Published in final edited form as:

Nat Neurosci. 2005 June ; 8(6): 776–781. doi:10.1038/nn1458.

Endocannabinoid signalling depends on the spatial pattern of synapse activation

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Abstract

The brain's endocannabinoid retrograde messenger system decreases presynaptic transmitter release^{1,2}, but its physiological function is uncertain. We show that endocannabinoid signalling is absent when spatially dispersed synapses are activated on rodent cerebellar Purkinje cells, but that it reduces presynaptic glutamate release when nearby synapses are active. This switching of signalling according to the spatial pattern of activity is controlled by postsynaptic metabotropic glutamate (mGluR1) receptors, which are activated disproportionately when glutamate spillover between synapses produces synaptic crosstalk. When spatially distributed synapses are activated, endocannabinoid inhibition of transmitter release can be rescued by inhibiting glutamate uptake to increase glutamate spillover. Endocannabinoid signalling initiated by mGluR1 is a homeostatic mechanism, which detects synaptic crosstalk and down-regulates glutamate release in order to promote synaptic independence.

Introduction

Plasticity of the glutamatergic granule cell to Purkinje cell synapses is important for cerebellar learning^{3,4}. Independent operation of these synapses would allow information to be stored in individual synapse strengths, thereby increasing the number of motor programmes that the cerebellum can store³⁻⁵. However, the high synapse density in the cerebellum can lead to crosstalk occurring by glutamate spillover between synapses⁶, and the effect on surrounding receptors of glutamate diffusing out of a synapse is expected to be larger when glutamate is also released at other synapses nearby because of non-linear summation of glutamate's effects, either via the receptors' dose-response curve or via local saturation of glutamate uptake. Since each Purkinje cell dendritic tree receives ~100,000 granule cell inputs, of which only 50 inputs need to be active to elicit an action potential⁷, physiological activity should involve synapses which are relatively scattered and hence isolated from each other. Despite that prediction, most studies of granule cell to Purkinje cell synaptic transmission have been performed, for convenience, by stimulating the parallel fibers, the granule cell axons which run through the molecular layer (ML) in a parallel geometry, so that activated synapses are adjacent.

We investigated the influence of the spatial pattern of synaptic activation on endocannabinoid-mediated plasticity of these synapses, by whole-cell clamping Purkinje cells in rodent cerebellar slices⁶ and comparing the effects of stimulating at different locations (Fig. 1a). Stimulating either the granule cell layer (GL) or the parallel fibres in the molecular layer (ML) activates the parallel fibre synapses onto Purkinje cells⁸ but, because the stimulated ascending granule cell axons rise to different levels in the molecular layer (Fig. 1a), GL stimulation should activate synapses that are more spatially dispersed than

occurs with ML stimulation. Indeed, although the synapses recruited by GL stimulation generate a fast AMPA receptor mediated synaptic current (EPSC_{fast}) similar to that evoked from the ML, they interact less because they are more isolated from each other⁶. We show that endocannabinoid signalling is absent when spatially dispersed synapses are activated, but that it reduces presynaptic glutamate release when glutamate spillover occurs between nearby active synapses.

Results

Endocannabinoid signalling depends on stimulation site

Tetanic stimulation of the parallel fibres activates mGluR1_{9,10} and produces a depression of synapses onto Purkinje cells by releasing endocannabinoids¹¹⁻¹⁴. However, whereas for parallel fibre stimulation tetani reliably depressed, for about 20 sec, the fast AMPA receptor mediated EPSP (EPSP_{fast}, initial amplitude 3.4 ± 0.5 mV; $n = 8$), and this was blocked by the CB1 receptor blocker AM251, in contrast, for granular layer stimulation evoking a similar EPSP_{fast} (3.9 ± 0.8 mV), tetani reliably potentiated the EPSP_{fast} ($n = 11$), and this was unaffected by cannabinoid receptor block (Fig. 1b,c,d,e). Even when granular layer stimulation was paired with climbing fibre stimulation, which potentiates endocannabinoid-mediated depression produced by parallel fibre stimulation¹⁵, no depression of the EPSC was seen (Fig. 1e). Thus, when spatially isolated parallel fibre synapses are activated no significant endocannabinoid signalling occurs.

Larger mGluR1 activation for parallel fibre stimulation

What causes the different effect of stimulating spatially adjacent and spatially dispersed synapses? This was not the result of a different density or effectiveness of cannabinoid or metabotropic receptors at the synapses activated from the different stimulation sites, since the cannabinoid agonist WIN 55,212-2, and the mGluR1 agonist DHPG, reduced EPSCs evoked by ML and GL stimulation by the same amount (Fig. 1f,g,h,i). Rather, it resulted from a different degree of activation of mGluR1. For an EPSC_{fast} of ~ 600 pA at -70 mV (~ 100 synapses active, see Methods), the mGluR1-mediated EPSC_{slow}¹⁰ evoked by GL stimulation was much smaller than that evoked in the same cell by ML stimulation of a similar number⁶ of synapses (Fig. 2a,b), both at 27°C in mouse (GL-evoked response was $15.4 \pm 4.6\%$ the size of the ML-evoked response in the same cell, $n = 7$, $P = 1.7 \times 10^{-6}$) and at 35°C in rat ($5.5 \pm 1.7\%$ of the ML-evoked response, $n = 7$, $P = 2.3 \times 10^{-9}$).

Synaptic crosstalk activates mGluR1 receptors

To investigate why mGluR1 activation is larger for activation of spatially adjacent synapses, we analyzed how the mGluR1-mediated EPSC_{slow} varied as a function of the number of synapses activated by ML stimulation. The size of the fast AMPA receptor mediated EPSC (EPSC_{fast}) in response to a single stimulus (Fig. 2c) was used to assess the number of synapses activated by a low or a high stimulation intensity⁶. The ratio of the variance to the mean of the EPSC_{fast} amplitude was independent of stimulus intensity (Fig. 2c, inset), consistent with the EPSC_{fast} amplitude being proportional to the number of activated synapses⁶. NBQX ($25 \mu\text{M}$) was then applied to block AMPA receptors, and trains of ten stimuli at 200 Hz (mimicking granule cell activity *in vivo*¹⁶) at the same two intensities were used to evoke an EPSC_{slow} (Fig. 2d). If all the synapses behaved independently, the EPSC_{slow} amplitude would be proportional to the number of activated synapses, and hence to the EPSC_{fast}. In fact, when the number of activated synapses (and the size of EPSC_{fast}) was increased, the EPSC_{slow} amplitude increased disproportionately (Fig. 2c-e), and the dependence of EPSC_{slow} amplitude on EPSC_{fast} amplitude had an exponent of $n = 1.4$ (Fig. 2f). Thus, synapses do not behave independently and part of the EPSC_{slow} is due to crosstalk between synapses.

We assessed quantitatively the importance of synaptic crosstalk for activating mGluR1 receptors. We estimate as ~6pA the mean EPSC_{fast} amplitude produced by a single activated synapse (see Methods). From Fig. 2f we deduce, therefore, that a single synapse activated by the 10-stimulus train used here would produce a mean EPSC_{slow} of ~34fA. Based on this, Fig. 2f shows a linear extrapolation (dotted line) predicting the EPSC_{slow} amplitude that would occur if synapses did not interact. The low and high stimulus intensities used evoked an EPSC_{slow} which was 4.7- and 8.3-fold higher than this prediction, showing that for ML stimulation the majority of the EPSC_{slow} is due to crosstalk. This provides an explanation for the much smaller EPSC_{slow} seen when spatially dispersed synapses are activated by GL stimulation, when crosstalk is minimal, as compared with parallel fibre stimulation, when crosstalk occurs (Fig. 2a,b).

mGluR1 receptors detect glutamate spillover

How does mGluR1 detect synaptic crosstalk? Crosstalk between synapses could occur intracellularly or extracellularly. The EPSC_{slow} may reflect direct activation of TRPC1 channels by mGluR1 receptors¹⁷, but ML stimulation also induces an mGluR1-mediated postsynaptic calcium increase, which may increase the EPSC_{slow}^{10,18,19}. We examined whether summation of $[Ca^{2+}]_i$ changes produced by adjacent synapses might mediate the EPSC_{slow} potentiation produced by synaptic crosstalk. Increasing the intracellular EGTA concentration 20-fold (while maintaining free $[Ca^{2+}]_i$ constant) should reduce mGluR1-evoked $[Ca^{2+}]_i$ changes, and thus reduce $[Ca^{2+}]_i$ -mediated EPSC_{slow} potentiation, but after 30 mins in whole-cell configuration the EPSC_{slow} when recording with 10mM EGTA (148 ± 24 pA, $n = 13$) was similar ($P = 0.27$) to that seen using 0.5mM intracellular EGTA (111 ± 21 pA, $n = 12$). Furthermore, the EPSC_{slow} amplitude still increased non-linearly with EPSC_{fast} amplitude (Fig. 2g). As previously reported¹⁰, the faster Ca^{2+} buffer BAPTA reduced the EPSC_{slow} amplitude by 64% (to 40 ± 25 pA, $P = 0.05$, $n = 8$; cf. ref 20) after 30 mins in whole-cell configuration. However, the EPSC_{slow} amplitude still increased non-linearly with EPSC_{fast} amplitude (Fig. 2h). We conclude that summation of $[Ca^{2+}]_i$ changes does not mediate the crosstalk detected by mGluR1.

Next we considered extracellular mediation of synaptic crosstalk. Two lines of evidence suggest that the detection of synaptic crosstalk by mGluR1 is due to glutamate spillover between synapses. First, the charge transfer of the AMPA-mediated EPSC_{fast} evoked by the stimulus trains used to evoke the EPSC_{slow} also exhibited a supralinear dependence on the number of activated synapses (Fig. 3a,b), consistent with increased spillover at higher stimulus intensities potentiating the glutamate receptor activation produced by the trains. Second, glutamate spillover is enhanced by suppression of the glial glutamate transporters GLAST and GLT-16, and the EPSC_{slow} was strongly potentiated by this manoeuvre. Knock-out of GLAST did not affect the magnitude of the EPSC_{fast}⁶, but it potentiated the EPSC_{slow} 2.2-fold (from 75 ± 27 pA in 9 wild-type cells to 165 ± 26 pA in 6 KO cells, $P = 0.03$; Fig. 3c) and less stimuli were needed to elicit a detectable EPSC_{slow} (Fig. 3d). These differences were not due to a higher expression of mGluR1 or its intracellular signalling since t-ACPD, an mGluR1 agonist, evoked a current that was not significantly different ($p=0.28$) in Purkinje cells from knock-out and wild-type mice (Fig. 3c, inset). Similarly, inhibiting GLT-1 with dihydrokainate in mice lacking GLAST potentiated the EPSC_{fast} by only 10% but produced a further $67 \pm 9\%$ potentiation of the EPSC_{slow} ($n = 3$, $P = 0.017$; Fig. 3c). The EPSC_{slow} is thus strongly increased (3.7 times for size and 4.7 times for charge transfer) when glutamate spillover is enhanced by suppression of glial glutamate transporters.

Promoting crosstalk rescues cannabinoid signalling

If mGluR1 activation and endocannabinoid signalling depend on glutamate spillover producing crosstalk between synapses, then promoting crosstalk would be expected to

rescue the EPSC_{slow} and endocannabinoid signalling that are normally greatly reduced when activating spatially dispersed synapses with GL stimulation. Consistent with this, although the EPSC_{slow} was nearly undetectable when spatially isolated synapses were activated by tetanic GL stimulation (EPSC_{slow} amplitude ~ 10 pA in rat at 35°C, Figs. 2b and 4a), it was restored when glutamate spillover was enhanced by partially inhibiting glutamate transporters⁶. In the presence of TBOA (200 μ M), GL stimulation evoked a large EPSC_{slow} (1646 ± 342 pA, $n = 3$) comparable in size to the one evoked by ML stimulation (Fig. 4b), and the post-tetanic potentiation seen normally when stimulating the granular layer (Fig. 1e) was converted into a cannabinoid mediated post-tetanic depression in all 5 cells studied (Fig. 4c).

Suppressing crosstalk prevents mGluR1 activation

Conversely, if mGluR1 mediated cannabinoid signalling is largely activated by synaptic crosstalk between adjacent activated synapses, and this signalling suppresses glutamate release sufficiently to prevent synaptic crosstalk, then subsequent activation of adjacent synapses should not activate mGluR1 receptors significantly. To test this prediction, we activated about 300 adjacent synapses with ML stimulation (4 stimuli at 200 Hz) and recorded the resulting AMPA receptor-mediated EPSC_{fast} and mGluR1-mediated EPSC_{slow} (Fig. 4d, -10 s), before switching to current clamp mode and stimulating the synapses with a train of 10 pulses at 200 Hz to activate endocannabinoid signalling. Ten seconds later (Fig. 4d, $+10$ s), the EPSC_{fast} recorded in voltage-clamp mode was reduced, but the EPSC_{slow} was almost abolished (Fig. 4e), and this abolition was blocked by AM251 (Fig. 4f,g). Thus, the mGluR1-cannabinoid signalling activated by synaptic crosstalk does indeed reduce glutamate release sufficiently to prevent crosstalk.

Discussion

We have shown that endocannabinoid signalling only occurs when nearby synapses are activated. This dependence on the spatial pattern of synapse activity is a result of mGluR1 activation being strongly dependent on crosstalk between synapses: when active synapses are spatially isolated little activation of mGluR1 is produced (Fig. 2) and hence little retrograde endocannabinoid signalling occurs (Fig. 1). However, mGluR1 is strongly activated in conditions enhancing crosstalk between synapses, such as excessive glutamate release from adjacent synapses (occurring physiologically, or during stimulation of the molecular layer as is commonly employed experimentally), or when glutamate spillover is artificially increased by blocking glutamate uptake^{6,21}. Synaptic crosstalk detection by mGluR1 results in a release of endocannabinoids which reduce presynaptic glutamate release for 20 sec, and thus will reduce synaptic crosstalk. This adjustment of glutamate release may not alter the overall level of excitation of the Purkinje cell, since retrograde cannabinoid signalling also suppresses GABA release^{22,23}. We conclude that mGluR1-mediated crosstalk detection is a homeostatic mechanism promoting synaptic independence. It will be of interest to determine whether conditions can be found that generate significant mGluR1 activation and endocannabinoid signalling without adjacent synapses needing to release sufficient glutamate to generate synaptic crosstalk.

It has recently been reported that, whereas activating the climbing fibre input to Purkinje cells while stimulating the parallel fibres leads to long term depression (LTD) of the parallel fibre EPSC, via a process dependent on mGluR1 activation⁴, no LTD is seen when the climbing fibre is stimulated while activating the granule cell axons by stimulating in the granule cell layer²⁴. The lack of LTD was attributed to the preferential activation of synapses on the ascending granule cell axon, but the lack of LTD and cannabinoid signalling seen for granular layer stimulation is unlikely to reflect a lack of mGluR1 receptors at ascending synapses since we found that the mGluR1 agonist DHPG depressed the synapses

activated from the granular layer by the same fraction as it depressed the synapses activated by stimulating the parallel fibres (Fig. 1h,i), and promoting glutamate spillover by blocking glutamate uptake rescued the mGluR1-mediated EPSC_{slow} and cannabinoid signalling when stimulating the granular layer (Fig. 4a-c). Our data suggest that the lack of LTD for granular layer stimulation may reflect the very limited activation of mGluR1 receptors that is produced by this stimulation (Fig. 2a,b), owing to the lack of synaptic crosstalk that occurs when spatially dispersed synapses are activated.

Methods

Purkinje cells were whole-cell clamped in parasagittal cerebellar slices from P18 rats or P14-21 mice at 27°C or 33-35°C (see text). Animal use was in accord with the UK Animals (Scientific Procedures) Act (1986). External solution contained (mM) NaCl 124, KCl 2.5, NaH₂PO₄ 1, NaHCO₃ 26, CaCl₂ 3, MgCl₂ 1, glucose 10, GABAzine 0.01 (to block GABA_A receptors), bubbled with 95% O₂/5% CO₂. Pipette solution contained (mM) Cs-gluconate 140, NaCl 4, HEPES 10, MgATP 4, Na₃GTP 0.5, Cs₂EGTA 0.5, CaCl₂ 0.1, pH adjusted to 7.3 with CsOH. When altering the Ca²⁺ buffer to 10mM EGTA or 13mM BAPTA, the CaCl₂ added was adjusted to maintain the free [Ca²⁺]_i at 30nM (calculated with the programme MaxChelate) and [Cs-gluconate] was decreased to maintain osmolarity. For current-clamp experiments (Fig. 1b-e, Fig. 4c-g), K⁺ replaced Cs⁺ in the pipette. For EPSP and EPSC_{slow} measurements the series resistance was not compensated but it was < 5MΩ when studying the effect of different Ca²⁺ buffering power to ensure good cell dialysis. For EPSC_{fast} measurements, series resistance was compensated to < 1MΩ. Stimuli were applied from a glass pipette placed in the slice below the Purkinje cell, either in the molecular layer to activate parallel fibres directly, or in the granular layer (which will also activate a beam of parallel fibre activity⁸); stimulus trains were applied every minute. The mean EPSC_{fast} amplitude produced by a single activated synapse (*p.i.*) at -70mV was estimated as ~6pA from the ratio of variance to mean measured for the EPSC_{fast} amplitude with the internal solution used here (Fig. 1a, inset) and the probability of release (*p* = 0.48) determined previously⁶, using $\text{variance}/\text{mean} = i_i(1-p)$, where *i_i* is the current produced when a vesicle is released. For GL stimulation the rapid time to peak of the EPSC_{fast} suggests that granule cells or ascending axons were stimulated rather than mossy fibres, which would have led to a disynaptic, delayed EPSC_{fast} (as was seen when stimulating the white matter: data not shown). Data are presented as mean ± s.e.m. Statistical comparisons were by Student's t-test.

Acknowledgments

We thank K. Tanaka for generously providing the KO mice, and B. Barbour, A. Gibb, D. Rossi, A. Silver and M. Hamann for comments on the manuscript. Supported by the EU, the Wellcome Trust and a Wolfson-Royal Society award.

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Figure 1.

Endocannabinoid-mediated plasticity seen for activation of nearby synapses is absent for stimulation of spatially dispersed synapses. (a) Stimulation sites in the molecular layer (ML) and granular layer (GL). (b,c) Effect of a 10 pulse train at 200Hz on the EPSPfast evoked at -70mV in the same rat Purkinje cell by a single stimulus, using ML (b) and GL (c) stimulation. Main traces: response to the train between EPSPfast responses to single stimuli. Insets: EPSPs 2s before and after the train. (d) Suppression of EPSPfast after the train (Ctr) for ML stimulation in 8 cells, and its block by the CB1 antagonist AM251 (5 cells). (e) For GL stimulation the train evokes a small potentiation (11 cells, filled circles) which is

unaffected by AM251 (5 cells), and pairing with mock-physiological climbing fibre stimulation¹⁵ (3 pulses delivered 100, 200 and 300msec after the start of GL stimulation) does not affect this (4 cells). **(f,g)** Effect of the cannabinoid agonist WIN 55,212-2 (5 μ M) on the EPSC_{fast} at -70mV evoked by ML **(f)** or GL stimulation **(g)**. The EPSC_{fast} was reduced to $15.3 \pm 1.4\%$ (s.e.m., 4 cells) and $19.0 \pm 2.3\%$ (4 cells) respectively (insignificantly different, $P = 0.22$). **(h,i)** Effect of the mGluR1 agonist DHPG (50 μ M) on the EPSC_{fast} at -70mV evoked by ML **(h)** or GL stimulation **(i)**. The EPSC_{fast} was reduced to $52.6 \pm 5.8\%$ and $59.8 \pm 4.2\%$ (10 cells) respectively ($P = 0.29$). DHPG suppression was measured after 1 min exposure to DHPG. All at 33°C.

Figure 2.

Absence of endocannabinoid signalling when activating spatially dispersed synapses results from mGluR1 being activated by synaptic crosstalk. **(a)** Train-evoked EPSC_{slow} (in NBQX, 27°C, -70 mV) for the same EPSC_{fast} amplitude evoked by interleaved ML and GL stimulation in mouse. **(b)** Mean EPSC_{slow} amplitude for 7 cells like (a) (EPSC_{fast} amplitude 596 ± 36 pA for ML and 572 ± 44 pA for GL stimulation; insignificantly different, *P* = 0.6) and for 7 rat cells (35°C, EPSC_{fast} amplitude 700 ± 114 pA for ML and 667 ± 67 pA for GL stimulation, *P* = 0.73). **(c)** AMPA receptor mediated EPSC_{fast} at -70 mV in a mouse Purkinje cell evoked by two intensities of ML stimulus (27°C, EPSC amplitudes are in f). Inset: Variance/mean of the EPSC_{fast} amplitude is independent of stimulus size. **(d)** In the same cell, with the same stimuli as c, the EPSC_{slow} evoked (in 25 μM NBQX, -70 mV) by 10 stimuli at 200 Hz increases more with stimulus strength than does the EPSC_{fast}. **(e)** Fractional change of EPSC_{slow} and EPSC_{fast}. **(f)** Dependence of EPSC_{slow} on EPSC_{fast} amplitude (proportional to number of fibres stimulated). Dotted line predicts relationship if synapses did not interact. **(g,h)** Fractional increase of EPSC_{fast} and EPSC_{slow} with Ca²⁺ buffering increased with EGTA (**g**, at 27°C) or BAPTA (**h**, at 35°C), with stimulus adjusted to increase EPSC_{fast} from 310 ± 20 to 1311 ± 50 pA (**g**) or from 323 ± 56 to 1164 ± 112 pA

(**h**, in 0.2 μ M NBQX to improve voltage-uniformity). All data except **b** from mouse Purkinje cells.

Figure 3.

Glutamate spillover mediates synaptic crosstalk detection by mGluR1 receptors. **(a)** AMPA receptor mediated currents in response to parallel fibre stimulus trains at two intensities (mouse Purkinje cell at 27°C). Inset: traces normalized by the amplitude of the first EPSC_{fast} of the train, demonstrating that post-train charge entry (shaded: grey, low stim; black, high stim) increases more than the number of fibres stimulated. **(b)** Dependence of charge transfer after the train on the amplitude of the first EPSC (proportional to number of fibres stimulated). Dotted line extrapolates relation calculated if synapses did not interact (as in Fig 2f). **(c)** Effect of deleting GLAST (-/-) (WT is wild-type control), and of blocking GLT-1 with dihydrokainate (DHK), on the EPSC_{slow} evoked in NBQX by trains of parallel fibre stimuli that produce a similar amplitude EPSC_{fast} (633 ± 48pA in 9 WT cells; 617 ± 36pA in 6 -/- cells; $P=0.8$). Insets: response of Purkinje cells at -70mV to 0.5μM AMPA (13 cells each) and 25μM ACPD (15 cells each) is similar in WT and -/- cells (ACPD also activates group II mGluRs, but activating group II mGluRs generates no current in Purkinje cells25). **(d)** Left: EPSC_{slow} response (in NBQX) of wild-type and GLAST KO Purkinje cells to trains of 2, 3, 5 or 10 stimuli applied to the parallel fibres at 200Hz. Right: Percentage of 9 wild-type and 10 KO cells that showed a detectable EPSC_{slow} after different numbers of stimuli.

Figure 4.

Endocannabinoid-mediated plasticity is rescued for stimulation of spatially-dispersed synapses by enhancing glutamate spillover, and inhibits glutamate release sufficiently to abolish crosstalk-activation of mGluR1. **(a-c)** Rescue of plasticity by promoting spillover. **(a)** Control response to ML and GL stimulus trains in NBQX (-70mV, EPSC_{fast} amplitude 497 ± 126 pA for ML and 560 ± 133 pA for GL, $P = 0.48$; TBOA does not affect EPSC_{fast} amplitude). **(b)** Response to same stimuli with glutamate spillover enhanced by blocking glutamate transporters with TBOA, and with CPCCOEt present to block mGluR1. **(c)** EPSP_{fast} amplitude after a train for GL stimulation in the absence (Ctr) and presence of 200µM TBOA: enhancing spillover rescues the endocannabinoid-evoked depression (5 cells). AM251 (2µM) blocked the depression rescued in TBOA (8 cells). **(d-g)** Cannabinoid signalling suppresses glutamate release sufficiently to prevent crosstalk-evoked mGluR1 activation. **(d)** EPSC_{fast} and EPSC_{slow} (in 0.2µM NBQX, to improve voltage uniformity, which reduced EPSC_{fast} amplitude by $65 \pm 4\%$, 5 cells) evoked by 4 stimuli (200 Hz) to the parallel fibres, 10 sec before (-10s) and after (+10s) a 10 stimulus train (200Hz) in current clamp mode. **(e)** After the train, suppression of glutamate release by cannabinoid signalling reduced the first EPSC_{fast} of a 4 pulse train by $44 \pm 12\%$, and reduced the peak EPSC_{fast} produced by the train by $36 \pm 6\%$, but reduced the EPSC_{slow} by $93 \pm 4\%$ (4 cells). **(f,g)** In AM251, there is little change in the EPSC_{fast} or EPSC_{slow} (10 cells). All in rat, 33°C.