Effects of ketamine on GABAergic and glutamatergic activity in the mPFC: biphasic recruitment of GABA function in antidepressant-like responses

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Running title: Biphasic recruitment of GABA activity in ketamine's actions

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Abstract

Major depressive disorder (MDD) is associated with disruptions in glutamatergic and GABAergic activity in the medial prefrontal cortex (mPFC), leading to altered synaptic formation and function. Low doses of ketamine rapidly rescue these deficits, inducing fast and sustained antidepressant effects. While it is suggested that ketamine produces a rapid glutamatergic enhancement in the mPFC, the temporal dynamics and the involvement of GABA interneurons in its sustained effects remain unclear. Using simultaneous photometry recordings of calcium activity in mPFC pyramidal and GABA neurons, as well as chemogenetic approaches in *Gad1-Cre* mice, we explored the hypothesis that initial effects of ketamine on glutamate signaling trigger subsequent enhancement of GABAergic responses, contributing to its sustained antidepressant responses. Calcium recordings revealed a biphasic effect of ketamine on activity of mPFC GABA neurons, characterized by an initial transient decrease (phase 1, <30 min) followed by an increase (phase 2, >60 min), in parallel with a transient increase in excitation/inhibition levels (10 min) and lasting enhancement of glutamatergic activity (30-120 min). Previous administration of ketamine enhanced GABA neuron activity during the sucrose splash test (SUST) and novelty suppressed feeding test (NSFT), 24 h and 72 h post-treatment, respectively. Chemogenetic inhibition of GABA interneurons during the surge of GABAergic activity (phase 2), or immediately before the SUST or NSFT, occluded ketamine's behavioral actions. These results indicate that time-dependent modulation of GABAergic activity is required for the sustained antidepressant-like responses induced by ketamine, suggesting that approaches to enhance GABAergic plasticity and function are promising therapeutic targets for antidepressant development.

Keywords: glutamate, GABA, mPFC, ketamine, depression, antidepressants, stress, plasticity

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Introduction

Major depressive disorder (MDD) is a recurring neuropsychiatric illness that has a lifetime prevalence of ~17% in the U.S., and is a leading cause of disability worldwide (1). Despite the significant economic and human impact, the effectiveness of treatments remains suboptimal, underscoring MDD's inherent heterogeneity and our limited comprehension of the molecular and functional mechanisms that underlie its etiology (2). Traditional antidepressants take weeks to months to induce a therapeutic response, and up to 33% of patients prescribed these medications are considered treatment resistant (3). Conversely, low doses of ketamine, an NMDA receptor (NMDA-R) blocker, can induce rapid (2 h) and sustained (up to 7 days) antidepressant effects in patients diagnosed with MDD, even in patients that are refractory to current antidepressant medications (4).

Human and rodent studies indicate that depression and chronic stress are linked to structural alterations in limbic brain areas, including the medial prefrontal cortex (mPFC), characterized by reduced volume, neuronal atrophy, and impaired excitatory synapse density and function (5-9). Conversely, accumulating evidence suggests that ketamine can reverse these deficits and produce rapid antidepressant effects through an initial, transient blockade of NMDA receptors (NMDA-R) in GABA interneurons, leading fast disinhibition of excitatory pyramidal neurons and triggering neuronal plasticity in the mPFC (10-12). An alternative hypothesis is that ketamine acts directly on pyramidal neurons to block NMDA-R activity driven by spontaneous glutamate release (13).

Furthermore, in addition to disruption in excitatory synapses, MDD subjects and animals exposed to chronic stress have reductions in cortical and plasma GABA levels, as well as several GABA markers in the PFC (10, 14-20). Since inhibitory inputs control network excitability,

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50   integration, and synchrony, deficits in GABA function compromise the signal-to-noise properties

51   of glutamatergic neurons and the integrity of circuit-level information transmission from the

52   mPFC to projection areas (5). Consistent with this idea, prefrontal cortical GABA abnormalities

53   are associated with hippocampal structural deficits in MDD subjects (9) and normalization of

54   GABA function is associated with remission of depressive symptoms (18, 21-27). Indeed,

55   following the initial enhancement of glutamate function, ketamine and other rapid

56   antidepressants also increase GABA signaling in the mPFC, potentially contributing to

57   reestablishing the integrity of excitatory and inhibitory signal efficiency and precision in

58   corticolimbic circuits (14-16, 19, 20, 28).

59   In this study, we hypothesized that ketamine might induce long-lasting changes in the

60   activity of GABA interneurons that could contribute to sustained antidepressant effects. To test

61   this, we imaged activity of mPFC pyramidal and GABA neurons simultaneously following

62   ketamine administration and during behavioral tests relevant to antidepressant efficacy. We then

63   employed chemogenetic approaches to investigate whether activity of GABA interneurons is

64   necessary and/or required for the behavioral actions of ketamine.

65

Materials and Methods

*Animal*s

Male glutamic acid decarboxylase 1 (*Gad1*)-*Cre* transgenic mice and WT littermates (8-12-week-old) on the C57BL/6 background were bred in-house as in previous studies (29, 30). Male mice were initially chosen to allow for comparison of the outcomes observed following ketamine administration with previous literature, predominantly described in male mice. All animals were group-housed with a 12/12h light-dark cycle and food and water *ad libitum*. Following surgery for viral infusion and/or optical fiber placement, animals were single housed for 4 weeks and remained isolated until the end of the experiments. All procedures were conducted in compliance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Yale Institutional Animal Care and Use Committee.

Viral constructs and surgery

Adeno-associated viruses AAV.CamKII.GCaMP6s.WPRE.SV40, pAAV.Syn.Flex.NES-jRCaMP1b.WPRE.SV40, $\geq 1 \times 10^{13}$ vg/ml, and AAV2-hSyn-DIO-hM4D(Gi)-mCherry (hM4DGi), $\geq 7 \times 10^{12}$ vg/ml, were obtained from Addgene (USA). To image activity of pyramidal neurons and GABA interneurons simultaneously, anesthetized *Gad1-Cre* mice (ketamine, 100 mg/kg; and xylazine, 10 mg/kg) received unilateral intra-mPFC infusion of a cocktail (1:1; 0.6 µl, 0.1µl/min) containing two calcium sensors with non-overlapping spectra: CaMKII-driven GCaMP6s (GCaMP6s) and Cre-driven jRCaMP1b (RCaMP) viruses (coordinates from bregma: anterior-posterior: +1.9 mm; medial-lateral: ± 0.4 mm; dorsal-ventral -2.7 mm), along with implantation of a fiber (stainless steel ferrule, 400 µm core, 0.50 NA, 2.5

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89   mm length, ThorLabs, Newton, New Jersey, USA) in the same region. The fiber was implanted

90   0.2 mm above the injection site and maintained in place with adhesive dental cement (C&B-

91   Metabond, Parkell, NY, USA). Animals received i.p. injections of carprofen (5 mg/kg)

92   immediately after the surgery and daily for the next 2 days. Following surgery, all animals

93   remained single-housed throughout the duration of the protocols. In this study, single housing

94   was used to prevent detachment of the optical fiber from the skull and to serve as a chronic mild

95   stressor to study inhibitory and excitatory neuronal activity. To preserve the integrity of the

96   cannula, including a control group not subjected to isolation, which would enable comparing

97   stress effects, was not feasible for practical reasons. For chemogenetic experiments, animals

98   received bilateral infusion of inhibitory hM4DGi (0.5 µl/side; 0.1µl/min) into the mPFC and

99   underwent the same post-surgery protocol as described, including single housing. Fiber

100   placement and viral efficiency were analyzed using a confocal microscope to obtain Z-stack

101   image sequences (Leica TSE-SPE) (30).

102

103   *Drug administration*

104   Ketamine (Sigma-Aldrich, 10 mg/kg, i.p.) was dissolved in saline. Clozapine-N-oxide (CNO, 0.5

105   or 1 mg/kg i.p., Enzo Life Sciences, Farmingdale, NY, USA) was administered at different time

106   points, as indicated, based on our previous studies indicating lack of behavioral and locomotor

107   effects at these low doses (30); however, to control for any off target effects that have been

108   reported at higher doses (5-10 mg/kg) (31, 32), both WT and *Gad1-Cre* groups received CNO.

109

110   *Behavioral studies*

111   Animals were habituated to testing rooms 30 min before each experiment. All behavioral tests

112   were video recorded and conducted between 10 a.m. and 4 p.m. Experiments were scored by an

113   experimenter blind to treatments.

114   *Sucrose Splash Test (SUST):* A 10% sucrose solution was squirted onto the dorsal coat of the

115   mouse, as has been described (33). Grooming time was measured for 5 min.

116   *Novelty-Suppressed Feeding Test (NSFT):* Mice were food deprived for 16 h and placed in a

117   dimly lit box (40 *x* 40 *x* 25) with a pellet of food in the center; the latency to feed was measured

118   with a time limit of 10 min, as described (30). Immediately after the test, home cage food intake

119   was measured over a 10 min period as a feeding control.

120

121   *Immunofluorescence*

122   *Gad-Cre* mice received a Cre-dependent hM4DGi viral infusion into the mPFC, as described,

123   and SST or PV co-localization with Gad-hM4DGi+ cells was assessed using primary antibodies

124   (mouse anti-SST #sc-74556, 1:200, Santa Cruz; rabbit anti-PV #ab181086, 1:1000, Abcam) and

125   appropriate secondary antibodies (AlexaFluor® 488 goat anti-rabbit or AlexaFluor® 647 goat

126   anti-mouse, 1:1000), as described (20, 30). For quantification, sections containing the mPFC

127   were analyzed using a Keyence BZ-X800 microscope equipped with an optical sectioning

128   module (20X magnification). The total number of Gad-hM4DGi$^+$, PV or SST neurons, as well as

129   Gad-hM4DGi$^+$ co-localized with PV$^+$ or SST$^+$ cells, were obtained within each section (3

130   sections/animal). The results were then averaged across animals (n = 3 mice) and expressed as a

131   percentage of co-localized cells (number of co-localized Gad-hM4DGi$^+$ and SST or PV

132   cells/total Gad-hM4DGi$^+$, PV or SST cells $\times$ 100).

133

134   *Fiber photometry*

135   Multichannel fluorescent signals were recorded with a Tucker RZ5P processor (Tucker-Davis

136   Technologies, Alachua, FL, USA) controlled by the Synapse software suite to display excitation

137   (465 nm for GCaMP, green, and 560 nm for RCaMP, red) and reference (405 nm, isobestic

138   control) signals, modulated at 531, 330 and 211 Hz, respectively. The LEDs (Doric, Quebec,

139   Canada) were adjusted to the photodetector (Newport 2151, Irvine, CA, USA) with excitation

140   light intensity of ~20 µW. Light was passed through a minicube (FMC6AE, Doric) that

141   contained excitation and emission filter sets. Animals were tethered to the system via fiber optic

142   patch cord (400 µM core, 0.50 NA, ThorLabs) connected to a head mounted fiber optic cannula

143   via a ceramic sleeve.

144

145   *Data processing and analysis*

146   Signals were low pass filtered with a frequency cutoff of 5 Hz using MATLAB. The excitation

147   signal for each channel was analyzed using an adaptive iteratively reweighted Penalized Least

148   Squares (airPLS) algorithm to correct bleaching and motion artifacts, and regressed against the

149   reference control signal, using the protocol developed by Martianova et al., 2019 (34) (available

150   at https://github.com/katemartian/Photometry_data_processing). The change in fluorescence

151   (dF/F) was calculated as dF/F (465 nm or 560 nm signal–fitted 405-nm signal)/fitted 405-nm

152   signal. To standardize signals across animals prior to analysis, results were normalized by z-

153   scoring and expressed as average z-scored dF/F. As it is currently not possible to use an isobestic

154   point for red-shifted sensors (RCaMP), the 405 nm signal was used as a reference for correcting

155   movement artifacts, as described (34). However, due to the nature of photobleaching associated

156   with long-term recordings shown in Figure 1 (~150 min), RCaMP fluorescence intensity

8

157 displayed a gradually decreasing trend evident in the vehicle-treated group (Supplementary

158 Figure 1). Therefore, bleaching in this experiment was additionally corrected applying the

159 Matlab linear function *detrend* (y = detrend(x, n), where n = 1; x – y = linear trend) to the

160 vehicle-treated group, and the resultant curve was subtracted from saline and ketamine signals,

161 as described (35). The *detrend* algorithm computes the least-squares fit of a straight line (or

162 composite line for piecewise linear trends) to the data and subtracts the resulting function from

163 the data. For this correction, we assumed that saline had no significant effects on calcium

164 fluorescence over time. Detrending was not required for the shorter recordings described in

165 Figures 2 and 3 (up to 60 min). To evaluate excitation/inhibition levels, baseline or 10-min

166 binned data were transformed into positive values by adding a constant, and the ratio of GCaMP

167 to RCaMP transients (dF/F) was calculated within each animal.

168 In the behavioral studies, z-scores of each epochs of interest (*e.g.,* time blocks following

169 ketamine treatment and during behavioral tests, and the bite event) were averaged across groups

170 and presented in bar graphs. In the NSFT, to account for variability in the time it takes for

171 different animals to bite the food (e.g., to remove a chunk of food from the pellet), the bite event

172 was defined as a 5-second period encompassing the food interaction and bite.

173

174 *Statistical analysis*

175 Results were subjected to repeated measures ANOVA (treatment *x* time) with Sidak's correction

176 for multiple analyses. Additionally, Student's two-tailed t-test or Two-way ANOVA (treatment *x*

177 genotype) followed by Duncan test were employed as appropriate. All distributions were tested

178 for homogeneity of variance using Levene's test and for normality using the Kolmogorov-

179 Smirnov test. Non-normal distributions were analyzed by Mann-Whitney U test (two-tailed) or

180   Friedman's two-way analysis of variances by ranks test. Sphericity was assessed by Mauchly's

181   test. In cases where the assumption of sphericity was violated, the data were analyzed using the

182   Greenhouse-Geisser correction. Across all analyses, differences were considered significant at p

183   ≤ 0.05. Sample sizes were chosen based on previous experience with the tests employed and

184   power analyses (Cohen's d power analysis, > 0.8 effect size) conducted following a pilot study.

185   For all analyses, we used the SPSS Software (v 29.0) or GraphPrism (v 9.5.1). The specific test

186   used for each experiment is described in the figure legend. Each experiment was replicated a

187   minimum of 2 times.

188

189   **Results**

190   *Photometry recordings immediately after treatment: ketamine enhances activity of excitatory*

191   *mPFC neurons and elicits a biphasic response in GABAergic neurons*

192   The rapid antidepressant actions of ketamine are thought to involve initial inhibition of GABA

193   interneurons, leading to a subsequent glutamate burst and enhancement of glutamatergic activity

194   in the mPFC (12, 30). Additionally, ketamine administration increases GABA-related synaptic

195   proteins in the mPFC 24 h post-administration (20). However, the temporal dynamics of

196   excitatory and inhibitory neuronal activity in the mPFC following ketamine administration has

197   not been measured. To address this, we infused CaMKII-driven GCaMP6s (green) and Cre-

198   driven jRCaMP1b (RCaMP, red) viruses into the mPFC of *Gad1-Cre* mice to record calcium

199   activity from both glutamatergic (CaMKII$^+$) and GABAergic (Gad$^+$) neurons simultaneously

200   (Figure 1A). Although previous studies indicate that the CaMKII promoter can lead to unspecific

201   expression in inhibitory neurons (36, 37), our analysis suggest no significant overlap (Figure

202   1B). Using this dual-channel strategy, we observed that ketamine administration increases the

203   activity of CaMKII$^+$ neurons beginning approximately 30 min after administration, and this

204   response persists until the end of the recording period (120 min, Figure 1C, F). In contrast,

205   ketamine decreases the activity of GABA neurons for the first 30 min after administration

206   compared to the vehicle-treated group. It is noteworthy that, since the injection procedure

207   initially produced some level of GABA activity evident in the vehicle-treated group during the

208   first 10 min post-injection (Supplementary Figure 2B), the reduction in calcium transients

209   produced by ketamine becomes apparent only when compared with the vehicle group but not

210   with the baseline (Figure 1D). This response is inverted after 60 min, such that there is a delayed

211   increase in GABA activity compared to baseline (60 min) and to the baseline and vehicle-treated

212   groups (90-120 min) (Figure 1D, G). Finally, because GABAergic and glutamatergic activity

213   shift in opposite directions immediately after ketamine treatment (Supplementary Figure 2), we

214   conducted further analysis to investigate the initial dynamics of ketamine's actions on E/I levels

215   within each animal. Given that ketamine reaches peak plasma and brain levels within 10 min of

216   intraperitoneal administration in mice (38), we assessed E/I activity during baseline and within

217   10 or 20 min post-treatment. Interestingly, our results revealed a rapid and transient increase in

218   E/I transients following ketamine administration (10 min) in comparison to its baseline and the

219   vehicle-treated group, which returned to baseline levels shortly thereafter (Figure 1E). These data

220   show that ketamine triggers a biphasic response in GABA interneurons, characterized by a

221   transient decrease followed by an increase of activity.

222

223   *Ketamine enhances activity of mPFC pyramidal and GABAergic neurons 24 h post-treatment*

224   *during the sucrose splash test*

11

225 Next, we investigated whether ketamine-induced rapid changes in glutamatergic and GABAergic

226 activity are long-lasting and engaged during stress-relevant behavioral tests. An independent

227 cohort of *Gad1-Cre* mice underwent surgery for virus infusion, along with implantation of an

228 optical fiber, as outlined. Twenty-four hours after vehicle or ketamine treatment, animals

229 underwent recording in the home cage to evaluate sustained effects of ketamine in the absence of

230 behavioral challenge (50 min, Supplementary Figure 3) and then during the SUST (5 min)

231 (Figure 2A). Ketamine administration did not alter baseline activity of GABAergic or

232 glutamatergic neurons 24 h after administration (50-min recording, Supplementary Figure 3A-

233 B). For visualization and statistical analyses, baseline traces in Figure 2 represent the last 300 sec

234 of baseline recordings. Ketamine increased grooming time during the SUST (300 sec) (Figure

235 2B), and increased calcium transients in both glutamatergic (Figure 2C, E-F) and GABAergic

236 cells (Figure 2D, E, G) in comparison to respective baselines and vehicle-treated groups.

237

238 *Ketamine enhances activity of GABAergic neurons 72 h post-treatment during the novelty*

239 *suppressed feeding test*

240   Animals then underwent recordings 72 h following ketamine treatment to evaluate

241 calcium transients in the home cage (50 min) and during the NSFT (10 min) (Figure 3A). As

242 expected, ketamine decreased the latency to feed in the NSFT 72 h following treatment

243 compared to the vehicle-treated group (Figure 3B). No change was observed in the home cage

244 food consumption (Supplementary Figure 4). The results indicate that ketamine did not alter

245 baseline activity of GABAergic or glutamatergic neurons 72 h after administration (0-50 min)

246 (Supplementary Figure 4A-B). For visualization and statistical analyses, baseline traces in Figure

247 3 represent the last 300 sec of baseline recordings. During the NSFT, ketamine administration

248   led to a strong trend towards increased calcium activity in glutamatergic cells compared to the

249   baseline condition, although it did not reach statistical significance (p = 0.06, Figure 3C).

250   Conversely, there was an increase in calcium transients in GABAergic neurons during the NSFT

251   in both vehicle- and ketamine-treated groups compared to the respective baseline groups (Figure

252   3F). However, ketamine produced a more robust increase in GABAergic activity during the first

253   150 s of test duration compared to the vehicle-treated group (Figure 3F). We also measured the

254   average z-scored dF/F paired specifically to the bite event (Figure 3D and G). Interestingly, these

255   results show that ketamine treatment results in activation of GABA interneurons but not

256   pyramidal neurons during the bite event, suggesting that ketamine-induced plasticity in

257   GABAergic neurons is long-lasting and manifest during an avoidance-related behavioral task.

258

259   *Chemogenetic inhibition of mPFC GABA neurons following ketamine treatment or during*

260   *behavioral tests at 24 h or 72 h occludes ketamine's behavioral actions*

261   Based on the biphasic GABAergic activity observed after ketamine treatment (Figure 1),

262   characterized by an initial decrease (phase 1) followed by an increase (phase 2) in calcium

263   transients, we sought to investigate whether this delayed enhancement of GABA function is

264   required for the sustained behavioral responses induced by ketamine. To address this, *Gad1-Cre*

265   and WT littermate controls were infused bilaterally with a Cre-dependent hM4DGi virus (Figure

266   4A-B) to selectively inhibit the activity of GABA interneurons in the mPFC during phase 2. To

267   investigate whether the hM4DGi virus expression is restricted to specific subpopulations of

268   interneurons or encompasses overall GABAergic cells in *Gad-Cre* mice, we conducted

269   immunostaining to co-label Gad- hM4DGi$^+$ cells with PV or SST. Our results indicate that

270   30.7% of the Gad- hM4DGi$^+$ cells express SST, while 27.1% express PV, revealing

271   heterogeneity within the *Gad-Cre* line (Figure 4C; Supplementary Figure 5). Although PV is

272   shown to be more expressed than SST in the neocortex (40% *vs* 30%, respectively) (39), most of

273   our injections are confined to layers II/III and, to a lesser extent, V of the mPFC, where PV and

274   SST cells are shown to be similarly expressed (39). Conversely, 18.1% and 25.6% of total

275   labeled PV or SST cells, respectively, were Gad- hM4DGi^{+}.

276   In this experimental setup, animals received either vehicle or ketamine (10 mg/kg),

277   followed by CNO (1 mg/kg) 40 min later (Figure 4A). This schedule of administration allows the

278   initial ketamine-induced blockade of GABA interneurons to occur (<30 min, phase 1), while

279   occluding the subsequent enhancement of GABA activity (phase 2). CNO has a short half-life in

280   mice (~1 h) (31), so an additional low dose of CNO (0.5 mg/kg) was administered after 2 h to

281   extend the effect. It is noteworthy that these low doses of CNO do not elicit behavioral or

282   locomotor responses 24 h or 72 h later when injected in hM4DGi-infused *Gad1-Cre* mice (30).

283   Ketamine increased grooming time in the SUST (24 h) and decreased the latency to feed in

284   NSFT (72 h) in WT mice, as expected, and these effects were blocked by chemogenetic

285   inhibition of GABA interneurons during phase 2 in *Gad1-Cre* mice (Figure 4D-E). No changes

286   were observed in home cage food consumption (Figure 4D). This suggests that activity at

287   GABAergic synapses is required for the sustained antidepressant-like effects of ketamine. In a

288   separate control experiment, we adopted an inverse approach where hM4DGi-infused *Gad1-Cre*

289   mice received CNO (1 mg/kg) 40 min *before* vehicle or ketamine, and then were tested in the

290   SUST or NSFT, 24 or 72 h post-treatment, respectively (Supplementary Figure 6). This protocol

291   allows us to evaluate CNO pre-treatment on ketamine's actions without affecting the delayed

292   GABAergic surge, as CNO would be largely eliminated by this time. As expected, prior CNO

293   treatment did not impact ketamine's behavioral effects in both the SUST (Supplementary Figure

294   6C) and NSFT (Supplementary Figure 6D), supporting our hypothesis that ketamine-induced

295   GABAergic activity is time-dependent and engaged at a later timepoint.

296   Based on the observation that GABAergic transients are increased *during* the SUST and

297   NSFT tests 24 and 72 h following ketamine treatment, respectively (Figure 2 and 3), we

298   determined whether activation of GABA interneurons *during* these tests is required for the

299   behavioral outcomes induced by ketamine. WT and *Gad1-Cre* mice were administered either

300   vehicle or ketamine, followed by CNO (1 mg/kg) 24 and 72 h later, injected 30 min before each

301   behavioral test (Figure 4F). Importantly, CNO does not produce any behavioral or locomotor

302   effects when administered 30 min before behavioral tests in hM4DGi-infused *Gad1-Cre* mice

303   (30). Previous ketamine administration increased grooming time in the SUST (24 h after

304   administration) and decreased the latency to feed in NSFT (72 h after administration) in WT

305   mice, and these effects were occluded by chemogenetic inhibition of GABA interneurons 30 min

306   before each test (Figure 4G-H). No differences were observed in home cage food consumption

307   among groups (Figure 4H). This suggests that ketamine-induced activity of GABAergic neurons

308   is long-lasting and engaged during stress-related behavioral tasks.

309

310   **Discussion**

311   In this study we provide direct evidence that ketamine exerts sustained antidepressant-like

312   actions through facilitation of GABA function. The results support the hypothesis that ketamine

313   modulates the activity of mPFC GABA interneurons in a biphasic manner, with an initial

314   decrease in activity (phase 1), accompanied by activation of pyramidal neurons, followed by an

315   increase in GABA function (phase 2) (Figure 5). Notably, chemogenetic data indicate that this

316   delayed enhancement of mPFC GABA activity is both required and necessary for the sustained

317 behavioral actions of ketamine. The increase in GABA signaling after ketamine administration is

318 also long-lasting and engaged during behavioral tests relevant to stress responses, suggesting it

319 may be involved in fine-tuning cortical circuits during stress-related behaviors.

320 A major hypothesis for the initial cellular trigger for ketamine's action it that it first

321 blocks NMDA-R expressed by cortical interneurons, notably, somatostatin (SST) and

322 parvalbumin (PV) GABA neuron subtypes (5, 12). Because these inhibitory neurons exhibit

323 tonic firing, they are thought to be more sensitive to NMDA-R blockade, as tonic activity

324 removes the Mg^{2+} block of the receptor, enabling ketamine to enter the channel pore and block

325 Ca^{2+} entry (12, 40). Inhibition of GABAergic interneuron firing decreases GABA activity,

326 resulting in disinhibition of excitatory pyramidal neurons, and leading to a glutamate burst that

327 drives activity-dependent BDNF release, activation of protein synthesis (via the mTORC1

328 pathway), new spine formation, and synaptogenesis (2). Supporting this hypothesis, NMDA-R

329 blockade leads to a reduction in the spontaneous firing of putative GABA interneurons in the

330 mPFC, coupled with an enhanced activity of pyramidal neurons at a delayed rate (40).

331 Furthermore, a low concentration of ketamine (1 μM), which approximates brain levels after *in*

332 *vivo* administration, rapidly decreases inhibitory- and increases excitatory postsynaptic currents

333 (IPSCs and EPSCs, respectively) onto pyramidal neurons in mPFC slices (12).

334 In this study we measured the dynamics in cellular responses to ketamine using

335 simultaneous dual photometry recordings of GABA neurons and glutamatergic cells in the

336 mPFC. Our findings indicate that ketamine initially produces a decrease in GABAergic neuron

337 activity compared to the vehicle group, and a transient increase in E/I levels (10 min), followed

338 by a long-lasting increase in pyramidal neuron activity (2 h). However, it remains unknown

339 whether the initial decrease in GABAergic transients directly lead to enhanced glutamatergic

16

340 activity, and more studies addressing cross-correlational dynamics and causality are needed. In a

341 previous study, increased GCaMP6f fluorescence in mPFC pyramidal neurons was observed 30

342 min after administering a lower dose of ketamine (3 mg/kg), and this study reported more

343 pronounced effects at 30 mg/kg, leading to an immediate increase in pyramidal activity lasting

344 up to 20 min post-injection (41). However, it is noteworthy that the interpretation of that study is

345 limited due to the absence of a vehicle control group. Selective knockdown of a key NMDA-R

346 subunit, GluN2B, in GABA interneurons, but not pyramidal (CaMKII$^+$) cells, occluded the

347 behavioral actions of ketamine (12). Optogenetic stimulation of GABA interneurons concurrent

348 with ketamine administration also occluded its behavioral effects (20). Similar cellular

349 mechanisms are observed with other rapid antidepressant candidates, including the muscarinic

350 acetylcholine receptor antagonist scopolamine (14, 29, 30). Stimulating GABA interneuron

351 activity before scopolamine administration blocks its rapid and sustained behavioral effects (30).

352 Likewise, selective knockdown or knockout of muscarinic type 1 (M1) receptors in SST

353 interneurons occludes scopolamine-induced behavioral responses and molecular plasticity at

354 GABAergic and glutamatergic synapses (14, 29). In the same direction, our previous studies

355 have shown that chemogenetic inhibition of either all GAD-positive neurons in the mPFC, or

356 only SST or PV interneuron subtypes, using a higher dose of CNO (2.5 mg/kg, 3x), resulted in

357 fast antidepressant-like responses and molecular changes, mimicking the effects of fast

358 antidepressants (30). Conversely, lower doses of CNO used in the present study (0.5 and 1

359 mg/kg) are not sufficient to produce behavioral effects on their own, suggesting that a more

360 robust silencing of GAD-positive neurons is required to recapitulate rapid antidepressant actions.

361 Interestingly, after the initial decrease in GABA activity, we observed a delayed

362 enhancement of GABA function starting 60 min post-ketamine administration, which was further

363 engaged during behavioral tests 24 h and 72 h later, suggesting that additional mechanisms

364 beyond glutamate-mediated plasticity might contribute to ketamine's actions (Figure 5). Indeed,

365 our chemogenetic experiments provided evidence supporting the involvement of GABAergic

366 interneurons in mediating the effects of ketamine. Notably, while the glutamatergic hypothesis

367 provides a conceptual model for the rapid changes produced by ketamine, it does not account for

368 the reductions in GABA levels and markers observed in cortical areas of human subjects with

369 MDD and chronically stressed animals, which can be restored by rapid antidepressant treatment

370 (10, 15-20, 28, 42-47). Several studies have reported decreases in the expression of SST and

371 proteins related to GABA signaling, such as GAD1—the primary enzyme responsible for

372 synthesizing GABA from glutamate—and multiple subunits of GABA$_A$ receptors in cortical

373 tissues obtained from both MDD subjects and stressed animals (48-51). Mice lacking SST (SST-

374 KO) exhibit increased stress-related behaviors, and reduced GAD1 gene expression (52).

375 Importantly, normalization of cortical and plasma GABA levels, as well as GAD1 expression,

376 following antidepressant treatment, is associated with remission of depressive symptoms (21-27).

377 Consistent with these findings, the antidepressant effects of ketamine are accompanied by

378 robust increase in GABA levels in the mPFC of patients diagnosed with MDD or obsessive-

379 compulsive disorder (19, 53). Ketamine can also restore impaired GABA release in the

380 hippocampus of stressed rats (54). Our previous findings suggest that ketamine and other fast-

381 acting drugs, including scopolamine, increase pre- and postsynaptic markers of GABA signaling

382 in the mPFC 24 h after administration, such as GAD1, the vesicular GABA transporter (VGAT)

383 and/or gephyrin, along with serotonin-induced IPSCs in pyramidal neurons (20). Ketamine can

384 also modulate GABA$_A$-R binding in human PFC and increase the activity of extrasynaptic

385 GABA$_A$-R in mouse cortex and hippocampus (55, 56). Combined administration of sub-effective

386   doses of muscimol, a selective agonist of GABA$_A$ receptors, and ketamine, produces

387   antidepressant-like effects in mice (57). Global deletion or mutation of γ2-GABA-R produces

388   stress-induced phenotypes, which are reversed by a single dose of ketamine or chronic

389   imipramine treatment (58). Ketamine also reverses GABAergic deficits in these animals,

390   increasing inhibitory postsynaptic currents (IPSCs) and gephyrin levels (58). Additionally,

391   disinhibition of somatostatin interneurons by deletion of γ2-GABA-R selectively from these cells

392   results in anxiolytic- and antidepressant-like effects, and confers resilience to stress in male

393   mice, mimicking the effects of ketamine (59, 60). It is worth noting that the experiments in the

394   present study were conducted in single-housed animals that underwent surgeries for cannula

395   placement and/or viral infusion. Social isolation in rodents has been shown to disrupt

396   corticolimbic structures and immune system function, contributing to stress-related behavioral

397   outcomes (61-63). Thus, the single housing may have contributed to baseline stress in these mice

398   that allowed robust changes in response to ketamine administration. However, further

399   experiments using well-validated animal models of stress, such as the CUS, are necessary to

400   evaluate the actions of ketamine compared to a control group (non-stressed).

401   While the precise mechanism by which ketamine enhances GABA activity remains

402   unclear, one hypothesis is that, following the glutamatergic burst, there is homeostatic self-

403   tuning adaptations to reestablish E/I balance in the mPFC (10). This local reorganization could

404   restore the integrity of signal transfer to target regions by re-establishing correct firing patterns,

405   and thereby promoting antidepressant effects (64) (Figure 5). Glutamate is the primary precursor

406   of GABA as a substrate for GAD, so strengthened inhibition may be a self-limiting mechanism

407   to prevent excitotoxicity in response to excessive excitation. Notably, activation of postsynaptic

408   NMDA-R or stimulation of glutamatergic neurons (CaMKII[+]) engages a positive feedback

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409   mechanism culminating in long-term potentiation of dendritic inhibition mediated by SST

410   interneurons in the mPFC (65). Concomitantly, disrupting the glutamate-glutamine cycle

411   depletes GABA neurotransmitter pools (66, 67), restoring balance through decreased inhibition.

412   Another possibility is that ketamine exerts direct effects on the GABAergic system, potentially

413   through its metabolites such as (2R, 6R)-HNK, which has shown ketamine-like antidepressant

414   effects without prompting psychotomimetic responses (38, 68).

415   Our findings also reveal enhancement of glutamate neuron activity 24 h post-ketamine

416   during the SUST; however, although there was a strong trend towards increased overall

417   glutamatergic activity 72 h later during the NSFT, no effect was found around the bite event,

418   raising the question of whether activity of mPFC CaMKII+ neurons is necessary for the

419   sustained effects of ketamine during specific avoidance-related tasks. In line with our hypothesis,

420   it is possible that activation of glutamatergic neurons is required for the initial cascade of cellular

421   events that culminate in a long-lasting enhancement of dendritic spine number and function (2,

422   69), while the activity of GABAergic neurons may be involved in fine-tuning these newly

423   formed microcircuits.

424   Consistent with the involvement of GABA mechanisms in antidepressant responses, new

425   classes of rapid-acting drugs targeting the $GABA_AR$ as positive or negative allosteric modulators

426   (PAMs and NAMs, respectively) have emerged in recent years. Brexanolone, a positive δ-

427   $GABA_AR$ modulator and an analogue of the neurosteroid allopregnanolone, has recently been

428   approved for the treatment of postpartum depression (70), marking a significant paradigm shift in

429   the field towards identifying innovative GABAergic compounds for depression therapy.

430   Furthermore, preclinical studies indicate that both PAMs and NAMs of the α5-containing

431    $GABA_A R$ demonstrate rapid antidepressant-like effects or prevent the behavioral responses

432   induced by chronic stress, comparable to the effects of ketamine (71-74).

433   Therefore, enhancement of both GABA and glutamate function is likely necessary to

434   restore signal integrity in the mPFC and other limbic regions, culminating in antidepressant

435   states (Figure 5). Our current GABAergic evidence builds upon the findings and conceptual

436   frameworks for antidepressant action described by Duman et al., 2019 (5) and Lusher et al., 2020

437   (16), providing support for a sequential glutamatergic-GABAergic hypothesis for the actions of

438   ketamine (Figure 5). While the current study provides novel insights into the GABAergic effects

439   of ketamine, we did not evaluate causality between these mechanisms and the enhancement of

440   glutamatergic function. Cross-correlational analyses of GABA and glutamatergic activity, as

441   well as recordings following chemogenetic manipulation of CaMKII^{+} neurons and ketamine

442   treatment, would be valuable additions to investigate the temporal dynamics that could result in

443   disinhibition. Also, further investigations are warranted to pinpoint the recruitment of specific

444   subpopulations of GABA interneurons (e.g., SST, PV) in this biphasic response, as well as

445   potential sex-dependent differences. In conclusion, the current findings suggest that modulating

446   GABAergic function holds promise for developing novel antidepressant medications.

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Author contributions

M.V.F. designed the study, performed the experiments, analyzed the data and wrote the manuscript. F.D. performed experiments and provided technical support. M.R.P. provided scientific input, was involved in data interpretation and analyses, edited and revised the manuscript.

Disclosures

The authors declare that they have no conflicts of interest to report.

Data Availability

Individual subject data are shown in the figures, and any unidentified raw data will be provided upon request. MATLAB code is available at

473   https://github.com/katemartian/Photometry_data_processing (34). Any additional information

474   will be provided upon request.

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675

676 **Figure legends**

677 **Figure 1. Photometry recordings from mPFC glutamatergic and GABAergic neurons**

678 **immediately after ketamine treatment.** (A) Time course for surgery, treatment and recording.

679 (B) Representative images of the mPFC from *Gad1-Cre* mice showing unilateral infusion of

680 CaMKII-driven GCaMP6s (CaMKII-GCaMP6s) and Cre-dependent jRCaMP1b (Gad-RCaMP)

681 virus, labeling pyramidal and GABAergic cells, respectively, along with implantation of an

682 optical fiber (magnification: 10X). (C) Ketamine increased calcium transients in glutamatergic

683 neurons (CaMKII+) compared to both the baseline (BL, - 20 to 0 min) and vehicle-treated

684 animals 30 min post-treatment, persisting until the end of the recording session ($F_{treatment\ 1,8}$ =

685 22.03; $F_{interaction\ 4,32}$ = 4.99, $p \leq 0.05$). (D) Ketamine produced a biphasic response in GABA

686 neuron calcium activity, in which there was an initial decrease (0 to 30 min) followed by an

687 increase in the photometry signal (60 to 120 min) ($F_{time\ 4,32}$ = 5.23; $F_{interaction\ 4,32}$ = 9.65, $p \leq 0.05$).

688 (E) Ketamine increased excitation/inhibition (E/I) levels within 10 min of administration, which

689 returned to baseline levels shortly thereafter ($F_{treatment\ 1,8}$ = 27.43; $F_{interaction\ 2,16}$ = 6.22, $p \leq 0.05$).

690 (F) Minimally processed traces representing pyramidal neurons (CaMKII+) expressing

691 GCaMP6s and (G) GABA neurons (Gad-Cre) expressing RCaMP in the mPFC from a vehicle-

692 or ketamine-treated *Gad1-Cre* mouse. The black arrow indicates the treatment time. Horizontal

693 dashed lines indicate z dF/F = 0. Photometry results per animal are computed as average z-scored

694 dF/F, and graphs are presented as mean ± standard error. Repeated measures ANOVA followed

695 by Sidak correction for multiple analysis; *$p \leq 0.05$ in comparison to the vehicle-treated group;

696 #$p \leq 0.05$ in comparison to the baseline (before treatment), n = 10/group. $F_{interaction}$ represents the

697 interaction between time and treatment factors.

698

29

699 **Figure 2. Photometry recordings from glutamatergic and GABAergic neurons 24 h after**

700 **ketamine treatment and during the sucrose splash test.** (A) Timeline for surgery, treatment,

701 recording and behavioral testing. (B) Ketamine increased the grooming time in the sucrose

702 splash test (SUST) 24 h after administration ($t_{16} = 3.01$, $p \leq 0.05$). (C-E) No change in baseline

703 (BL, -300 to 0 sec) activity of pyramidal or GABA neurons was observed 24 h after ketamine

704 administration ($p > 0.05$). (C) There was an enhancement of calcium transients in both pyramidal

705 ($F_{time\ 1,8} = 25.16$; $F_{interaction\ 1,8} = 6.51$, $p \leq 0.05$) and (D) GABA neurons ($F_{time\ 1,8} = 9.63$; $F_{interaction}$

706 $_{1,8} = 24.86$, $p \leq 0.05$) in ketamine-treated animals during the SUST (0 to 300 sec) compared to

707 both the baseline and vehicle groups. (E) 60-sec binned representation of the grooming time

708 (orange), GCaMP6s (green, z dF/F) and RCaMP (red, z dF/F) during the SUST duration. (F)

709 Representative traces of pyramidal neurons (CaMKII+) expressing GCaMP6s and (G) GABA

710 neurons (Gad-Cre) expressing RCaMP in the mPFC from vehicle- and ketamine-treated *Gad1-*

711 *Cre* mice before and during the SUST. Photometry results per animal are computed as average z-

712 scored dF/F, and graphs are presented as mean ± standard error. Repeated measures ANOVA

713 followed by Sidak correction for multiple analysis; *$p \leq 0.05$ in comparison to the vehicle-

714 treated group; #$p \leq 0.05$ in comparison to the baseline (before test), n = 9/group. $F_{interaction}$

715 represents the interaction between time and treatment factors.

716

717 **Figure 3. Photometry recordings from glutamatergic and GABAergic neurons 72 h after**

718 **ketamine treatment and during the novelty suppressed feeding test.** (A) Timeline for

719 surgery, treatment, recording and behavioral testing. (B) Ketamine decreased the latency to feed

720 in the novelty suppressed feeding test (NSFT) 72 h after administration (U = 16, $p \leq 0.05$). (C)

721 Ketamine did not change baseline (BL, -300 to 0 sec) activity of pyramidal cells 72 h after

722 administration (p > 0.05) and produced a trend towards increased pyramidal activity during the

723 NSFT (150-sec time blocks; vehicle: $\chi^2_{(4)}= 2.22$, p > 0.05; ketamine: $\chi^2_{(4)}= 9.24$, p = 0.06). (D)

724 No change was observed during the bite event ($t_{16} = 0.19$, p > 0.05). (E) Representative traces of

725 pyramidal neurons (CaMKII+) expressing GCaMP6s in the mPFC from vehicle- and ketamine-

726 treated *Gad1-Cre* mice before and during the NSFT. (F) Ketamine did not change baseline

727 activity of GABA neurons 72 h after administration (-300 to 0 sec, p > 0.05). However, exposure

728 to the NSFT resulted in an enhancement of GABA neuron activity in both vehicle- and

729 ketamine-treated groups ($F_{time\ 4,32} = 58.83$; $F_{interaction\ 4,32} = 3.23$, $p \leq 0.05$), with a more robust

730 activity observed in the ketamine- compared to the vehicle-group in the first 150 sec of the test

731 (0-150 sec, $p \leq 0.05$). (G). Ketamine increased calcium activity in GABA neurons during the bite

732 event ($t_{16} = 2.45$, $p \leq 0.05$). (H) Representative traces of GABA neurons (Gad-Cre) expressing

733 RCaMP in the mPFC from vehicle- and ketamine-treated *Gad1-Cre* mice before and during the

734 NSFT. Black arrows represent a bite event. Photometry results per animal are computed as

735 average z-scored dF/F and graphs are presented as mean ± standard error. Repeated measures

736 ANOVA followed by Sidak correction for multiple analysis, Friedman's two way analysis of

737 variance by ranks or Mann-Whitney U test; *$p \leq 0.05$ in comparison to the vehicle-treated group;

738 #$p \leq 0.05$ in comparison to the baseline (before test), n = 9/group. $F_{interaction}$ represents the

739 interaction between time and treatment factors.

740

741 **Figure 4. GABA neuron activity is necessary for the sustained behavioral actions of**

742 **ketamine.** (A) Timeline for surgery, treatments, and behavioral testing. (B) Representative

743 images of the mPFC from *Gad1-Cre* and WT (Cre-negative littermate) mice that received

744 bilateral infusions of hM4DGi virus (magnification: 10X; inset: 60X). (C) Co-labeling

31

745 percentage of PV or SST cells with hM4DGi virus in mPFC sections from *Gad-Cre* mice

746 (magnification: 20X; inset: 40X). (D) CNO administration 40 min (1 mg/kg) and 2 h (0.5 mg/kg)

747 post-ketamine treatment blocked its behavioral effects in the sucrose splash test (SUST, 24 h

748 post-ketamine; $F_{genotype\ 1,3} = 13.42$, $F_{treatment\ 1,3} = 6.33$, $F_{interaction\ 1,3} = 14.52$, $p \leq 0.05$) and (E)

749 novelty suppressed feeding test (NSFT, 72 h post-ketamine; $F_{interaction\ 1,3} = 4.67$, $p \leq 0.05$). There

750 was no change in home cage food consumption ($F_{interaction\ 1,3} = 0.22$, $p > 0.05$). (F) Timeline for

751 surgery, treatments, and behavioral testing. (G) CNO administration (1 mg/kg) 30 min before the

752 behavioral test occluded the behavioral effects of ketamine in the sucrose splash test (SUST, 24 h

753 post-ketamine; $F_{treatment\ 1,3} = 4.34$, $F_{interaction\ 1,3} = 4.21$, $p \leq 0.05$) and (H) novelty suppressed

754 feeding test (NSFT, 72 h post-ketamine; $F_{interaction\ 1,3} = 4.66$, $p \leq 0.05$). There was no change in

755 home cage food consumption ($F_{interaction\ 1,3} = 0.30$, $p > 0.05$). Graphs are presented as mean $\pm$

756 standard error. Two-way ANOVA followed by Duncan; $*p < 0.05$ in comparison to the WT

757 vehicle-treated group; $\#p < 0.05$ in comparison to the WT ketamine-treated group. $F_{interaction}$

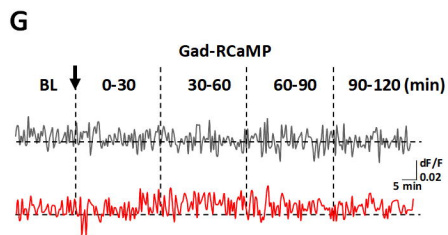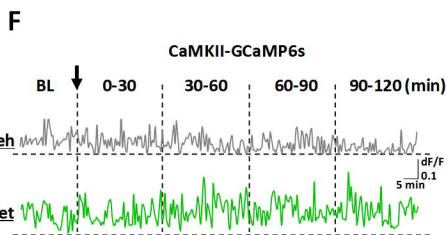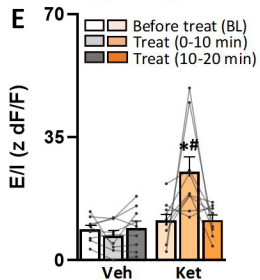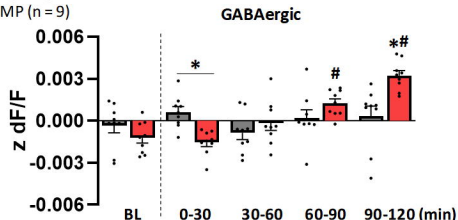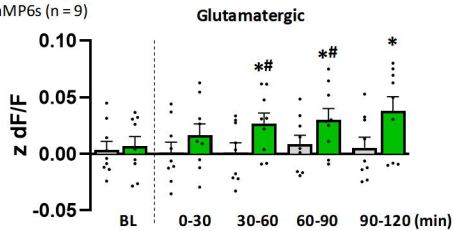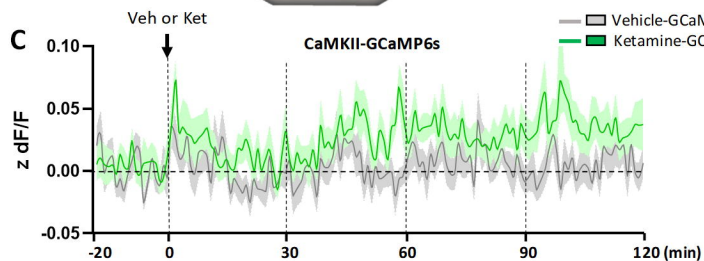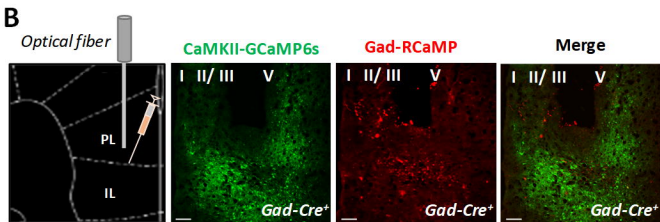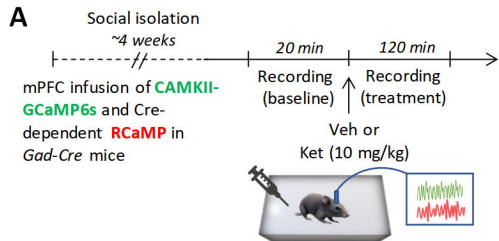758 represents the interaction between genotype and treatment factors.

759

760 **Figure 5. Biphasic glutamatergic-GABAergic model for ketamine action.** (A) Major

761 depressive disorder (MDD) and chronic stress impair glutamate and GABA function in the

762 mPFC, leading to altered connectivity and network dysfunction in corticolimbic brain regions, as

763 suggested by previous studies (5, 14, 18, 20, 60, 69). (B) Ketamine restores these deficits by

764 acting through two phases: (1a) In phase 1 (acute), ketamine blocks the activity of GABA

765 interneurons via NMDA-R, disinhibiting pyramidal cells and generating a glutamate burst and

766 increasing excitation/inhibition (E/I) levels (1b). The glutamate burst triggers a cascade of

767 cellular events culminating in synaptic plasticity and fast antidepressant responses, as suggested
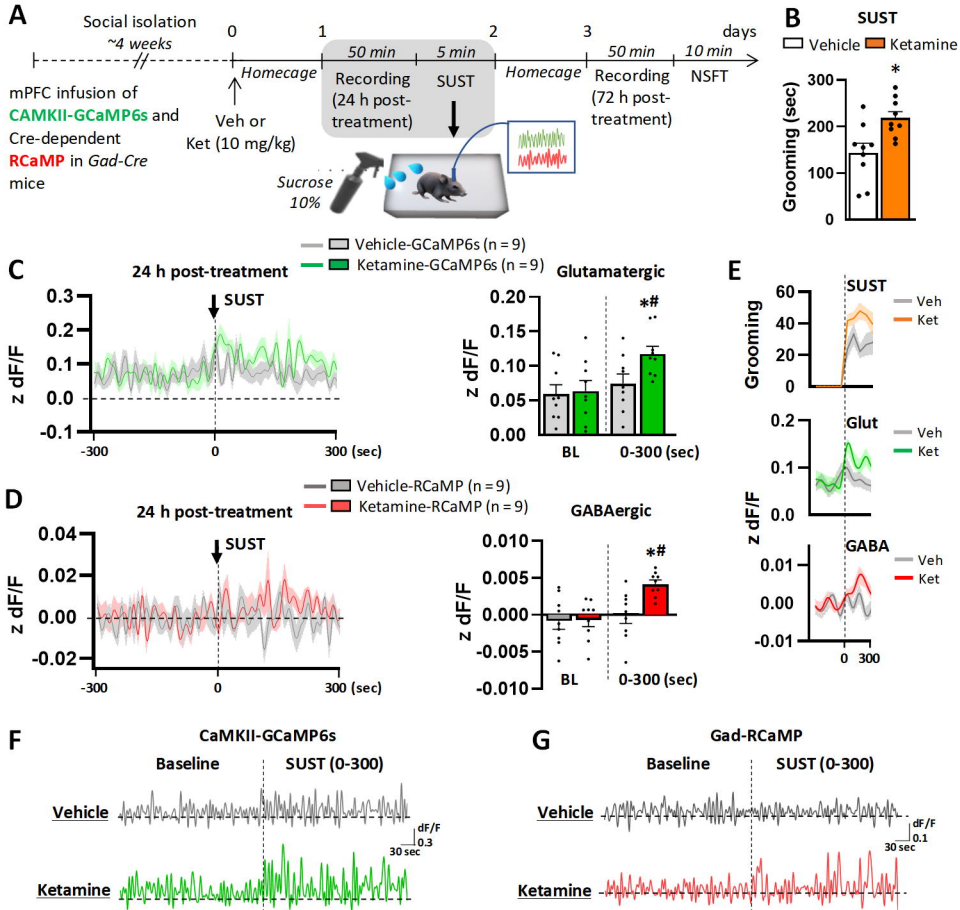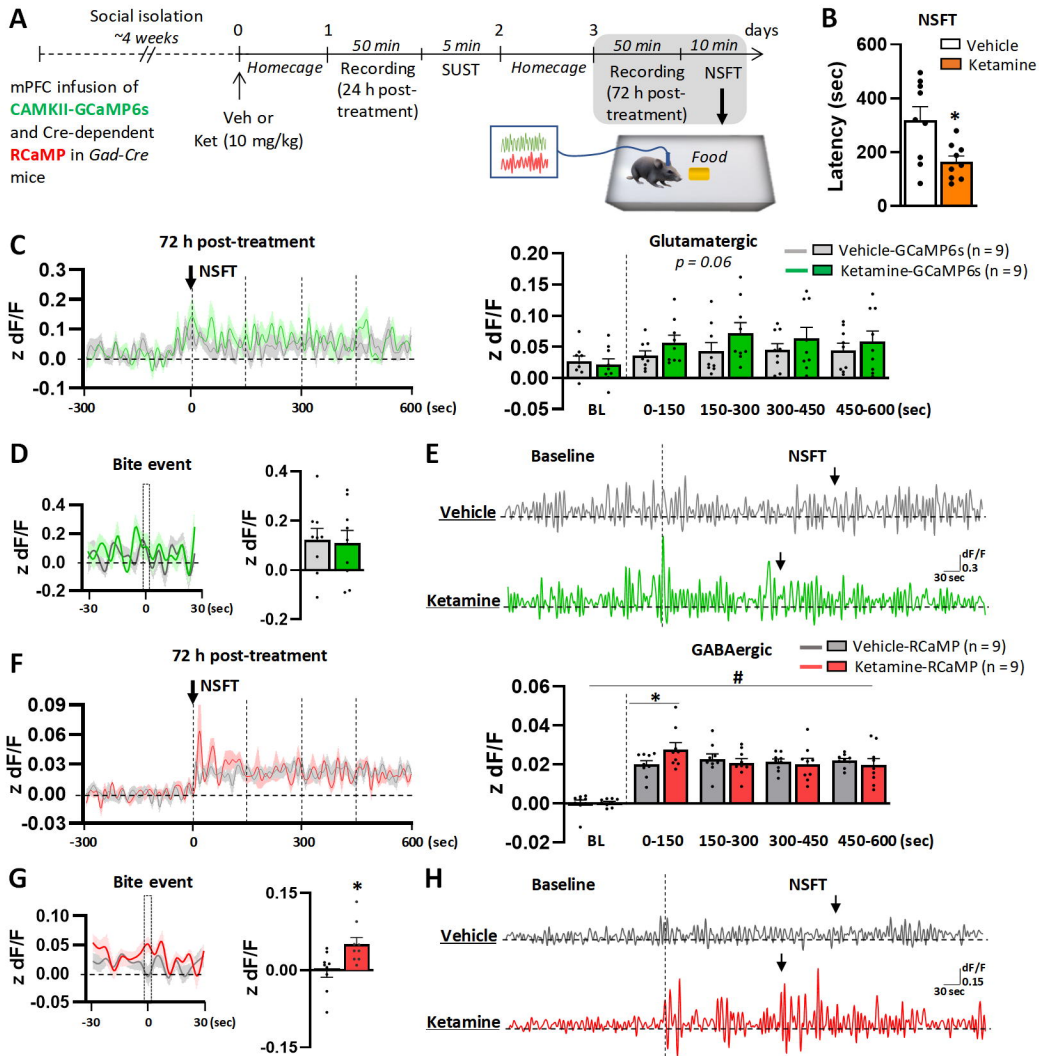
32

768   by previous studies (12, 20, 30, 69). In a second phase (sustained), there is a self-tuning

769   adjustment to reach E/I balance in the mPFC, leading to potentiation of GABAergic function

770   (2a) and restored circuit connectivity (2b). The recruitment of long-lasting increases in GABA

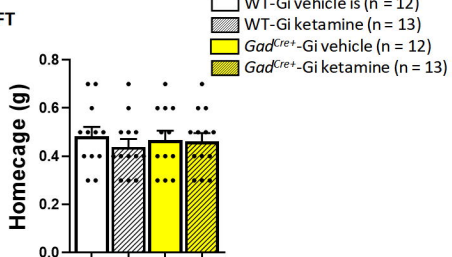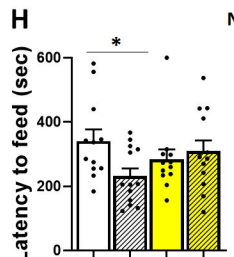771   neuron activity contributes to sustained antidepressant effects. Created with BioRender.com.

772

773

A Social isolation ~4 weeks

mPFC infusion of CAMKII-GCaMP6s and Cre-dependent RCaMP in *Gad-Cre* mice

Veh or Ket (10 mg/kg)

Homecage | Recording (24 h post-treatment) | SUST | Homecage | Recording (72 h post-treatment) | NSFT

Sucrose 10%

B SUST
☐ Vehicle ■ Ketamine

C 24 h post-treatment — Glutamatergic
Vehicle-GCaMP6s (n = 9)
Ketamine-GCaMP6s (n = 9)

D 24 h post-treatment — GABAergic
Vehicle-RCaMP (n = 9)
Ketamine-RCaMP (n = 9)

E SUST — Veh / Ket
Glut — Veh / Ket
GABA — Veh / Ket

F CaMKII-GCaMP6s
Baseline | SUST (0-300)
Vehicle
Ketamine

G Gad-RCaMP
Baseline | SUST (0-300)
Vehicle
Ketamine

A Social isolation ~4 weeks

Bilateral mPFC Infusion of Cre-dependent Gi-DREADD in *Gad-Cre* mice

0 — 40 min — 2 h — 1 — 2 — 3 days

Veh or Ket (10 mg/kg) — CNO (1 mg/kg) — CNO (0.5 mg/kg) — SUST — NSFT

B

AAV2-hM4DGi

Cg1 / PL / IL

Gad-Cre hM4DGi — I II/III V

WT hM4DGi — I II/III V

C

Gad-hM4DGi (%)

Others 42.2% / SST 30.7% / PV 27.1%

Gad-Cre hM4DGi

SST — I II/III V

PV — I II/III V

D SUST

Grooming (sec)

E NSFT

Latency to feed (sec)

Homecage (g)

☐ WT-Gi vehicle (n = 14)
▨ WT-Gi ketamine (n = 14)
▨ *Gad^{Cre+}*-Gi vehicle (n = 12)
▨ *Gad^{Cre+}*-Gi ketamine (n = 12)

F Social isolation ~4 weeks

Bilateral mPFC Infusion of Cre-dependent Gi-DREADD in *Gad-Cre* mice

0 — 1 — 30 min — 2 — 3 — 30 min — days

Veh or Ket (10 mg/kg) — CNO (1 mg/kg) — SUST — CNO (1 mg/kg) — NSFT

G SUST

Grooming (sec)

H NSFT

Latency to feed (sec)

Homecage (g)

☐ WT-Gi vehicle is (n = 12)
▨ WT-Gi ketamine (n = 13)
▨ *Gad^{Cre+}*-Gi vehicle (n = 12)
▨ *Gad^{Cre+}*-Gi ketamine (n = 13)

A **Chronic Stress/MDD**

B **Rapid antidepressants**

Phase 1 (rapid)   Phase 2 (sustained)

Adaptive state
Homeostasis
Disease state

GABA   Glutamate

Glutamate   GABA   Glutamate

GABA

GABA

GABA function

GABA IN

Neuronal atrophy
Spine formation
and function

Pyr

Impaired signal transfer
within the mPFC and to
projection areas: stress-
related responses

1b. Pyramidal neuron
disinhibition
(increased E/I balance)

Spine number and
function
Synaptogenesis

2b. Restored connectivity

2a. Increase of
GABA activity

Ketamine

1a. Transient GABA
interneuron blockade

Enhanced signal transfer
within the mPFC and to
projection areas:
antidepressant effects