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Negative allosteric modulation of $GABA_A$Rs at α5 subunit-containing benzodiazepine sites reverses stress-induced anhedonia and weakened synaptic function in mice

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Abstract

Background: Abnormal reward processing, typically anhedonia, is a hallmark of human depression and is accompanied by altered functional connectivity in reward circuits. Negative allosteric modulators of $GABA_A$ receptors (GABA-NAMs) have rapid antidepressant-like properties in rodents and exert few adverse effects, but molecular targets underlying their behavioral and synaptic effects remain undetermined. We hypothesized that GABA-NAMs act at the benzodiazepine site of $GABA_A$Rs containing α5 subunits to increase gamma oscillatory activity, strengthen synapses in reward circuits, and reverse anhedonia.

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Methods: Anhedonia was induced by chronic stress in male mice and assayed by preferences for sucrose and female urine (n=5-7 mice/group). Hippocampal slices were then prepared for electrophysiological recording (n=4-6/mouse). EEG power was quantified in response to GABA-NAM and ketamine administration (n=7-9 mice/group).

Results: Chronic stress reduced sucrose and female urine preferences, and hippocampal temporoammonic-CA1 synaptic strength. A peripheral injection of the GABA-NAM MRK-016 restored hedonic behavior and AMPA:NMDA ratios in wildtype mice. These actions were prevented by pretreatment with the benzodiazepine site antagonist flumazenil. MRK-016 administration increased gamma power over the prefrontal cortex in wildtype mice, but not α5 KO mice, whereas ketamine promoted gamma power in both genotypes. Hedonic behavior and AMPA:NMDA ratios were only restored by MRK-016 in stressed wildtype mice, but not α5 KO mice.

Conclusions: Alpha5 selective GABA-NAMs exert rapid anti-anhedonic actions and restore the strength of synapses in reward regions by acting at the benzodiazepine site of α5-containing $GABA_A$Rs. These results encourage human studies using GABA-NAMs to treat depression by providing readily translatable measures of target engagement.

Keywords

GABA; antidepressant; ketamine; depression; gamma; hippocampus

Major depressive disorder (MDD, depression) afflicts an estimated 264 million people worldwide. The cardinal symptoms of MDD are chronically depressed mood and the inability to experience pleasure from previously rewarding stimuli, or anhedonia. Feelings of anhedonia are strongly correlated to suicidal ideation[1,2] and 66 - 90% of all completed suicides are carried out by those with mood disorders[3].

Selective serotonin reuptake inhibitors (SSRIs) and other monoamine-modulating drugs are the standard treatment for MDD, but SSRIs typically require 4 to 8 weeks to elicit symptomatic relief, produce remission in only half of patients[4], and exert significant side effects[5]. (*S*)-Ketamine, an NMDA antagonist, is the first FDA-approved fast-acting antidepressant, with relief of symptoms occurring within hours and persisting for days-to-weeks[6,7]. Ketamine, however, has significant risks for abuse and produces a dissociative alteration of consciousness. Because ketamine antagonizes NMDARs throughout the entire brain, it is difficult to minimize these undesirable effects while retaining its antidepressant actions.

Ketamine may exert its beneficial effects, at least in part, by acutely decreasing the excitation of inhibitory interneurons, thereby promoting synchronous neuronal discharges in pyramidal cells[8-11] and activating several activity-dependent synaptic strengthening processes[12-16]. Negative allosteric modulators of $GABA_A$ receptors (GABA-NAMs) that contain α5 (alpha5) subunits represent a promising alternative to ketamine because they produce disinhibition directly and thereby promote increased excitatory glutamatergic transmission[17-20]. Drugs that target α5 subunit-containing $GABA_A$Rs are uniquely attractive because expression of this class of receptor is greatly enriched in the hippocampus and

prefrontal cortex[21-23], two stress-sensitive brain regions implicated in regulating emotion and motivation.

Two common α5-preferring GABA-NAMs, MRK-016 and L655,708, have been shown to exert rapid and persistent beneficial behavioral effects in rodent models of stress-induced anhedonia[24,25] and in tests of potential antidepressant efficacy, such as the forced swim test[25-29]. GABA-NAMs bind $GABA_A$Rs with high affinity at the interface of an α and γ subunit, a site that is shared with benzodiazepine positive allosteric modulators[30]. GABA-NAMs bind to recombinant human $GABA_A$Rs containing different α subunits with a range of affinities, selectivities, and potencies[30-32]. L655,708, for example, has 50-100-fold selectivity for binding to $GABA_A$Rs containing α5 subunits, but only inhibits GABA responses by ca. 25%[33]. MRK-016, in contrast, binds with comparable nM-affinity to $GABA_A$Rs containing α1, α2, α3, and α5 subunits, but potently inhibits responses only at α5-containing $GABA_A$Rs (>50% inhibition)[31]. To advance α5-selective GABA-NAMs to clinical trials for human MDD, it is essential to determine whether they exert their antidepressant-like actions through engaging their predicted target.

We therefore used the potent GABA-NAM, MRK-016, to test the hypothesis that α5-preferring GABA-NAMs exert their antidepressant-like behavioral and synaptic actions by acting at the benzodiazepine binding site of α5-containing $GABA_A$Rs. If true, then we predicted that both the non-subunit specific antagonist of the benzodiazepine site, flumazenil, and the genetic deletion of the α5 subunit, would be sufficient to prevent the rapid restoration of stress-induced deficits in hedonic behavior and synaptic strength.

Materials and Methods

Additional methodological details are provided in Supplemental Materials.

Animals and housing:

Male mice were group-housed with *ad libitum* access to water and standard rodent chow. Mice were 8 weeks-old at the start of chronic multimodal stress (CMMS) and were transferred to clean caging, where they remained singly housed through the duration of the experiment.

$GABA_A$R α5 knockout (KO) mice:

Initial breeding pairs of global $GABA_A$R α5 KO mice on a C57BL/6J background[34] were transferred from UR's colony at McLean Hospital to the University of Maryland.

Drug Treatment:

MRK-016 (Tocris Bioscience, R&D Systems, Minneapolis, MN) was prepared in 100% DMSO and injected at 3mg/kg intraperitoneally (i.p.), a dose shown to produce 85% receptor occupancy in rats[31] (injection volume = 40-50 μL). Flumazenil (Tocris Bioscience) was prepared in 100% DMSO and injected at 20mg/kg. This dose was calculated to be sufficient to antagonize the benzodiazepine site based on half-life in the rodent brain and its ability to restore the benzodiazepine-induced loss of righting-reflex in mice[35] for the

duration of MRK-016's rodent elimination half-life[31]. (*R,S*) ketamine was purchased from Sigma-Aldrich, prepared in 0.9% saline and injected at 10mg/kg i.p. (100-150 μL).

Behavioral Protocols

Chronic multi-modal stress (CMMS):

Mice were singly housed in a fresh cage at the onset of the stress protocol. Starting at 10 AM, each animal was immobilized in a translucent plastic restraint tube and subjected to white noise and strobe lighting for 4 hours a day for 10 concurrent days. All mice were returned to their home cages and singly housed for the duration of the experiment.

Sucrose preference test (SPT):

Bottles containing either 1% sucrose solution or tap water were placed in the home cage overnight. Sucrose preference was calculated by dividing the weight of 1% sucrose solution consumed by the total weight consumed from both bottles. This was repeated the following night with the two nightly sucrose preference values averaged. Mice displaying a sucrose preference of >65% at baseline which decreased by at least 10% to a value of <65% following stress were considered stress-susceptible and included in further sucrose preference tests.

Female urine sniff test (FUST):

Two cotton swabs individually soaked in male urine and urine from females in estrus were placed at opposite ends of the cage. Time spent sniffing each swab during a 3 min trial was quantified by a trained observer blinded to treatment and position of each swab. Female urine preference was calculated as the total time a mouse spent sniffing the female urine swab over the total time sniffing both swabs. Mice displaying a female urine preference of greater than 50% at baseline that decreased by at least 10% following stress were considered stress-susceptible and used in the FUST arm of the study.

Electrophysiology

Extracellular recording:

Mice were anesthetized with isoflurane and decapitated. The hippocampus was rapidly excised, mounted into a 3% agar block, and sectioned into 400μm transverse slices while submerged in ice-cold ACSF comprised of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose, and bubbled with 95% O_2/5% CO_2. All slices were kept in a humidified recovery chamber for at least one hour prior to recording. Hippocampal region CA3 was removed with a razor and slices were transferred to an immersion recording chamber. Glass recording electrodes were placed in stratum lacunosum-moleculare (SLM), with concentric bipolar tungsten stimulating electrodes placed approximately >500 μm away in the temporo-ammonic (TA) pathway.

Field EPSPs (fEPSPs) were recorded extracellularly in room temperature (20-22°C) Mg^{2+}-free ACSF perfused at a rate of 1mL/min. Picrotoxin (100 μM) and CGP54626

(2μM) (Tocris Bioscience) were added to the bath to block $GABA_A$Rs and $GABA_B$Rs, respectively. Sequential 15-minute wash-ins of DNQX (50μM) and D,L - APV (80μM) (Tocris Bioscience) were used to isolate the components of the fEPSP mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, respectively[36]. The initial slope (1.5ms after fEPSP onset prior to DNQX wash-in) was used to quantify the AMPAR-mediated component of the fEPSP and the slope 4.0ms after fEPSP onset, following DNQX wash-in, was used to quantify the NMDAR-mediated component. The slopes were normalized to their fiber volley amplitude. Traces at each stimulation intensity were averaged, and pairs of responses recorded at the same intensities before and after DNQX wash-ins with an NMDA component closest to 0.1mV/ms were divided to calculate an AMPA:NMDA ratio. Analysis was performed blind to animal genotype or treatment. Individual data points represent the average of all AMPA:NMDA ratios collected from slices from one individual animal (n=1-6 slices per animal), where responses in all individual slices are shown in the AMPA slope and NMDA slope graphs.

EEG recording:

12-week-old male $GABA_A$R α5 KO mice and their wildtype littermate controls were anesthetized with 3% isoflurane, with anesthesia maintained using 1.5% isoflurane. An F20-EET radiotelemetric transmitter (Data Sciences International, Minneapolis, MN, USA) was embedded subcutaneously between the scapulae. Transmitter leads were implanted unilaterally above the prefrontal cortex. Mice were allowed to recover seven days prior to experiments.

EEG signals were recorded with the Ponemah software suite (Data Sciences International, Minneapolis, MN, USA). Baseline activity was recorded for 3 hours during the light cycle. Mice were then treated with a volume-equivalent dose of 100% DMSO vehicle (i.p., 40-50 μL) and the EEG was recorded for 90 minutes. The mice were then given 3 mg/kg MRK-016 (i.p.) and EEG recordings continued for another 90 minutes. Subsequent injections of saline and 10 mg/kg (*R,S*) ketamine were given 24 hours later using the same protocol. Power analysis was conducted in Neuroscore (DSI, Harvard Bioscience) and spectrograms were generated in MathWorks MATLAB with oscillations at delta frequencies defined as 1-3Hz, theta at 4-7Hz, alpha at 8-12Hz, beta at 13-29Hz and gamma at 30-80Hz. Post-treatment powers were normalized to their average power during the last 30 minutes of the baseline recording.

Statistical analysis:

All one-, two- and three-way ANOVA, Student’s t-tests, linear regressions and correlations were performed using GraphPad Prism 9 software. Three-way ANOVAs were calculated as uncorrected values and Šidák corrections were performed manually in Microsoft Excel. One and two-way ANOVAs employed Holm-Šidák corrections for multiple comparisons. Figures represent group average ± SEM.

Results

Pretreatment with the benzodiazepine site antagonist flumazenil prevents the effects of MRK-016 on reward behavior and synaptic strength

We first asked whether MRK-016 mediates its anti-anhedonic actions by acting at the benzodiazepine binding site of $GABA_A$Rs. Flumazenil is a functionally neutral, competitive antagonist of benzodiazepine agonists[37] and MRK-016[31], and has minimal impact on $GABA_A$R-mediated currents at physiological concentrations of GABA[38]. We therefore tested whether flumazenil would block the behavioral and synaptic actions of MRK-016 in chronically stressed mice.

After measuring baseline behaviors, 8-week-old male C57BL/6J mice were exposed to a 10-day period of chronic multimodal stress (CMMS) (Fig 1A). Mice subjected to CMMS displayed significant decreases in their responses in two different hedonic behavioral assays: the sucrose preference test (SPT), measuring consumption of 1% sucrose solution compared to consumption of tap water, and the female urine sniffing test (FUST), measuring time spent interacting with cotton swabs soaked in urine from either female mice in estrous or male mice (Fig. 1B,C). Mice susceptible to CMMS displayed significant reductions in preference for both sucrose and female urine. A single 3mg/kg i.p. injection of MRK-016 significantly improved preferences in both SPT and FUST assays 24-48 hours later, compared to post-stress values. No significant change in reward behaviors was observed in mice treated with an equivalent volume of vehicle. In contrast, mice pretreated with 20mg/kg flumazenil, followed 5 minutes later by administration of either 3mg/kg of MRK-016 or an equivalent volume of vehicle showed no significant improvement in preferences in either the SPT and FUST assays, compared to post-stress values. No significant changes in reward behaviors were observed in mice treated with flumazenil alone.

Following behavioral testing, these same mice were euthanized, and hippocampal slices were prepared in order to determine whether flumazenil administration had any effect on the ability of MRK-016 to restore the strength of TA-CA1 hippocampal synapses (Fig 2A,B). Concomitant with restoration of reward behavior, significantly higher TA-CA1 AMPA:NMDA ratios were observed in hippocampal slices taken from mice given 3 mg/kg MRK-016, compared to stressed mice treated with vehicle alone. AMPA:NMDA ratios in slices from stressed mice that received MRK-016 were not different to those from unstressed mice, with or without MRK-016 administration. Mice that were pretreated with flumazenil prior to MRK-016 administration displayed significantly smaller AMPA:NMDA ratios than those pretreated with vehicle prior to MRK-016. The increase in TA-CA1 AMPA:NMDA ratio was mediated by a selective increase in the slope of the AMPA-mediated component of the fEPSP (Fig 2C,D). There was a significant positive correlation between behavioral responses in the SPT and FUST of individual mice with their calculated AMPA:NMDA ratio (Fig 2E,F).

These behavioral and electrophysiological actions of MRK-016 are consistent with previous observations following chronic stress and administration of GABA-NAMs in rats[24]. We conclude that the ability of MRK-016 to produce an anti-anhedonic behavioral response

and to strengthen stress-sensitive synapses requires its binding to the benzodiazepine site of $GABA_ARs$.

The behavioral and electrophysiological effects of MRK-016 require the $GABA_AR$ α5 subunit

Benzodiazepine binding sites can be formed by most $GABA_AR$ α subunits and MRK-016 has comparable affinity for several α subunits. We therefore asked whether an action at α5 subunits was required for the behavioral and electrophysiological effects of MRK-016 using mice with global knockout (KO) of the α5 subunit gene[34].

Acute increases in electroencephalogram (EEG) power in the high frequency gamma band (30-80Hz) are a hallmark of a number of rapid acting antidepressant interventions in mice and humans[15,39,40] and are believed essential to their persistent actions. We have previously demonstrated that MRK-016 administration also promotes increases in EEG gamma power quantified over the PFC[27]. We therefore first tested whether MRK-016 would increase EEG gamma power in α5 KO mice.

Cohorts of 12-week-old male α5 KO mice and wildtype (WT) littermate control mice were given DMSO vehicle injections (i.p.) followed 90 minutes later by 3mg/kg of MRK-016. Wildtype mice demonstrated significantly higher levels of gamma oscillatory activity 60 minutes following MRK-016 administration compared to α5 KO mice (Fig 3A,B,E,F). As a positive control for the ability of the α5 KO mice to generate gamma activity, these same mice were treated 24 hours later with saline vehicle followed by 10mg/kg (*R,S*) ketamine 90 minutes later. Both WT and α5 KO mice displayed significantly higher EEG gamma power 60 minutes after ketamine administration compared to their saline values (Fig 3C,D,G,H). We conclude that MRK-016 requires $GABA_ARs$ containing α5 subunits to generate increases in EEG gamma power.

Concomitant with the increase in gamma power, MRK-016 produced a significant decrease in EEG power lasting >30 min within the theta (4-7 Hz), alpha (8-12 Hz), and beta (13-29 Hz) bands in wild type mice, but not in α5 KO mice (Fig S1). Decreases in low frequency EEG power were also observed in response to ketamine, however these decreases were more transient than those elicited by MRK-016 and were equivalent in both wild type and α5 KO mice (Fig S2).

We next tested whether MRK-016 could reverse deficits in hedonic behavior and synaptic strength in stressed α5 KO mice. A cohort of 8-week-old male α5 KO mice and WT littermate controls were subjected to 10 days of CMMS. Stress-susceptible mice were treated with a single i.p. injection of 3mg/kg MRK-016 or an equivalent volume of vehicle (Fig 4A). Significant restoration of both SPT and FUST preferences compared to post-stress values was observed only in wildtype mice treated with MRK-016 (Fig 4B,C). No significant difference between post-stress and post-treatment values was observed in α5 KO mice given MRK-016 or in mice treated with vehicle alone.

Following behavioral testing, hippocampal slices were prepared for electrophysiological recording of TA-CA1 synaptic strength. Significantly greater AMPA:NMDA ratios

were observed in stressed WT mice given MRK-016, compared to stressed WT mice receiving vehicle (Fig 5A,B). In contrast, there was no significant difference between the AMPA:NMDA ratios of stressed α5 KO mice given either MRK-016 or vehicle. The increase in TA-CA1 AMPA:NMDA ratio was mediated by a selective increase in the slope of the AMPA-mediated component of the fEPSP(Fig 5C,D). An individual animal's TA-CA1 synaptic strength was positively correlated with hedonic behavior in these experiments (Fig 5E,F).

To confirm the hedonic behavior of α5 KO mice is not innately refractory to rapid-acting antidepressant compounds, a separate cohort of α5 KO mice and WT littermate controls were chronically stressed for 10 days and given three treatments: an MRK-016 dose-equivalent volume of vehicle, 3mg/kg MRK-016, and 10mg/kg (R,S) ketamine, each spaced 72 hours apart (Fig S3). When given MRK-016, only the wildtype cohort showed significant restoration of hedonic behavior. Both SPT and FUST preferences in the α5 KO mice were significantly improved compared to post-stress values after administration of ketamine.

MRK-016 does not alter hedonic behavior or synaptic strength in unstressed mice

A separate cohort of unstressed, 10-week-old, male α5 KO mice and WT littermates were given 3mg/kg of MRK-016 or an equivalent volume of vehicle (Fig S4). Sucrose preferences remained unchanged following treatment with MRK-016 or vehicle, regardless of genotype. Hippocampal slices were prepared from these mice and TA-CA1 AMPA:NMDA ratios were found to be the same across genotypes and treatments.

Discussion

Negative allosteric modulators of α5 subunit-containing $GABA_A$Rs are a promising class of compounds for the treatment of major depressive disorder. In preclinical models, GABA-NAMs produce a rapid and persistent restoration of reward behaviors in rats with stress-induced anhedonia[24], a finding we replicate here in mice, while exerting fewer potential side effects than ketamine[26,27], the only fast-acting antidepressant currently approved by the FDA. A critical next step in advancing GABA-NAMs from preclinical studies to FDA approval is to ensure that they exert their actions by engaging their expected target. This is especially important for putative α5-selective GABA-NAMs because many of them have comparable affinity at multiple $GABA_A$ receptor α subunits[31]. Here we tested the hypothesis that their behavioral and electrophysiological actions are mediated at the benzodiazepine binding site of $GABA_A$Rs formed by α5 subunits, as predicted by *in vitro* binding assays[31]. We found that both the benzodiazepine site antagonist flumazenil and deletion of the α5 gene led to a failure of the GABA-NAM MRK-016 to exert an anti-anhedonic response in two assays of reward behavior or restore the strength of stress-weakened excitatory synapses in the hippocampus. We conclude the behavioral and electrophysiological actions of α5-selective GABA-NAMs require binding to the benzodiazepine site on α5 subunit-containing $GABA_A$Rs.

Utility of stress-induced anhedonia as a readout of efficacy

Depression is a complex, uniquely human psychological disorder with a range of symptoms, including the two cardinal symptoms of persistently depressed mood and anhedonia. Preclinical studies have better predictive outcomes when they are based on models that are both based on shared putative causes and result in behavioral changes that are analogous to human symptoms. Chronic stress is known to be positively correlated with the likelihood of human depression. For example, depressed patients report more stressful life events than non-depressed subjects, including physical illness, troubled family relationships, and financial difficulty[41]. In the human brain, depression is associated with altered brain structure and functional connectivity across a range of regions, including reward processing regions[42-44]. In rodents, stress also has a number of deleterious effects on excitatory synaptic function, including dendritic atrophy[45], decreases in dendritic spine density[46,47], decreases in glutamate receptor expression[36,48], and reduced expression of the synaptic growth-promoting factors, such as BDNF[49]. Our finding that the binding of GABA-NAMs to their appropriate target is required for their anti-anhedonic and synaptic actions in these models thus offers preclinical endpoints with the best available predictive reliability for future human trials.

We note that α5-selective positive allosteric modulators (GABA-PAMs) have been suggested as potentially useful antidepressant compounds[50], which would be inconsistent with our proposed model. This suggestion was based on prior immunocytochemical and Western blotting studies of postmortem tissue samples from depressed subjects[51,52] and preclinical stress models[53], suggestive of GABAergic interneuron hypofunction that could be reversed by GABA-PAM administration. Effects of an α5-selective GABA-PAM after chronic stress were not seen in male mice, and were observed only for an anxiety behavior in stressed female mice[50], suggestive of anxiolytic rather than anti-anhedonic actions.

Mechanisms underlying GABA-NAM action

Negative allosteric modulators of $GABA_A$Rs decrease the ability of GABA to open the ion channel, thereby promoting cell and network excitability by effectively diminishing the “brake” on the depolarization of excitatory pyramidal cells[54-56]. While diminishing the efficacy of GABA action nonspecifically promotes seizures[57], α5 subunit-selective GABA-NAMs do not produce or promote seizures in mice or human subjects[31]. This is likely because α5-containing $GABA_A$Rs represent a small subset of all $GABA_A$Rs, even in the hippocampus and PFC, where they are expressed at the highest levels[21-23]. Alpha5-selective GABA-NAMs do not produce significant off-target effects in preclinical models[27,58] or in limited human studies[31], perhaps because the ratio of α5-containing to α5-lacking $GABA_A$Rs is much lower outside of the hippocampus and prefrontal cortex.

Alpha5-containing $GABA_A$Rs contribute to both tonic and phasic inhibition[18,19,59] of pyramidal cells in the hippocampus and PFC, and are also expressed at synapses formed by dendrites targeting inhibitory interneurons[20,55,56]. By reducing both forms of inhibition, α5 subunit-selective GABA-NAM can be predicted to promote pyramidal cell discharge, dendritic NMDAR activation, and plasticity of excitatory synapses[55,60]. Therefore, α5-selective GABA-NAMs provide the means to selectively and safely alter the balance of

excitation and inhibition primarily within the hippocampus and PFC in a transient manner that leads to a persistent improvement in circuit function. Alpha5-containing $GABA_A$Rs are also expressed at synapses between inhibitory interneurons[61]. We have not tested whether the critical α5-containing $GABA_A$Rs contributing to anti-anhedonic action are expressed by pyramidal cells[18,19,59] or interneurons[34].

Our results suggest that α5-selective GABA-NAMs exert their anti-anhedonic actions through the following mechanism (Fig 6). GABA-NAM – induced increases in the excitability of pyramidal cells favor mutual excitation locally and promote excitatory drive in efferent projections to subcortical targets. Indeed, MRK-016 promotes an increase in EEG power in the gamma frequency band and decreases in power in the lower frequency bands over the PFC in wildtype mice, but not in mice lacking α5 subunits. Because ketamine increases gamma power in α5 KO mice, we can conclude that promotion of gamma oscillations by GABA-NAMs is specifically triggered at the benzodiazepine site formed by α5 $GABA_A$R subunits, rather than a defect in the function of critical circuits in the absence of α5-containing $GABA_A$Rs. Alpha5-containing $GABA_A$Rs have been implicated in maintaining cortical functional connectivity through the generation of high frequency EEG gamma power[17], however we observed no difference in EEG gamma power generated *in vivo* between wildtype and global α5 KO mice following ketamine administration. There is considerable evidence in preclinical models and humans for structural and functional deficits in synaptic connectivity in depression[13,14,42,43,62]. Such deficits in reward circuits could explain the symptom of anhedonia. GABA-NAM – induced increases in synchronous high frequency discharge is likely to be a powerful activator of a range of activity-dependent synaptic strengthening processes, including increased BDNF-trkB signaling, increased expression of AMPARs, or mTORC1 activation, much like ketamine[12,14,16,29]. It is widely believed that these mechanisms can then act to restore the function of stress-weakened synapses, both within the PFC and hippocampus, as well as in downstream targets receiving their synchronized output, such as the NAc[63]. Improved function in reward circuits could explain the anti-anhedonic actions of GABA-NAMs. These mechanisms can also account for the persistence of the therapeutic actions long after the compounds have been eliminated[13,15,64]. Indeed, increases in EEG gamma power are a hallmark of putative rapid-acting antidepressants, such as ketamine and its metabolites (e.g. hydroxynorketamines) in rodents[65] and humans[40,66].

Clinical potential of α5-selective GABA-NAMs

Originally developed for their nootropic potential, α5-selective GABA-NAMs represent promising tools for the treatment of MDD, as well as other disorders with significant comorbid mood dysregulation. They may also exert potential therapeutic actions in a variety of other conditions in which there is a pathological imbalance between synaptic inhibition and excitation[30]. For example, there is strong evidence that α5-selective GABA-NAMs can improve cognitive function by dampening excess inhibition in preclinical models of Downs syndrome[20,67], although initial clinical trials have failed to detect benefits (ClinicalTrials.gov ID NCT02024789). They may also improve postsurgical cognitive outcomes following the use of common general anesthetics[68,69].

We conclude that the rapid-acting antidepressant-like effects of MRK-016 are mediated via negative allosteric modulation of the $GABA_A$ receptor at an α5-containing benzodiazepine site, thereby promoting transient generation of EEG gamma power and increased hippocampal synaptic strength. Our findings directly link MRK-016's target engagement with its downstream synaptic effects and the ultimate restoration of reward-seeking behaviors to a physiological normal. They also suggest two powerful approaches to ensure target engagement in future human trials, which will aid in their clinical development. First, displacement of radiolabeled $GABA_AR$ ligands can be readily performed in human subjects[61] and EEG or MEG recording of changes in gamma power[66] can be used to determine appropriate dosing and ensuring target engagement. These preclinical mechanistic results may provide a model for developing additional rapid-acting antidepressant-like compounds, as well as accelerating the use of GABA-NAMs in human clinical trials for mood disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements and disclosures

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TAT and SMT conceived and designed the project and wrote the manuscript. TAT, PZ and PG performed all behavioral experiments. TAT performed all electrophysiological experiments. PZ, PG and TDG assisted with design and performance of behavioral and EEG experiments. UR provided α5 KO mice and assisted with genotyping. All authors contributed to the editing of the manuscript. Data from this manuscript was previously shown in posters at 2020 meetings of The Society for Neuroscience and American College of Neuropsychopharmacology under the title of "The $GABA_AR$ α5 subunit is required for the fast antidepressant-like actions of MRK-016 on stress-induced anhedonia and weakened synaptic function."

The University of Maryland Baltimore has a patent pending (USPTO application number 15/300,984), on which SMT is listed as an inventor, covering the use of α5-selective GABA-NAMs to treat psychiatric disease. T.D.G. is listed as a co-author of patent applications related to the pharmacology and use of (2R,6R)-hydroxynorketamine in the treatment of depression, anxiety, anhedonia, suicidal ideation and post-traumatic stress disorder. TDG has received research funding from Allergan and Roche Pharmaceuticals and served as a consultant for FSV7 LLC during the preceding 3 years. TT, PZ, PG, and UR declare that they have no biomedical financial interests or potential conflicts of interest.

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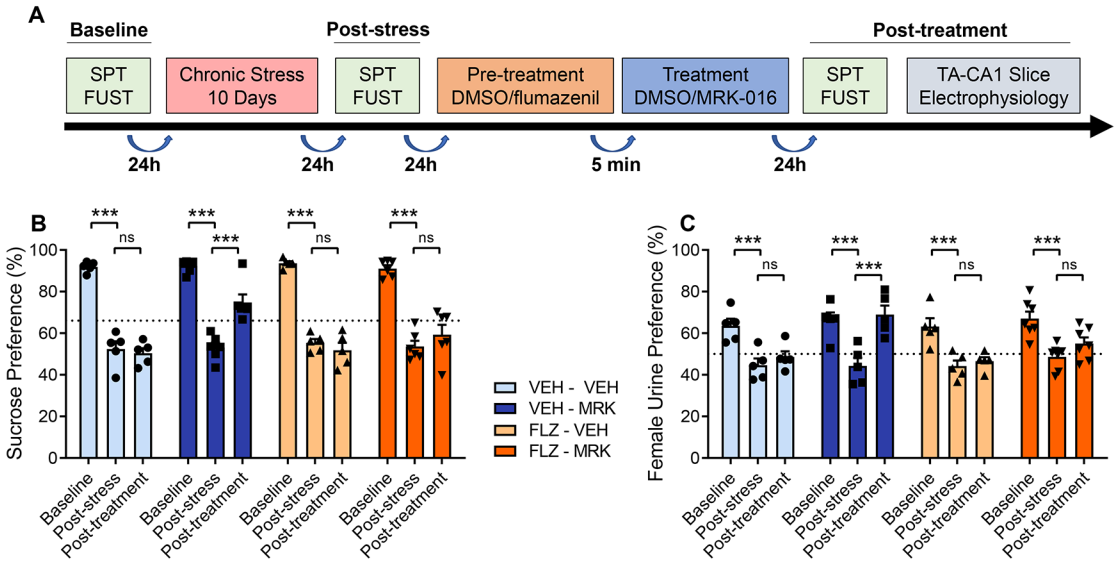

Figure 1.

Flumazenil prevents restoration of hedonic behavior by MRK-016 in stressed mice. (**A)** Timeline of hedonic behavior assessment in relation to chronic stress and drug treatment. **(B)** 10 days of CMMS significantly decreased sucrose preference compared to baseline in all groups (vehicle-vehicle, n=5, p=0.0012; vehicle-MRK-016, n=6, p=0.0012; flumazenil-vehicle, n=5, 0.0012, MRK-016, n=5, p=0.0012). Administration of 3mg/kg MRK-016 significantly increased sucrose preference over post-stress values (p=0.0012) in vehicle pretreated mice. Pretreatment with 20mg/kg flumazenil prevented significant restoration of sucrose preference from their post-stress values (p=0.1256). Neither vehicle (p=0.6120) or flumazenil alone (p=0.6130) improved sucrose preferences following stress. Three-way ANOVA revealed a significant effect of stress ($F_{2,36}$ = 242.3, p<0.0001), MRK-016 ($F_{1,36}$ = 242.3, p<0.0001) and stress × MRK-016 ($F_{2,36}$ = 12.98, p<0.0001), but not stress × flumazenil × MRK ($F_{2,36}$ = 2.35, p=0.1098). **(C)** CMMS decreased female urine preference in each group (vehicle-vehicle, n=5, p=0.0012; vehicle-MRK-016, n=5, p=0.0012; flumazenil-vehicle, n=5, p=0.0012, MRK-016, n=7, p=0.0012). MRK-016 significantly increased female urine preferences following stress (p=0.0012), but this was prevented by flumazenil pretreatment (p=0.1869). Female urine preference was not significantly different following administration of vehicle (p=0.6678) or flumazenil (p=0.5281) alone. Three-way ANOVA revealed a significant effect of stress ($F_{2,36}$ = 59.44, p<0.0001), MRK-016 ($F_{1,18}$ = 6.661, p=0.0188) and stress × MRK-016 ($F_{2,36}$ = 7.272, p=0.0022), but not stress × flumazenil × MRK ($F_{2,36}$ = 3.038, p=0.0604). p<0.005 ***, ns: not significant.

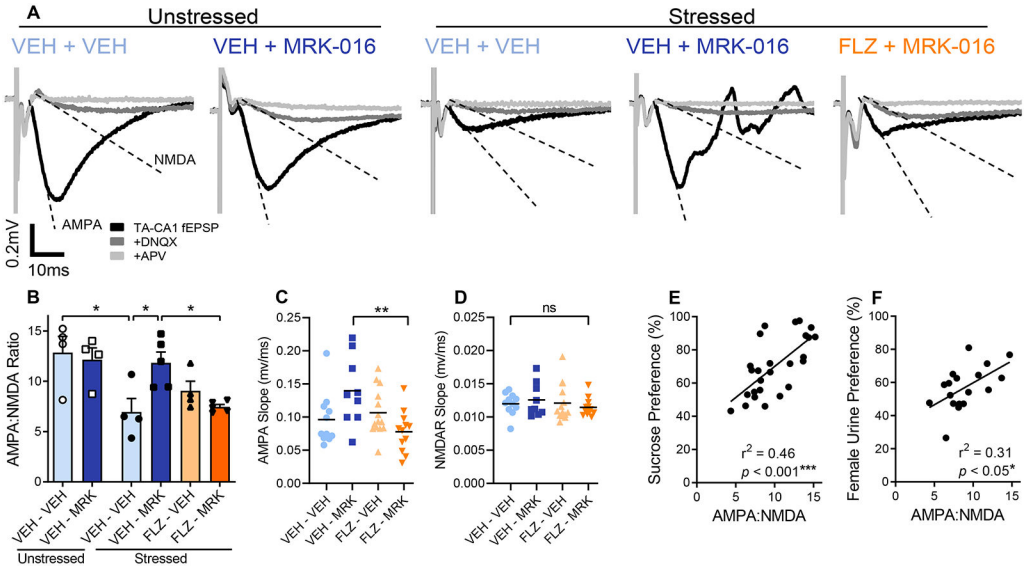

Figure 2.

Flumazenil prevents restoration of synaptic strength by MRK-016 in stressed mice. **(A)** Representative traces comparing AMPA:NMDA ratios in stressed animals given MRK-016 with or without flumazenil pretreatment. **(B)** CMMS decreases TA-CA1 synaptic strength. Stressed DMSO vehicle treated mice had a significantly lower TA-CA1 AMPA:NMDA ratio than unstressed mice (n=4 animals, p=0.0298). Among stressed mice, those administered 3mg/kg MRK-016 (n=5) have significantly higher AMPA:NMDA ratios than those receiving vehicle alone (n=4, p=0.0167) or MRK-016 in conjunction with flumazenil pretreatment (n=4, p=0.0199). Two-way ANOVA of treated stress-susceptible animals indicated significant pretreatment × treatment interaction ($F_{1,14}$ = 11.50, P=0.0044). **(C)** MRK-016 increases the AMPA-mediated component of the TA-CA1 fEPSP in stress-sensitive animals, which is prevented by flumazenil (n=individual slices from Fig. 2B, p=0.0079). One-way ANOVA indicated significant effect of treatment on AMPAR-mediated slope ($F_{3,42}$ = 4.47, P=0.0084). **(D)** MRK-016 administration does not change the NMDAR-mediated component of the TA-CA1 fEPSP. One-way ANOVA indicated no effect of treatment on NMDAR-mediated slope ($F_{3,42}$ = 0.4757, P=0.7009). **(E)** TA-CA1 synaptic strength correlates with an animal's sucrose preference (n=26, r=0.6763, p=0.0001. Line of fit: Y = 0.1220*X + 1.465). **(F)** TA-CA1 synaptic strength correlates with an animal's female urine sniffing preference (n=18, r=0.5561, p=0.0164. Line of fit: Y = 0.1167*X + 2.272). p<0.05 *, p<0.01 **, p<0.005 ***, ns: not significant

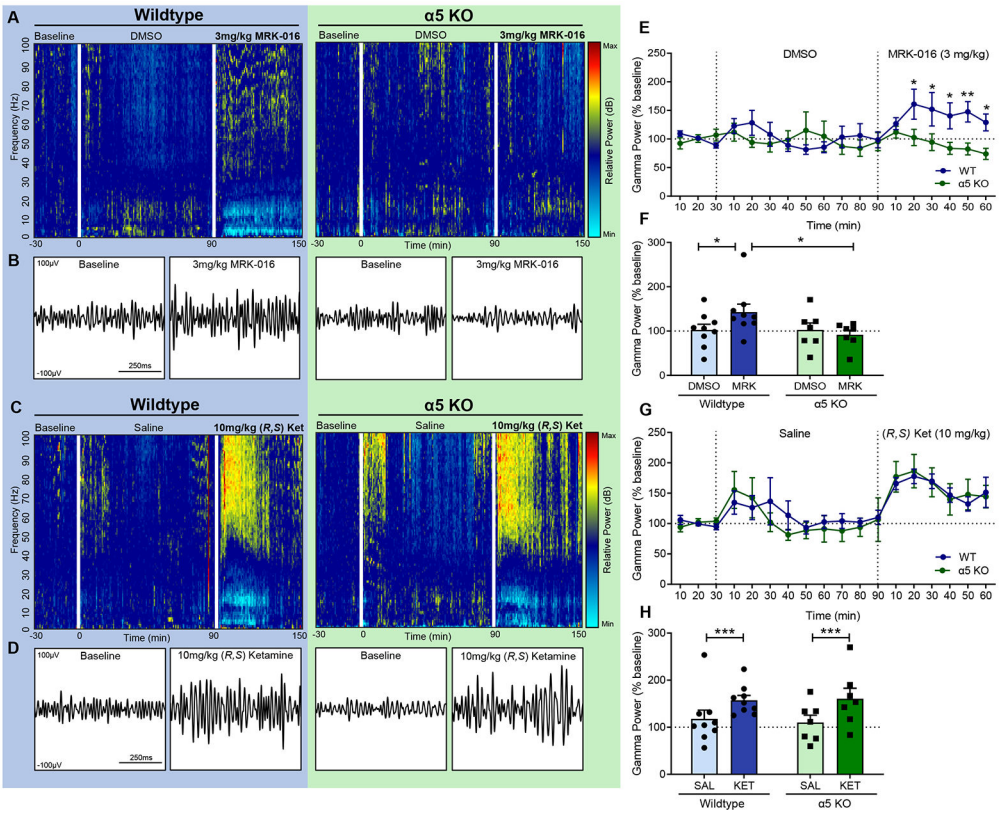

Figure 3.

Induction of EEG gamma activity by MRK-016 is absent in mice lacking $GABA_AR$ α5 subunits. **(A, C)** Representative EEG spectrograms showing oscillatory activity normalized to baseline for wildtype and α5 KO mice respectively. More relative activity at a given frequency range is indicated by “warmer” colors, and less by “cooler” colors. **(B, D)** Representative EEG gamma activity (750ms) at baseline and 30 minutes after treatment, filtered to 30-80Hz. **(E)** EEG gamma powers (30-80hz) were normalized to the last 30 minutes of baseline gamma activity following DMSO vehicle and MRK-016 administration. MRK-016 produces significantly higher cortical gamma power in wildtypes vs α5 KO animals beginning 20 minutes after administration. 2-way ANOVA identifies significant effect between time × genotype ($F_{17,238} = 2.678$, p=0.0005). **(F)** There is no significant difference in gamma powers at any time point between wildtype or α5 KO animals in response to ketamine. Time × genotype ($F_{17,238} = 0.5589$, p=0.9193). (**G)** MRK-016 promotes significant increases in gamma power over a 60-minute period after treatment compared to DMSO vehicle in wildtype (n=9, p=0.0462) but not α5 KO animals (n=7, p=0.9962). Wildtype animals generate significantly more gamma power compared to α5 KOs following MRK-016 administration (p=0.0390). 2-way ANOVA identifies significant effect between treatment × genotype ($F_{1,14} = 5.052$, p=0.0412). **(H)** Normalized EEG gamma power quantified for 60 minutes after 10mg/kg (*R,S*) ketamine (i.p.) administration was significantly greater than after volume-equivalent saline vehicle for both wildtype (n=9, p=0.0033) and α5 KO cohorts (n=7, p=0.0027). 2-way ANOVA identifies significant

treatment effect ($F_{1,14} = 28.35$, p=0.0001), but no significant interaction between treatment × genotype ($F_{1,14} = 0.4164$, p=0.5292). p<0.05 *, p<0.005 ***.

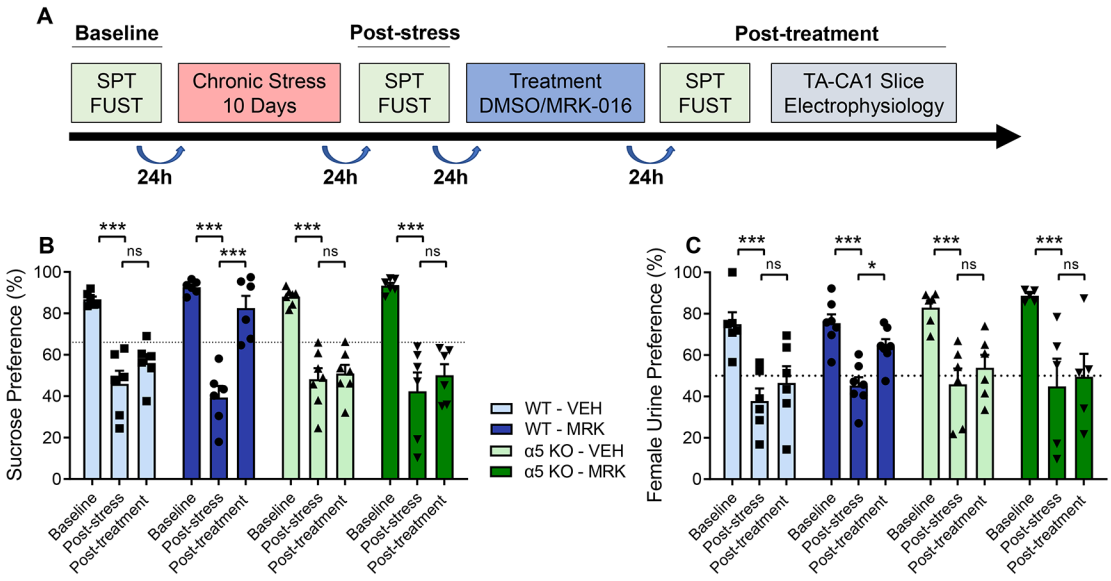

Figure 4.

Restoration of hedonic behavior by MRK-016 is absent in mice lacking $GABA_AR$ α5 subunits. **(A)** Timeline outlining the experimental protocol of assessing hedonic behavior and chronic multimodal stress. **(B)** 10 days of CMMS significantly decreased sucrose preference compared to baseline among all groups and genotypes (wildtype-vehicle, n=6, p=0.0012; wildtype-MRK-016, n=6, p=0.0012; α5 KO-vehicle, n=7, 0.0012, α5 KO-MRK-016, n=6, p=0.0012). Wildtype animals given 3mg/kg MRK-016 demonstrated significantly increased sucrose preferences compared to post-stress values (p=0.0012), but this effect was absent in the α5 KO cohort (p=0.5079). Administration of DMSO vehicle did not significantly alter sucrose preference values after stress in either the wildtype (p=0.5472) or α5 KO groups (p=0.6923). Three-way ANOVA revealed a significant effect of stress ($F_{2,42} = 83.35$, p<0.0001), stress × treatment ($F_{2,42} = 3.493$, p=0.0395), but not stress × treatment × α5 KO ($F_{2,42} = 2.360$, p=0.1069). **(C)** CMMS significantly lowered female urine preferences in all groups compared to baseline values (wildtype-vehicle, n=6, p=0.0012; wildtype-MRK-016, n=7, p=0.0012; a5 KO-vehicle, n=6, 0.0012, α5 KO-MRK-016, n=5, p=0.0012). MRK-016 significantly increases female urine preference following stress in wildtype (p=0.0296) but not α5 KO (p=0.5505) groups. Vehicle did not significantly improve female urine preferences following stress in either wildtype (p=0.5345) or α5 KO groups (p=0.4576). Three-way ANVOA showed a significant effect of stress ($F_{2,40} = 58.46$, p<0.0001). Stress × treatment ($F_{2,40} = 2.019$, p=0.1462). Stress × treatment × a5 KO ($F_{2,40} = 1.839$, p=0.1722). p<0.05 *, p<0.005 ***, ns: not significant.

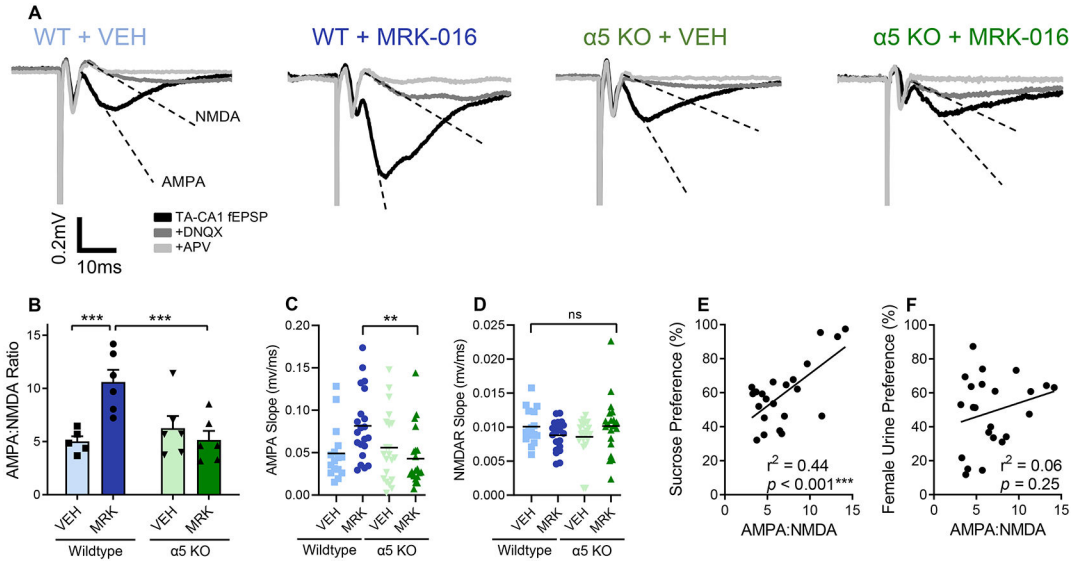

Figure 5.

Restoration of synaptic strength by MRK-016 is absent in mice lacking $GABA_AR$ α5 subunits. **(A)** Representative traces showing larger AMPA:NMDA ratios in wildtype animals compared to α5 KOs. **(B)** CMMS decreases TA-CA1 synaptic strength. Among stress-sensitive wildtype mice, 3mg/kg MRK-016 (n=6 animals) significantly increased AMPA:NMDA ratios compared to vehicle (n=5) treated animals (p=0.0045). AMPA:NMDA ratios in stress-sensitive α5 KO mice were not significantly different between vehicle (n=6) or MRK-016 (n=6) groups (p=0.7749). Wildtype mice displayed significantly higher AMPA:NMDA ratios than α5 KO mice after MRK-016 administration (p=0.0044). Two-way ANOVA indicated significant interaction of genotype × MRK-016 ($F_{1,19}$ = 11.62, P=0.0029). **(C)** MRK-016 increases the AMPA-mediated component of the TA-CA1 fEPSP in stress-sensitive wildtype but not α5 KO mice (n=individual slices from Fig. 5B, p=0.0079). One-way ANOVA indicated significant effect of treatment on AMPAR-mediated slope ($F_{3,75}$ = 4.211, P=0.0083). **(D)** MRK-016 administration does not change the NMDAR-mediated component of the TA-CA1 fEPSP. One-way ANOVA indicated no effect of treatment on NMDAR-mediated slope ($F_{3,75}$ = 1.476, P=0.2279). **(E)** TA-CA1 synaptic strength correlates with an animal's sucrose preference (n=23, r=0.6625, p=0.0006. Line of fit: Y = 3.791*X + 33.20). **(F)** TA-CA1 synaptic strength positively trends with an animal's female urine sniffing preference (n=23, r=0.2481, p=0.2536. Line of fit: Y = 1.637*X + 37.66). p<0.01 **p<0.005 ***, ns: not significant.

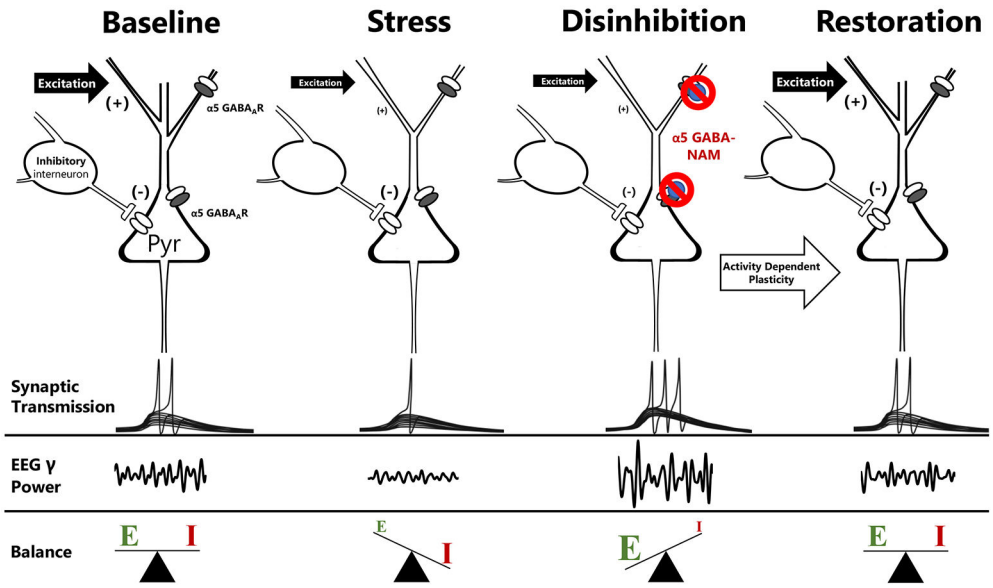

Figure 6.

Mechanisms underlying the antidepressant actions of α5 GABA-NAMs. Excitability and function in reward circuits is determined by the balance of excitatory and inhibitory transmission. Chronic stress has numerous deleterious effects on excitatory synaptic function, including reductions in net excitability, spine density, and dendritic branching. This shifts the balance of excitation and inhibition and contributes to the genesis of the anhedonic state. Negative allosteric modulators of α5-containing $GABA_A$Rs bind to benzodiazepine allosteric sites on α5-containing $GABA_A$Rs, including perisomatic extrasynaptic receptors and synaptic receptors on distal dendrites, to reduce GABAergic inhibition acutely, thereby promoting glutamatergic transmission and favoring high-frequency gamma frequency oscillations. These oscillations promote endogenous activity dependent plasticity mechanisms, thereby strengthening stress-weakened synapses, restoring excitation/inhibition balance, and rescuing hedonic behavior.

KEY RESOURCES TABLE

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Add additional rows as needed for each resource type	Include species and sex when applicable.	Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use “this paper” if new.	Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources.	Include any additional information or notes if necessary.
Chemical Compound or Drug	MRK-016	Tocris Bioscience	Cat. No. 3817	
Chemical Compound or Drug	flumazenil	Tocris Bioscience	Cat. No. 1328	
Chemical Compound or Drug	picrotoxin	Tocris Bioscience	Cat. No. 1128/1G	
Chemical Compound or Drug	CGP54626	Tocris Bioscience	Cat. No. 1088/10	
Chemical Compound or Drug	DNQX	Sigma-Aldrich	Cat. No. D0540	
Chemical Compound or Drug	APV	Tocris Bioscience	Cat. No. 0105/10	
Chemical Compound or Drug	ketamine	Sigma-Aldrich	Cat. No. K2753	
Organism/Strain	alpha5 knock-out mice (males)	PMCID: PMC4571505		