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***Fmr1* deletion enhances and ultimately desensitizes CB₁ signaling in autaptic hippocampal neurons**

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Abstract

Fragile X Syndrome (FXS) is a heritable form of mental retardation caused by a non-coding trinucleotide expansion of the FMR1 gene leading to loss of expression of this RNA binding protein. Mutations in this gene are strongly linked to enhanced Group I metabotropic glutamate receptor (mGluR) signaling. A recent report found that mGluR5-dependent endogenous cannabinoid signaling is enhanced in hippocampal slices from *fmr1* knockout mice, suggesting a link between FXS and cannabinoid signaling. Alterations in cannabinoid signaling have an impact on learning and memory and may therefore be linked to some aspects of the FXS phenotype.

We have used autaptic hippocampal neurons cultured from *fmr1* knockout mice to further explore the interaction between endocannabinoid signaling and FMRP. These neurons express several robust forms of retrograde endocannabinoid signaling including depolarization induced suppression of excitation (DSE) and a metabotropic form (MSE) that results from Group I mGluR activation.

We now report that young *fmr1* neurons exhibit considerably enhanced DSE, likely via increased production of 2-AG, rather than enhanced mGluR-MSE. We find that depolarizations as brief as 50ms, which do not ordinarily produce DSE, routinely inhibited glutamate release. Furthermore, as neuronal cultures mature, CB₁-receptor signaling strongly desensitizes. Our results suggest that loss of FMRP broadly affects the endocannabinoid signaling system, possibly through local 2-AG over production. Furthermore, the net effect of the loss of FMRP may actually be diminished cannabinoid signaling due to receptor desensitization as an adaptation to 2-AG overproduction.

Keywords

Fmr1; Fragile X; cannabinoid; mGluR; CB₁

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INTRODUCTION

Fragile X Syndrome (FXS) is the most common heritable form of mental retardation, arising from an expanded and hypermethylated non-coding trinucleotide repeat of the FMR gene (Verkerk *et al.*, 1991). This leads to a deficiency of fragile X mental retardation protein (FMRP), an RNA binding protein. In addition to significant mental retardation, FXS may be associated with neurological abnormalities including seizures (Kerbeshian *et al.*, 1984). Mutations in this gene are linked to enhancement of metabotropic glutamate receptor or (mGluR) Group I signaling (Huber *et al.*, 2002) a change that may be due to increased mGluR protein synthesis (Bear *et al.*, 2004; Dolen *et al.*, 2007; Pfeiffer *et al.*, 2006). The evidence for an mGluR Group I link to FXS gave rise to an “mGluR theory” of FXS (Bear *et al.*, 2004).

The cannabinoid signaling system includes cannabinoid CB₁ receptors that are the target of exogenous phytocannabinoids such as Δ^9 -THC (Gaoni *et al.*, 1964; Matsuda *et al.*, 1990), the chief psychoactive ingredient in marijuana and hashish. This signaling system also includes endogenous cannabinoids, including 2-arachidonoyl glycerol (Stella *et al.*, 1997), a lipid messenger, and the enzymes to produce and break down this messenger (Bisogno *et al.*, 2003; Dinh *et al.*, 2002). Functionally, this CB₁-based signaling system has been shown to serve as a retrograde signaling mechanism in many neurons throughout the CNS (Kano *et al.*, 2009; Wilson *et al.*, 2001). 2-AG is produced in response to activation of diacylglycerol lipases in dendrites, either via depolarization (increasing intracellular calcium) or activation of metabotropic receptors (Bisogno *et al.*, 2003). When targeting excitatory neurons, the former form of plasticity is known as depolarization induced suppression of excitation (DSE) whereas the latter is metabotropic suppression of excitation (MSE). The consequence of DSE or MSE in glutamatergic neurons is an inhibition of glutamate release. Analogous processes targeting inhibitory neurons are referred to as DSI/MSI. DSE/DSI typically lasts for tens of seconds and occurs via inhibition of presynaptic calcium channels. MSE/I is driven by post-synaptic G_q-coupled receptor activation of phospholipase C beta (PLC β). The best-studied G_q-coupled G protein coupled receptors (GPCRs) that drive MSE/I are Group I mGluR's (which include mGluR5) and muscarinic receptors. The association between FXS and group I mGluR's as well as the association between group I mGluR's and endocannabinoids, suggests that forms of endocannabinoid-mediated synaptic plasticity involving group I mGluR's may be altered in models of FXS. Indeed, using hippocampal slices from *fmr1*^{-/-} mice, Zhang & Alger showed that mGluR MSI and iLTD (a form of mGluR5/endocannabinoid-mediated long term depression) but not DSI were enhanced in *fmr1*^{-/-} mice. In contrast, Jung et al. (Jung *et al.*, 2012) report an uncoupling of mGluRs from DGLs in the striatum and prefrontal cortex of *fmr1*^{-/-} mice.

To further explore the mechanisms of altered endocannabinoid signaling in *fmr1*^{-/-} mice early in development we made use of autaptic hippocampal neurons from these mice. These neurons express all the machinery necessary for robust DSE and MSE, and have been extensively characterized (Straiker *et al.*, 2009a; Straiker *et al.*, 2005; Straiker *et al.*, 2007). We have found that cultured *fmr1*^{-/-} hippocampal neurons do not have altered mGluR5-based MSE. Instead they initially exhibit enhanced DSE, which declines with time as the cultures age. Likely this is due to excessive 2-AG production with progressive CB₁ receptor desensitization and eventual inhibition of CB₁-mediated synaptic plasticity.

Materials & Methods

Animals and Cell culture

All animal care and experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Indiana University and conform to the

Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse (FVB.129P2-*Pde6b*⁺ *Tyrc-ch Fmr1tm1Cgr/J*, Jackson Laboratory, Bar Harbor, ME, or WT (CD1)) hippocampal neurons isolated from the CA1–CA3 region were cultured on microislands as previously described (Bekkers *et al.*, 1991; Furshpan *et al.*, 1976). Neurons were obtained from animals (at postnatal day 0–2, killed via rapid decapitation without anesthesia) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison *et al.*, 1991). Cultures were grown in high-glucose (20 mM) minimum essential media containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than 3 h after removal from culture medium (Straiker *et al.*, 2005). All electrophysiological experiments were performed exclusively on excitatory neurons. All tests were made on neurons from at least two different preparations.

Electrophysiology

When a single neuron is grown on a small island of permissive substrate, it forms synapses – or ‘autapses’ – onto itself. All experiments were performed on isolated autaptic neurons. Whole-cell, voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA, USA). The extracellular solution contained (mM) NaCl 119, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 30 and HEPES 20. Continuous flow of solution through the bath chamber (2 mL·min⁻¹) ensured rapid drug application and clearance. Drugs were typically prepared as a stock then diluted into extracellular solution at their final concentration and used on the same day. Recording pipettes of 1.8–3 MΩ were filled with solution containing (mM): potassium gluconate 121.5, KCl 17.5, NaCl 9, MgCl₂ 1, HEPES 10, EGTA 0.2, MgATP 2 and LiGTP 0.5. Access resistance was monitored and only cells with a stable access resistance were included for data analysis.

EPSC sizes were lower for *fmr1*^{-/-} neurons in both young and older cultures, with the difference increasing with age (EPSC peak amplitude for young wild type neurons: 2.9 ± 0.04 nA, n=21; for young *fmr1*^{-/-} neurons: 2.1 ± 0.05 nA, n=9; for older wild type neurons: 3.8 ± 0.5 nA, n=14; for older *fmr1*^{-/-} neurons: 2.6 ± 0.3 nA, n=9). However the difference was not statistically significant (p=0.11 for older wild type vs. older *fmr1*^{-/-} neurons).

To test for an effect of the lithium salt used in the internal solution we obtained response curves for DSE and mGluR agonist DHPG using a NaGTP salt and found that the baseline responses (EC₅₀ and maximal) were unaltered (DHPG EC₅₀ LiGTP: 1.6 μM (1.4–1.7); NaGTP 2.6 μM (1.7–3.9); DSE EC₅₀ LiGTP: 0.76 sec (0.65–0.91); NaGTP: 0.74 sec (0.65–0.84)). We also examined whether passive membrane properties in the form of leak conductance differed between wild type and mutant neurons. We found that leak conductance was unaltered (wild type: 9.0 ± 1.8 nS; *fmr1*^{-/-}: 10.2 ± 2.8 nS).

Data analysis

Data are reported as mean $\pm$ SEM (except EC₅₀ and t_{1/2} data, which are reported as mean (95% CI)). Non-linear regression was used to fit the concentration response curves. Treatment effects on evoked EPSCs were evaluated using one-way ANOVA with Bonferroni multiple comparison tests where indicated. Statistical significance is indicated as follows: *p < 0.05. All graphs and statistical analyses were generated using GraphPad Prism 4.0 software (Hearne Scientific Software, Chicago, IL, USA).

RESULTS

Depolarization of autaptic hippocampal neurons can result in a form of retrograde inhibition termed depolarization induced suppression of excitation (DSE (Straiker *et al.*, 2005)). This can be quantified by a series of successively longer depolarizations (50ms, 100ms, 300ms, 500ms, 1sec, 3 sec, 10 sec) resulting in progressively greater inhibition of neurotransmission (Straiker *et al.*, 2012). This produces a “depolarization-response curve” that permits the characterization of some pharmacological properties of cannabinoid signaling including the calculation of an effective-depolarization 50 (ED50), corresponding in this case to the duration of depolarization that results in 50% of the maximal response. We found that *fmr1*^{-/-} neurons between 8 and 10 days in culture were much more sensitive to brief depolarizations than wild-type neurons. As a consequence the ED50 was shifted from 760ms (Fig. 1, 95% CI: 645–906) to 234ms (95% CI: 201–274). The maximal effect was unaltered (Fig. 1., Relative EPSC charge after 10 sec depolarization (1.0 = no inhibition) in WT: 0.38 ± 0.06 , n=8; and *fmr1*^{-/-}: 0.36 ± 0.04 , n=7). In principle, enhanced endocannabinoid signaling may reflect increased 2-AG production, decreased 2-AG degradation, or increased sensitivity of pre-synaptic CB₁ signaling. To test whether the sensitivity of presynaptic CB₁ signaling was increased we applied increasing concentrations of 2-AG to WT and *fmr1*^{-/-} neurons. We found that the concentration-response curves for 2-AG were similar (WT EC50 (95% CI): 261nM (240–283); *fmr1*^{-/-}: 185nM (106–323), overlapping 95% CI). This indicates that the enhancement of cannabinoid signaling is likely not due to increased CB₁ receptor sensitivity. In excitatory autaptic cultures 2-AG is primarily degraded by monoacylglycerol lipase (MGL)(Straiker *et al.*, 2009a). Decreased MGL activity will prolong the time course of DSE (Straiker *et al.*, 2009a), thus we tested whether the DSE recovery time courses were altered in young WT vs. *fmr1*^{-/-} cultures. However, the half-lives ($t_{1/2}$) of DSE in cultures from the two genotypes were identical ($t_{1/2}$ for young WT neurons: 42.2 sec (95% CI: 37–49); for *fmr1*^{-/-} neurons: 41.5 sec (95% CI: 36–48)). Thus, the most likely explanation for the increased DSE in *fmr1*^{-/-} cultures is an increase in 2-AG production.

As DHPG-induced MSI has been reported to be enhanced in hippocampal slices from *Fmr1*^{-/-} mice (2–4 months of age), we also tested autaptic Group I mGluR MSE (Straiker *et al.*, 2007). In contrast to Zhang & Alger who found that the Group I mGluR agonist DHPG more potently elicited MSI, the effect of a low concentration of DHPG (1μM) was unaltered in the autaptic neurons. Surprisingly, the EC50 for DHPG-induced MSE exhibited a small, but statistically significant rightward shift in *fmr1*^{-/-} cultures (EC50 in WT: 1.56μM (95% CI: 1.40–1.74); in *Fmr1*^{-/-}: 2.52μM (95% CI: 2.06–3.07)). This suggests that increased Group I mGluR signaling is unlikely to explain the increased sensitivity of DSE. Autaptic MSE in cultures from *fmr1*^{-/-} continued to be mediated by mGluR5 as it was eliminated by the mGluR5 antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP; 5μM, Figure 2).

In perhaps our most notable finding, we observed that DSE responses diminished as the neuronal cultures from *fmr1*^{-/-} mice aged. Maximal responses to depolarization after 14–17 days in culture were substantially diminished in *Fmr1*^{-/-} cultures relative to young (8–10 days in culture) *fmr1*^{-/-} or WT counterparts (Fig. 3A; Relative EPSC charge after 10 sec depolarization: 0.48 ± 0.09 , n=5). The ED50 was shifted to the right (ED50 older culture: 1.32 sec (95% CI: 0.82–2.11 sec); younger culture: 0.23 sec (95% CI: 0.20–0.27)), as was the EC50 for 2-AG (Fig 3B; EC50 older culture: 1.72μM (95% CI: 1.51–1.96μM); younger culture: 185nM (95% CI: 106–322nM). EPSC amplitudes of WT and *fmr1*^{-/-} neurons did not change with age in culture (see Methods). However, inhibition of glutamate release by other presynaptic G_{i/o} G protein-coupled receptors remained intact, with GABA_B and

adenosine A1 responses unchanged with age in culture (Fig 3C, D). This indicates that CB₁ responses desensitize, presumably as a consequence of increased 2-AG production.

DISCUSSION

Our chief findings are that CB₁-mediated DSE, a form of depolarization-dependent retrograde inhibition, is profoundly enhanced in young *fmr1*^{-/-} cultures. This is most likely due to enhanced 2-AG production, with the result that CB₁ receptors ultimately desensitize as the cultures age. This finding is surprising in several respects. First, the net effect of an absence of FMR protein in this model system is in fact a diminishment of CB₁-dependent cannabinoid signaling. Second, we see a clear enhancement of DSE despite the absence of an effect on DSI in brain slices (Zhang *et al.*, 2010). Third, our results run counter to the mGluR model insofar as we see no statistically significant alteration in maximal responses for autaptic mGluR Group I dependent MSE.

The reported enhancement of Group I mGluR signaling in FXS models, and the enhancement of endocannabinoid signaling (mGluR MSI) in slices from *fmr1*^{-/-} mice (Zhang *et al.*, 2010) led us to the expectation that autaptic mGluR-dependent MSE would also be enhanced. In particular, Zhang & Alger (2010) studied potential alterations of three forms of cannabinoid signaling in wild type vs. *fmr1*^{-/-} acute hippocampal slices: DSI, mGluR-initiated eCB short term-depression of evoked IPSCs (MSI) and eCB-dependent LTD of IPSCs (eCBiLTD). DSI was unaltered but MSI was increased and the *fmr1*^{-/-} neurons were more susceptible to eCB-iLTD. Because levels of mGluR1, mGluR5 and CB₁ protein were unaltered, the authors concluded that the changes in MSI and iLTD occurred as a result of enhanced coupling of mGluR's to eCB mobilization. We also observed an enhancement of 2-AG mobilization, but in contrast to Zhang & Alger it was *DSE* that was markedly sensitized, while mGluR-dependent signaling was at most modestly altered. Furthermore, with increasing time in culture, the 2-AG responses desensitized, decreasing the influence of endocannabinoids on synaptic signaling.

Given the difference in our findings, enhanced metabotropic 2-AG mobilization in hippocampal slices vs. enhanced depolarization-dependent 2-AG mobilization in cultured hippocampal neurons, it is worth reviewing qualitative differences between these forms of 2-AG mobilization. Under normal circumstances, DSE and MSE each mobilize 2-AG via activation of diacylglycerol lipases (DGLs), but they differ in the way that they bring about DGL activation (Bisogno *et al.*, 2003; Hashimoto *et al.*, 2007). MSI/MSE occurs via G_q-coupled Group I mGluR and PLC β activation (Hashimoto *et al.*, 2005; Maejima *et al.*, 2001). PLC β in turn forms the 2-AG precursor DAG and IP₃, which may contribute to release of calcium from internal stores. In contrast, DSE occurs via depolarization-induced postsynaptic calcium increases that activate DGL, without obligatory PLC β activation (Brenowitz *et al.*, 2003; Gao *et al.*, 2010; Hashimoto *et al.*, 2008), though autaptic DSE includes calcium release from intracellular stores (Straiker *et al.*, 2005). Our confirmation that GABA_B and adenosine A1 signaling remain intact in the older *fmr1*^{-/-} neurons shows that G_{i/o}-mediated inhibition of neurotransmission is not fundamentally altered by the absence of the FMR protein. Instead, in terms of presynaptic modulation, the consequences may be specific to the CB₁-based cannabinoid signaling system.

Our finding of potentiated signaling may be seen as complementary to those of Zhang & Alger, who studied cannabinoid modulation of GABA-based inhibitory signaling as opposed to glutamate-based excitatory signaling. Recent work by Jung *et al.* (Jung *et al.*, 2012) offers a completely different profile in *fmr1*^{-/-} mice, albeit at excitatory synapses in prefrontal cortex and striatum. Instead of potentiation of 2-AG mobilization they found that mGlu5-dependent 2-AG mobilization was *compromised* in the *fmr1*^{-/-} mice. Their findings were

supported by evidence that loss of FMRP leads to mistargeting of DGL α mRNA, increasing the distance between mGluR5/PLC β , and DGL α , which reduces mGluR5—DGL α functional coupling and markedly diminishes endocannabinoid-dependent LTD. However, in our studies with cultured hippocampal neurons, the mGluR-coupling remains intact at excitatory synapses. It is possible that other factors may account for the observed differences. For instance, the *fmr1*^{-/-} strain used in this work (FVB) differed from that used by Zhang et al. (C57), while Jung et al. used a mix of C57 and FVB-based strains. It should also be noted that our own work made use of two different strains of mice (FVB and CD1), however the core observation of desensitization of CB₁ signaling occurred within strain; nor did we observe desensitization in C57 cultures of increasing age (data not shown) indicating that in two wild type strains CB₁ desensitization does not occur on its own. It is also possible that DSE is intrinsically more prone to desensitization than DSI as it requires higher concentrations of 2-AG to produce an equivalent suppression of synaptic currents in some preparations (Ohno-Shosaku *et al.*, 2002), though not in autaptic neurons (Straiker *et al.*, 2009b). We have found that the rate of recovery of fast autaptic DSI is due to the cooperative breakdown of 2-AG by monoacylglyceride lipase (MGL) and cyclooxygenase 2 (COX2) in contrast to the slower DSE that relies on MGL. As such, the cellular machinery supporting DSE may be less well equipped to deal with overproduction of 2-AG before CB₁ desensitization occurs. It is additionally possible that the age at which autaptic neurons are harvested (early postnatal) contributes to the observed profile and to the differences between our results and other studies. Zhang et al. and Jung et al. both used adult animals (2–4, and 1–3 months, respectively). Because FXS is a developmental disorder, there are advantages to understanding the alterations in endocannabinoid signaling during early stages of postnatal development as was done here. Lastly, it is possible that differences between autaptic DSE and DSE/DSI described thus far in brain slices may go some way to explaining the differences seen. In particular, autaptic DSE appears to rely more heavily on calcium from internal calcium stores rather than on post-synaptic influx through calcium channels. This stands in contrast to characterizations of DSE/DSI in hippocampal slices (Brenowitz *et al.*, 2003; Kreitzer *et al.*, 2001). As a consequence, autaptic DSE may share some qualities in common with mGluR-dependent MSE. The differences between our results and those of Jung et al. and Zhang et al., whether due to the brain region, developmental stage, the preparation, the strain, temperature or some other cause, nonetheless underscores the diversity of consequences of *fmr1* deletion. Perhaps this diversity should be expected given that FMR protein serves as a regulator of protein expression, and is consequently well-placed to alter the function of numerous proteins (Bear *et al.*, 2008), which will have diverse cellular effects, even on a signaling pathway utilizing a single neuromodulator.

The modulation of cannabinoid signaling is of particular interest for FXS research, partly because of the ubiquity of cannabinoid CB₁ receptors (Herkenham *et al.*, 1990), but also because of their extensive role in neuromodulation and their implication in important physiological processes including learning and memory and seizures (Kano *et al.*, 2009; Lutz, 2004). As such, alterations of the cannabinoid signaling system may participate in the expression of FXS and modulating endocannabinoid signaling may serve as a therapeutic avenue to ameliorate some behavioral symptoms, as recently reported (Jung *et al.*, 2012). In addition, a desensitization-induced decline in cannabinoid inhibition of excitatory synapses may favor an increased propensity to uncontrolled neuronal firing and an increased risk for seizures.

In summary, we have found that initially cannabinoid inhibition of excitatory neurotransmission is enhanced in autaptic hippocampal neurons cultured from *fmr1* knockout mice. However, counter to expectations, this enhancement is independent of mGluR5 signaling, instead occurring when endocannabinoids are produced by depolarization (DSE). The long term consequence of this enhanced CB₁ signaling is

desensitization of CB₁ receptors and a *decline* in (endo)cannabinoid signaling. By identifying a distinct alteration of cannabinoid signaling by *fmr1* deletion, our results underscore the breadth of consequences of *fmr1* deletion and also offer a novel mechanism for FMRP regulation of neurotransmission.

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Abbreviations

DSE	depolarization-induced suppression of excitation
FXS	Fragile X Syndrome
mGluR	metabotropic glutamate receptor
FMRP	fragile X mental retardation protein
MSE	metabotropic suppression of excitation
2-AG	2-arachidonoyl glycerol
EPSC	excitatory postsynaptic current
DHPG	dihydroxyphenyl glycine
MPEP	2-methyl-6-(phenylethynyl)-pyridine
CPA	cyclopentyladenosine
LTD	long-term depression.

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Highlights

- In a mouse model of Fragile X syndrome (FXS), endocannabinoid signaling is enhanced.
- However, this ultimately leads to CB1 cannabinoid receptor desensitization.
- The net impact of *fmr1* deletion is *diminished* cannabinoid signaling.
- This is the first evidence that *fmr1* deletion affects endocannabinoid-mediated DSE.
- Because mGluR signaling is unaltered in this model, this runs counter to the ‘mGluR’ model of FXS.

Figure 1.

DSE is enhanced in *fmr1*^{-/-} autaptic hippocampal neurons with no change in CB₁ receptor sensitivity. A) Depolarization-response curves for WT and *fmr1*^{-/-} autaptic neurons. B) Sample time courses for DSE in response to 500ms depolarization in WT and *fmr1*^{-/-} neurons. Inset shows sample EPSCs from same neurons (WT, top; *fmr1*^{-/-} bottom) at time points indicated. C) Concentration-response curves for 2-AG in WT and *fmr1*^{-/-} neurons.

Figure 2.

Group I mGluR responses are non-significantly increased in *fmr1*^{-/-} vs. WT autaptic neurons. Concentration response curves for inhibition in response to varying concentrations of Group I mGluR agonist DHPG in WT (black squares) and *fmr1*^{-/-} (red circle) neuronal cultures. Black triangle shows DHPG (10 μ M) response in presence of the mGluR5 blocker, MPEP (5 μ M) in *fmr1*^{-/-} cultures. *fmr1*^{-/-} points are non-significantly different vs. WT at each concentration tested, 2-way ANOVA with Bonferroni posthoc test.

Figure 3.

CB₁ receptors desensitize with age in *fmr1*^{-/-} neurons. A) Depolarization-response curve in young (8–10 days in culture (DIC)) vs. old (14–17 DIC) *fmr1*^{-/-} neuronal cultures. B) Concentration response curve for 2-AG in older *fmr1*^{-/-} cultures. WT and *fmr1*^{-/-} young cultures shown from Fig 1 for reference. C) EPSC inhibition in young vs. old cultures for the indicated concentrations G_{i/o}-coupled GPCR agonists: CB₁ (2-AG), GABAB (baclofen), and Adenosine A1 (CPA). p<0.05 1 way ANOVA with Bonferroni posthoc test for 2-AG in young vs. older cultures. D) Sample time course from a neuron after 14 days in cultures showing little EPSC inhibition in response to increasing concentrations of 2-AG (1nM–5μM, steps indicate increasing concentrations) followed by robust inhibition from baclofen (25μM) and cyclopentyladenosine (CPA, 1μM).