P* = 0.12).

F) Sample waveforms of isolated NMDA receptor-mediated epsps before and after glutamate alone or DPCPX + glutamate. Calibrations: 1mV, 10ms.

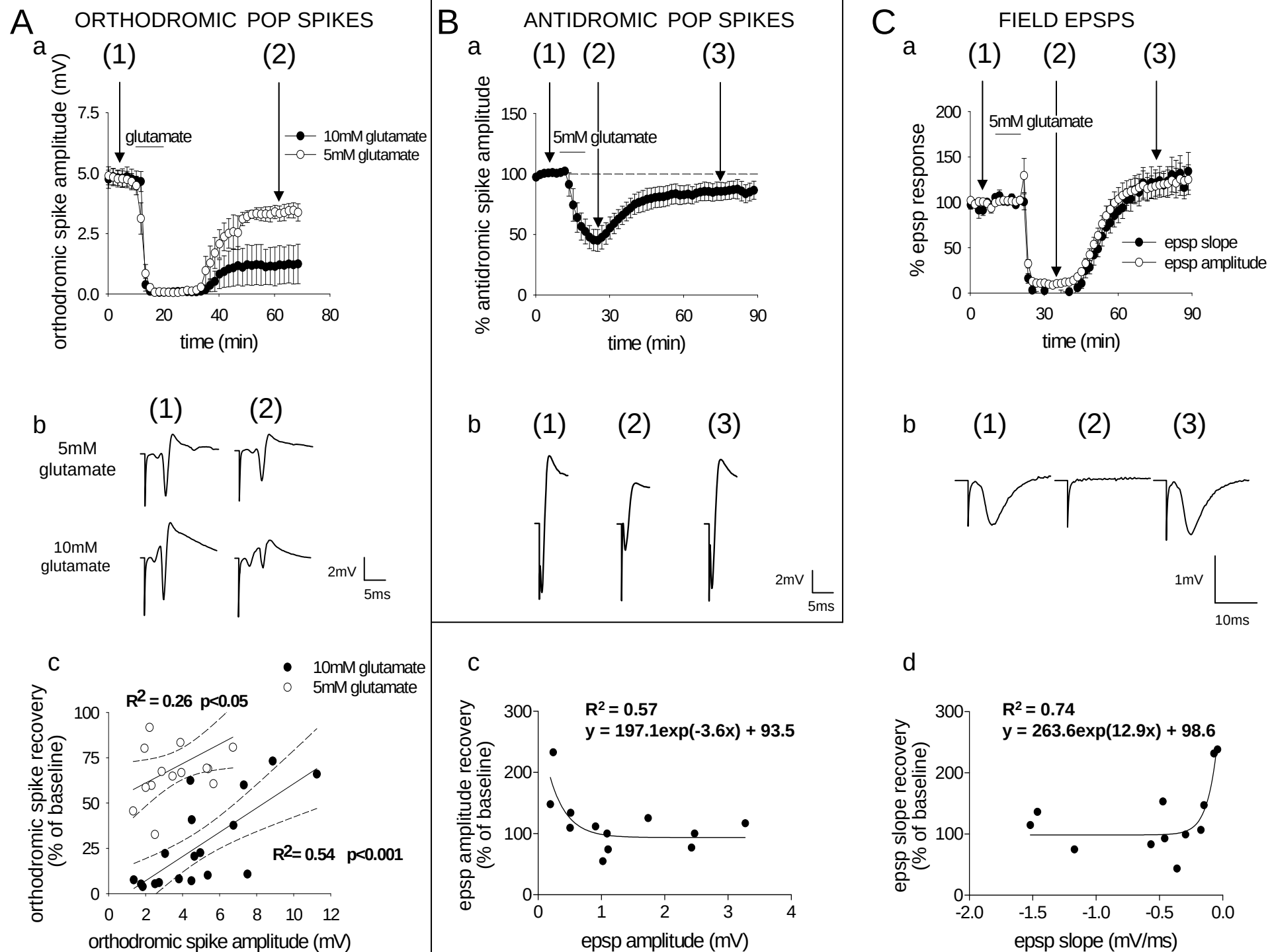
Figure 5

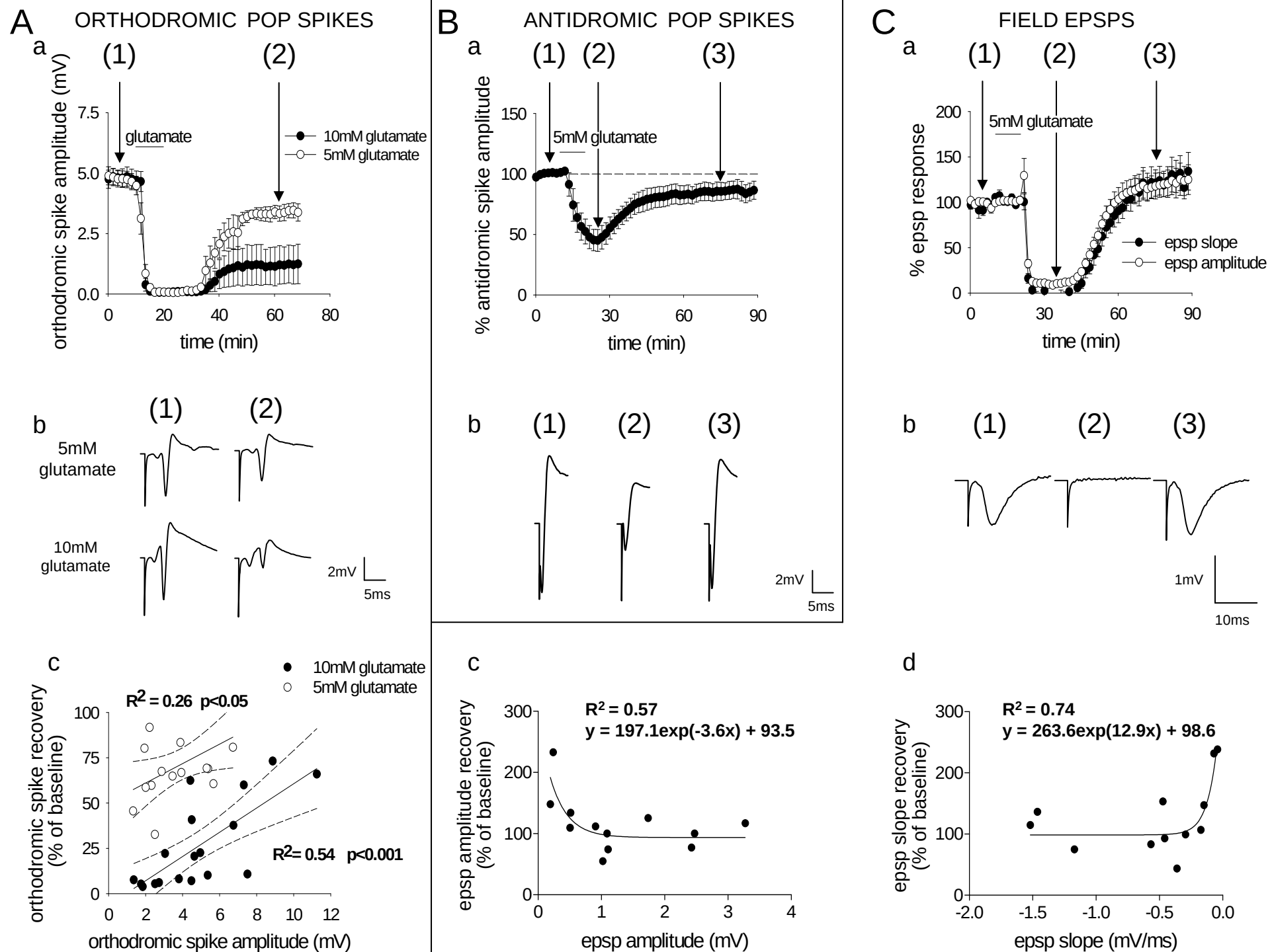
Intracellular recording of epsps and spike probability

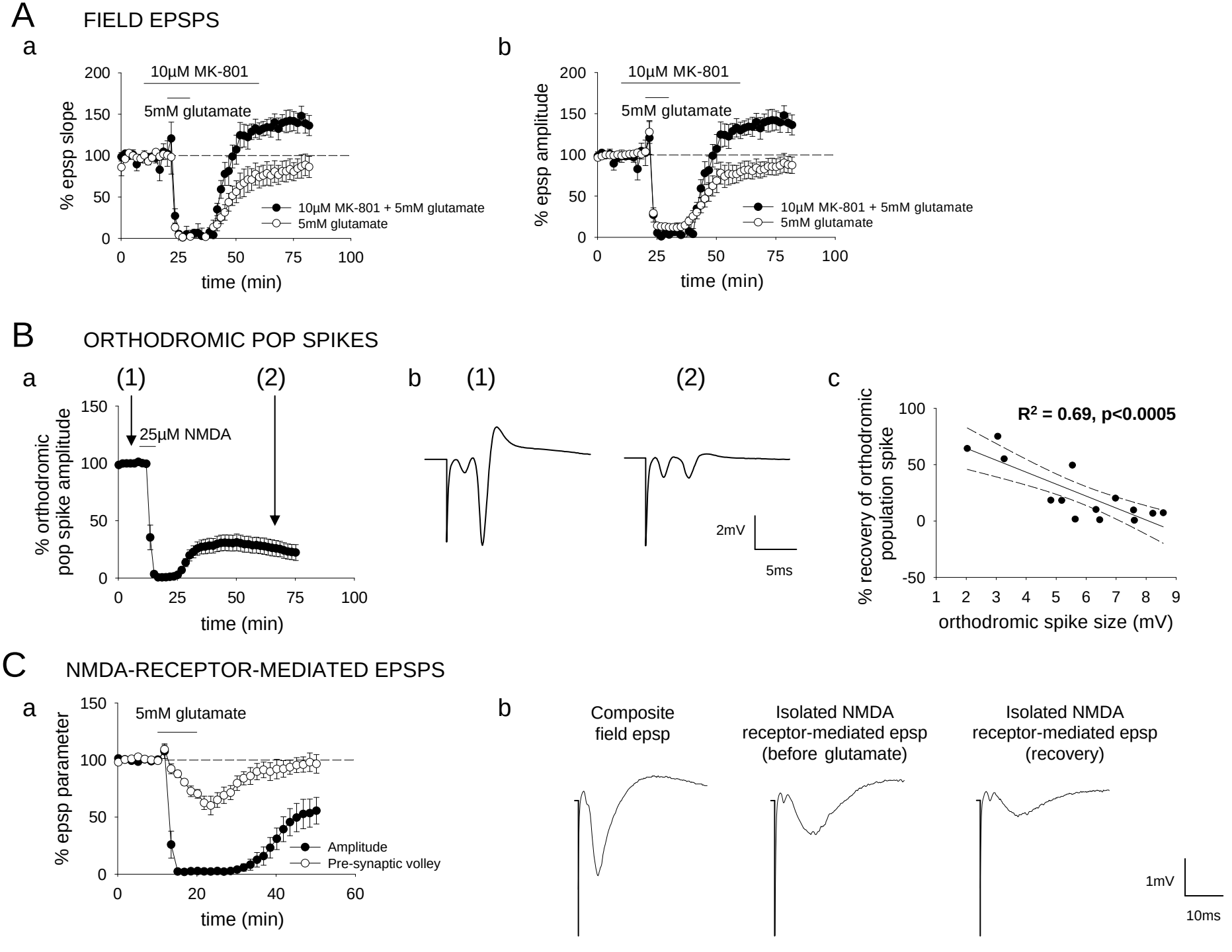
A) Panel (a) illustrates recordings from a pyramidal neurone under control conditions. The stimulus used here to activate the Schaffer collateral and commissural axons was 80μA, which gave approximately 50% probability of epsp-induced spike firing (11 successes, 13 failures). Fifty minutes after the application of glutamate but in the presence of the adenosine A1 receptor blocker DPCPX, the same stimulus still evoked epsps which triggered spikes with a high probability (7 successes, 14 failures). compared with the greatly reduced probability noted after glutamate alone.

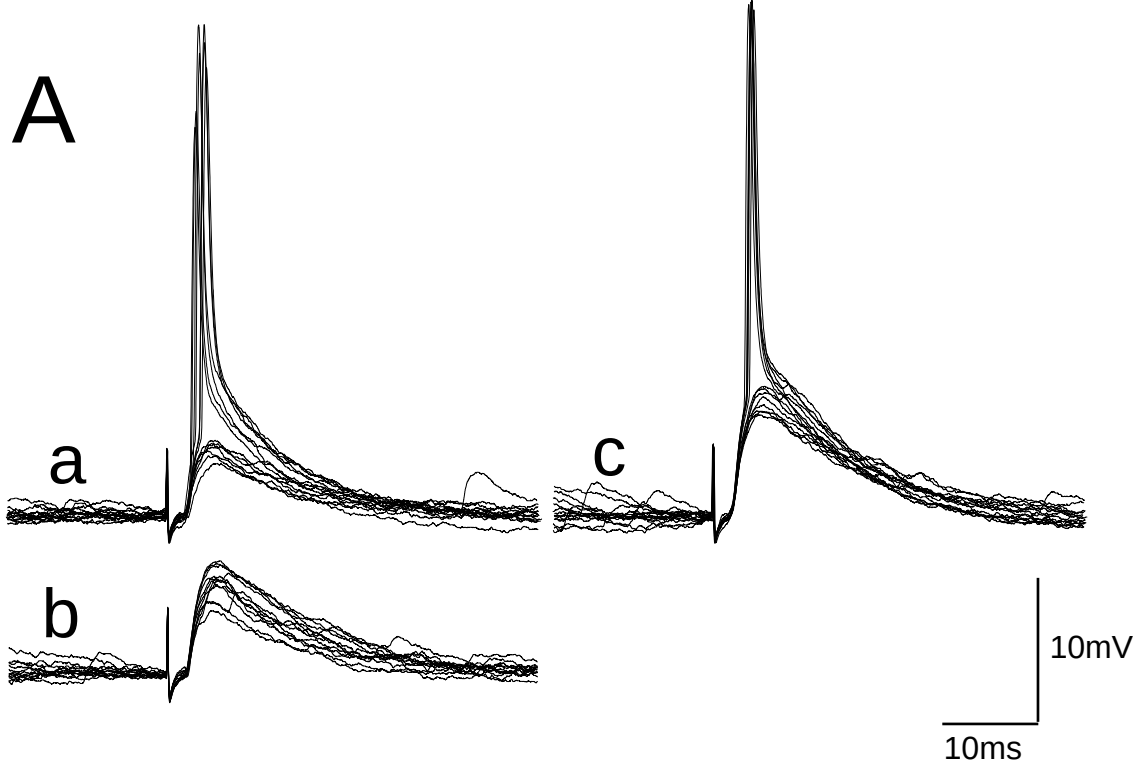
B) summarises the intracellular data, plotting the probability of spike firing at different stimulus intensities. Data are shown as mean ± s.e.m (n = 8 neurons). At only one stimulus intensity was there a just significant difference between the mean probabilities. Calibrations: 10mV and 10ms. The amplitudes of the spikes are restricted by the recording bandwidth.

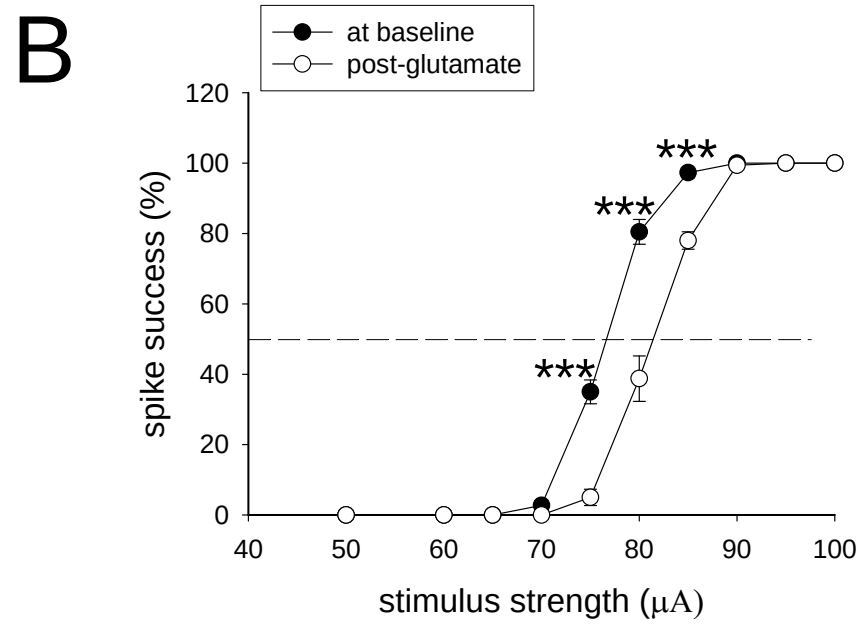
*P < 0.05 between baseline and post-glutamate probabilities.

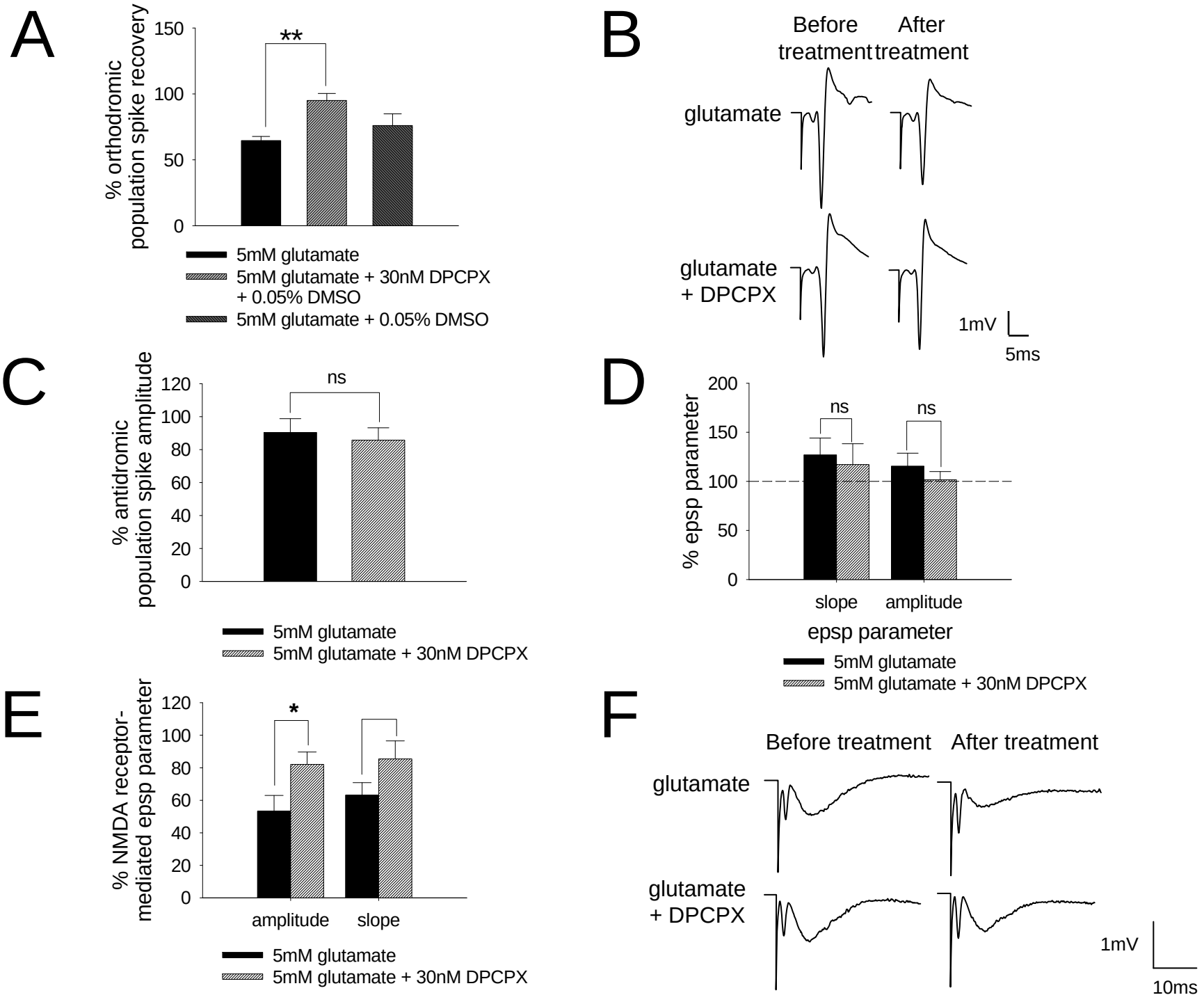

A
ORTHODROMIC POP SPIKES
a
(1)
(2)
orthodromic spike amplitude (mV)
glutamate
10mM glutamate
5mM glutamate
time (min)
b
5mM glutamate
10mM glutamate
2mV
5ms
c
10mM glutamate
5mM glutamate
R2 = 0.26 p<0.05
R2= 0.54 p<0.001
orthodromic spike recovery (% of baseline)
orthodromic spike amplitude (mV)
B
ANTIDROMIC POP SPIKES
a
(1) (2) (3)
% antidromic spike amplitude
5mM glutamate
time (min)
b
2mV
5ms
c
R2 = 0.57
y = 197.1exp(-3.6x) + 93.5
epsp amplitude recovery (% of baseline)
epsp amplitude (mV)
C
FIELD EPSPS
a
(1) (2) (3)
% epsp response
5mM glutamate
epsp slope
epsp amplitude
time (min)
b
1mV
10ms
d
R2 = 0.74
y = 263.6exp(12.9x) + 98.6
epsp slope recovery (% of baseline)
epsp slope (mV/ms)

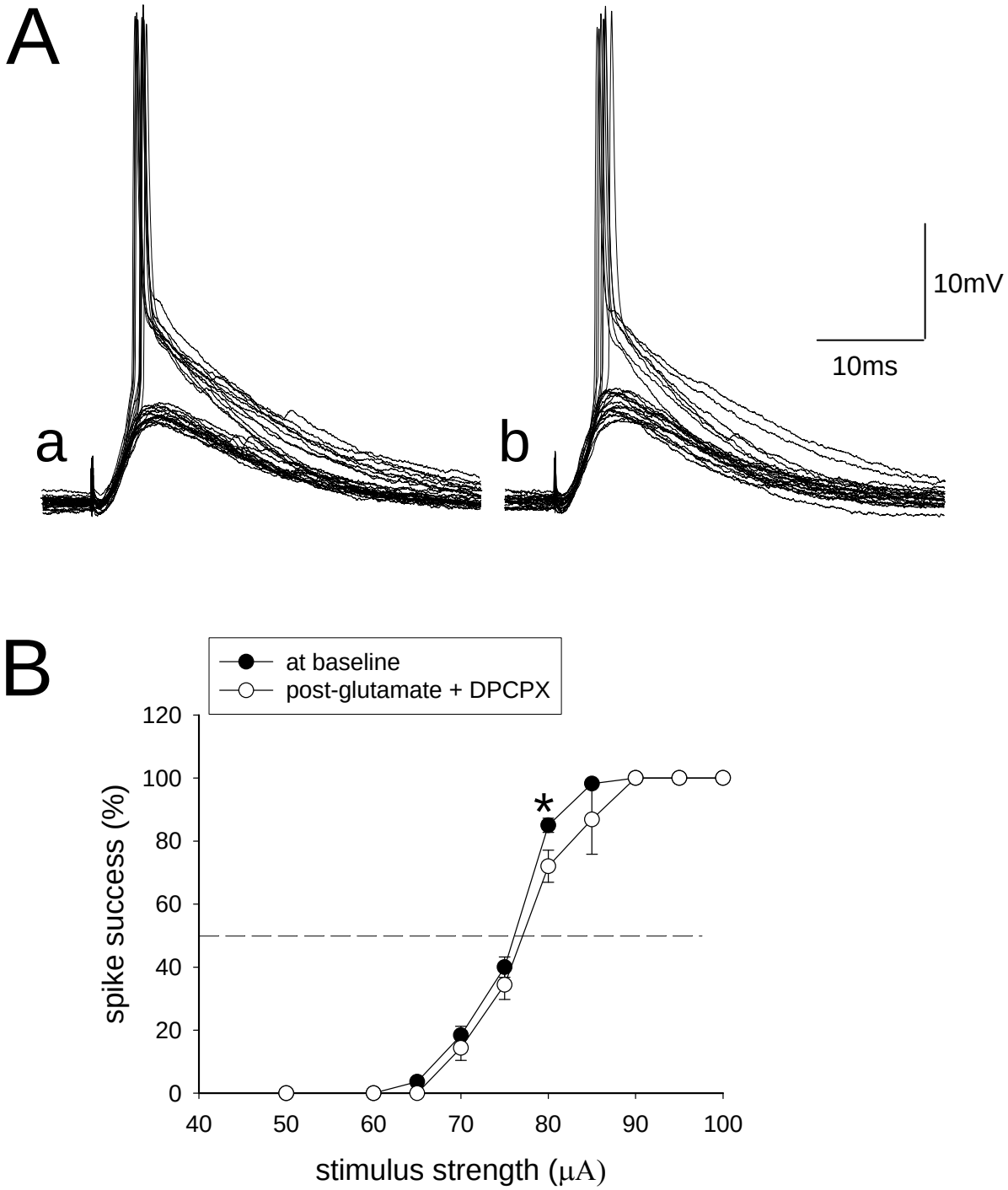

A
ORTHODROMIC POP SPIKES
a
(1)
(2)
orthodromic spike amplitude (mV)
glutamate
10mM glutamate
5mM glutamate
time (min)
b
5mM glutamate
10mM glutamate
2mV
5ms
c
10mM glutamate
5mM glutamate
R2 = 0.26 p<0.05
R2= 0.54 p<0.001
orthodromic spike recovery (% of baseline)
orthodromic spike amplitude (mV)
B
ANTIDROMIC POP SPIKES
a
(1) (2) (3)
% antidromic spike amplitude
5mM glutamate
time (min)
b
2mV
5ms
c
R2 = 0.57
y = 197.1exp(-3.6x) + 93.5
epsp amplitude recovery (% of baseline)
epsp amplitude (mV)
C
FIELD EPSPS
a
(1) (2) (3)
% epsp response
5mM glutamate
epsp slope
epsp amplitude
time (min)
b
1mV
10ms
d
R2 = 0.74
y = 263.6exp(12.9x) + 98.6
epsp slope recovery (% of baseline)
epsp slope (mV/ms)


A
FIELD EPSPS
a
10μM MK-801
5mM glutamate
% epsp slope
time (min)
10μM MK-801 + 5mM glutamate
5mM glutamate
b
% epsp amplitude
B
ORTHODROMIC POP SPIKES
a
(1)
(2)
25μM NMDA
% orthodromic pop spike amplitude
b
2mV
5ms
c
R² = 0.69, p<0.0005
% recovery of orthodromic population spike
orthodromic spike size (mV)
C
NMDA-RECEPTOR-MEDIATED EPSPS
a
% epsp parameter
Amplitude
Pre-synaptic volley
b
Composite field epsp
Isolated NMDA receptor-mediated epsp (before glutamate)
Isolated NMDA receptor-mediated epsp (recovery)
1mV
10ms


A
a
b
c
10mV
10ms


B
at baseline
post-glutamate
spike success (%)
stimulus strength (μA)
***
***
***
0
20
40
60
80
100
120
40
50
60
70
80
90
100


A
% orthodromic population spike recovery
150
100
50
0
**
5mM glutamate
5mM glutamate + 30nM DPCPX + 0.05% DMSO
5mM glutamate + 0.05% DMSO
B
Before treatment
After treatment
glutamate
glutamate + DPCPX
1mV
5ms
C
% antidromic population spike amplitude
120
100
80
60
40
20
0
ns
5mM glutamate
5mM glutamate + 30nM DPCPX
D
% epsp parameter
200
150
100
50
0
ns
ns
slope
amplitude
epsp parameter
5mM glutamate
5mM glutamate + 30nM DPCPX
E
% NMDA receptor-mediated epsp parameter
120
100
80
60
40
20
0
*
amplitude
slope
5mM glutamate
5mM glutamate + 30nM DPCPX
F
Before treatment
After treatment
glutamate
glutamate + DPCPX
1mV
10ms


A
a
b
10mV
10ms
B
at baseline
post-glutamate + DPCPX
spike success (%)
120
100
80
60
40
20
0
*
40
50
60
70
80
90
100
stimulus strength (μA)