FMRF regulates an ethanol-dependent shift in GABA_B R function and expression with rapid antidepressant properties

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Alcohol promotes lasting neuroadaptive changes that may provide relief from depressive symptoms, often referred to as the self-medication hypothesis. However, the molecular/synaptic pathways that are shared by alcohol and antidepressants are unknown. In the current study, acute exposure to ethanol produced lasting antidepressant and anxiolytic behaviours. To understand the functional basis of these behaviours, we examined a molecular pathway that is activated by rapid antidepressants. Ethanol, like rapid antidepressants, alters "gamma-aminobutyric acid type B receptor (GABA_B R) expression and signalling, to increase dendritic calcium. Furthermore, new GABA_B Rs are synthesized in response to ethanol treatment, requiring fragile-X mental retardation protein (FMRF). Ethanol-dependent changes in GABA_B R expression, dendritic signalling, and antidepressant efficacy are absent in Fmr1-knockout (KO) mice. These findings indicate that FMRF is an important regulator of protein synthesis following alcohol exposure, providing a molecular basis for the antidepressant efficacy of acute ethanol exposure.
The presence of major depression increases the risk of alcohol use disorders (AUDs) by ~2-fold (and vice versa). The self-medication hypothesis suggests AUDs may develop when the initial antidepressant actions of alcohol are shifted to depressive allostatic states with chronic abuse. The molecular mechanism underlying the initial antidepressant effects of alcohol is unknown.

A major advance in understanding and treating depression is the recognition that NMDA receptor (NMDAR) antagonists act as rapid and effective antidepressant drugs. A single injection of an NMDAR antagonist or ‘rapid antidepressant’ is effective within 2 h and has sustained antidepressant efficacy for 2 weeks. These long-lasting properties depend on the activity of mammalian target of rapamycin (mTOR)3,6, a serine/threonine kinase essential for messenger RNA translation7. Recently, we demonstrated that activation of mTOR-dependent protein synthesis by NMDAR antagonists requires a shift in GABA\(_{\text{B}}\)R signalling from opening potassium channels to facilitating an increase in dendritic calcium6,8. Interestingly, both acute ethanol and rapid antidepressants block NMDARs3,9. In light of these data, we propose that ethanol has lasting antidepressant efficacy, shares the same downstream molecular signalling events as rapid antidepressants, and requires de novo protein synthesis (Supplementary Fig. 1).

Studies suggest that antidepressant efficacy requires two phases—an induction phase and a sustained phase1,10,11. Notably, GABA\(_{\text{B}}\)R-mediated, mTORC1-dependent protein synthesis is required for the long-lasting sustained phase of rapid antidepressants. Our previous work indicates that both new protein synthesis and an increase in protein stability are required for the GABA\(_{\text{B}}\)R shift in function necessary to increase mTORC1 activity8. However, the mechanism that initiates such dynamic changes in protein expression by rapid antidepressants remains unclear.

FMRP is an RNA-binding protein that has been characterized as a repressor of mRNA translation. Some forms of synaptic activity trigger FMRP to release its targets, allowing them to be translated12,13. Moreover, degradation and new protein synthesis of FMRP creates a window for the translation of specific mRNAs, facilitating long-lasting changes in synaptic function14,15. Complete loss of FMRP results in Fragile-X syndrome (FXS), the single most common genetic cause of autism16. Moreover, reduced levels of FMRP, caused by a pre-mutation, lead to a higher incidence of tremors, ataxia, memory loss, and neuronal neuropathy in older men17. These findings argue that precise levels of FMRP protein and its target mRNAs are required for normal neuronal function.

Drugs of abuse promote profound changes in gene expression, mRNA translation rates and synaptic protein composition18,19. Some studies suggest that drugs and alcohol hijack the molecular mechanisms that underlie synaptic plasticity20,21. In agreement with this premise, FMRP has been implicated in cocaine addiction22. However, little is known about the mRNA targets and the signalling mechanisms involved. Here we describe a critical role for FMRP in mediating GABA\(_{\text{B}}\)R synthesis and plasticity following acute ethanol exposure, a mechanism required for antidepressant efficacy.

Results
Antidepressant and anxiolytic effects of ethanol on behaviour.
To determine if acute alcohol has antidepressant properties, as predicted by the self-medication hypothesis, we first assessed the efficacy of alcohol on antidepressant- and anxiolytic-like effects on behaviour. The forced swim test (FST) is a rodent behavioural test predictive of antidepressant activity in humans23. Rodents treated with a single injection of NMDAR antagonists or rapid antidepressants swim longer and thus have reduced immobility relative to controls. Notably, these positive effects on behaviour last long after the drug has been metabolized24,25. Therefore, we considered the possibility that ethanol, which blocks NMDARs9, could also act like an antidepressant at 24 h, well beyond the intoxication period25. To test this, C57BL/6 mice were injected with ethanol (2.5 g kg\(^{-1}\), intraperitoneal (i.p.)), a concentration that is achieved during self-administration in mice26. Twenty-four hours after injection, the immobility of ethanol-treated mice was reduced by ~15% relative to controls (Fig. 1a), similar to our previous observation in mice that had been exposed to the rapid antidepressant Ro-25-6981 (refs 6,8). These results demonstrate that acute ethanol elicits a lasting antidepressant effect on behaviour similar to that seen with rapid antidepressants8.

As another measure of antidepressant effect of ethanol on behaviour, we assessed the grooming behaviour of mice using the splash test after ethanol or saline administration. The splash test measures latency to groom and dedicated grooming time as indicators of self-care and motivation to behaviour27,28. Lack of self-care is often observed in humans with depressive disorder29. We have previously shown that mice receiving a single i.p. injection of the rapid antidepressant Ro-25-6981 spend more time grooming compared with control mice8. We hypothesized that ethanol would produce similar effects on grooming behaviour. Indeed, ethanol-treated mice spent more time grooming and displayed shorter latency to initiate grooming relative to controls (Fig. 1b,c).

Ethanol is a well-known anxiolytic substance30. However, the anxiolytic effect of a single dose of ethanol 24 h after administration has not been determined. We subjected ethanol- and saline-injected mice to the open field test to assess the influence of ethanol on anxiety-like behaviours after 24 h. Mice that spend more time in the centre of the open field are scored as having reduced anxiety-like behaviour relative to mice that remain close to the perimeter31. Indeed, mice that received a single dose of ethanol (2.5 g kg\(^{-1}\), i.p.) had reduced anxiety-like behaviour, spending ~40% more time in the centre relative to controls (Fig. 1d). There was no significant difference in total distance travelled or average speed between the groups (Fig. 1e,f). These data suggest that the anxiolytic effects of ethanol last up to 24 h post injection.

Acute ethanol increases GABA\(_{\text{B}}\)R2 and surface GABA\(_{\text{B}}\)Rs.
Both ethanol and rapid antidepressants block NMDARs in the hippocampus4,9. One of the first events triggered by NMDAR antagonism is increased dendritic GABA\(_{\text{B}}\)R2 protein expression3. GABA\(_{\text{B}}\)R2s are obligate heteromultimers, consisting of GABA\(_{\text{B}}\)R1 and R2. GABA\(_{\text{B}}\)R2 is required for expression of receptors at the surface by masking an endoplasmic reticulum retention sequence on GABA\(_{\text{B}}\)R1 (ref. 32). Similarly, treatment with a rapid antidepressant leads to (1) increased dendritic expression of GABA\(_{\text{B}}\)R2 but not GABA\(_{\text{B}}\)R1 (ref. 8) and (2) a corresponding increase in surface expression of GABA\(_{\text{B}}\)R1 (refs 6,8).

To determine if acute ethanol exposure in vivo rapidly increases the levels of GABA\(_{\text{B}}\)R1 and/or GABA\(_{\text{B}}\)R2, hippocampal synaptoneurosomes were isolated from mice that had been injected with a single dose of ethanol (2.5 g kg\(^{-1}\), i.p.) or saline for western blot analysis. The hippocampi were collected within the initiation phase (30 min post injection), a phase where molecular changes facilitate increased downstream mTORC1 activity11. Consistent with rapid antidepressants, acute ethanol injection increased the protein expression of GABA\(_{\text{B}}\)R2 by ~37% in the hippocampus with no significant change in GABA\(_{\text{B}}\)R1 (Fig. 2a–c; uncropped blots, Supplementary Fig. 7a).
were measured in the open field test 24 h post injection. Ethanol-treated (2.5 g kg$^{-1}$) mice spent more time in the centre, while speed and distance were unaffected compared with vehicle-treated (saline, i.p.) mice, indicating an ethanol-induced anxiolytic effect without altering mobility. Total centre time: Veh $= 327.5 \pm 35.62$ s, n = 6; ETOH $= 459.2 \pm 19.13$ s, n = 6. Average speed: Veh $= 0.042 \pm 0.002$ m s$^{-1}$, n = 6; ETOH $= 0.041 \pm 0.002$ m s$^{-1}$, n = 6. Total distance: Veh $= 75.35 \pm 3.92$ m, n = 6; ETOH $= 73.00 \pm 3.35$ m, n = 6. Significance determined by one-tailed t-test. Values represent mean $\pm$ s.e.m.

To further identify the subcellular localization of ethanol-induced increase in GABA$_B$R2, we examined GABA$_B$R expression in cultured hippocampal neurons. GABA$_B$R1 and R2 were immunostained and quantified in the dendrites. A concentration of 30 mM ethanol was chosen, as it has been shown to reduce NMDAR activity in hippocampal neurons and reflects that achieved in vivo following i.p. injection$^{9,33}$. Acute ethanol exposure (30 mM, 2 h) increased the dendritic levels of GABA$_B$R2 by $\sim 47\%$, but did not affect GABA$_B$R1 levels (Fig. 2d–g). We did not observe a difference in the diameter of the primary dendrites between vehicle- and ethanol-treated neurons, demonstrating that ethanol does not modify dendritic calibre (Supplementary Fig. 2). These in vivo and in vitro findings establish a role for ethanol in increasing GABA$_B$R2 protein expression.

Since GABA$_B$R2 is required for the surface expression of the heteromultimeric receptor, we predicted that the ethanol-induced elevation in GABA$_B$R2 levels would increase expression of receptors at the surface. We measured the surface expression of dendritic GABA$_B$R using an antibody directed against the extracellular domain of GABA$_B$R1 in unpermeabilized hippocampal neurons. The surface signal was normalized by the total dendritic GABA$_B$R1 levels after permeabilization$^6$. As predicted, surface expression of GABA$_B$R1s in ethanol-treated neurons was significantly higher ($\sim 66\%$ increase) relative to controls (Fig. 2h,i). This ethanol effect was consistent with what we previously observed following rapid antidepressant treatment of cultured hippocampal neurons$^6$. Collectively, these results suggest that ethanol promotes the surface expression of GABA$_B$R1s, and this is likely achieved by increasing GABA$_B$R2 protein levels.

**FMRP regulates GABA$_B$R1 and GABA$_B$R2 expression.** Next, we sought to identify the mechanism by which NMDAR antagonism increases GABA$_B$R2 expression. GABA$_B$R2 mRNA is present in the dendrites of hippocampal neurons$^{34}$, suggesting that this mRNA may be locally regulated at the translational level. Thus, we examined RNA-binding factors that may regulate GABA$_B$R2 mRNA expression in dendrites. Notably, both GABA$_B$R1 and GABA$_B$R2 mRNAs are reported targets of FMRP, an RNA-binding protein that stalls translational elongation of its targets$^{35,36}$. To test the hypothesis that FMRP regulates GABA$_B$R mRNA translation, we first verified that (1) GABA$_B$R mRNAs bind to FMRP, and that (2) the absence of FMRP in knockout mice results in aberrant expression of GABA$_B$Rs. Using a specific antibody against FMRP, bound mRNAs were isolated using RNA immunoprecipitation (RIP). GABA$_B$R1 and GABA$_B$R2 binding were assessed by reverse transcription (RT) and quantitative PCR. Indeed, GABA$_B$R1 and GABA$_B$R2 mRNAs were detected in the
immunoprecipitate, along with CaMKIIα, a well-known FMRP mRNA target (Fig. 3a,b; uncropped representative qPCR gels, Supplementary Fig. 7d). The calcium channel accessory subunit Cacna2δ2 mRNA is not a reported target for FMRP35 and was used as a negative control. Cacna2δ2 mRNA was not detected in the FMRP RIP (Fig. 3b). In parallel, we used lysates isolated from brains of mice with a genetic deletion of the Fmr1 gene. We did not observe amplification of any of the mRNAs in Fmr1 KO brains, providing additional evidence for specific binding of FMRP to GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 mRNAs (Fig. 3a–c; uncropped blots, Supplementary Fig. 7b).

Next, we determined if FMRP regulates GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 protein levels. Genetic deletion of Fmr1 leads to the constitutive translation of FMRP target mRNAs and the loss of activity-dependent translation16. Protein levels of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 were compared in hippocampal synaptoneurosomes from Fmr1 KO and wild-type (WT) mice (Fig. 3c–g). GABA<sub>B</sub>R2 basal protein levels were elevated by ∼53% in Fmr1 KO hippocampi (Fig. 3g). GABA<sub>B</sub>R1 protein levels also increased, albeit to a lesser extent than GABA<sub>B</sub>R2 (Fig. 3f). Collectively, these data suggest that FMRP regulates the expression of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2.
Ethanol and rapid antidepressants reduce dendritic FMRP. As an initial test to determine if FMRP-regulated translation is linked to alcohol exposure, we compared FMRP target mRNAs with mRNAs that are differentially expressed in the hippocampus of alcohol-dependent humans. Remarkably, 225 or ~25% of verified FMRP target mRNAs overlap with mRNAs that are altered in alcohol-dependent individuals, suggesting a role for FMRP in aberrant protein levels observed in humans with AUD (Fig. 4a)35,37. We then determined whether exposure to acute ethanol (30 mM, 2 h) or Ro-25-6981 (10 μM, 2 h) alters FMRP expression in the dendrites of hippocampal neurons. Using immunofluorescence, we found that ethanol and Ro-25-6981 reduced FMRP levels by ~38% and 45%, respectively (ethanol: Fig. 4b,c; Ro-25-6981: Supplementary Fig. 3). These data suggest that ethanol and Ro-25-6981 alter protein expression in an FMRP-dependent manner.

Ethanol-induced synthesis of GABA_B2 requires FMRP. Due to the ethanol-induced decreases in FMRP, we hypothesized that FMRP is required for ethanol-induced expression of GABA_B2.

Specifically, if expression of GABA_B2 is constitutive and unregulated in Fmr1 KO mice, then ethanol-induced changes in GABA_B2 expression should be absent in Fmr1 KO mice. Hippocampal synaptoneurosomes were isolated from WT and Fmr1 KO mice 30 min after i.p. injection of ethanol (2.5 g kg^-1). Western blot analysis indicated that both GABA_B1 and GABA_B2 expression remained constant in vehicle- and ethanol-treated Fmr1 KO mice. As observed in Fig. 2, WT hippocampal synaptoneurosomes showed an ~23% increase in GABA_B2 but no change in GABA_B1 expression (Fig. 5a–c; uncropped blots, Supplementary Fig. 7c). These data suggest that ethanol-induced changes in GABA_B2 expression are dependent on FMRP translational regulation.

To determine whether protein synthesis is essential for the FMRP-dependent changes in GABA_B2 expression, we measured ethanol-induced GABA_B2 in the presence of cycloheximide (CHX), a protein synthesis inhibitor. As demonstrated previously, ethanol did not influence the dendritic expression of GABA_B1; however, co-treatment with cycloheximide increased GABA_B1 expression by ~22%. FMRP deletion did not affect the basal, ethanol-, or cycloheximide-induced dendritic protein expression of GABA_B1 (Fig. 5d,e,h). For GABA_B2, we again saw a significant ~28% increase in dendritic expression with acute ethanol treatment; however, in the presence of cycloheximide the ethanol-induced increase was abolished. Notably in Fmr1 KO cultures, no change was observed with ethanol or ethanol + cycloheximide (Fig. 5f,g,i).

We next examined the requirement for protein synthesis and FMRP in ethanol-dependent surface expression of GABA_B2. Using WT and Fmr1 KO hippocampal neurons, we measured ethanol-induced surface expression of GABA_B1 with or without cycloheximide. Co-assembly of GABA_B1 and GABAB2 is required to express GABAB2 heterodimers in the membrane32. Thus, we predicted that the ethanol-induced increase in surface GABA_B2 would require FMRP-regulated synthesis of GABA_B2. Again,

**Figure 3 | GABA_B1 and GABA_B2 mRNAs are FMRP targets.** (a,b) RNA immunoprecipitation (RIP) for FMRP was performed using brains from wild type (WT) and Fmr1 KO male mice. (a) Gels showing RT-qPCR amplified product of input sample, FMRP RIP, and IgG control for GABA_B1 and GABA_B2. (b) Relative fold-enrichment as determined by real-time qPCR relative to input control (ΔΔCt = 2^(- (Ct FMRP RIP – Ct IgG input)) – (Ct FMRP RIP – Ct IgG input)). FMRP binds GABA_B1, GABA_B2, and the positive control CaMKII mRNA as detected in the RIP sample by real-time qPCR. Cacnn262 expressed as detected in the RIP sample by real-time qPCR. Cacnn262 served as a negative control and was not detected above background. WT: GABA_B1 = 2.66 ± 0.2488, n = 2; GABA_B2 = 2.19 ± 0.08, n = 2; CaMKII = 3.72 ± 0.94, n = 2; Cacnn262 = 0.11 ± 0.6, n = 2. Fmr1 KO: GABA_B1 = 0.01 ± 0.0002, n = 2; GABA_B2 = 0.02 ± 0.00006, n = 2; CaMKII = 0.04 ± 0.01, n = 2; Cacnn262 = 0.012 ± 0.005, n = 2. (c–g) Western blot analysis of hippocampal synaptoneurosomes isolated from C57BL/6 WT and Fmr1 KO mice on a C57BL/6 background indicates the absence of (c) FMRP and increased protein expression of (d) GABA_B1 and (g) GABA_B2. Representative western blots are pseudocoloured to indicate intensity of bands, and the normalized optical density for each band is indicated below blot (Lookup table, below western blot). Western blots were normalized to the loading control, α-Tubulin. WT: FMRP = 1.00 ± 0.10; GABA_B1 = 1.00 ± 0.06; GABA_B2 = 1.00 ± 0.08. Fmr1 KO: FMRP = 0.03 ± 0.01; GABA_B1 = 1.27 ± 0.08; GABA_B2 = 1.54 ± 0.17. Experiment was repeated three times. Significance determined by Student’s t-test. Values represent mean ± s.e.m. Uncropped versions of qPCR gel, with size markers, are available in Supplementary Fig. 7d. Uncropped version of western blots, with size markers are available in Supplementary Fig. 7b.
Ethanol-induced GABA<sub>B</sub>R plasticity requires FMRP. We previously demonstrated that rapid antidepressants shift GABA<sub>B</sub>R signalling from opening potassium channels to increasing dendritic calcium<sup>6</sup>. To determine whether ethanol (30 mM, 2 h) causes the same plasticity in GABA<sub>B</sub>R signalling, we performed fluorescence calcium imaging in cultured WT and <i>Fmr1</i> KO hippocampal neurons. A transient rise or fall in calcium in dendritic compartments can be detected using a fluorescent indicator that exhibits changes in fluorescent properties depending on the amount of bound calcium<sup>39</sup>. We used baclofen, a GABA<sub>B</sub>R agonist, to activate GABA<sub>B</sub>Rs in the presence or absence of ethanol. After establishing a baseline measurement, baclofen reduced dendritic calcium fluorescence in saline-treated WT neurons by ~11%, a characteristic signature of GABA<sub>B</sub>R signalling increasing outward potassium conductance<sup>8</sup>. However, in ethanol-treated WT neurons, baclofen induced distinct calcium waves and an overall averaged increase in calcium signal of ~9% (Fig. 7a–c and Supplementary Fig. 4a,c). These results recapitulate our previous observations with NMDAR antagonists<sup>6</sup>. In addition, these findings in WT mouse neurons are consistent with what we observed in rat cultured hippocampal neurons treated with ethanol or the clinically relevant rapid antidepressant Ro-25-6981 (Supplementary Fig. 5). Unexpectedly, GABA<sub>B</sub>R activation in saline-treated <i>Fmr1</i> KO neurons failed to reduce the calcium signal. Moreover, in ethanol-treated <i>Fmr1</i> KO neurons, GABA<sub>B</sub>R activation failed to increase dendritic calcium signal (Fig. 7b,c and Supplementary Fig. 4b,c). These findings suggest that the loss of FMRP in <i>Fmr1</i> KO dendrites decouples GABA<sub>B</sub>Rs from potassium channels. These results also suggest that the dynamic, ethanol-induced plasticity in GABA<sub>B</sub>R signalling, which is observed with rapid antidepressants, requires FMRP<sup>6</sup>.

To further substantiate that FMRP regulates ethanol-dependent GABA<sub>B</sub>R plasticity, we overexpressed FMRP in rat hippocampal neurons. Overexpression of FMRP did not alter the GABA<sub>B</sub>R activation in saline-treated neurons because baclofen reduced the dendritic calcium signal. However, in ethanol-treated neurons, overexpressing FMRP blocked the ethanol-induced GABA<sub>B</sub>R plasticity (Fig. 7d,e and Supplementary Fig. 4d,e). These results provide additional evidence that the dynamic reduction of FMRP with ethanol exposure is important for the expression of GABA<sub>B</sub>R plasticity.

Antidepressant effect of ethanol on behaviour requires FMRP. Since FMRP is important for ethanol-induced GABA<sub>B</sub>R plasticity, we examined antidepressant and anxiolytic-like effects of ethanol on behaviour in <i>Fmr1</i> KO mice. Interestingly, ethanol
Figure 5 | Fmr1 KO prevents ethanol-induced altered GABA<sub>R</sub> expression. (a–c) Western blot analysis of GABA<sub>R</sub>1 and GABA<sub>R</sub>2 in wild type (WT) and Fmr1 KO C57BL/6 hippocampal synaptoneurosomes after vehicle (Veh; saline i.p., 30 min) or ethanol (ETOH; 2.5 g kg<sup>-1</sup> i.p., 30 min) treatment. (a) Pseudocoloured representative western blots showing band intensity, and normalized optical densities to WT–vehicle are reported below each image (lookup table, below western blot). Western blots were normalized to the loading control, α-Tubulin. No change was found in b GABA<sub>R</sub>1 after ethanol treatment in either genotype as shown by ethanol/vehicle comparison. A significant increase in c GABA<sub>R</sub>2 expression was observed in WT mice after ethanol, but no change was observed in Fmr1 KO mice (shown as ethanol/vehicle). WT ETOH/Veh: GABA<sub>R</sub>1 = 1.15 ± 0.21; GABA<sub>R</sub>2 = 1.22 ± 0.01. Fmr1 KO ETOH/Veh: GABA<sub>R</sub>1 = 0.94 ± 0.04; GABA<sub>R</sub>2 = 0.95 ± 0.03. Experiment was repeated three times. Significance determined by Student’s t-test. Values represent mean ± s.e.m. Representative immunofluorescent images (d–g) and quantification summaries (h, i) of dendritic expression of GABA<sub>R</sub>1 and GABA<sub>R</sub>2 from WT and Fmr1 KO primary mouse hippocampal cultures normalized to MAP2. (h) GABA<sub>R</sub>1 expression was not changed in either genotype after 2-h treatment with vehicle (Veh; H<sub>2</sub>O), ethanol (ETOH; 30 mM), or ethanol and cycloheximide (30 mM ETOH + 50 μM CHX). WT GABA<sub>R</sub>1: Veh = 1.00 ± 0.04, n = 44 dendrites; ETOH = 1.07 ± 0.04, n = 29 dendrites; ETOH + CHX = 1.22 ± 0.05, n = 34 dendrites. Fmr1 KO GABA<sub>R</sub>1: Veh = 1.09 ± 0.03, n = 72 dendrites; ETOH = 1.12 ± 0.03, n = 41 dendrites; ETOH + CHX = 1.42 ± 0.07, n = 43 dendrites. (i) GABA<sub>R</sub>2 expression in WT neurons increased after ethanol (ETOH; 30 mM, 2 h) treatment, and was rescued with co-treatment of cycloheximide (CHX; 50 μM, 2 h). GABA<sub>R</sub>2 expression in Fmr1 KO dendrites was not significantly altered between neurons treated with Veh, ETOH, or ETOH + CHX. WT GABA<sub>R</sub>2: Veh = 1.00 ± 0.03, n = 41 dendrites; ETOH = 1.28 ± 0.06, n = 40 dendrites; ETOH + CHX = 0.99 ± 0.05, n = 33 dendrites. Fmr1 KO GABA<sub>R</sub>2: Veh = 1.46 ± 0.05, n = 73 dendrites; ETOH = 1.56 ± 0.04, n = 45 dendrites; ETOH + CHX = 1.63 ± 0.07, n = 36 dendrites. Significance determined by two-way analysis of variance with Tukey’s post test. Value represent mean ± s.e.m. Scale bars, 5 μm. Uncropped version of western blots, with size markers are available in Supplementary Fig. 7c.
Figure 6 | New GABAB2 protein and surface expression requires FMRP. Immunofluorescent images and quantification summary of GABAB1 surface expression in wild type (WT) and Fmr1 KO primary hippocampal cultures normalized to MAP2 as volume control. (a, b) Representative images of immunostaining. (c) Increased expression of surface GABAB1 in WT dendrites after ethanol (ETOH: 30 mM, 2 h) compared with vehicle (Veh: H2O, 2 h) or ethanol–cycloheximide (30 mM ETOH + 50 μM CHX, 2 h) treatment. No significant change in surface GABAB1 expression in Fmr1 KO cultures treated with ETOH or ETOH + CHX was observed. WT surface GABAB1: Veh = 1.00 ± 0.08, n = 28 dendrites; ETOH = 1.76 ± 0.17, n = 37 dendrites; ETOH + CHX = 1.22 ± 0.1, n = 36 dendrites. Fmr1 KO surface GABAB1: Veh = 1.36 ± 0.13, n = 39 dendrites; ETOH = 1.44 ± 0.15, n = 42 dendrites; ETOH + CHX = 0.91 ± 0.09, n = 29 dendrites. (d–i) BONCAT combined with PLA, a method to detect newly synthesized proteins. Representative images for GABAB1 and GABAB2 expression. Pixels were equally dilated by 1 using ImageJ software for enhanced visualization as described by Cajigas et al.34. In WT and Fmr1 KO primary hippocampal cultures (h) GABAB1 synthesis in dendrites was not altered by ethanol (30 mM, 2 h) compared with vehicle (H2O, 2 h) treatment normalized to MAP2. WT GABAB1: Veh = 1.00 ± 0.09, n = 47 dendrites; ETOH = 0.82 ± 0.08, n = 39 dendrites. Fmr1 KO GABAB1: Veh = 0.64 ± 0.09, n = 38 dendrites; ETOH = 0.48 ± 0.05, n = 36 dendrites. (i) In contrast, ethanol induced a significant increase in new GABAB2 synthesis in WT hippocampal dendrites but not in Fmr1 KO dendrites. WT GABAB2: Veh = 1.00 ± 0.06, n = 21 dendrites; ETOH = 1.39 ± 0.11, n = 25 dendrites. Fmr1 KO GABAB2: Veh = 1.48 ± 0.10, n = 32 dendrites; ETOH = 1.23 ± 0.07, n = 41 dendrites. Significance determined by two-way analysis of variance with Tukey’s post test. Values represent mean ± s.e.m. Scale bars, 5 μm.
administration did not affect the behaviours of Fmr1 KO mice in the splash and open field tests compared with saline-treated mice (Supplementary Fig. 6a–e). Surprisingly, the basal state of the splash and open field tests compared with saline-treated mice administration did not affect the behaviours of injected WT mice (Fig. 8). To explore this paradox, we examined the requirement of GABA<sub>B</sub>R activation in ethanol-induced decreases in immobility by using CGP-35348 to inhibit postsynaptic GABA<sub>B</sub>Rs. We previously showed that GABA<sub>B</sub>R antagonism blocked the antidepressant-like behaviour produced by NMDAR antagonist in the FST<sup>6</sup>. GABA<sub>B</sub>R inhibition alone did not affect the immobility of saline-injected WT mice in the FST, similar to what we observed previously (Fig. 8)<sup>6</sup>. CGP-35348, however, abolished the ethanol-induced antidepressant behaviour, demonstrating a requirement for GABA<sub>B</sub>R activation in ethanol-triggered reduction of immobility. Neither ethanol, CGP-35348, nor ethanol + CGP-35348 treatment in Fmr1 KO mice produced immobility scores that were significantly different.

**Figure 8 | Ethanol's antidepressant effect requires GABA<sub>B</sub>R activation.**
Wild type (WT) C57BL/6 and Fmr1 KO male mice were subjected to the forced swim test (FST) 24 h post injection of vehicle (Veh: saline), ethanol (ETOH: 2.5 g kg<sup>–1</sup>), CGP-35348, a GABABR antagonist (CGP: 100 mg kg<sup>–1</sup>) or ethanol + CGP-35348. Ethanol-induced decrease in immobility was absent in Fmr1 KO mice. WT: Veh = 100 ± 3.19 s, n = 9 mice; ETOH = 72.97 ± 6.23 s, n = 7 mice; CGP-35348 = 98.38 ± 4.2 s, n = 10; ETOH + CGP-35348 = 94.73 ± 3.77 s, n = 7 mice. Fmr1 KO: Veh = 58.75 ± 10.33 s, n = 9; ETOH = 69.02 ± 8.99 s, n = 3; CGP-35348 = 88.00 ± 9.56 s, n = 3; ETOH + CGP-35348 = 77.78 ± 16.04 s, n = 3. Significance determined by two-way analysis of variance Tukey’s multiple comparison test. Values represent mean ± s.e.m.

**Figure 7 | GABA<sub>B</sub>R plasticity and signalling is absent in Fmr1 KO mice.**
(a,c) Mouse hippocampal cultured neurons were pre-treated for 2 h with either vehicle (Veh: H<sub>2</sub>O) or ethanol (ETOH: 30 mM). Line graphs represent the average fluorescent calcium signal in dendrites over time from (a) wild type (WT) and (b) Fmr1 KO mice. Baseline was established for 1 min before the addition of GABA<sub>B</sub>R agonist baclofen (Bac: 50 μM) in vehicle- or ethanol-exposed neurons. Baclofen was allowed to equilibrate as indicated by the break between dotted lines. (c) Summary graph shows significant increase in dendritic calcium signal (ΔF/F) with the addition of baclofen in WT neurons pre-treated with ethanol, which was not observed in Fmr1 KO neurons. WT: Veh + Bac = −11.82 ± 3.55, n = 8; ETOH + Bac = 9.10 ± 1.65, n = 14. Fmr1 KO: Veh + Bac = 2.81 ± 1.48, n = 12; ETOH + Bac = 3.74 ± 1.30, n = 12. (d) Dendritic calcium imaging was performed as before in hippocampal cultured neurons infected with either vector (rAAV:mSYN-tdTomato) or FMRP overexpression (rAAV:mSYN-FMRP and rAAV:mSYN-tdTomato). Ethanol-induced increase in dendritic calcium is prevented by FMRP overexpression. Vector: Veh + Bac: −6 ± 1.6, n = 17 dendrites; ETOH + Bac = 2.5 ± 2.5, n = 17 dendrites. FMRP overexpression: Veh + Bac: −4 ± 2, n = 11 dendrites; ETOH + Bac: −4.7 ± 1.4, n = 27 dendrites. Significance determined by two-way analysis of variance, followed by Tukey’s multiple comparison. Values represent mean ± s.e.m.
FROM saline-treated Fmr1 KO mice. These findings collectively demonstrate that GABA<sub>B</sub>Rs and FMRP are necessary to elicit the ethanol-mediated antiedpressant response.

**Discussion**

Emerging behavioural and molecular evidence demonstrate that NMDAR antagonists act as rapid antidepressants<sup>3,6,8,24,40</sup>. Because it has long been speculated that individuals with major depressive disorders self-medicate with alcohol, we examined whether ethanol, which blocks NMDARs<sup>8</sup>, acts through the same synaptic pathways as NMDAR antagonists. Until this study, the molecular mechanisms shared by alcohol and antidepressants were unexplored. Here we provide molecular and behavioural evidence that acute alcohol exposure elicits antiedpressant-like behaviours that persist up to 24 h after administration (Fig. 1), supporting the hypothesis that ethanol initiates lasting antidepressant activity. We have previously demonstrated that NMDAR inhibition by rapid antidepressants induces two key molecular changes that are responsible for the antidepressant response, namely (1) an increase in GABA<sub>B</sub>R protein synthesis and (2) a shift in GABA<sub>B</sub>R function that increases dendritic calcium signalling<sup>6,8</sup>. Our current work shows that these same signature changes are produced by acute ethanol exposure (Figs 5–7).

Surface expression of functional GABA<sub>B</sub>Rs requires the dimerization of GABA<sub>B</sub>R1 and R2 subunits. Without GABA<sub>B</sub>R2, GABA<sub>B</sub>R1 is retained in the endoplasmic reticulum<sup>32</sup>. Our current studies show that the release of GABA<sub>B</sub>R2 mRNA translational repression by FMRP is necessary for the ethanol-induced increase in surface GABA<sub>B</sub>Rs with NMDAR blockade (Figs 4.6 and 8; Supplementary Fig. 3). Reduction of FMRP, as seen in animal models of FXS, is associated with elevated protein synthesis normally seen in neurobasal A medium supplemented with B27, glutamine, and 1% fetal bovine serum. Cultures were plated at a density of ~100,000 cells per 12 mm on glass coverslips that had been coated overnight with 50 μg ml<sup>−1</sup> poly-d-lysine and 25 μg ml<sup>−1</sup> laminin in borate buffer. Cultures were fed after 1 day in vitro (DIV), and media was replaced approximately once a week with either fresh rat culture media (neurobasal A supplemented with B27, glutamine and 3 μM AraC) or fresh mouse culture media (glial-conditioned media with 3 μM AraC) until cultures were used at DIV 14–21.

**Methods**

**Cell culture.** Primary hippocampal neurons were prepared as previously described by Niere et al.<sup>44</sup> Briefly, hippocampi were extracted from postnatal day 1–3 Sprague–Dawley rat pups, WT C57BL/6 mouse pups, or Fmr1-knockout (Fmr1 KO) mouse pups on a C57BL/6 background. The tissue was dissociated and plated in neurobasal A medium supplemented with R27, glutamine, and 1% fetal bovine serum. Cultures were plated at a density of ~100,000 cells per 12 mm glass coverslips that had been coated overnight with 50 μg ml<sup>−1</sup> poly-d-lysine and 25 μg ml<sup>−1</sup> laminin in borate buffer. Cultures were fed after 1 day in vitro (DIV), and media was replaced approximately once a week with either fresh rat culture media (neurobasal A supplemented with B27, glutamine and 3 μM AraC) or fresh mouse culture media (glial-conditioned media with 3 μM AraC) until cultures were used at DIV 14–21.

**In vitro pharmacology.** Primary hippocampal neurons were treated in ethanol vapour chambers according to a method adapted from Chandler et al.<sup>29</sup> Ethanol vapour chambers were prepared by placing a reservoir of 31.5 mM ethanol (105% of the desired ethanol concentration, that is, 30 mM) in a plastic container with 24-well culture plates containing neuronal cultures in which 30 mM ethanol was added to the culture media. Chambers were filled with 95% O<sub>2</sub>/5% CO<sub>2</sub> and cultures were incubated for 2 h at 37 °C. Cultures treated with vehicle (H<sub>2</sub>O) were incubated in the same manner but in the absence of ethanol. For calcium imaging, ethanol was added directly to HEPES-based artificial cerebral spinal fluid (ACSF in mM: 100 NaCl, 10 HEPES (pH 7.4), 3 KCl, 2 Ca<sub>2</sub>O<sub>3</sub>, 1 MgCl<sub>2</sub>, 10 glucose) that was adjusted to match the osmolality of cell culture media for live imaging. For GABA<sub>B</sub>R activation neurons were treated with (R)-baclofen (50 μM, Tocris) for 10 min before ethanol treatment. For Supplementary Fig. 3, neurons were treated with Ro-25-6981 (10 μM, Tocris) or Veh (H<sub>2</sub>O) for 2 h. Cultures were treated at 14–21 DIV. Following treatment, cultures were immediately fixed or live imaged.

**In vivo pharmacology.** Male C57BL/6 mice (Charles Rivers) or Fmr1-knockout (Fmr1 KO) mice on a C57BL/6 background (at least 7 weeks old) were given i.p.
Live calcium imaging. Dissected hippocampal cultures were prepared from WT and Fmr1 KO mice as described46. Neurons at 14–21 DIV were used for live calcium imaging. Neurons were treated as outlined in in vitro pharmacology above. Before imaging, cells were incubated in ACSF with Oregon Green 488 BAPTA-1 AM (OGB, 200 μM; 30 min; 37°C; ThermoFisher) as described. After OGB incubation, cells were transferred to fresh ACSF (37°C) for imaging (1 frame per 20 s). Baseline calcium signal was imaged (1 min), after which (R)-baclofen (50 μM, Tocris) or vehicle (H2O) was added. For ethanol-treated cells, the neurons were incubated in OGB with and imaged in ACSF containing ethanol (30 mM). Neurons were imaged for 800 s at room temperature. Quantification of the calcium signal was performed using Metamorph (Molecular Devices) as described. Briefly, dendritic regions of interest (ROI) that were at least 5 μm from the soma were analysed. The mean intensity values for each ROI at each time were averaged as baseline (F0). The ROI intensity values obtained at each time point after the addition of baclofen or vehicle were averaged (F). The equation, ΔF/F = (F – F0)/F0, was used to measure the change in signal and data were plotted as a percentage of the baseline.

Western blot analysis. Protein was isolated from hippocampal synaptoneurosomess prepared from male mice age 7–8 weeks treated with ethanol or vehicle as previously described. Synaptoneurosomes were prepared by homogenizing hippocampal tissue in homogenization buffer (20 mM HEPES pH 7.4, 5 μM EDTA pH 8.0, and protease inhibitor cocktail). Homogenate was filtered through a 100-μm filter and centrifuged by a 5-min spin at 150,000 g at 4°C (ref. 62). The pellet was resuspended in RIPA buffer (150 mM NaCl; 10 mM Tris pH 7.4, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA and protease inhibitor cocktail) and centrifuged for 15 min. The supernatant was used for western blot analysis. Protein was separated on a 4–20% gradient SDS/Tris-glycylamide gel. The gel was then transferred to a nitrocellulose membrane, blocked in 5% non-fat dry milk in tris-buffered saline and 0.1% Tween20 (TBST) for 1 h, and incubated with primary antibody in blocking buffer overnight at 4°C. The blot was washed in TBST three times for 10 min each, incubated in secondary antibody in blocking buffer 3 times for 10 min at room temperature, and washed four times for 10 min with PBS before mounting in Fluoromount with DAPI to counterstain cell nuclei (invitrogen, A-10144).

RNA immunoprecipitations. Cortices from 6-week-old C57BL/6 and Fmr1 KO male mice were prepared and flash-frozen on dry ice. RIP was performed by modified method of Jain et al. and Keene et al.45,46. Tissue was homogenized and lysed with a cordless pestle motor and disposable piston mixers (WVR) in polysome lysis buffer (10 mM HEPES pH 7.0, 100 mM KCl, 25 mM EDTA, 1 mM MgCl2, 1 mM DTT, 0.5% NP-40) in a 1:1 tissue-buffer ratio. RNaseOUT (Thermo) and protease/phosphatase inhibitors (Halt Protease and Phosphate Cocktail, Pierce Biotechnology) were freshly added to samples. Samples were rotated for 10 min at 4°C to induce swelling and then flash-frozen on dry ice. Samples were thawed by brief vortexing and centrifuged to remove debris that precipitated at 3,000g for 10 min. Lysates obtained above were pre-cleared by adding 50 μl of washed magnetic bead slurry (Protein A Dynabeads, Thermo) and rotating for 30 min at 4°C. To bind the antibody to the beads, 50 μl of magnetic beads slurry was washed and then resuspended in eight volumes of NT-2 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM DTT, 0.5% NP-40) in 1:1 microtube-buffer ratio. RNaseOUT (Thermo) and protease/phosphatase inhibitors (Halt Protease and Phosphate Cocktail, Pierce Biotechnology) were freshly added to samples. 10 μl of each antibody-MS-IP-Dye 800 (1/5,000 dilution excluding tubulin at 1/10,000 dilution; LICOR, 96-32210) and anti-rabbit Alexa680 (1/5,000 dilution; Invitrogen, A-21084).

Microscopy and analysis. Images were acquired with a Leica SP5 confocal microscope using a × 63 oil immersion lens for fixed tissue or a × 63 water immersion lens for live imaging. Max projected images were used for immuno-staining analysis from 10 μm Z-stacks of 1,024 × 1,024 pixels obtained using a 400-Hz scan rate60. For each experiment, all images were collected using the same settings. Fixed images were analysed using NIH imaging software ImageJ, and live imaging quantification was performed with Metamorph (Molecular Devices, Sunnyvale, CA). Background signal was determined by shifting the ROI adjacent to the dendrite being traced, but void of all processes. Dendritic signal was background subtracted and averaged every 10 μm using a customized R script.
temperature. Beads were quickly washed six times in ice-cold NT-2 buffer and immediately resuspended in 350 μl TRI Reagent Solution (Ambion) for 10 min at room temperature. Beads were pelleted and the supernatant was removed and resuspended in 350 μl of absolute ethanol. RNA was extracted by applying ethanol-resuspended samples to spin column from the Direct-zol RNA MiniPrep Kit (Zymogen) according to the manufacturer's instructions. Eluted RNA (25 μl) was DNase treated using the TURBO DNA-free kit (Thermo).

cDNA synthesis and quantitative real-time PCR. DNase-treated RNA samples were reverse-transcribed to complementary DNA using the iScript cDNA Synthesis Kit (Bio-Rad) in a 20 μl volume according to the manufacturer’s instructions. Quantitative real-time PCR (qRT–PCR) was performed in 20 μl reaction volume using the iQ SYBR Green Supermix (Bio-Rad) and primers for GABAB1 (GeneCopoeia, MQP031832), GABAB2-R1 (GeneCopoeia, MQP026008), CaMKIIα (GeneCopoeia, MQP028785), and CaMKIIβ (GeneCopoeia, MQP032309). qRT–PCR was run with the following protocol: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min, 95 °C for 1 min, and 55 °C for 1 min. Total sample enrichment was determined by the equation ΔΔCt = 2−[(CT FMRP input – CT IgG input) (CT FMRP input – CT IgG input) (ref. 65).

Forced swim test. Male mice were tested in the forced swim test 24 h post injection as described previously66. Mice were individually placed into a cylinder containing 1 l of water (25 °C) for 6 min. Each session was video recorded and the last 4 min of the sessions were later scored blindly for immobility. Animals were scored for escape-directed behaviours. The water was replaced between animals. Experiments were repeated by three independent experimenters. Data were normalized by experimenter. Power analysis was performed in R Programming66 to predict sample size for all behavioural tests. This sample size was used as a guideline for the WT animals, however since transgenic animals were used, the sample size. Prism software (GraphPad) was used for all statistical analyses.

Data availability. All relevant data are available from the authors upon request. Accession numbers for deposited data used in Fig. 4a: GSE26809 (ref. 35) and SRA029279 (ref. 37).

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**Author contributions**

S.A.W., E.R.W., R.A.H. and K.F.R.-G. designed the experiments. S.A.W. performed the experiments and analysis for Figs 2.5 and 6 and Supplementary Figs 1.2 and 3. E.R.W. performed the experiments and analysis for Supplementary Fig. 5. C.F.H. performed the experiments and analysis for Fig. 1. S.N. and E.R.W. contributed the experiments and analysis for Figs 4.5 and 5. E.R.W. contributed the experiments and data analysis for Figs 1, 2, 6 and Supplementary Fig. 6. C.F.H., E.R.W. and S.A.W. contributed the experiments and analysis for Fig. 8. Data analysis for Figs 1, 2 and Supplementary Fig. 6 was performed by C.F.H., E.R.W. and S.A.W. L.P.C. contributed to Figs 5–7 and Supplementary Fig. 4. B.V.Z. generated rAAV for Fig. 7 and Supplementary Fig. 4. M.R.D helped design and guide the experiments outlined in Figs 1 and 8, and Supplementary Fig. 6. K.F.R.-G. and S.A.W. wrote the manuscript. K.F.R.-G., S.A.W., R.A.H. and F.N. edited the manuscript.

**Additional information**

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