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Muscarinic M1 Receptor Modulation of Synaptic Plasticity in Nucleus Accumbens of Wild-Type and Fragile X Mice

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Supporting Information

ABSTRACT: We investigated how metabotropic acetylcholine receptors control excitatory synaptic plasticity in the mouse nucleus accumbens core. Pharmacological and genetic approaches revealed that M1 mAChRs (muscarinic acetylcholine receptors) trigger multiple and interacting forms of synaptic plasticity. As previously described in the dorsal striatum, moderate pharmacological activation of M1 mAChR potentiated postsynaptic NMDARs. The M1-potentiation of NMDAR masked a previously unknown coincident TRPV1-mediated long-term depression (LTD). In addition, strong pharmacological activation of M1 mAChR induced canonical retrograde LTD, mediated by presynaptic CB1R. In the fmr1+/- mouse model of Fragile X, we found that CB1R but not TRPV1 M1-LTD was impaired. Finally, pharmacological blockade of the degradation of anandamide and 2-arachidonylglycerol, the two principal endocannabinoids restored fmr1+/− LTD to wild-type levels. These findings shed new light on the complex influence of acetylcholine on excitatory synapses in the nucleus accumbens core and identify new substrates of the synaptic deficits of Fragile X.

KEYWORDS: Synaptic plasticity, endocannabinoid, acetylcholine, muscarinic receptors, CB1R, TRPV1R, accumbens, fragile X

INTRODUCTION

Acetylcholine is a major neurotransmitter and modulator in the CNS acting via ionotropic nicotinic and metabotropic muscarinic receptors. It is involved in a plethora of cognitive and executive functions.1

Five distinct muscarinic acetylcholine receptor (mAChR) subtypes (M1−M5) are expressed in the brain,2,3 and quantitative autoradiographic studies have demonstrated that the striatum has one of the highest concentrations of muscarinic receptors,4 highlighting the importance of muscarinic signaling in the basal ganglia. The role of dorso-striatal cholinergic transmission in the control of voluntary movement is well established.5 The ventral part of the striatum, the nucleus accumbens, has been conceptualized as the “gatekeeper” of the basal ganglia, because it is ideally positioned to integrate signals originating from limbic and cortical areas and modulate reward-related motor output.6 The accumbens has been extensively studied in the context of drug abuse and addiction related behaviors.7,8 More recently, its role in rewarding social behaviors and social interactions has been highlighted.9−11 Muscarinic and nicotinic receptors in the accumbens are necessary for the acquisition of appetitive tasks,12 food and drug satiety.13 How cholinergic inputs modulate glutamatergic synaptic transmission onto medium spiny neurons (MSN) remains poorly understood.

M1 mAChR activation triggers long-term depression (LTD) in the perirhinal cortex,14 the visual cortex,15 the hippocampus,16,17 the prefrontal cortex18 as well as axonal signal processing.19 In contrast to the dorsal striatum, how mAChR modulate synaptic plasticity in the accumbens remains largely unknown.

Cholinergic dysfunction has been implicated in the pathophysiology of schizophrenia, mood disorders, as well as neurodegenerative disorders including Alzheimer’s and Parkinson’s diseases.5,20,21 Fewer studies have addressed the implication of the cholinergic system in Fragile X syndrome (FXS), the most common monogenic cause of inherited intellectual disability and a leading cause of autism.22−24 The disease is caused by mutation of a single X-linked gene called fmr1.25 The Fragile X mental retardation protein (FMRP) is a 71 kDa protein which regulates the transport and translation of

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Figure 1. Direct pharmacological activation of M1 AChR triggers STD and LTD in the nucleus accumbens core. (A) Representative field recording showing the effects of 10 μM and 100 μM Carbachol. The lowest dose (10 μM) induced a strong but transient depression of synaptic responses (short-term depression, STD). The highest concentration of Carbachol (100 μM) induced a robust LTD. (B) Averaged fEPSPs for three different Carbachol concentrations (1 μM, n = 8; 10 μM n = 9; 100 μM n = 13). All three concentrations induced STD but only 100 μM Carbachol induced LTD. (C) Pearson’s correlation showed no dependence of LTD magnitude on STD. (D) 100 μM Carbachol mediated LTD was highly sensitive to the M1 antagonist VU0225035. n = 12, *P < 0.05.

RESULTS

Direct Activation of Muscarinic M1 Receptors Induces LTD in the Accumbens Core. Acute cholinergic stimulation induces synaptic plasticity in several cortical areas.18,37 Striatal medium spiny neurons (MSN) receive cholinergic innervation from the brain stem38 and local giant cholinergic interneurons.39 We first tested the hypothesis that G-protein coupled muscarinic acetylcholine receptors (mAChRs) can modulate excitatory synapses in the accumbens.

Recording field EPSPs from MSN in the accumbens core, we first challenged slices from adult wild-type mice with brief (10 min) applications of the large spectrum muscarinic agonist Carbachol. Figure 1A shows the individual field responses in two representative experiments. Bath perfusion with 10 μM Carbachol induced a short lasting and fully reversible depression (STD), which returned to baseline levels after 20 min. In contrast, bath-perfusion of 100 μM Carbachol induced a sustained LTD of synaptic efficacy in the accumbens core. Figure 1B summarizes the average field responses for the three different concentrations tested. All three concentrations induced significant STD in comparison to baseline response (1 μM: 79.27 ± 5.373, p = 0.006, n = 8; 10 μM: 52.89 ± 3.730, p < 0.0001, n = 9; 100 μM: 42.22 ± 3.249, p < 0.0001, n = 13; one-sample t-test). There was a concentration-dependent difference in the amount of STD (one-way ANOVA p < 0.0001; with Holm-Sidak’s multiple comparisons test: 1 μM vs 10 μM p = 0.0002; 1 μM vs 100 μM p < 0.0001; 1 μM vs 10 μM; 10 μM vs 100 μM p = 0.0216). From our results, it is clear that LTD was triggered solely in response to the highest dose of Carbachol (1 μM: 96.48 ± 6.677, p = 0.6146; 10 μM: 107.3 ± 4.661, p = 0.1555; 100 μM: 76.90 ± 4.190, p < 0.0001; one-sample t-test). Figure 1C shows the lack of correlation between

more than 850 mRNAs in the brain and especially in synapses.26−28 In humans with FXS, the loss of FMRP results in a variety of neurological symptoms widely associated with dysfunctional synaptic plasticity in critical brain regions such as the cortex, hippocampus, and amygdala.29,30 In the fmr1−/− mice model of FXS, structural and functional deficits have been reported in multiple brain areas, most notably the hippocampus, cortex but also the striatum and accumbens.31−35 Although acetylcholine plays a key role in arousal and reward and FXS patients commonly show symptoms in associated behaviors,36,37 how acetylcholine-accumbens plasticity is affected in fmr1−/− mice is currently not known.

Here we used pharmacological methods to explore acetylcholine-dependent synaptic plasticity and its underpinnings in the accumbens core region of wild-type and fmr1−/− mice. We report that two types of M1 mAChR-mediated LTD and one long-term potentiation (LTP) cohabit at excitatory synapses onto accumbens core MSN. Moderate pharmacological activation of M1 mAChR induces both a TRPV1-mediated LTD and a potentiation of NMDAR, two phenomena that occlude mutually. In response to strong activation, M1 mAChRs induce a CB1R-mediated retrograde LTD. Finally, we show that CB1R-mediated but not TRPV1-mediated M1-LTD was affected in fmr1−/− mice and that pharmacological blockade of the degradation of anandamide and 2-arachidonoylglycerol, the two principal endocannabinoids (eCBs), restored LTD in the Fragile X mouse model.

The results provide a previously unidentified link between M1 mAChR-mediated accumbal synaptic plasticity and cognitive dysfunction in Fragile X and suggest the cholinergic system as a novel therapeutic target.
shows the average NMDAR field recordings, and the inset illustrates two averaged field responses (pre- and post-Carbachol application): 10 μM Carbachol induced a rapid short-term depression of NMDAR mediated fEPSPs (66.23 ± 4.884, n = 5, p = 0.0023, one-sample t test; Figure 2B) followed by a LTP after drug washout (137 ± 12.6, p = 0.0425, one-sample t test), whereas 100 μM Carbachol also induced rapid short-term depression of NMDAR fEPSPs (43.58 ± 10.67, n = 8, p = 0.2156, one-sample t test) followed by a trend toward LTD after drug washout (81.22 ± 13.79, p = 0.2156, one-sample t test). Thus, NMDAR-mediated synaptic responses in the accumbens are potentiated by mAChR, as previously reported in the dorsal striatum.4,51

We hypothesized that LTP of NMDAR synaptic potentials might mask the LTD of AMPAR fEPSPs. We reasoned that NMDAR antagonism could unmask LTD in slices perfused with low Carbachol. In support of this scenario, bath perfusion of 10 μM Carbachol in the presence of the NMDAR antagonist D-AP5 (50 μM) now induced a significant LTD (85.28 ± 3.365, n = 15, p = 0.0006, one-sample t test). This LTD was blocked in accumbens slices incubated with the M1 specific receptor antagonist VU0225035 (10 μM) (94 ± 3.103, n = 8, p = 0.1126, one-sample t test, Figure 2B).

Together our data show that moderate activation of M1 mAChR with 10 μM Carbachol induces concomitant AMPAR LTD and NMDAR LTP. Although “low Carbachol” largely modulates AMPAR and NMDAR function, the change in synaptic transmission can only be unmasked by blocking NMDAR.

**TRPV1 Receptors, not CB1R, Mediate “Low Carbachol” LTD.** Different LTD pathways allow a single neuron to engage either presynaptic CB1R or postsynaptic TRPV1 receptors.42 It has been long established that M1 mAChR can engage either presynaptic CB1R or postsynaptic TRPV1 receptors.43,44 In the bed nucleus stria terminalis42 and also the accumbens, eCB engage presynaptic CB1R and/or postsynaptic TRPV1R depending on cell type and stimulation patterns.43,44

Hence, we explored the locus of LTD expression and the mechanism of the low Carbachol LTD. A series of experiments was performed in the presence of D-AP5 to block NMDAR and unmask LTD (Figure 3). We found that induction of low Carbachol LTD was abolished in slices incubated with the selective TRPV1 receptor antagonist AMG9810 (98.21 ± 5.984, n = 5, p = 0.7801, Student’s t test; Figure 3). However, low Carbachol LTD was unaffected by the CB1R antagonist SR141716A (83.64 ± 5.932, n = 10, p = 0.0220, Student’s t test; Figure 3C). We verified the locus of LTD by simply quantifying the changes in the paired-pulse ratio from the field excitatory responses (Figure 3A, lower trace). The paired-pulse ratio quickly returned to baseline (p = 0.5818, Student’s t test), pointing toward a postsynaptic expression mechanism of LTD expression. Taken together, these data strongly suggest that low Carbachol induces a postsynaptic LTD mediated by TRPV1 receptors.

**“High Carbachol” LTD Depends on Endocannabinoid Retrograde Signaling and CB1R.** We next examined whether CB1R and/or TRPV1 were responsible for high Carbachol/M1 mAChR LTD. The CB1R antagonist SR14176A (5 μM) efficiently blocked high Carbachol LTD (96.44 ± 5.061, n = 7, p = 0.5082, Student’s t test Figure 4) whereas the TRPV1 receptor antagonist AMG9810 (10 μM) did not prevent the expression of LTD (89.38 ± 1.966, n = 7, p = 0.00398). 

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**Potentiated Synaptic NMDA Currents Mask “Low Carbachol” LTD.** In contrast with previous studies, including one from our laboratory demonstrating LTD induced by low concentrations of Carbachol in the prefrontal cortex (PFC)18,40 we were surprised to observe that bath application of 10 μM Carbachol was not sufficient to induce LTD. Such a discrepancy could be due to low M1R expression or poor M1R-coupling efficiency to downstream effectors or result from multiple compensating/agonizing M1R-mediated synaptic effects. Noteworthy, activation of M1R potentiates NMDAR currents and offsets LTD in the dorsal striatum.4,51 To test if a similar process occurs in the accumbens core, we simply re-registered NMDAR-mediated fEPSP in artificial cerebrospinal fluid (ACSF) containing 0 Mg2+ and 100 μM CNQX to block ionotropic glutamate receptors fast synaptic potentials (i.e., mediated by AMPAR/KAR). fEPSP recordings were chosen to allow for the direct comparison with our LTD experiments and their robustness to pharmacological treatments. Figure 2A

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**Figure 2. NMDAR modulates mAChR-induced synaptic plasticity.** (A) Averaged field recordings of NMDAR responses. In response to bath-application of 10 μM Carbachol, transient depression was followed by a marked LTP of NMDAR-fEPSP. In response to bath-application of 100 μM Carbachol, the transient depression was followed by a trend toward LTD of NMDAR-fEPSP. Inset: representative field response before and after 10 μM Carbachol application. (B) Average field recordings of AMPAR responses. Ten μM Carbachol mediated LTD was unmasked after blocking NMDAR with dAPV. This LTD was blocked with the M1 specific receptor antagonist VU0225035 (50 μM). *p < 0.05.
We conclude that high Carbachol LTD requires CB1R, not TRPV1. In the extended amygdala and accumbens, both mGluR1 and mGluR5 participate to eCB-LTD.42,46 In striking contrast, neither the mGluR5 specific antagonist MPEP nor the mGluR1 specific antagonist CPCCoEt prevented from high Carbachol LTD ($p = 0.0142; n = 8$, Student’s t test, data not shown).

We verified that high Carbachol LTD had a presynaptic locus of expression as typically expected if CB1R were implicated.45 Indeed, high Carbachol LTD was paralleled by a significant enhancement of the paired-pulse ratio ($p = 0.0142; n = 8$, Student’s t test, data not shown). Together these data suggest that M1 mAChR LTD induced by high Carbachol is mediated by eCB acting at presynaptic CB1R.

CB1R-Mediated but Not TRPV1-Mediated LTD Is Impaired in fmr1-/y Mice. The postsynaptic mGluR5/eCB signaling complex is impaired at accumbens synapses of fmr1-/y mice.33,35 M1 mAChR and mGluR1/5 are Gq/11-protein coupled receptors with common downstream effectors including eCB.32 Having established that activation of M1 mAChR receptors triggers eCB-mediated LTD via CB1R or TRPV1, we next tested low and high Carbachol LTD in adult fmr1-/y mice. As for Figure 3, the experiments to characterize low Carbachol/TRPV1R-dependent LTD were performed in the presence of D-AP5 to block NMDAR and unmask LTD. The data show that low Carbachol/TRPV1R-dependent LTD was readily induced in fmr1-/y mice (84.21 ± 3.9, $n = 5$, $p = 0.0155$, Student’s t test; Figure 5A) and not different from controls ($p = 0.743$, one-way ANOVA).

On the contrary, high Carbachol/CB1R-mediated LTD was not abolished (94.55 ± 2.084, $n = 17$, $p = 0.0187$, Student’s t test) but significantly reduced in fmr1-/y mice compared to WT littermates (Figure 5B, $p = 0.0029$, unpaired t test).

In the fmr1-/y mouse model, enhancing 2-AG levels by blocking its degradation with the selective monoacylglycerol lipase inhibitor JZL184, normalized synaptic and behavioral impairments.32 We attempted a similar strategy to rescue deficient high Carbachol LTD in fmr1-/y. Indeed, blocking 2-AG degradation with JZL184 restored high Carbachol LTD in

Figure 3. Postsynaptic TRPV1 mediates low carbachol LTD. (A) Averaged field recordings of AMPAR responses showing that preincubation with the TRPV1R antagonist AMG 9810 (10 μM) completely prevented the induction of LTD by 10 μM Carbachol. (B) Example traces of average field response before and after Carbachol application. (C) Summary bar graph of the pharmacological experiments characterizing low (10 μM) LTD. LTD was blocked by the application of the TRPV1R antagonist AMG9810 (10 μM) but not the CB1R antagonist SR141716a (5 μM). Error bars represent mean ± SEM. §$p < 0.05$, Student’s t test. The number in each bar indicates the number of experiments.

Figure 4. Presynaptic CB1R mediates high carbachol LTD. (A) Averaged field recordings of AMPAR responses. In slices preincubated with the CB1R antagonist SR141716A (5 μM), 100 μM Carbachol induced STD but not LTD. (B) Example traces of average field response before and after Carbachol application. (C) Summary bar graph of all pharmacological experiments characterizing the effects of high-Carbachol: LTD was blocked by the application of the CB1R antagonist SR141716a but not by the TRPV1R antagonist AMG9810 (10 μM). Error bars represent mean ± SEM. *$p < 0.05$, unpaired t test; §$p < 0.05$, Student’s t test. The number in each bar indicates the number of experiments.
rescues high Carbachol LTD via enhancing CB1R but not TRPV1 signaling.

Neither selective monoacylglycerol lipase inhibitor JZL184 nor the FAAH inhibitor URB-597 had a significant effect on high Carbachol LTD in wildtype littermates (see Supplementary Figure 1).

**DISCUSSION**

The principal results of this study are (1) that, at accumbens core synapses, M1 mAChR control two forms of endocannabinoid mediated LTD that differ in their signaling pathways and locus of depression and (2) that lack of FMRP expression selectively impairs CB1R-mediated plasticity.

Low concentration of Carbachol engaged TRPV1 receptors and caused a reduction in postsynaptic AMPAR. These results are in agreement with a previous report showing that activation of TRPV1 via the endocannabinoid anandamide induces LTD in the accumbens.59 Indeed, anandamide has been demonstrated to be an endogenous TRPV1 agonist.52

It is important to note that the low Carbachol LTD was unmasked when blocking NMDAR. Several mechanisms could explain the mAChR-mediated synaptic potentiation of NMDAR in accumbens MSN. First, Calabresi et al. have demonstrated that elevation of endogenous acetylcholine increases the conductance of NMDAR.5 Second, M1-dependent inhibition of SK channels boosts synaptic potentials.41 Although we cannot unequivocally determine which of these mechanisms mediates NMDAR potentiation, we clearly demonstrate that blocking this potentiation unmasks "low Carbachol" LTD.

The high Carbachol LTD required CB1R-mediated presynaptic inhibition of glutamate release. In the nucleus accumbens core, eCB-LTD implicates postsynaptic mGluR5, the production of 2-AG that retrogradely activates presynaptic CB1R.44,45 Both mGluR5 and M1 mAChR are Gq-coupled receptors, that engage similar downstream plasticity mechanisms.53 M1 mAChR regulate inhibitory and excitatory synapses via 2-AG and CB1R.54–58 Thus, the current data add to the growing list of central synapses where 2-AG is the principal mediator of eCB mediated GPCR synaptic plasticity.

Whether low and high Carbachol LTD are induced simultaneously in response to strong M1 mAChR stimulation or whether they exclude each other is not completely clear: the slight decrease in LTD after application of a TRPV1 antagonist, which would demonstrate a summation of plasticity did not reach statistical significance (see Figure 4C). The two forms of LTD could engage different signaling pathways by recruiting anandamide for postsynaptic LTD and 2-AG for presynaptic LTD. Indeed, that both eCBs are engaged in M1-LTD is supported by the present observation that LTD in fmr1-/y mice is rescued by blocking the degradation of either anandamide or 2-AG. How the activation of M1 can lead to the engagement of two different endocannabinoid signaling pathways remains to be determined.

Although the production of both endocannabinoids has been shown to depend on GPCR activation, this production can also be state dependent, e.g., depend on activation of voltage gated calcium channels59 which could bias the production of one eCB over the other depending on the degree of activation. The two forms of plasticity could also be expressed separately in the two subtypes of medium spiny neurons (i.e., D1R- or D2R-expressing). Although subtype specific synaptic plasticity mechanisms have been reported with various induction
protocols, the animal models used have been questioned. The unimodal distribution of LTD observed in patch clamp experiments does not support the idea that CB1R and TRPV1R are expressed in different MSN subtypes (Supplementary Figure 2).

In fmr1−/− mice, only high CB1R-mediated LTD was ablated, and TRPV1R-mediated LTD was normal. Inhibition of either 2-AG or anandamide degradation restored CB1R-LTD. Our results are compatible with recent reports showing that blocking the FAAH inhibitor with URB-597 improves performance in the passive avoidance test and social impairments in fmr1−/− mice.

The complex regulation of synaptic plasticity in the accumbens by M1 mAChR supports the idea that the cholinergic system is a substrate of arousal and emotional deficits observed in Fragile X.

METHODS

Animals. Animals were treated in compliance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were housed with 12 h light/dark cycles and access to food and water ad libitum.

Slice Preparation. Adult male fmr1−/− mice on a C57Bl6/J genetic background aged between 60 and 95 postnatal days were used, with wild-type littermates and C57Bl6/J mice purchased from Janvier Laboratories France used as control group. They were anesthetized with isoflurane and decapitated according to institutional regulations. The brain was sliced (300 μm) in the coronal plane with a vibratome (Integraslice, Campden Instruments, Loughborough, UK) in a sucrose-based solution at 4 °C (in mM: 87 NaCl, 75 sucrose, 25 glucose, 2.5 KCl, 4 MgCl2, 0.5 CaCl2, 23 NaHCO3 and 1.25 NaH2PO4). Immediately after cutting, slices were stored for 1 h at 32 °C in a low calcium artificial cerebrospinal fluid (low Ca2+ ACSF) that contained (in mM) 130 NaCl, 11 glucose, 2.5 KCl, 2.4 MgCl2, 1.2 CaCl2, 23 NaHCO3, 1.2 NaH2PO4, and was equilibrated with 95% O2/5% CO2. Slices were maintained at room temperature until the time of recording.

Electrophysiology. Field potential recordings were made in coronal slices containing the accumbens core as previously described. Recordings were made in the medial ventral accumbens core close to the anterior commissure.

For recording, slices were placed in the recording chamber chamber and superfused (1.5−2 mL/min) with ACSF (same as low Ca2+ ACSF with the following exception: 2.4 mM CaCl2 and 1.2 mM MgCl2). All experiments were done at 25 °C. Picrotoxin (100 μM) was added to the superfusion medium to block gamma-aminobutyric acid type A (GABA-A) receptors. All drugs were added at the final concentration and incubated for 20 min before starting the experiments. At least 3−12 animals were used for each single experimental condition. The Shapiro−Wilk test confirmed the normal distribution of data sets. Therefore, depending on the experimental condition, statistical differences were assessed using t tests and one-way ANOVA post hoc tests. A confidence level of P < 0.05 was considered significant.

ASSOCIATED CONTENT

Supporting Information

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GraphPad Prism (GraphPad Software Inc, La Jolla, CA). All values are given as mean ± standard error. N indicates the number of experiments. At least 3−12 animals were used for each single experimental condition. The Shapiro−Wilk test confirmed the normal distribution of data sets. Therefore, depending on the experimental condition, statistical differences were assessed using t tests and one-way ANOVA post hoc tests. A confidence level of P < 0.05 was considered significant.

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Author Contributions

D.N. and O.J.M. designed research; D.N. and O.L. performed research; D.N. and O.L. analyzed data; D.N. and O.J.M. wrote the paper.

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Notes

The authors declare no competing financial interest.

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Table 1. Drug Suppliers, Final Concentrations, and Incubation Times

| Drug                          | Supplier          | Concentration (μM) | Incubation Time (min) | Preincubation Time (min) |
|-------------------------------|-------------------|--------------------|-----------------------|--------------------------|
| picrotoxin (GABA-A antagonist) | Sigma-Aldrich     | 100                | 20                    | 20                       |
| CNQX (AMPA/ KainateR antagonist) | NIMH              | 100                | 0                     | 0                        |
| D-AP5 (NMDAR antagonist)      | NIMH              | 50                 | 20                    | 20                       |
| Carbachol (AChR agonist)      | Tocris            | 1−100              | 10                    | 10                       |
| VU0225035 (M1R antagonist)     | Tocris            | 10                 | 45                    | 20                       |
| SR141716A (CB1R antagonist)   | NIMH              | 5                  | 45                    | 20                       |
| AMG9810 (TRPV1R antagonist)    | Tocris            | 10                 | 120                   | 20                       |
| JZL184 (MAGLα inhibitor)      | NIMH              | 1                  | 45                    | 45                       |
| URB597 (FAAH inhibitor)       | Tocris            | 2                  | 45                    | 45                       |
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