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Extrasynaptic localization is essential for $\alpha 5$ GABAA receptor modulation of dopamine system function

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1 **Title: Extrasynaptic localization is essential for α 5GABA_A receptor modulation of dopamine system function**

2 **Abbreviated title: Extrasynaptic α 5GABA_A modulation of dopamine**

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22 **Competing Interests:**

23 E.S., T.D.P and J.C. are co-inventors or listed on US patent applications that cover GABAergic ligands, including GL-II-73,
24 and their use in brain disorders. E.S. is co-founder, CEO and CSO, and T.D.P is Director of Operation of DAMONA
25 Pharmaceuticals, a biopharmaceutical company dedicated to treat cognitive deficits in brain disorders. All remaining
26 authors have nothing to disclose.

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28 **Abstract:**

29 Dopamine system dysfunction, observed in animal models with psychosis-like symptomatology, can be restored by
30 targeting Gamma-Aminobutyric Acid type A receptors (GABA_AR) containing the $\alpha 5$, but not $\alpha 1$, subunit in the ventral
31 hippocampus (vHipp). The reason for this discrepancy in efficacy remains elusive; however, one key difference is that
32 $\alpha 1$ GABA_ARs are primarily located in the synapse, whereas $\alpha 5$ GABA_ARs are mostly extrasynaptic. To test whether receptor
33 location is responsible for this difference in efficacy, we injected a small interfering ribonucleic acid (siRNA) into the vHipp
34 to knock down radixin, a scaffolding protein that holds $\alpha 5$ GABA_ARs in the extrasynaptic space. We then administered GL-
35 II-73, a positive allosteric modulator of $\alpha 5$ GABA_ARs ($\alpha 5$ -PAM) known to reverse shock-induced deficits in dopamine system
36 function, to determine if shifting $\alpha 5$ GABA_ARs from the extrasynaptic space to the synapse would prevent the effects of
37 $\alpha 5$ -PAM on dopamine system function. As expected, knockdown of radixin significantly decreased radixin-associated
38 $\alpha 5$ GABA_ARs and increased the proportion of synaptic $\alpha 5$ GABA_ARs, without changing the overall expression of $\alpha 5$ GABA_ARs.
39 Importantly, GL-II-73 was no longer able to modulate dopamine neuron activity in radixin-knockdown rats, indicating that
40 the extrasynaptic localization of $\alpha 5$ GABA_ARs is critical for hippocampal modulation of the dopamine system. These results
41 may have important implications for clinical use of GL-II-73, as periods of high hippocampal activity appear to favor
42 synaptic $\alpha 5$ GABA_ARs, thus efficacy may be diminished in conditions where aberrant hippocampal activity is present.

43

44 **Significance Statement: Currently available treatments for psychosis, a debilitating symptom linked with several brain**
45 **disorders, are inadequate. While they can help manage symptoms in some patients, they do so imperfectly. They are**
46 **also associated with severe side effects that can cause discontinuation of medication. This study provides preclinical**
47 **evidence that the drug, GL-II-73, possesses the ability to modulate dopamine activity, a key player in psychosis**
48 **symptoms, and further provides some mechanistic details regarding these effects. Overall, this work contributes to the**
49 **growing body of literature suggesting that GL-II-73 and similar compounds may possess antipsychotic efficacy.**

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52 Introduction

53 Gamma-Aminobutyric Acid type A receptors containing the $\alpha 5$ subunit ($\alpha 5\text{GABA}_A\text{Rs}$) have received considerable attention
54 as a therapeutic target for multiple disorders involving hippocampal pathology, likely due to their enhanced expression
55 within CA1 and CA3 regions of the hippocampus (Fritschy & Mohler, 1995; Olsen & Sieghart, 2009; Sur et al., 1999). Of
56 particular interest to the pharmaceutical industry are positive allosteric modulators (PAMs) selective for $\alpha 5\text{GABA}_A\text{Rs}$
57 because of their low propensity for side effects compared to nonselective benzodiazepines, which are known to cause
58 sedation through actions mediated by $\alpha 1$ subunits (Sieghart & Savić, 2018; Sigel & Ernst, 2018). Preclinical studies using
59 $\alpha 5$ -PAMs have demonstrated a range of beneficial effects when given acutely including: anxiolytic, antidepressant, and
60 pro-cognitive effects (Prevot et al., 2019, 2020). When administered chronically, $\alpha 5$ -PAMs can reverse stress- or age-
61 related neuronal atrophy in the hippocampus and prefrontal cortex (Bernardo et al., 2022; Prevot et al., 2020).
62 Additionally, $\alpha 5$ -PAMs have shown promise in preclinical studies as antipsychotics (Gill et al., 2011; McCoy et al., 2022;
63 Perez et al., 2022). These results suggest that $\alpha 5$ -PAMs may have therapeutic utility for multiple disorders, especially those
64 in which aberrant hippocampal activity is present.

65 Though the dopamine hypothesis asserts that psychosis is driven by excessive dopamine, convergent evidence suggests
66 that dopamine dysregulation is secondary to aberrant hippocampal output, which drives the dopamine system
67 dysfunction (Lodge & Grace, 2007, 2008b, 2011). Indeed, increased hippocampal activity has been observed in humans
68 with psychosis (Schobel et al., 2009) and in rodent models (Lodge & Grace, 2007, 2008b, 2011). Further, we have
69 previously demonstrated that decreasing hippocampal activity using pharmacological (Perez & Lodge, 2018), cell-based
70 (Donegan et al., 2017; Perez & Lodge, 2013), or surgical (Perez et al., 2013) approaches effectively normalizes dopamine
71 system function and related behaviors in rodent models with schizophrenia-like symptomatology. Thus, we posit that
72 augmenting the function of $\alpha 5\text{GABA}_A\text{Rs}$ in the ventral hippocampus (vHipp) will likely have the same effect and normalize
73 dopamine system function and behavior in a stress-based model displaying psychosis-like pathology. Indeed, we have
74 previously demonstrated that this is the case in animal models used to study both schizophrenia and post-traumatic stress
75 disorder (PTSD) where aberrant dopamine system function is present (Donegan et al., 2019b; McCoy et al., 2022; Perez et
76 al., 2022). This evidence suggests that $\alpha 5\text{GABA}_A\text{Rs}$ may represent a viable therapeutic target for the treatment of psychosis
77 across multiple disorders.

78 Interestingly, nonspecific positive allosteric modulation of GABA_ARs or selectively targeting hippocampal α 1GABA_ARs are
79 largely ineffective at reversing aberrant dopamine system function (Donegan et al., 2019b; Perez et al., 2022). One crucial
80 difference between α 5- and α 1GABA_ARs is that α 5GABA_ARs can dynamically travel between the extrasynaptic space, where
81 they regulate tonic inhibition (Bonin et al., 2007; Caraiscos et al., 2004; Glykys et al., 2008) and the synapse, where they
82 mediate phasic inhibition (Davenport et al., 2021), whereas α 1GABA_ARs are almost exclusively synaptic (Brünig et al., 2002;
83 Crestani et al., 2002). Unlike typical extrasynaptic receptors that are diffuse in the membrane, α 5GABA_ARs form clusters
84 (Brünig et al., 2002; Hausrat et al., 2015; Loebrich et al., 2006). This clustering is mediated through an interaction with
85 radixin, a scaffolding protein that anchors the receptor to actin, concentrating receptors in the extrasynaptic space (**Figure**
86 **1**; Loebrich et al., 2006). The radixin- α 5 interaction is phosphorylation-dependent, such that dephosphorylation of radixin
87 decouples the two proteins, allowing the receptor to diffuse freely through the membrane (Hausrat et al., 2015). In mutant
88 mice that express a phosphorylation incompetent radixin, α 5GABA_ARs co-localize with gephyrin, the inhibitory synaptic
89 scaffolding protein, suggesting that, in the absence of a radixin interaction, α 5GABA_ARs will move into the synapse and
90 interact with gephyrin (Hausrat et al., 2015; Loebrich et al., 2006). Furthermore, the shifting of α 5GABA_ARs into the
91 synapse appears to be physiologically relevant, as induction of long-term potentiation in the hippocampus can also
92 increase synaptic relocation of α 5GABA_ARs (Davenport et al., 2021). Indeed, it has been hypothesized that the purpose
93 of α 5GABA_ARs clustering is to serve as a readily releasable pool of GABA_ARs to rapidly adjust to perturbations of
94 excitatory/inhibitory balance, with periods of high activity increasing the contribution of α 5GABA_ARs to inhibitory post
95 synaptic potentials (Davenport et al., 2021).

96 Given the remarkable difference in antipsychotic-like efficacy between targeting α 5- and α 1- GABA_ARs (Donegan et al.,
97 2019b; Perez et al., 2022), we sought to examine if receptor location (extrasynaptic vs synaptic) of α 5GABA_ARs could
98 influence the effects of a selective α 5-PAM, GL-II-73, on dopamine system function and sensorimotor gating (prepulse
99 inhibition of startle; PPI), a dopamine-dependent behavior often affected in psychosis (Swerdlow et al., 2001). We injected
100 small interfering RNA (siRNA) targeting radixin or a scrambled siRNA, as a control, directly into the vHipp of adult rats.
101 Under these conditions, we examined the effects of GL-II-73 on dopamine neuron population activity in the ventral
102 tegmental area (VTA) and on PPI. Exposure to an inescapable shock (IS) for two days is a validated model used to study
103 PTSD-like pathology in rodents (Van Dijken et al., 1992), a condition often comorbid with psychosis (Compean & Hamner,
104 2019). In this model, we have demonstrated that the rats exhibit psychosis-like symptomatology, such as robust

alterations in dopamine neuron activity and deficits in PPI (Elam et al., 2021) that can be reversed by GL-II-73 (McCoy et al., 2022). Here, we now report that this reversal was blunted following radixin knockdown, causing $\alpha 5\text{GABA}_A\text{Rs}$ to shift into the synapse. These findings establish a clear relationship between $\alpha 5\text{GABA}_A\text{R}$ localization and the antipsychotic-like efficacy of GL-II-73. Such information is critical for clinical use of $\alpha 5\text{-PAMs}$, as $\alpha 5\text{GABA}_A\text{R}$ location appears to be activity dependent (Davenport et al., 2021), and conditions in which hippocampal hyperactivity is present (e.g. epilepsy-induced psychosis) may promote a synaptic shift of $\alpha 5\text{GABA}_A\text{Rs}$ and would decrease antipsychotic efficacy in these individuals.

1. Materials and Methods

All experiments were performed in accordance with the guidelines outlined in the USPH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and the Use Committees of UT Health San Antonio and U.S. Department of Veterans Affairs.

2.1 Animals

Adult, male (350-400g) and female (250-300g) Sprague Dawley rats purchased from Envigo (Indianapolis, IN, USA) were used for all experiments. Rats were kept on a 12 h light/dark cycle. Food and water were provided *ad libitum*. GL-II-73 or vehicle (85% H_2O , 14% propylene glycol, 1% Tween80) were administered directly into the vHipp (100ng/ μL ; 0.75 μL ; AP - 5.3 mm, ML \pm 5.0 mm, from Bregma DV -6.0 mm from brain surface) at a rate of approximately 0.5 $\mu\text{L}/\text{min}$ 20 minutes prior to electrophysiology or behavior. This dose and timing was selected based on previous characterization (Prevot et al., 2019) as well as our own data (McCoy et al., 2022; Perez et al., 2022).

2.2 siRNA mediated knockdown of Radixin

Rats were anesthetized with 2-4% isoflurane prior to placement in a stereotaxic apparatus using blunt atraumatic ear bars. Bilateral indwelling cannulas (Protech International, Roanoke, VA, C317G, D/V -6 mm below plate) were implanted in the vHipp (A/P -5.3 mm M/L \pm 5.0 mm from Bregma D/V -6.0 mm from brain surface) and fixed in place with dental cement and four anchor screws. Rats received the analgesic ketoprofen (5 mg/kg, *s.c.*) and allowed to recover, individually housed, for a minimum of one week before experimentation. Injectors extending 1 mm past the end of the guide cannula were utilized for microinjections. Guide cannulas were kept patent with dummy cannulas. Rats were injected with either siRNA targeting RDX (0.2 $\mu\text{g}/\mu\text{L}$; 0.75 μL) or a non-targeting, scrambled siRNA as a control at a rate of approximately 0.5 $\mu\text{L}/\text{min}$. This concentration was selected based on published data (Mitchnick et al., 2015). The four RDX targeting sequences in the siRNA SMARTpool are as follows: GAAUCAGUUAUAACGUUUA; CCAAUAAAUGUAAGAGUAA; CCUUAUUGCUAAAAGAAUC;

132 CUCUAAUUUUGGAUAAU. Accell siRNA (Dharmacon, Lafayette, CO, USA) was chosen specifically as it was designed to
133 incorporate into cells that are difficult to transfect, such as neurons, without the use of a transfection agent and results in
134 peak knockdown within 3-4 days (Jarome et al., 2018; Webb et al., 2017).

135 *2.3 Inescapable foot shock stress*

136 Rats were randomly assigned to control (no shock) or to the shock groups that received two consecutive days of
137 inescapable foot shock stress (IS) as previously described (Elam et al., 2021; McCoy et al., 2022). The two-day IS paradigm
138 consisted of placing the rats in a 30.5 x 25.4 x 30.5 cm conditioning chamber with a stainless-steel grid shock floor
139 (Coulbourn Instruments, Whitehall, PA, USA). One session of IS consists of 60 x 15s, 0.8 mA foot shocks with an average
140 inter-trial interval (ITI) of 30 seconds with a 25% deviation (± 7.5 seconds) and lasted approximately 40 minutes. Control
141 rats were handled daily but not exposed to conditioning chambers. Electrophysiology and behavioral experiments were
142 conducted 24 hours after the last day of IS as previously described (Elam et al., 2021; McCoy et al., 2022).

143 *2.4 In vivo extracellular dopamine recordings*

144 Rats were anesthetized with 8% chloral hydrate (400 mg/kg, *i.p.*) and placed in a stereotaxic apparatus (Kopf Instruments,
145 Tujunga, CA, USA). This anesthetic was specifically chosen as it does not significantly alter dopamine neuron activity
146 compared to freely moving animals (Hyland et al., 2002) and also produces analgesia (Ward-Flanagan & Dickson, 2023).
147 Extracellular glass microelectrodes (impedance ~ 6 -10 M Ω) were lowered into the VTA (from bregma: AP -5.3 mm, ML ± 0.6
148 mm, and DV -6.5 to -9.0 mm) using a hydraulic micro-positioner (Model 640, Kopf). Multiple areas within the VTA were
149 sampled by making multiple vertical passes ("tracks"), separated by 200 μ m, in a predetermined pattern. Spontaneously
150 active dopamine neurons within a track were identified using open filter settings (low-frequency cutoff: 30 Hz; high-
151 frequency cutoff: 30 kHz) according to previously established electrophysiological criteria (Grace & Bunney, 1983; Ungless
152 & Grace, 2012). Three parameters of dopamine activity were measured and analyzed: 1.) the number of dopamine
153 neurons firing spontaneously per track (population activity (Lodge & Grace, 2011)) 2.) firing rate, and 3.) proportion of
154 action potentials occurring in bursts (defined as the incidence of spikes with < 80 ms between them; termination of the
155 burst is defined by > 180 ms between spikes). Analysis of dopamine neuron activity was performed using LabChart software
156 (ADInstruments, Sydney, Australia). Immediately following, rats were rapidly decapitated, and brains were extracted. A
157 subset of brains was used to verify electrode and canula placement, while the remaining brains were used for molecular
158 analysis.

159 *2.5 Prepulse inhibition of startle (PPI)*

160 Rats were placed into a sound-attenuated chamber (SD Instruments, San Diego, CA, USA) and allowed to acclimate to
161 65dB background noise for 5 minutes. Rats were then exposed to 10 startle-only trials [40ms, 120dB, 15s average inter-
162 trial intervals (ITI)]. Next, rats were exposed to 24 trials where a pre-pulse (20ms at 69dB, 73dB and 81dB) is presented
163 100ms before the startle pulse. Each prepulse + startle pulse trial was presented 6 times in a pseudo-random order (15s
164 average ITI). The startle response was measured from 10-80ms after the onset of the startle pulse and recorded and
165 analyzed using SR-LAB Analysis Software (SD Instruments). PPI was calculated for each prepulse intensity and averaged
166 across the three intensities.

167 *2.6 Immunoprecipitation*

168 Immediately following completion of electrophysiology or behavior, rats were euthanized by rapid decapitation, and the
169 hippocampus was dissected out on ice and separated into dorsal and ventral portions. Samples were homogenized with
170 lysis buffer and centrifuged at 14,000g for 2 minutes. Supernatants were collected and stored at -80 until $\alpha 5$ subunit and
171 its binding partners were immunoprecipitated using SureBeads protein G magnetic beads according to the manufacturer's
172 protocol (Biorad, Hercules, CA, USA). Western blots (*detailed in section 2.8*) were used to quantify $\alpha 5$, RDX, and GEPH
173 levels in both the immunoprecipitated samples and hippocampal homogenates.

174 *2.7 Chemical Cross-linking Assay*

175 A chemical cross-linking assay was performed in a subset of rats as previously described (Boudreau et al., 2012; Tomoda
176 et al., 2022). Briefly, rats were rapidly decapitated, and brains extracted. The whole hippocampus was dissected out over
177 ice, separated into dorsal and ventral portions, and minced into small pieces using a razor blade. The vHipp from one
178 hemisphere was incubated in Dulbecco's phosphate buffered saline (PBS) with calcium chloride and magnesium chloride
179 (Sigma) containing bis(sulfosuccinimidyl)suberate (BS3) cross-linker (2mM, ThermoFisher, Waltham, MA, USA) for 2 hours
180 at 4C on a shaker. The other hemisphere was incubated in Dulbecco's PBS as a control. All samples were quenched by
181 adding 100mM glycine and rotating another 10 minutes at 4C. Samples were then centrifuged (20,000g for 2 minutes at
182 4C) and supernatants were discarded. A lysis buffer containing 0.1% Triton-X-100 and peptidase inhibitors was added and
183 tissues were homogenized (PowerGen 125, Fisher Scientific) and centrifuged for 2 minutes (20,000g at 4C). The
184 supernatants were collected and stored at -80 until analyses by western blot.

185 *2.8 Western Blot*

186 Proteins in the lysates were separated in an SDS-PAGE gel followed by blotting onto a 0.2 μ m nitrocellulose membrane.
187 Membranes were incubated with an antibody against α 5GABA_ARs (1:1000), Radixin (1:1000), Gephyrin (1:3000), or GAPDH
188 (1:1000) in 2.5% BSA in TBST, overnight at 4C. They were then washed with Tris-buffered saline with 0.1% Tween 20 (TBST)
189 prior to incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit; 1: 10,000 or horse
190 anti-mouse; 1:5000) for 1 hour at room temperature. Membranes were washed with TBST (3x for 10 min each) and
191 incubated with a Pierce enhanced chemiluminescence kit (Thermofisher) followed by exposure to X-ray film for detection.
192 Blots were stripped using a commercially available stripping buffer, washed, blocked, and re-probed no more than once.
193 Densitometry analyses of immunoreactive bands were performed using the NIH Image J software from the scanned films.
194 Densitometric arbitrary units were normalized to GAPDH, except in immunoprecipitation studies in which radixin and
195 gephyrin measures were normalized to α 5GABA_AR levels.

196 *2.9 Histology*

197 To verify electrode and cannula placement, brains were fixed for at least 24 hours (4% phosphate buffered formaldehyde),
198 and cryoprotected (10% w/v sucrose in phosphate-buffered saline) until saturated. Brains were coronally sectioned (25
199 μ m) using a cryostat (Leica, Buffalo Grove, IL, USA). Sections containing electrode or cannula tracks were mounted onto
200 gelatin-coated slides, stained with neutral red (0.1%) and thionin acetate (0.01%), and cover-slipped with DPX Mountant
201 for histochemical confirmation within the VTA (electrode) or vHipp (cannula) (**Figure 2A**).

202 *2.10 Materials*

203 The proprietary compound, GL-II-73, was synthesized by the University of Wisconsin-Milwaukee and supplied by the
204 Centre for Addiction and Mental Health, Campbell Family Mental Health Research Institute (Toronto, ON, CA),. Chloral
205 hydrate (C8383), propylene glycol (P4347), and Tween80 (P1754) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
206 Antibodies were from R&D Systems, (Minneapolis, MN USA,) #PPS027 (α 5) or Abcam (Cambridge, UK) ab5249 (radixin)
207 ab181382 (gephyrin) #9484 (GAPDH) or Cell signaling (Davers, MA, USA) #7074 (anti-rabbit-HRP) #7076 (anti-mouse-HRP).
208 Accell siRNA were purchased from Dharmacon.

209 *2.11 Statistical analysis*

210 Data are represented as mean \pm SEM and n values representing either the number of rats or neurons as indicated. In all
211 experiments, data were analyzed by three-way ANOVA (electrophysiology and PPI; factors: stress x drug x siRNA), two-
212 way ANOVA (α 5 surface expression; factors: siRNA x crosslinker) or t-test (Western Blot) and plotted using Prism software

213 (GraphPad Software Inc.; San Diego, CA, USA). When significant main effects or interactions were detected the Holm–
214 Sidak post-hoc test was used. All tests were two-tailed, and significance was determined at $p < 0.05$. While both sexes
215 were represented, we were not powered to detect sex differences and therefore did not explicitly test for this. Raw
216 electrophysiology data were analyzed using LabChart version 8 (ADInstruments, Colorado Springs, CO, USA) and PPI data
217 were analyzed using SR Labs Analysis software (SD Instruments).

218 2. Results

219 3.1 The therapeutic effects of intra-vHipp administration of GL-II-73 on dopamine system function are blocked by radixin 220 knockdown

221 To evaluate dopamine system function, we measured dopamine neuron activity in the VTA using *in vivo* extracellular
222 electrophysiology. Consistent with previous findings (Elam et al., 2021; McCoy et al., 2022), inescapable footshock stress
223 elicited a significant increase in population activity ($n = 6$ rats; 1.733 ± 0.109 cells per track; three-way ANOVA; $F_{\text{Shock}(1,48)} =$
224 73.860 ; $p < 0.0001$; $F_{\text{siRNA}(1,48)} = 8.135$; $p = 0.006$; Holm–Sidak; $t = 6.153$, $p < 0.0001$; **Figure 2A**) when compared to non-
225 shocked vehicle rats ($n = 6$ rats; 0.900 ± 0.089 cells per track). This shock-induced increase in dopamine neuron activity was
226 completely reversed by intra-hippocampal administration of GL-II-73 ($n = 7$ rats; 1.071 ± 0.078 cells per track; Holm–Sidak;
227 $t = 5.072$, $p = 0.0001$) and had no effect in non-shocked rats who received intra-hippocampal GL-II-73 ($n = 8$ rats;
228 1.013 ± 0.069 cells per track). Further, knocking down radixin in non-shocked rats had no effect on population activity in
229 vehicle ($n = 6$ rats; 0.967 ± 0.061 cells per track) and GL-II-73 treated rats ($n = 8$ rats; 1.113 ± 0.088 cells per track). Again,
230 consistent with observations in rats who received the scrambled siRNA, shock produced a significant increase in dopamine
231 neuron activity, ($n = 7$ rats; 1.571 ± 0.121 cells per track; Holm–Sidak; $t = 1.780$, $p = 0.669$) in radixin knockdown rats.
232 Interestingly, increasing synaptic $\alpha 5\text{GABA}_A\text{R}$ localization by knocking down radixin blocked the ability of intra-hippocampal
233 GL-II-73 to restore dopamine system function in shocked rats ($n = 8$ rats; 1.788 ± 0.081 cells per track). As expected, no
234 significant differences were observed in the average firing rate (**Figure 2B**; $F_{\text{Shock}(1,404)} = 0.953$; $p = 0.330$; $F_{\text{siRNA}(1,404)} = 0.304$;
235 $p = 0.582$ $F_{\text{drug}(1,404)} = 0.033$; $p = 0.856$) or burst firing (**Figure 2C**; $F_{\text{Shock}(1,404)} = 3.427$; $p = 0.065$; $F_{\text{siRNA}(1,404)} = 0.044$; p
236 $= 0.833$; $F_{\text{drug}(1,404)} = 2.200$; $p = 0.139$). Representative traces from control (left) and shock (right) animals are shown in
237 **Figure 2D**.

238 3.2 Increased synaptic $\alpha 5\text{GABA}_A\text{R}$ expression does not prevent the effects of GL-II-73 in prepulse inhibition of startle

239 To evaluate sensorimotor gating, we measured prepulse inhibition of the acoustic startle response (**Figure 3**). Previous
240 studies measuring PPI in rats exposed to IS reported a significant decrease in the %PPI following inescapable footshock
241 stress (Elam et al., 2021; McCoy et al., 2022). Here, we observed a significant main effect of shock (n= 9-11 rats per group;
242 three-way ANOVA; $F_{\text{shock}} (1, 71) = 14.310$; $p = 0.0003$) and of intra-vHipp administration of GL-II-73 ($F_{\text{drug}} (1, 71) = 8.765$;
243 $p=0.004$); however, *post hoc* tests revealed no significant differences between groups.

244 3.3 Radixin knockdown increased markers of synaptic $\alpha 5\text{GABA}_A\text{R}$ but did not alter $\alpha 5\text{GABA}_A\text{R}$ expression within the vHipp

245 To validate the successful knockdown of radixin, we measured radixin associated with $\alpha 5\text{GABA}_A\text{R}$ using
246 coimmunoprecipitation in control rats. We observed a significant difference between radixin knockdown and control
247 groups (**Figure 4A**, t test; $t = 2.629$, $p = 0.017$). Additionally, co-immunoprecipitation of $\alpha 5\text{GABA}_A\text{R}$ and gephyrin revealed
248 a significant increase in gephyrin levels in rats that had radixin knocked down compared to controls (**Figure 4B**, t test;
249 $t=3.069$, $p=0.008$), suggesting an increase in synaptic $\alpha 5\text{GABA}_A\text{Rs}$. Finally, to ensure any electrophysiology and behavioral
250 results were not due to degradation or internalization of $\alpha 5\text{GABA}_A\text{Rs}$ when radixin is knocked down, we also measured
251 surface and total $\alpha 5\text{GABA}_A\text{R}$ using a chemical crosslinking assay. While there was an expected significant difference
252 between crosslinked samples and homogenate (**Figure 3C**, two-way ANOVA, $F_{\text{crosslinker}} (1, 16) = 11.300$; $p = 0.004$) there were
253 no significant differences due to radixin knockdown ($F_{\text{siRNA}} (1, 16) = 0.173$; $p = 0.683$).

254 Discussion

255 Psychosis is debilitating symptom that accompanies many neurological disorders, including PTSD (Compean & Hamner,
256 2019; van den Berg et al., 2016). The dopamine hypothesis states that aberrant dopamine neuron activity underlies
257 psychosis symptoms, yet currently available antipsychotics that target dopamine D2 receptors are not always effective
258 and often result in intolerable side effects (i.e. dyskinesias and metabolic disorders) (Lieberman et al., 2005). This has led
259 some to suggest that indirectly modulating dopamine neuron activity through manipulating activity in upstream brain
260 regions may be an effective treatment strategy that produces fewer adverse effects. The hippocampus is a brain region
261 that can modulate dopamine neuron activity through a multisynaptic pathway starting in the nucleus accumbens (Lodge
262 & Grace, 2007, 2008b). Using a multitude of techniques, we and others have demonstrated that attenuating vHipp activity
263 can restore dopamine neuron population activity and related behaviors in animal models used to study psychosis (Lodge
264 & Grace, 2007; Perez et al., 2013; Perez & Lodge, 2013, 2018; Valenti et al., 2011) . An effective and translational approach

265 to inhibiting vHipp activity is by using $\alpha 5$ -PAMs. Indeed, we and others have previously shown that targeting $\alpha 5$ GABA_ARs
266 can improve physiological and behavioral alterations associated with psychosis (Donegan et al., 2019b; Gill et al., 2011;
267 McCoy et al., 2022; Perez et al., 2022), suggesting that $\alpha 5$ -PAMs may possess antipsychotic efficacy. Interestingly, the
268 efficacy of PAMs appears to be specific to those selective for the $\alpha 5$ -subunit, as targeting vHipp $\alpha 1$ GABA_ARs does not
269 appear to modulate VTA dopamine neuron activity (Donegan et al., 2019b; Perez et al., 2022). A major delineation
270 between these receptor types is their cellular location, with $\alpha 5$ GABA_ARs existing both in the synapse and extrasynaptic
271 space whereas $\alpha 1$ GABA_ARs are limited to the synapse. Here, we examined if the observed differences in antipsychotic-like
272 efficacy were due to receptor location (extrasynaptic vs synaptic). Based on our previous findings with targeting synaptic
273 $\alpha 1$ GABA_ARs, we posited that GL-II-73 would no longer be able to modulate dopamine neuron activity when $\alpha 5$ GABA_ARs
274 are shifted into the synapse. These studies have important implications, as previous studies have determined that high
275 periods of hippocampal activity, often observed in psychosis (Lodge & Grace, 2007, 2008b; Schobel et al., 2009), can
276 promote movement of $\alpha 5$ GABA_ARs into the synapse.

277 Aberrant dopamine neuron activity is central to the pathology of psychosis and is observed in both patients (Abi-Dargham,
278 2004; Howes et al., 2009; Laruelle & Abi-Dargham, 1999) and rodent models (Lodge, 2013). To examine dopamine system
279 function, we use *in vivo* electrophysiology to measure the number of spontaneously active dopamine neurons in the VTA,
280 referred to as population activity (Lodge & Grace, 2011). We consistently find that animal models used to study psychosis
281 have elevated dopamine neuron activity (Donegan et al., 2017; Perez et al., 2013; Perez & Lodge, 2019). Here, we report
282 that IS exposure, a common rodent model to study PTSD, induced aberrant dopamine neuron population activity, a finding
283 consistent with previous literature (Elam et al., 2021; McCoy et al., 2022). This was reversed by GL-II-73. However, in
284 conditions of knocking down radixin, which caused a shift of $\alpha 5$ GABA_ARs to the synapse, this effect of GL-II-73 was lost.
285 These results suggest that the ability of GL-II-73 to modulate VTA dopamine neuron activity is dependent on the
286 extrasynaptic localization of $\alpha 5$ GABA_ARs.

287 Patients with PTSD and patients with psychosis both display deficits in sensorimotor gating (Bakshi et al., 2012; Kohl et al.,
288 2013; Meteran et al., 2019), a behavioral dimension that is readily assessed in rodents using PPI (Braff & Geyer, 1990).
289 Indeed, rodent models used to study both PTSD and psychosis display deficits in PPI (Elam et al., 2021; Perez et al., 2019),
290 which can be reversed by GL-II-73 (McCoy et al., 2022). In the current study we demonstrated that IS decreases PPI and
291 that intervention with GL-II-73 attenuates this, regardless of the radixin knockdown (i.e., regardless of the localization of

292 $\alpha 5$ GABA_ARs). While PPI is a dopamine-dependent behavior, it is mediated by other circuits and not exclusively controlled
293 by dopamine (Swerdlow et al., 2001). This may suggest that the reliance of GL-II-73 on extrasynaptic receptors is limited
294 to modulation of dopamine neuron activity, and may not apply to the antidepressant-like effects (Prevot et al., 2019) or
295 the pro-cognitive effects (Prevot et al., 2020).

296 Nonselective benzodiazepines, or derivatives that primarily act on $\alpha 1$ GABA_ARs are ineffective as antipsychotics (Easton &
297 Janicak, 1991; Gillies et al., 2005). This is in line with our previous studies demonstrating that selectively targeting
298 $\alpha 1$ GABA_ARs or nonselectively targeting GABA_ARs in the vHipp does not affect dopamine neuron activity (Donegan et al.,
299 2019a; Perez et al., 2022). Taken with the findings presented in the current study, it appears that this is due to targeting
300 of synaptic GABA_ARs. However, an open question remains as to why synaptic GABA_ARs do not modulate VTA dopamine
301 neuron activity in the way that extrasynaptic ones can. One possibility is that the type of inhibition produced by
302 extrasynaptic receptors (tonic) is more effective at maintaining a decrease in hippocampal activity than synaptic receptors
303 (phasic). It is possible that the fast, transient nature of phasic inhibition is insufficient to produce changes in downstream
304 dopamine activity, whereas the relatively slower and more persistent effects of $\alpha 5$ -mediated tonic inhibition has a more
305 robust effect (Koniaris et al., 2011; Schulz et al., 2018). Although this explanation only partially accounts for earlier studies
306 which demonstrate that dampening excitatory transmission in the vHipp using tetrodotoxin can also restore healthy
307 dopamine system function in animal models used to study psychosis (Lodge & Grace, 2008a; Perez & Lodge, 2018; Valenti
308 et al., 2011).

309 The loss of efficacy when moved into the synapse may also be explained by a change in receptor functionality. While it
310 has been shown that synaptic $\alpha 5$ GABA_ARs can successfully contribute to IPSCs (Davenport et al., 2021; Hausrat et al., 2015;
311 Loebrich et al., 2006), differences in structure caused by loss or gain of protein-protein interactions may prevent GL-II-73
312 from modulating $\alpha 5$ GABA_AR when they move to the synapse. For example, it is known that $\alpha 5$ GABA_ARs interact with
313 auxiliary subunits, such as Shisa7, which can modify receptor kinetics (Castellano et al., 2022) and trafficking (Wu et al.,
314 2022) and appear to be critical for tonic currents (Wu et al., 2021). Alterations in protein interactions may change receptor
315 function enough to negate the effects of GL-II-73 in the situation of $\alpha 5$ GABA_ARs being localized to the synapse.

316 We confirmed that decoupling $\alpha 5$ GABA_ARs from radixin did not reduce membrane expression of $\alpha 5$ GABA_ARs, suggesting
317 that the absence of an effect of GL-II-73 in radixin knockdown rats is not due to a reduction in receptor availability.
318 However, a limitation of this study is that we did not measure actual levels of synaptic and extrasynaptic receptors. Rather,

319 we measured markers of $\alpha 5\text{GABA}_A\text{Rs}$ localization through association with radixin and gephyrin. Thus, while our
320 coimmunoprecipitation studies suggest that knockdown of radixin increases synaptic $\alpha 5\text{GABA}_A\text{Rs}$, we acknowledge the
321 caveat associated with measuring proxies for localization. Future studies should more rigorously examine the dynamics of
322 $\alpha 5\text{GABA}_A\text{R}$ relocalization and pinpoint the biological processes resulting in the dramatic loss of efficacy we observed here,
323 despite the incomplete knockdown of radixin. Functional studies demonstrating the impact of radixin knockdown on tonic
324 and phasic GABAergic currents would be helpful, and the absence of these experiments represents a limitation of this
325 study. We emphasize the importance of follow-up studies, as the results obtained here may have important clinical
326 implications, especially as interest in $\alpha 5\text{-PAMs}$ from the pharmaceutical industry increases. Indeed, $\alpha 5\text{GABA}_A\text{R}$ localization
327 appears to be dynamically modulated by hippocampal activity levels (Davenport et al., 2021). It is possible that certain
328 conditions where hippocampal activity is dramatically altered, the proportion of synaptic $\alpha 5\text{GABA}_A\text{Rs}$ could increase,
329 diminishing the ability of GL-II-73 and perhaps other $\alpha 5\text{-PAMs}$ as well. Although the results obtained here suggest that
330 this is limited to modulation of dopamine neuron activity, as PPI was unaffected by radixin knockdown. This study
331 highlights the importance of testing novel therapeutics in multiple disease states and/or models, a concept of particular
332 importance for GL-II-73, which has shown promising therapeutic potential for a variety of psychiatric conditions (McCoy
333 et al., 2022; Perez et al., 2022; Prevot et al., 2019, 2020).

334 **Author Contributions**

335 A.M.M. made contributions to the design of the work, acquisition, analysis and interpretation of the data, as well as
336 drafting and editing the manuscript. T.D.P. and E.L.S. assisted in interpretation of the data and revising and editing the
337 manuscript content. M.Y.M. and D.S. synthesized GL-II-73 under the supervision of J.C. A.N.A. contributed to acquisition
338 of the data. D.J.L. contributed to the concept and design of the study, analysis and interpretation of the data, revising and
339 editing manuscript content, as well as providing final approval of the document.

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535 **Figure Legends:**

536 **Figure 1. Schematic of radixin knockdown.** Diagram of an inhibitory synapse and surrounding extrasynaptic area under
537 (A) baseline conditions and (B) when radixin is knocked down. Figure made using Biorender.

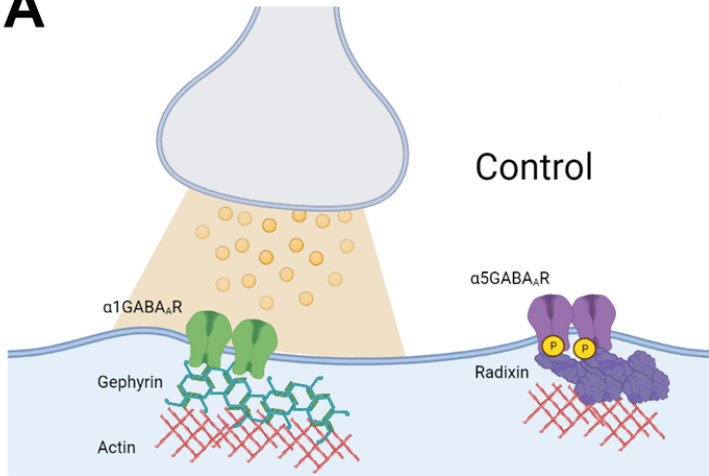
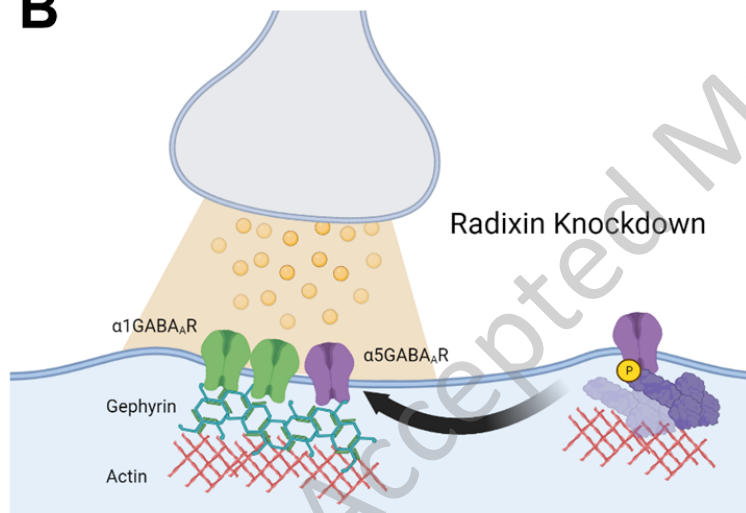
538 **Figure 2. GL-II-73 was unable to restore dopamine system function when radixin is knocked down.** *In vivo* extracellular
539 electrophysiology was used to measure dopamine cell activity in the ventral tegmental area. A) *Left*, Inescapable shock
540 exposure significantly increased the number of spontaneously active cells/track (population activity), which was reversed
541 by intra-ventral hippocampus injection of GL-II-73 (100ng/μL; 0.75 μL), but not when radixin was knocked down. *Right*,
542 Representative brain slice with electrode placement in the ventral tegmental area (VTA) (black arrow) and cannula
543 placement for drug administration in the ventral hippocampus (vHipp, white arrow), with corresponding schematics of
544 the brain section (-5.40 mm posterior to bregma) with box indicating the area in which tracks were found. Neither (B)
545 firing rate nor (C) burst firing was affected by siRNA, shock, or drug treatment. (D) representative traces from control (left)
546 and shocked (right) rats. n=6-8/group, males and females represented as circles and squares, respectively *** $P = 0.0001$,
547 **** $P < 0.0001$, RDX = Radixin

548 **Figure 3. Radixin knockdown does not alter prepulse inhibition.** Two days of inescapable shock had a significant main
549 effect on PPI as did treatment with GL-II-73 ($p = 0.0042$), however, post hoc analysis revealed no relevant group
550 differences. n=9-11/group, males and females represented as circles and squares, respectively. ** $P < 0.005$, RDX = radixin.

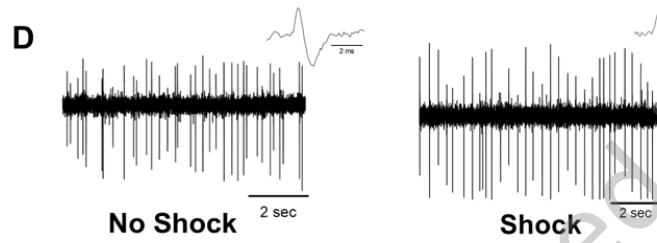
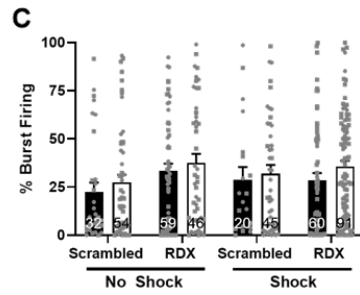
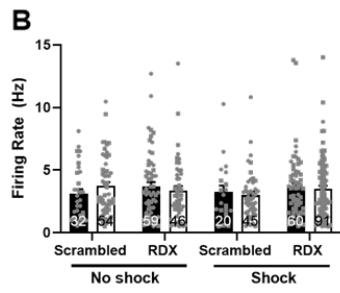
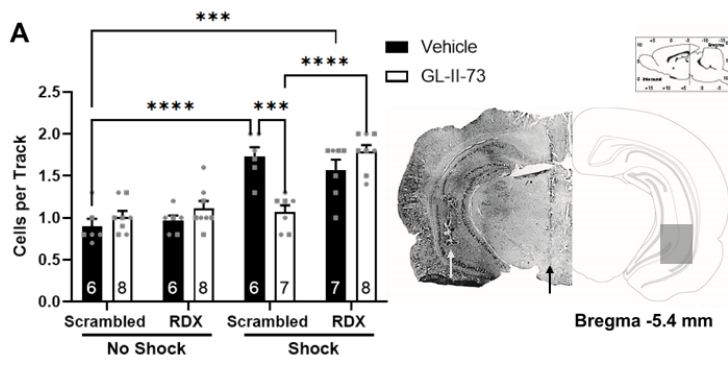
551 **Figure 4: Radixin knockdown increases synaptic $\alpha 5$, without changing surface or total $\alpha 5$ expression.** A) Co-
552 immunoprecipitation of $\alpha 5$ and radixin revealed a significant decrease in $\alpha 5$ -associated radixin in rats that received radixin-
553 targeted siRNA compared to those that received scrambled siRNA. Representative image of bands below. n=10/group. B)
554 Conversely, co-immunoprecipitation of $\alpha 5$ and gephyrin revealed a significant increase in gephyrin levels in the radixin
555 knockdown group. Representative image of bands below. n=9-11/group C) Treatment with the crosslinking agent caused
556 a significant decrease in optical density of $\alpha 5$ immunoreactive bands, but no differences in total $\alpha 5$ (homogenate) or

557 surface (crosslinked) were observed between rats that received scrambled siRNA or radixin-targeted siRNA.
558 Representative image of bands below graphs. n=5/group, males and females represented as circles and squares,
559 respectively. *p<0.05, **p<0.01 n = 10, RDX = Radixin, OD = optical density.

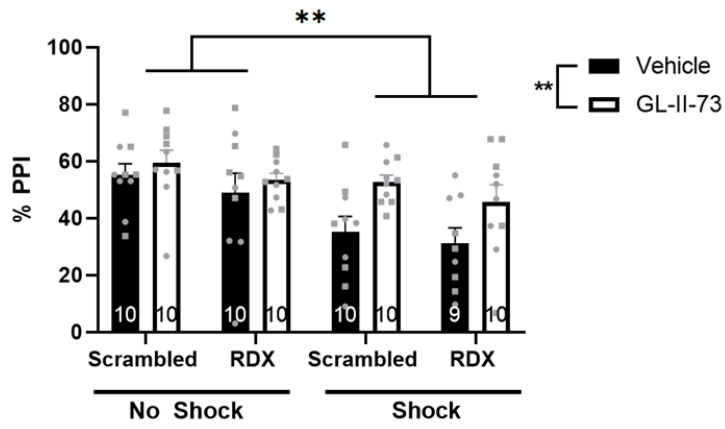
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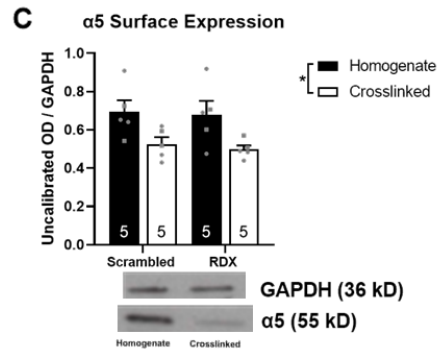
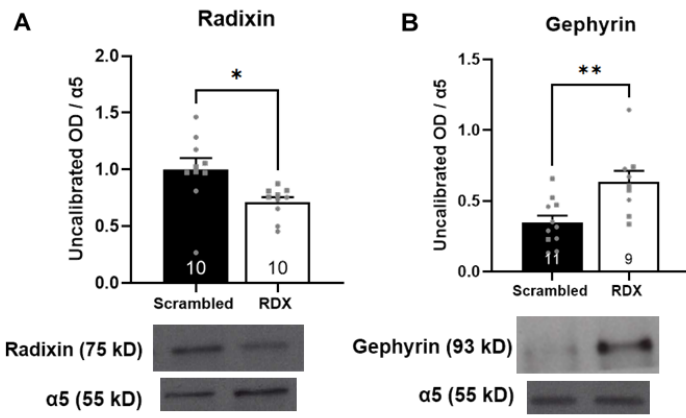
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