European Journal of Neuroscience, Vol. 23, pp. 581–586, 2006 doi:10.1111/j.1460-9568.2005.04588.x

SHORT COMMUNICATION
Long-term depression of neuron to glial signalling in rat cerebellar cortex

Tomas C. Bellamy[1,2] and David Ogden[1]
[1]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[2]The Babraham Institute, Babraham, Cambridge CB2 4AT, UK

Keywords: astrocyte, Bergmann glial cell, cerebellum, glutamate, synaptic plasticity

Abstract

Bergmann glial cells enclose synapses throughout the molecular layer of the cerebellum and express extrasynaptic AMPA receptors and glutamate transporters. Accordingly, stimulation of parallel fibres leads to the generation of inward currents in the glia due to AMPA receptor activation and electrogenic uptake of glutamate. Elimination of AMPA receptor Ca^{2+} permeability leads to the withdrawal of glial processes and synaptic dysfunction, suggesting that AMPA receptor-mediated Ca^{2+} signalling is essential for glial support of the neuronal network. Here we show that glial extrasynaptic currents (ESCs) exhibit activity-dependent plasticity, specifically, long-term depression during repetitive stimulation of parallel fibres at low frequencies (0.033–1 Hz) – conditions in which Purkinje neuron excitatory postsynaptic currents (EPSCs) remain stable. Both the rate of onset and the magnitude of ESC depression increased with stimulation frequency. Depression was reversible following brief periods of stimulation, but became increasingly persistent as the duration of repetitive stimulation increased. All glial currents – AMPA receptors, glutamate transporter and a recently discovered slow 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX)-sensitive current – were depressed. Increasing presynaptic release probability by doubling external Ca^{2+} concentration did not affect the time course of depression, suggesting that neither decreased release probability nor fatigue of release sites contribute to depression. Inhibition of glutamate uptake caused a dramatic enhancement of the rate of depression, implicating glutamate in the underlying mechanism.The strength of neuron to glial signalling in the cerebellum is therefore dynamically regulated, independently of adjacent synapses, by the frequency of parallel fibre activity.

Introduction

Astroglial cells are essential for the function of the CNS, providing support to neurons through supply of metabolic intermediates, buffering of neuronal K^+ efflux and uptake of neurotransmitters from the extracellular space (Kettenmann & Ransom, 1995). In addition, astrocytes express receptors that are activated during synaptic transmission, triggering Ca^{2+} signalling pathways (Verkhratsky *et al*., 1998; Araque *et al*., 2001) that lead to the release of neurotransmitters and neuromodulators to feed back onto neurons (Haydon, 2001). In this way, astrocytes play a role in information processing in the CNS. Relatively little is known, however, about the plasticity of such neuron to glial signalling pathways, despite synaptic plasticity being recognized as a key computational property of neuronal networks (Sejnowski, 1999).

The Bergmann glial cell of the cerebellar cortex – a specialized astrocyte – is closely associated with the principal output cell of the cortex, the Purkinje neuron. Stimulation of each of the two inputs to the Purkinje neuron, the climbing fibre and the parallel fibres, results in the generation of currents in the Bergmann glia through activation of AMPA receptors and glutamate transporters (Bergles *et al*., 1997; Clark & Barbour, 1997).

Glial glutamate transporters clear glutamate from the extracellular space, preventing synaptic crosstalk, protecting against excitotoxicity and regulating the activation of perisynaptic metabotropic receptors (Danbolt, 2001). Bergmann glial cell AMPA receptors are Ca^{2+} permeable, due to the absence of the GluR2 subunit in these cells (Hollmann *et al*., 1991; Burnashev *et al*., 1992; Muller *et al*., 1992). The importance of Ca^{2+} signalling through this pathway has been demonstrated by introducing the GluR2 subunit into glia with an adenoviral vector, eliminating Ca^{2+} permeability (Iino *et al*., 2001). This manipulation caused glial processes to withdraw from Purkinje neuron synapses, resulting in a prolongation of the time course of synaptic transmission and the reinnervation of Purkinje neurons with additional climbing fibres. The implication is that neuron to glial signalling is necessary for the maintenance of proper synaptic connectivity in the molecular layer.

In this study, we show that Bergmann glial cell AMPA receptors and glutamate transporters exhibit activity-dependent long-term plasticity during parallel fibre stimulation. It was found that glial extrasynaptic currents (ESCs) are depressed during repetitive stimulation, even at frequencies low enough to have no effect on synaptic strength (0.1–1 Hz). Thus, the strength of neuron to glial signalling is dynamically regulated, independently of the strength of adjacent synapses.

Correspondence: Dr T.C. Bellamy, [2]The Babraham Institute, as above.
E-mail: tom.bellamy@bbsrc.ac.uk

Received 31 October 2005, revised 30 November 2005, accepted 1 December 2005

Materials and methods

Whole-cell patch-clamp recordings were obtained from Bergmann glial cells in 300-μm transverse cerebellar slices from Wistar rats aged 16–20 days (killed by cervical dislocation), as previously described (Bellamy & Ogden, 2005). Briefly, slices were perfused with a bath solution containing (in mM): NaCl, 135; KCl, 3; HEPES, 10; $MgSO_4$, 1; $NaHCO_3$, 2.5; $CaCl_2$, 2; glucose, 25; pH 7.4 (with 1 M NaOH) at room temperature, and gassed with 99.5% O_2/0.5% CO_2. In Purkinje neuron experiments, the bath solution was supplemented with 20 μM bicuculline methiodide to inhibit γ-aminobutyric acid $(GABA)_A$ receptors.

Micropipette internal solution contained (in mM): Kgluconate, 110; KCl, 5; HEPES, 50; EGTA, 0.05; $MgSO_4$, 4; ATP, 4; GTP, 0.2; phosphocreatine, 9; pH 7.35 (with 1 M KOH). In normal bath solution, the liquid junction potential was 12 mV, pipette negative, and was corrected for. Series resistance ranged from 5 to 15 MΩ, and was not compensated.

Parallel fibres were stimulated with a patch pipette (1–2 MΩ) filled with bath solution, positioned within the molecular layer ~150–300 μm from the recorded cell. Stimulation intensity ranged from ~5 to 25 μA, with 100-μs pulses delivered from a constant voltage-isolated stimulator (Digitimer, Welwyn, UK).

Results

ESCs in Bergmann glial cells

Stimulation of parallel fibres in a transverse cerebellar slice evokes an ESC in Bergmann glial cells (Clark & Barbour, 1997; Bellamy & Ogden, 2005), composed of a rapid AMPA receptor current, an intermediate current due to electrogenic uptake of glutamate, and a slower component due to an 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX)-sensitive and G-protein-coupled current that has only recently been described (Fig. 1A and B) (Bellamy & Ogden, 2005). The ESC is preceded by a rapid triphasic current due to changes in the extracellular field potential generated by the parallel fibre volley (Clark & Barbour, 1997; Bellamy & Ogden, 2005), which is truncated in most records shown.

Paired-pulse stimulation of parallel fibres leads to pronounced facilitation of ESCs (Fig. 1B and C). Inhibition of glutamate transporters with 100 μM D,L-threo-β-benzyloxyaspartate (TBOA) enhances the slow G-protein-coupled current, such that it forms a second peak distinct from the fast AMPA receptor current during paired-pulse stimulation (Fig. 1B).

Persistent depression of glial ESCs at low stimulation frequencies

After initiating whole-cell recording from Bergmann glia, parallel fibres were stimulated with pairs of pulses separated by 100 ms at a frequency of 0.2 Hz, a protocol used in many studies of parallel fibre–Purkinje neuron transmission. With this protocol, the amplitude of Bergmann glial ESCs decreased progressively, to about 35% of the initial level, with a half-time of approximately 2 min (Fig. 1C). To test whether the decline was simply 'rundown' of cell responsiveness caused by diffusion of cytosolic factors into the pipette solution, a hiatus of 5 min was introduced into the stimulation regime. ESC amplitude had recovered to 65% of control after this period (Fig. 1C), indicating that depression was an activity-dependent effect.

When the baseline frequency was decreased to 0.033 Hz, the decline in ESC amplitude over time was much reduced (Fig. 1D). Raising stimulation frequency from 0.033 Hz to 0.2 Hz caused a

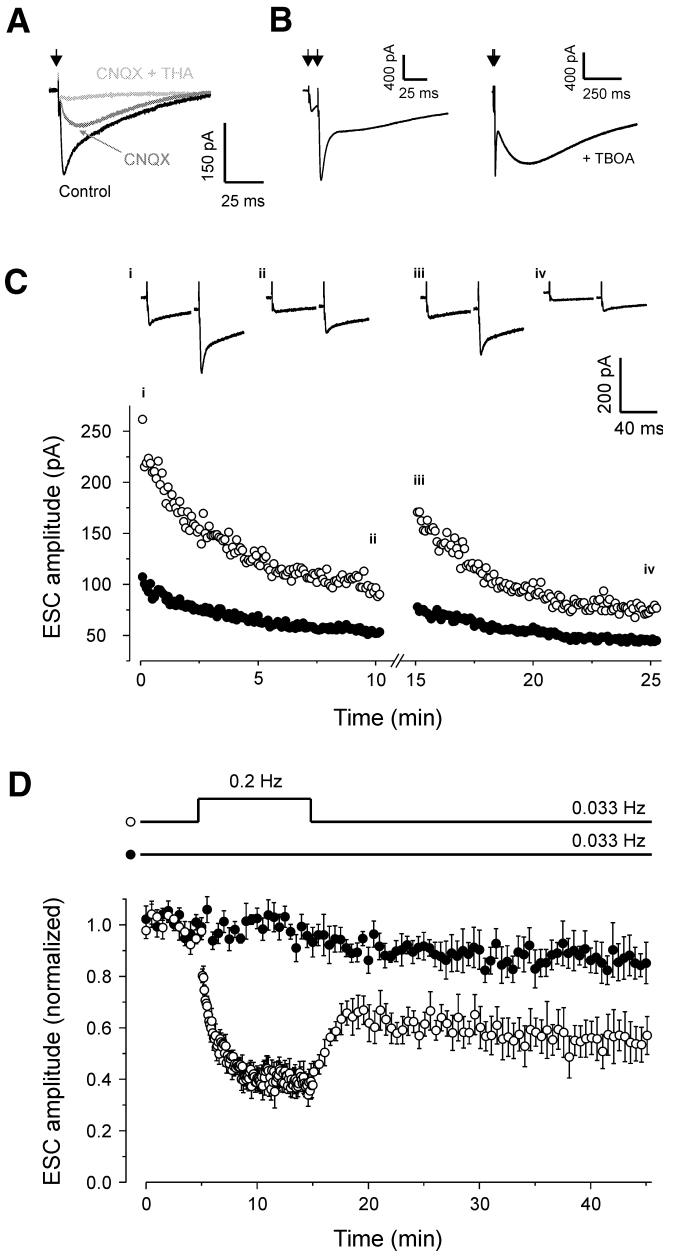

FIG. 1. Depression of glial extrasynaptic currents (ESC) at low frequencies. (A) A typical ESC evoked by parallel fibre stimulation (at arrow), composed of an AMPA receptor current [blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)] and an electrogenic glutamate uptake current [blocked by 100 μM L-threo-β-hydroxyaspartic acid (THA)]. A second, slow 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX)/CNQX-sensitive, G-protein-coupled current is also present in Bergmann glia (Bellamy & Ogden, 2005). (B) Left panel: paired-pulse stimulation of parallel fibres (pulse interval 10 ms) causes facilitation of ESC. Right panel: the slow NBQX- and GDP-β-S-sensitive current is enhanced by inhibition of glutamate transporters with D,L-threo-β-benzyloxyaspartate (TBOA, 100 μM). (C) ESCs recorded during paired-pulse stimulation (100 ms pulse interval; first pulse: filled circles; second pulse: open circles) of parallel fibres at 0.2 Hz. Traces are averages of five ESCs recorded at times indicated by roman numerals. A 5-min period without stimulation was introduced after 10 min of stimulation, before resuming stimulation for a further 10 min. (D) Long-term depression of ESC amplitude after raising stimulation frequency from 0.033 Hz to 0.2 Hz for 10 min (open circles) compared with control stimulation at 0.033 Hz (filled circles). Data are mean ± SEM of the amplitude of the second pulse in a pair with 100-ms interval, normalized to baseline (n = 5–6 cells).

decrease in ESC amplitude, reaching a stable level that was 43 ± 4.0% of control after 5 min. Returning to 0.033 Hz resulted in recovery to ~60% of control amplitude. Thereafter, depression was sustained at this level for at least another 30 min, with no indication of further recovery.

To test for an influence of paired-pulse stimulation on depression, the experiment was repeated with single pulses. The result was indistinguishable (data not shown), confirming that onset of ESC depression did not depend on short-term effects due to paired-pulse facilitation. Access resistance did not vary during the recording period, and the amplitude of the fibre volley was stable (data not shown), indicating that changes in the number of active parallel fibres did not account for the depression.

The dependence of ESC depression on stimulation frequency was examined over the range 0.1–1 Hz, from a baseline of 0.033 Hz (paired-pulses, 100 ms interval). Increasing frequency led to a progressive decrease in ESC amplitude (Fig. 2A). Both the rate of decline and the magnitude of depression increased with increasing frequency in this range.

The persistence of depression was examined by raising stimulation frequency from 0.033 Hz to 1 Hz for different periods, ceasing stimulation for 10 min to allow some recovery (see Fig. 1C), and then resuming stimulation at 0.033 Hz (Fig. 2B). ESC amplitude recovered to 73 ± 6.6% of control amplitude after 1 Hz stimulation for 2 min, to 56 ± 5.6% following 5 min at 1 Hz, and to only 31 ± 3.6% after 10 min at 1 Hz. Thus, the recovery of ESCs from depression was reduced as the duration of raised frequency stimulation increased.

This long-term depression of glial ESC was unanticipated, as the frequency range of 0.033–0.2 Hz is generally used as baseline for stimulation of Purkinje neurons. Accordingly, the effect on Purkinje neuron excitatory postsynaptic currents (EPSCs) of raising frequency above 0.033 Hz was examined (Fig. 2C). The amplitude of neuronal EPSCs did not change significantly ($P > 0.05$, paired t-test, $n = 6$) after raising the frequency from 0.033 Hz to 0.2 or 1 Hz.

Origin of ESC depression

Selective pharmacological agents were used to isolate the components of the ESC (Fig. 1A) and investigate their susceptibility to long-term depression. Inhibition of AMPA receptors with 10 μM NBQX isolates the glutamate transporter current. Raising stimulation frequency from 0.033 to 1 Hz led to depression of uptake currents (Fig. 3A). The time course of depression was slower for NBQX-treated cells than control cells, suggesting that AMPA receptor currents depress more rapidly than transporter currents. To obtain the time course of AMPA receptor depression, the mean data from Fig. 3A were normalized to the ESC amplitude before NBQX treatment, and then subtracted from the mean data in Fig. 2A (Fig. 3A, inset). At 1 Hz stimulation, depression of AMPA receptor currents is effectively complete within ~60–120 s. The time course could not be well fitted with a single exponential, however, suggesting that the onset of depression may involve several processes.

A second NBQX-sensitive current was recently described in Bergmann glia (Bellamy & Ogden, 2005), which is G-protein-coupled, but not linked to glial AMPA receptor activation. From its sensitivity to antagonists, it is thought to arise indirectly via the release of a paracrine messenger linked to activation of neuronal AMPA receptors (Bellamy & Ogden, 2005). The amplitude of this slow current is enhanced by inhibition of glutamate uptake with TBOA (Fig. 1B). Raising the stimulation frequency from 0.033 Hz to either 1 Hz or 0.2 Hz (Fig. 3B) in the presence of TBOA (100 μM) caused a rapid decrease in ESC amplitude, within two pairs of pulses at the higher frequency. Both the initial fast AMPA receptor current and slower G-protein-linked current were abruptly depressed in the presence of TBOA (Fig. 3B and inset). Furthermore, the ESC evoked at 0.033 Hz was less stable than under control conditions, with progressive depression evident even at this low frequency (Fig. 3B). Thus, prolonging the extrasynaptic glutamate concentration transient with uptake inhibitors increases the rate of ESC depression, implying that glutamate-dependent signalling underlies the mechanism of depression. However, neither the metabotropic glutamate receptor antagonists 7-hydroxyiminocyclopropan[b]chromen-1α-carboxylic acid ethyl ester (CPCCOEt, 20 μM) or (*RS*)-α-methyl-(4-carboxyphenyl)glycine (MCPG, 2 mM), nor the *N*-methyl-D-aspartate (NMDA) receptor antagonist AP5 (50 μM) blocked depression (data not shown). Furthermore, when GDP-β-S (5 mM) was included in the pipette solution to inhibit the slow current (Bellamy & Ogden, 2005), the abrupt depression of fast AMPA receptor currents in the presence of TBOA was still observed (mean ESC amplitude = 0.43 ± 0.07 after two pairs of pulses at 0.2 Hz in four cells treated with GDP-β-S), indicating that the slow current is not itself linked to the mechanism of depression. Additionally, the $GABA_A$ receptor antagonist bicuculline (20 μM) and the CB1 receptor antagonist AM 251 (2 μM) also failed to block ESC depression.

The finding that the three main components of glial ESCs are all depressed by repetitive stimulation of parallel fibres is suggestive of a common mechanism, such as a reduction in the supply of glutamate from presynaptic terminals to extrasynaptic glia. Recent evidence has indicated that AMPA receptors on Purkinje neurons and Bergmann glia respond to glutamate released from different sites (Matsui & Jahr, 2003, 2004). It is therefore feasible that decreased release probability at the ectopic sites that release glutamate onto Bergmann glial cell membranes (Matsui *et al*., 2005) or fatigue of these sites could cause ESC depression without influencing synaptic transmission.

Changes in presynaptic release probability (P_R) are often inferred from changes in the paired-pulse ratio of measured currents (Atluri & Regehr, 1996; Zucker & Regehr, 2002). Increasing stimulation frequency from 0.033 Hz to 0.2 or 1 Hz caused a decrease in paired-pulse ratio of ESC amplitudes (Fig. 3C). The decrease in paired-pulse ratio was also observed in the presence of NBQX (Fig. 3C inset). According to the residual Ca^{2+} hypothesis, a decrease in paired-pulse ratio indicates an increase in P_R. The situation may be complicated at the parallel fibre synapse, however, because glutamate concentration increases during paired-pulse facilitation at both synaptic (Coesmans *et al*., 2004) and extrasynaptic (Bellamy & Ogden, 2005) sites, meaning factors other than presynaptic Ca^{2+} concentration may contribute to paired-pulse ratio.

To test the hypothesis further, P_R was increased by doubling external Ca^{2+} concentration. ESCs were recorded from a cell at 2 mM Ca^{2+} during parallel fibre stimulation, until a stable baseline was established. Stimulation frequency was then raised to 0.2 Hz for 2 min. Thereafter, stimulation was ceased for 10 min allowing complete recovery of ESC amplitude (data not shown). External Ca^{2+} concentration was raised to 4 mM, increasing ESC amplitude 3.04 ± 0.77-fold (mean ± SEM, $n = 7$) for the first pulse, and 1.97 ± 0.19-fold (mean ± SEM, $n = 7$) for the second pulse. Stimulation at 0.2 Hz was resumed for another 2 min. Finally, a further 10-min recovery was followed by restoring Ca^{2+} concentration to 2 mM, to check the reversibility of P_R enhancement. The mean data from seven cells treated in this way are summarized in Fig. 3D.

If depression arises from decreased P_R, increasing external Ca^{2+} would be predicted to decrease the rate of ESC depression due to the highly cooperative relationship between Ca^{2+} concentration and P_R (Atluri & Regehr, 1996). Conversely, if depression arose from fatigue

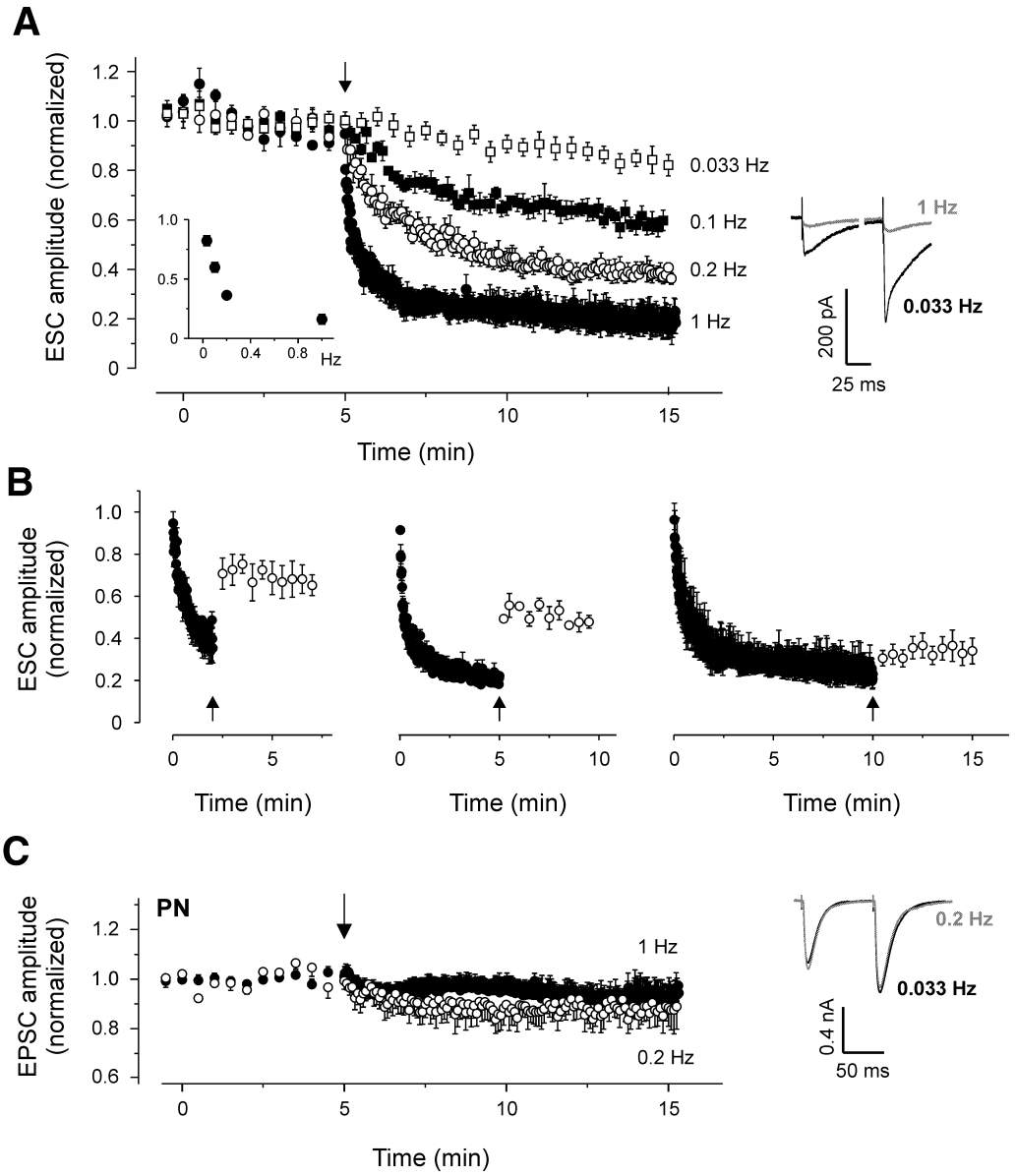

FIG. 2. Frequency dependence of extrasynaptic current (ESC) depression. (A) Mean ESC amplitude after raising stimulation frequency (at arrow) from 0.033 Hz to 0.1, 0.2 or 1 Hz as indicated. Inset: mean ESC amplitude after 10-min stimulation against stimulation frequency (Hz). The trace shows ESCs recorded immediately before (black) and 10 min after (grey) 1 Hz stimulation ($n = 5$–12). (B) Recovery of ESC amplitude from depression. Cells were stimulated at 0.033 Hz until a stable baseline was established. Stimulation was raised to 1 Hz for 2, 5 or 10 min (filled circles), and then ceased for 10 min (arrow). Stimulation was restarted at 0.033 Hz (open circles) to determine recovery from depression. (C) The amplitude of EPSCs recorded in Purkinje neurons was unaffected by changing baseline stimulation frequency to 0.2 Hz (open circles) or 1 Hz (filled circles). Data are mean amplitudes ± SEM ($n = 7$) for the second pulse in a pair. Right panel: representative excitatory postsynaptic currents (EPSCs) recorded immediately before (black trace) and 10 min after (grey trace) the stimulation frequency was raised to 0.2 Hz.

of presynaptic sites, increasing P_R should hasten the onset of depression. However, in both 2 mM and 4 mM Ca^{2+}, the time course of ESC depression was well fitted by a biexponential decay with time constants of $\tau_1 = 2.16$ s and $\tau_2 = 135$ s (Fig. 3D).

Discussion

In recent years, a role for neuron to glial signalling pathways in CNS function has gained prominence. The detection of synaptic activity by extrasynaptic receptors on astroglial cells has been well established, and feedback from glia to modulate neuronal activity has been described in diverse systems (Carmignoto, 2000; Haydon, 2001). However, the plasticity of neuron to glial signalling pathways has received less attention.

In cerebellar slices, Bergmann glial cells enclose parallel fibre synapses onto Purkinje neurons, and stimulation of these inputs results in glutamate-evoked ESCs through (at least) three pathways (Fig. 1). We have found that these glial ESCs are highly plastic and operate independently of the synapses that the glia enclose.

Repetitive stimulation of parallel fibres, even at low frequencies that are without effect on Purkinje neuron EPSCs (0.1–1 Hz), causes depression of all three components of the ESC; AMPA receptors, glutamate transporters and the unidentified G-protein-coupled channels. ESC depression was frequency and time dependent; persistent

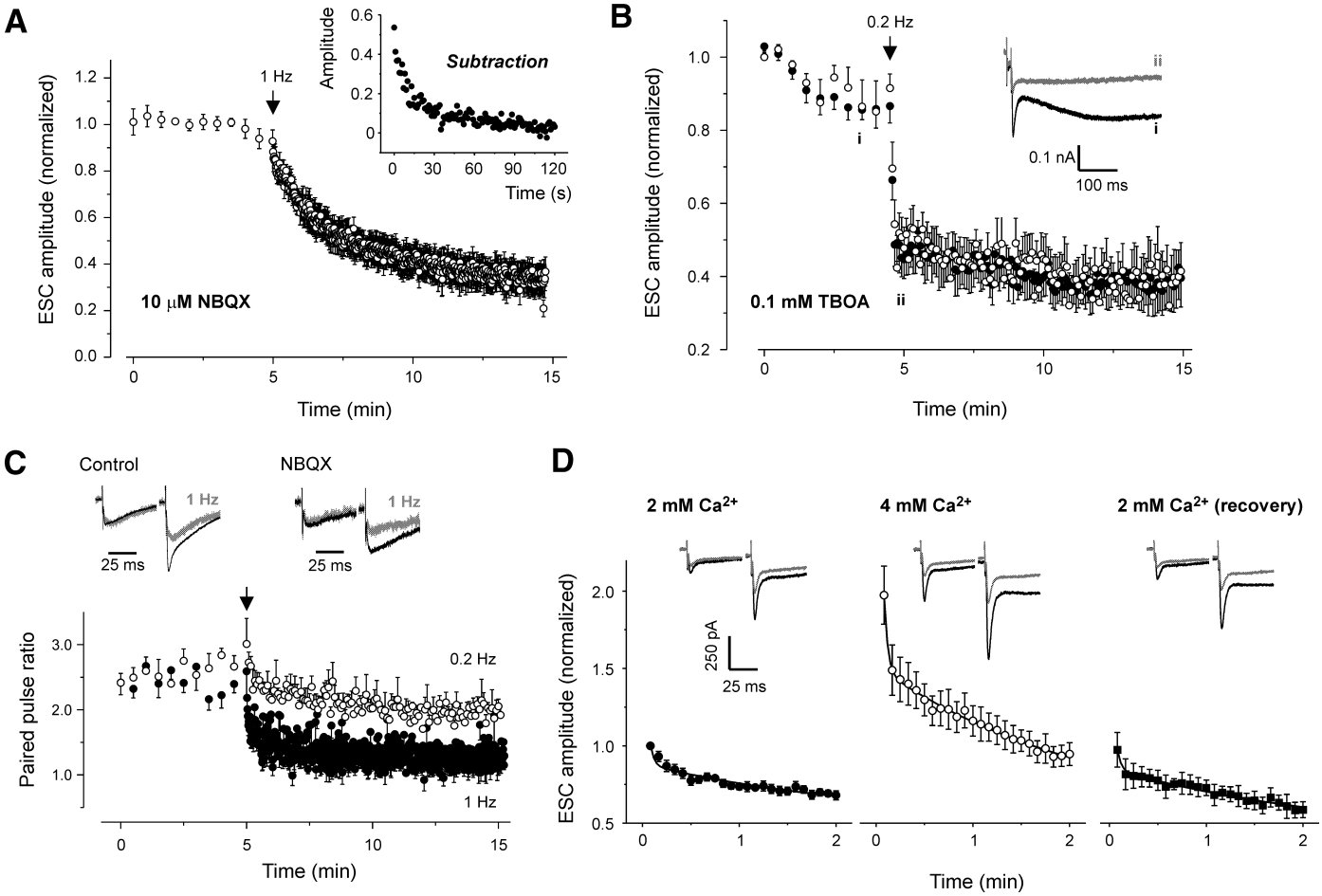

FIG. 3. Origin of extrasynaptic current (ESC) depression. (A) Depression of ESC in the presence of 10 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX), after raising the baseline frequency to 1 Hz at arrow ($n = 4$). Inset: time course of AMPA receptor depression obtained by subtracting the mean data in the presence of NBQX (normalized to control ESC amplitude) from the mean data in the absence of NBQX (from Fig. 2A). (B) Inhibition of transporters with 100 μM D,L-threo-β-benzyloxyaspartate (TBOA) greatly increases the rate of depression of both fast (filled circles) and slow (open circles) NBQX-sensitive currents by 0.2 Hz stimulation ($n = 7$). Inset: ESCs recorded immediately before (*i*, black line) and after (*ii*, grey line) switching to 0.2 Hz stimulation. (C) Mean paired-pulse ratio after raising the stimulation frequency from 0.033 Hz to 0.2 Hz (open circles; $n = 6$) or 1 Hz (filled circles; $n = 6$) at arrow. A significant decrease in ratio occurs at both frequencies ($P < 0.05$; paired *t*-test; average ratio of five pairs immediately before and 10 min after arrow). Traces show control (left) and NBQX-incubated (right) cells, before (black) and after (grey) 10-min stimulation at 1 Hz, scaled to the peak of the first pulse in a pair. (D) The effect of external Ca^{2+} concentration on time course of ESC depression. ESCs were recorded during parallel fibre stimulation at 0.2 Hz for 2 min (paired-pulses, 100-ms interval), separated by 10-min intervals for recovery, at 2 mM and 4 mM external [Ca^{2+}] as indicated, and normalized to the amplitude of the first pair of pulses ($n = 7$). All time courses were fitted with a biexponential decay with $\tau_1 = 2.16$ s and $\tau_2 = 135$ s. The areas of the exponentials were as follows: 2 mM Ca^{2+} ($a_1 = 1.28$, $a_2 = 0.44$); 4 mM Ca^{2+} ($a_1 = 5.01$, $a_2 = 1.00$); 2 mM Ca^{2+} recovery ($a_1 = 1.34$, $a_2 = 0.36$). The inset traces show the first (black) and last (grey) ESCs for each concentration of Ca^{2+} from a representative cell.

stimulation for 5–10 min resulted in long-term depression, whereas brief stimulation allowed recovery of ESC amplitude.

The mechanism underlying ESC depression has not been identified, but may be linked to glutamate signalling, based on the observation that inhibition of glutamate uptake dramatically increased the rate of ESC depression (Fig. 3B, cf. Figs 2A and 3D). Recent reports have highlighted the complexity of glutamate release at parallel fibre synapses. The existence of ectopic release sites targeting Bergmann glia (Matsui *et al*., 2005), the decreased potency of low-affinity AMPA receptor antagonists during paired-pulse stimulation (Bellamy & Ogden, 2005), and discrepancies in paired-pulse ratio when stimulating single or multiple fibres (Isope & Barbour, 2002) all suggest that multiple sources of glutamate can summate in the extrasynaptic space. Identification of the pathways involved in ESC depression may therefore require elucidation of the glutamate dynamics inside and outside the parallel fibre synapse.

Ca^{2+} influx through glial AMPA receptors appears necessary for glia to interact with synapses and support normal transmission (Iino *et al*., 2001). The plasticity described in this report suggests that this interaction may be dynamically regulated depending on the pattern of synaptic activity. Evidence from *in vivo* studies suggests that parallel fibres tend to fire in high-frequency trains (Hartmann & Bower, 2001; Chadderton *et al*., 2004). Such intermittent bursts of activity would not be associated with long-term depression of glial ESCs, and so these synapses are likely to mount the extrasynaptic Ca^{2+} signals necessary for glial association. In contrast, those parallel fibres that fire repetitively would cause depression of neuron to glial signalling pathways, which may be associated with withdrawal of the glial sheath from the parallel fibre synapse. As glutamate transporter currents also decrease (Fig. 3A), this withdrawal may decrease the stringency with which synapses are isolated by glial transporters (Marcaggi *et al*., 2003) and increase the activation of perisynaptic metabotropic

glutamate receptors associated with synaptic plasticity (Neale *et al*., 2001). In this way, the observed glial plasticity may have a role to play in modulating the strength of parallel fibre to Purkinje neuron synaptic transmission.

Acknowledgements

This work was supported by the Medical Research Council, UK, and the Biotechnology and Biological Sciences Research Council, UK. T.C.B. holds a BBSRC David Phillips Fellowship.

Abbreviations

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic currents; ESC, extrasynaptic current; GABA, γ-aminobutyric acid; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide; P_R, presynaptic release probability; TBOA, D,L-threo-β-benzyloxyaspartate; THA, L-threo-β-hydroxyaspartic acid.

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