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ARTICLE

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Suppression of pyramidal neuron G protein-gated inwardly rectifying K+ channel signaling impairs prelimbic cortical function and underlies stress-induced deficits in cognitive flexibility in male, but not female, mice

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Imbalance in prefrontal cortical (PFC) pyramidal neuron excitation:inhibition is thought to underlie symptomologies shared across stress-related disorders and neuropsychiatric disease, including dysregulation of emotion and cognitive function. G protein-gated inwardly rectifying K^+ (GIRK/Kir3) channels mediate excitability of medial PFC pyramidal neurons, however, the functional role of these channels in mPFC-dependent regulation of affect, cognition, and cortical dynamics is unknown. We used a viral-cre approach in male and female mice harboring a "floxed" version of the kcnj3 (Girk1) gene, to disrupt GIRK1-containing channel expression in pyramidal neurons within the prelimbic cortex (PrL). In males, loss of pyramidal GIRK1-dependent signaling differentially impacted measures of affect and impaired working memory and cognitive flexibility. Unexpectedly, ablation of PrL GIRK1-dependent signaling did not impact affect or cognition in female mice. Additional studies used a model of chronic unpredictable stress (CUS) to determine the impact on PrL GIRK-dependent signaling and cognitive function. CUS exposure in male mice produced deficits in cognition that paralleled a reduction in PrL pyramidal GIRK-dependent signaling akin to viral approaches whereas CUS exposure in female mice did not alter cognitive flexibility performance. Stress-induced behavioral deficits in male mice were rescued by systemic injection of a novel, GIRK1-selective agonist, ML297. In conclusion, GIRK1-dependent signaling in male mice, but not females, is critical for maintaining optimal PrL function and behavioral control. Disruption of this inhibition may underlie stress-related dysfunction of the PrL and represent a therapeutic target for treating stress-induced deficits in affect regulation and impaired cognition that reduce quality of life.

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INTRODUCTION

Dysfunction of the prefrontal cortex (PFC) is an underlying factor in both affect and cognition-related behavioral deficits that co-occur across neuropsychiatric disorders [1–12]. Similar symptomologies are observed in individuals with chronic psychosocial or self-perceived chronic stress [13–16]. Converging evidence indicates that prolonged exposure to environmental stressors can increase the risk and severity of these neuropsychiatric disorders, highlighting a need to identify neural substrates that contribute to optimal PFC function and behavioral control.

The prelimbic cortical subdivision (PrL) of the medial PFC (mPFC) is involved in top-down regulation of behavior related to anxiety, motivation, stress, and coordination of working memory and flexible decision-making (e.g., strategy shifting) [17–26]. Optimal function of the PrL relies on coordinated activity of principle output glutamatergic pyramidal neurons. This activity is critically dependent on a dynamic balance of cell excitation: inhibition mediated largely by intrinsic (physiological) membrane properties that influence the cellular response to synaptic input [27–31]. Accordingly, imbalances in pyramidal neuron excitation: inhibition are thought to contribute to numerous symptoms observed in neuropsychiatric disorders [4, 28, 32–40].

PrL pyramidal neuron excitability and spike firing is modulated by activity of G protein-gated inwardly rectifying K^+ (GIRK/Kir3) channels which produce a slow hyperpolarizing current that acts as a neuronal "off switch" in both males and females [41–44]. GIRK channels are the primary postsynaptic downstream effector for inhibitory metabotropic receptors, including perisomatic $GABA_B$ receptor ($GABA_B$)—a major target of local GABA neurons [45, 46]. Converging clinical and preclinical data highlights a link between hypofunction of $GABA_B$-GIRK signaling with cognitive disabilities, abnormalities in affect, and susceptibility to neuropsychiatric disease [44, 47–56], however the anatomical locus of this hypofunction remains unclear. Importantly, what is known about

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GIRK-dependent signaling physiology and behavior have often been done in males, with little known about the role of GIRK channels in females. As the role of GIRK signaling in PFC-dependent behavior has not been characterized in either males or females, this study focused on testing the hypothesis that PrL GIRK1-dependent signaling is a critical locus of affect and cognitive flexibility. Further, we hypothesized that chronic unpredictable stress (CUS) would result in reductions in PrL GIRK-dependent signaling and that GIRKs represent a therapeutic target to treat stress-induced deficits.

METHODS

Animals

Experiments were approved by the Institutional Animal Care and Use Committee at Marquette University. Girk1$^{flox/flox}$ stock mice generated on a C57/BL6 background as described [57, 58], with experimental mice bred in house (91 ± 1 PD at start of food training for cognitive testing). For stress-related studies, adult male and female mice (91 ± 1 PD) were a combination of C57BL/6 bred in house or purchased directly from Jackson Laboratories, or in some instances BAC transgenic mice expressing tdTomato or enhanced green fluorescent protein in D1R- and D2R-MSNs, as our findings show no baseline differences in these strains compared to wild-type mice [59, 60]. Mice were housed in a temperature and humidity-controlled room with a 12 h/12 h light/dark cycle. All procedures were conducted during the light phase. Food and water were available ad libitum except where described.

Viral ablation of GIRK1 signaling

Adult male and female Girk$^{flox/flox}$ mice were anesthetized with isoflurane and received a bilateral infusion of either AAV8:CamkII:Cre with a GFP or mCherry fluorescent tag (UNC) or AAV8:CamkII:GFP (UNC) into the PrL (AP: Male: + 1.80, Female: +1.75, ML: ± 0.40, DV:−2.30) using a 5 µL flat Hamilton syringe (0.5 µL/infusion; 0.1 µL/min). Syringes were left in place for 5 min to reduce backflow then drawn to the surface over 5 min. Mice recovered for a period of at least three weeks to ensure full viral expression.

Behavioral testing

Elevated Plus Maze (EPM) and Forced Swim Test (FST). Tests were conducted as previously described [60, 61]. For EPM, mice were individually placed in the center of a lit (~50 lux) maze facing an open arm and allowed for exploring for 5 min. The percent time in the open arm which was recorded and analyzed using AnyMaze (Stoelting Company) was calculated as the total time in the open arms divided by the total time in the maze. For FST, mice were individually placed in a beaker filled with 25 ± 2 °C tap water and allowed to habituate for 2 min followed by 4 mi of testing during which the time spent immobile and the number of immobile episodes was tracked using a side-mounted camera and AnyMaze software with immobility sensitivity of 85% and a minimum immobility episode time of 250 ms.

Forced alternation T-maze paradigm. A subset of cre- and cre+ male and female mice underwent 3 days of habituation and training prior to testing in a T-maze based on previous literature [62]. Following food restriction and context habituation, mice were allowed to explore the maze and consume a reward (50ul of 50% diluted in tap water liquid vanilla Ensure®) in each arm for 5 min (×5 trials). On day 3, mice underwent forced run training with only one arm baited and the other blocked for 12 trials (6/arm). During the testing period, animals underwent 12 daily trials with access to one reward arm and allowed to consume the reward. Following a 20-s period, mice were given access to both arms with the previously unentered arm baited (correct choice). The percent correct for each day was calculated, as well as, the percent correct for each trial across all days.

Attention set shifting. Operant attentional set-shifting task (ASST) procedures were performed in sound-attenuating boxes (Med Associates, Inc.) based on previously developed rat protocols [26, 63] and on our previous work [61]. Male and female mice were food-deprived 85–90% of their free-feeding weight and underwent initial food training where the left and/or right lever required pressing a combined 50 times within a 3-h and subsequent 30-min session. During food training, all lever presses resulted in 20-s access to a liquid dipper (Med-Associates ENV-302) containing 50% liquid Ensure®. *Lever training* was conducted with the left or right lever presented in a pseudorandom order for a total of 90 trials (45 trials/lever). Response on the presented lever resulted in access to Ensure® for 20-s and no response within 10-s counted as an omission (criterion is ≤5 omissions on 2 consecutive days). Lever training was conducted to make certain all groups had similar lever responses prior to testing. Once a mouse reached lever training criterion, lever preference was assessed in a *bias test* where both the left and right lever were presented and reinforced for a total of 7 trials.

In initial groups, ASST test days were ran as follows: visual cue, extradimensional shift (ED Shift), and reversal tests. Criterion for each test was ten consecutive correct responses in a row with a minimum of 25 trials (or until 150 trials were conducted). If criterion was not reached in 150 trials, the test was conducted again the following day. During the *visual cue test*, a light was illuminated above the right or the left lever for 3-s followed immediately by presentation of both levers, with the correct response being the lever under the visual cue resulting in a reward. In the *ED Shift* and *response* testing, the cue was presented but the correct response was the lever opposite the bias regardless of cue location. In a subset of male mice, 20 mg/kg of the GIRK1-selective agonist ML297 was injected 30 minutes prior to the ED shift. During *reversal testing*, the reinforced lever was opposite to the correct lever during the ED Shift. In another experiment, a subset of male mice did not receive lever training but rather received 'visual cue training' which was conducted as described above. Lastly, another subset of male mice went through lever training as described above, but then received a response test during which the correct response was the lever opposite of bias regardless of cue location. Once reaching criterion, male mice then received a visual cue test as described above. In instances where there was a significant difference in errors to criterion, the error type was further investigated by dividing tests into blocks of 16 trials (excluding omissions) and initial/perseverative errors were those made until less than 16 errors were made in a block or were characterized as regressive errors which were all subsequent errors [61, 63].

Progressive ratio. Following the conclusion of ASST, male and female mice remained at ~85–90% free feeding weight. Responses on the left lever were reinforced and the responses required to obtain the next reward progressively increased with each reward obtained ($(5e^{.2*n})-5$; [64]). Testing continued until 30 min elapsed without a response or a total of 1.5 h. It should be noted that a majority of mice were still responding at the end of the test session and is a potential limitation to the interpretation of these findings.

Slice electrophysiology

Male and female mice were anesthetized with isoflurane and brains sliced in oxygenated sucrose ACSF slush as described [60]. All recordings were performed using borosilicate electrodes (2.5–4.5 MΩ) filled with a K-Gluconate solution [41, 60]. Virus-infected cells were verified through the use of the mCherry or GFP fluorescent tags. For rheobase and spike frequency, a 20-pA 1-s current-step injection was used (0–200 pA). For ML297 and baclofen recordings, a consistent holding current was obtained (<20% fluctuation), followed by bath application of the selective GIRK1 agonist ML297 (10 µM; Dr. David Weaver, Vanderbilt School of Medicine) in 0.04% DMSO or the $GABA_B$ agonist baclofen (200 µM; Sigma-Aldrich). Evoked currents were reversed using bath application of 0.30 mM barium chloride (Fisher Scientific). Recordings were filtered at 2 kHz and sampled at 20 kHz, with series resistance (<40 MΩ) monitored throughout all recordings.

Assessment of viral expression

Viral expression was assessed using an mcherry or GFP fluorescent tag which was either confirmed in slices immediately prior to slice electrophysiology studies or in 100 µm fixed tissue (4% paraformaldehyde for 24–48 h). Only mice with bilateral viral expression primarily confined to the PrL were used in analyses.

Chronic unpredictable stress

Male and female mice received four weeks of CUS of twice-daily stressors or were handled twice daily (controls), as previously described [60]. Briefly, stressors varied in the location, length, intensity, and timing of stress and included stressors such as cage tilt, forced swim, cold room, food

deprivation, restraint, and lights on overnight. Mice began training in ASST 3-5d following the last stress exposure. Following completion of testing (22–25 days post-stress), a subset of mice was euthanized for assessment of $GABA_B$-GIRK signaling using slice electrophysiology.

Data analysis

Data are presented as mean ± SEM. Statistical analyses were performed using SigmaPlot. Independent-samples t-tests, one-way ANOVA, or repeated-measures were used; when parametric normality tests were violated, a Kruskal-Wallis or Mann-Whitney nonparametric test was used. Student–Newman–Keuls method for multiple post-hoc comparisons were used when applicable. Statistical outliers (±2 SD) were excluded from analyses (10 data points from all experiments/groups). Mice that did not pass lever training within 15 days were not tested in ASST or progressive ratio and a subset was also not tested in FST. Mice that had an >25 number of omissions during any attention set-shift test except visual cue training were excluded from analyses as this may be due to equipment-related issues or motoric changes. Electrophysiology sample sizes are denoted as *n* for the number of recordings/cells and *N* for the number of mice.

RESULTS

Characterization of PrL pyramidal neuron GIRK knockout in males and females

GIRK channels containing the GIRK1 subunit mediate a majority of GIRK- and $GABA_B$-dependent signaling in PrL layer 5/6 (L5/6) pyramidal neurons and loss of this signaling leads to enhanced excitability [41]. To validate our model in males, we began by evaluating $GABA_B$ and GIRK1 somatodendritic currents in L5/6 pyramidal neurons in $GIRK1^{flox/flox}$ male mice bilaterally infused with a cre-expressing or GFP-expressing adeno-associated virus (AAV) driven by the CaMKII promoter (Fig. 1a). In GFP-expressing cells (cre-), bath application of the $GABA_B$ agonist, baclofen (200 µM), evoked an outward current ($I_{Baclofen}$) that was reversed by bath application of barium chloride (Ba^{2+}; 0.30 mM). Fluorescently identified pyramidal neurons from $Girk1^{flox/flox}$ mice expressing cre (cre+; $n = 8/N = 5$) had a significant reduction in $I_{Baclofen}$ amplitude compared to cre- ($n = 6/N = 4$) pyramidal neurons ($t_{(12)} = 2.33$, $p = 0.038$; Fig. 1b). Similarly, GIRK currents evoked by bath application of the GIRK1-selective agonist, ML297, were significantly reduced (~84%) in cre+ ($n = 6/N = 4$) pyramidal neurons compared to cre- ($n = 6/N = 5$) pyramidal neurons ($t_{(10)} = 8.67$, $p < 0.001$; Fig. 1c). The residual I_{ML297} in cre+ pyramidal neurons may be due to activation of residual GIRK channels or due to non-selective current activation produced by the DMSO vehicle in which ML297 was diluted in. In a subset of controls cells, bath application of DMSO vehicle alone produced a current similar in amplitude to residual I_{ML297} in cre+ pyramidal neurons ($n = 3$, 37.7 ± 5.8 pA; data not shown), suggesting that the remaining current is largely vehicle-dependent rather than due to residual GIRK channels.

Having established that $GABA_B$ and GIRK-dependent signaling is reduced in pyramidal neurons following viral cre treatment in males, we next investigated whether viral-mediated reduction in GIRK1-dependent signaling in male mice is sufficient to increase intrinsic excitability. Cre+ pyramidal neurons ($n = 8/N = 6$) exhibited a reduction in current required to fire an action potential (rheobase) compared to cre- ($n = 10/N = 7$) pyramidal neurons ($t_{(16)} = 3.63$, $p = 0.002$; Fig. 1d). Assessment of current-spike relationship showed a significant virus by current interaction ($F_{(10,150)} = 2.23$, $p = 0.02$; cre- $n = 9/N = 6$; cre+ $n = 8/N = 6$) with significant post-hoc comparisons at 100–180 pA indicating increased action potential frequency in cre+ pyramidal neurons (Fig. 1e). Collectively, these findings verify the ability of viral-mediated approaches to suppress GIRK-dependent inhibition and increase excitability of PrL L5/6 pyramidal neurons.

Whole-cell recordings in $Girk1^{flox/flox}$ female mice showed that similar to PrL pyramidal neuron GIRK knockout in male mice (Fig. 3a) [41, 65], $I_{Baclofen}$ was reduced in female cre+ ($n = 7/N = 4$) versus cre- ($n = 6/N = 4$) pyramidal neurons ($t_{(11)} = 2.33$, $p = 0.04$; Fig. 3b). External application of ML297 resulted in a reduced evoked current in cre+ ($n = 6/N = 3$) compared to cre- ($n = 6/N = 3$) L5/6 pyramidal neurons ($t_{(10)} = 4.09$, $p = 0.002$; Fig. 3c). Consistent with males, this aligned with a significant reduction in firing threshold ($t_{(23)} = 3.66$, $p = 0.001$; cre-: $n = 15/N = 8$; cre+: $n = 8/N = 6$; Fig. 3d) and a condition by current interaction ($F_{(10,190)} = 4.47$, $p < 0.001$; cre- $n = 10/N = 4$; cre+ $n = 11/N = 6$), with cre+ exhibiting an increase in spike frequency compared to cre- at 60–200 pA (Fig. 3e).

PrL pyramidal neuron GIRK knockout variably influences male performance in EPM, FST, and progressive ratio

Percent time spent in the open arms of an EPM, time immobile in the FST, and breakpoint during a progressive ratio test were measured to initially determine the impact of PrL pyramidal neuron GIRK knockout in male mice (Fig. 2a). Cre+ male mice spent a greater percentage of time in the open arm compared to cre- ($t_{(25)} = 2.10$, $p = 0.0456$; Fig. 2b) while maintaining similar locomotion as evidenced by no difference in number of entries into the center of the maze ($t_{(25)} = -0.90$, $p = 0.38$; data not shown). Alternatively, cre+ mice exhibited an increase in time spent immobile compared to cre- in the FST ($t_{(24)} = -3.07$, $p = 0.005$; Fig. 2c) and had fewer immobile episodes ($t_{(24)} = 2.81$, $p = 0.01$; data not shown) indicating a reduction in active coping behaviors. Using a progressive ratio test to assess motivation, we found no differences in total active lever responses ($t_{(20)} = -0.96$, $p = 0.35$; data not shown) or breakpoint ($U = 36.50$, $p = 0.12$; Fig. 2d). These data indicate that reducing PrL pyramidal neuron GIRK1-dependent signaling in males reduces normal anxiety-like responses and escape-related strategies suggestive of an anxiolytic and pro-depressive phenotype [66, 67], without altering appetitive reward motivation.

Working memory in PrL pyramidal neuron GIRK knockout male mice

The most consistently documented cognitive deficits in neuropsychiatric disease and stress pathologies includes impaired behavioral flexibility and working memory [68, 69], thus we examined whether loss of GIRK promotes similar impairments. Using a forced alternation T-maze paradigm to assess working memory, we found no effect of day ($F_{(5,65)} = 0.24$, $p = 0.95$), or condition (virus) by day interaction ($F_{(5,65)} = 0.66$, $p = 0.65$) on the percent of correct trials for each day. A main effect of condition was observed ($F_{(1,13)} = 22.46$, $p < 0.001$), with cre+ male mice showing an overall reduction in percent correct choices compared to cre- controls (Fig. 2e). When averaging performance of each trial across days, there was a significant condition by trial interaction ($F_{(11,143)} = 2.10$, $p = 0.24$; Fig. 2f), with post-hoc comparisons indicating that the cre+ male mice performed significantly worse on trials 2 ($p = 0.004$), 7 ($p = 0.018$), 9 ($p = 0.034$), 10 ($p = 0.008$), and 11 ($p = 0.008$). These data indicate that PrL pyramidal neuron GIRK1-dependent signaling in male mice plays a key role in information processing related to working memory.

Impact of PrL pyramidal neuron GIRK knockout on cognitive flexibility in male mice

To determine if PrL pyramidal GIRK channels play a role in complex forms of PFC-dependent cognition, we examined the impact of PrL GIRK1 suppression on cognitive flexibility. We used a modified operant-based ASST [61, 63] that is reliant on PrL function [24, 70]. This task resembles the Wisconsin Card Sorting Task in its sensitivity to distinct components of decision making such as suppression of irrelevant strategies, acquisition and generation of novel strategies, and maintenance of effective strategies. This is the first known study to investigate cognitive flexibility in $GIRK1^{flox/flox}$ mice, therefore we wanted to determine if $GIRK1^{flox/flox}$ mice exhibit a baseline phenotype. We first compared $GIRK1^{flox/flox}$ mice

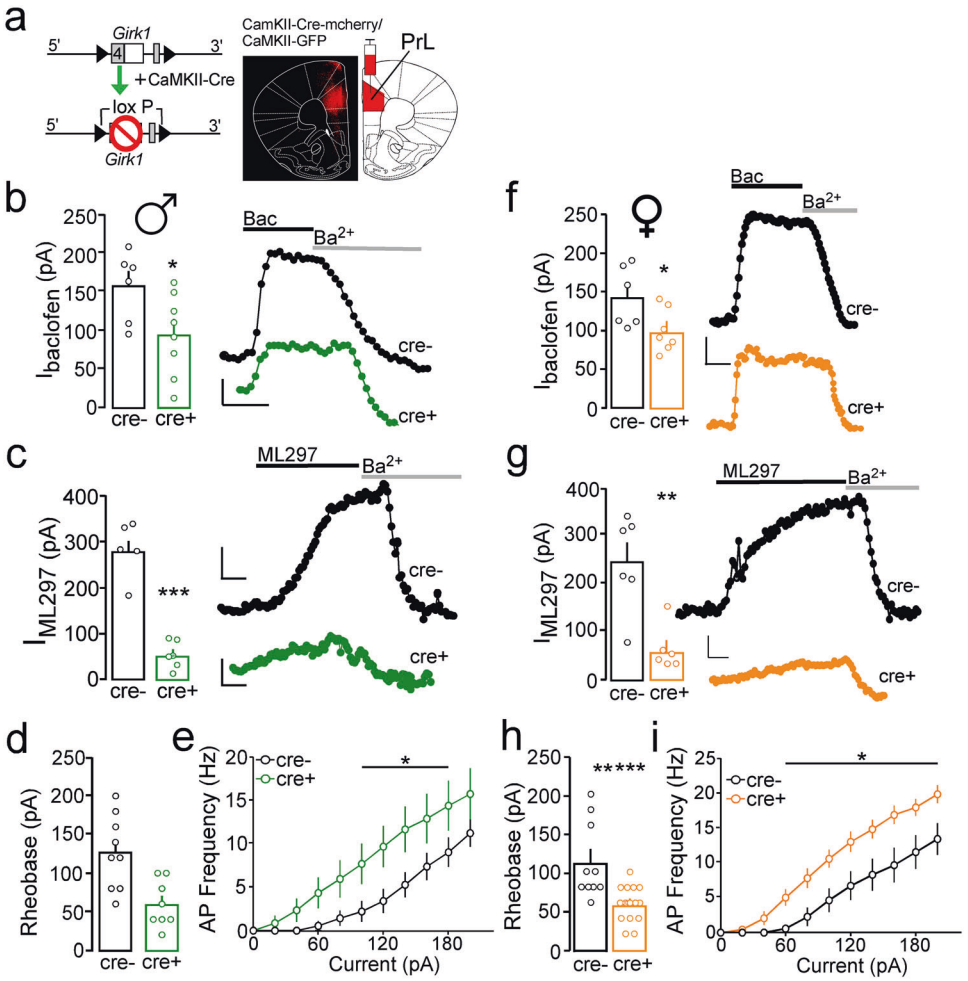

Fig. 1 Characterization of PrL pyramidal neuron GIRK knockout in males and females. a Schematic showing knockdown of GIRK1 in PrL pyramidal neurons through bilateral infusion of a cre-expressing or GFP-expressing AAV driven by the CaMKII promotor and noting whole-cell recordings in PrL L5/6 pyramidal cells (**b**–**e**, males; **f**–**l**, females). **b** Outward currents evoked by bath application of baclofen ($I_{baclofen}$; 200 µM) in Girk1$^{flox/flox}$ male mice were significantly reduced in cre + (green) versus cre- (black) PrL L5/6 pyramidal neurons. $I_{baclofen}$ was reversed in the presence of external Ba^{2+} (0.30 mM). **c** Outward current evoked by bath application of the selective GIRK1 agonist, ML297 (I_{ML297}) was reduced in male cre+ PrL L5/6 pyramidal neurons compared to cre-. **d** Threshold to fire an action potential (rheobase) in PrL L5/6 pyramidal neurons was reduced in male cre+ compared to cre- **e** Current-spike plots from Girk1$^{flox/flox}$ PrL L5/6 pyramidal neurons showed an increase in spike frequency in male cre+ cells compared to cre- at 100–180 pA. Similar to males, baclofen- (**f**) and (**g**) ML297-evoked currents were smaller in female cre+ mice (orange) versus cre- (black) and reversed with subsequent Ba^{2+} application. **h** was also reduced in female cre+ compared to cre- mice. **i** Current-spike plots from showed a condition by current interaction, with female cre+ exhibiting an increase in spike frequency compared to cre- at 60–200 pA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bars: 50 pA,100 s.

to C57BL/6 mice receiving sham surgery and found no difference in their performance on any measure in ASST (lever train: $t_{(11)} = -0.98$, p = 0.35; VC trials: $t_{(11)} = -0.35$, $p = 0.74$; ED trials: $t_{(11)} = -0.31$, $p = 0.76$; REV trials: $t_{(11)} = -1.27$, $p = 0.23$; data not shown), thus data were combined for further analyses. There was no difference in days to criterion for lever training between cre- and cre + ($t_{(19)} = -1.04$, $p = 0.31$; Fig. 2g). Following lever training, comparison of performance in the visual cue test showed that cre+ male mice required significantly greater number of trials ($t_{(19)} = -2.52$, $p = 0.02$; Fig. 2h) and errors ($t_{(19)} = -2.56$, $p = 0.019$; Supplementary 1a) to reach criterion compared to cre- but did not differ on omissions ($U = 45.00$, $p = 0.58$; Supplementary Table 1). Further investigation of error type revealed no difference in initial errors ($U = 51.00$, p = 0.94) however the cre+ male mice had significantly more regressive errors compared to cre- control mice ($t_{(19)} = -2.87$, p = 0.01; data not shown). During the ED Shift, cre- and cre+ mice required a similar number of trials ($U = 28.50$, $p = 0.09$; Fig. 2i), errors ($t_{(19)} = 0.95$, $p = 0.35$; Supplementary 1b) and omissions to reach criterion ($t_{(19)} = 1.17$, $p = 0.26$; Supplemental Table 1). Similarly, no difference was observed in trials ($t_{(19)} = -0.35$, $p = 0.73$; Fig. 2j), errors ($t_{(19)} = -0.87$, $p = 0.40$; Supplementary 1c) or omissions ($t_{(19)} = -1.42$, $p = 0.17$; Supplementary Table 1) to criterion in a subsequent reversal learning test.

Past studies have shown that pharmacological inhibition of the PrL does not impact performance in an operant or maze-based visual cue discriminative learning task in rats [63, 71]. To ensure that impaired performance during the visual cue test in male GIRK1-knockout mice did not reflect deficits in general attention to a cue or visual acuity, we next trained a new cohort of mice on the visual cue test only (i.e., no lever training) with similar criterion to test sessions (i.e., 10 consecutive correct responses). During visual cue training, there were no differences in the trials ($t_{(16)} = 0.95$, $p = 0.36$; Fig. 2k), errors ($t_{(16)} = 0.33$, $p = 0.75$; Supplementary 2a), or omissions ($U = 32.00$, $p = 0.48$; Supplementary Table 2) to reach criterion between cre+ and cre- mice indicating that the cre+ male mice do not have deficits in the

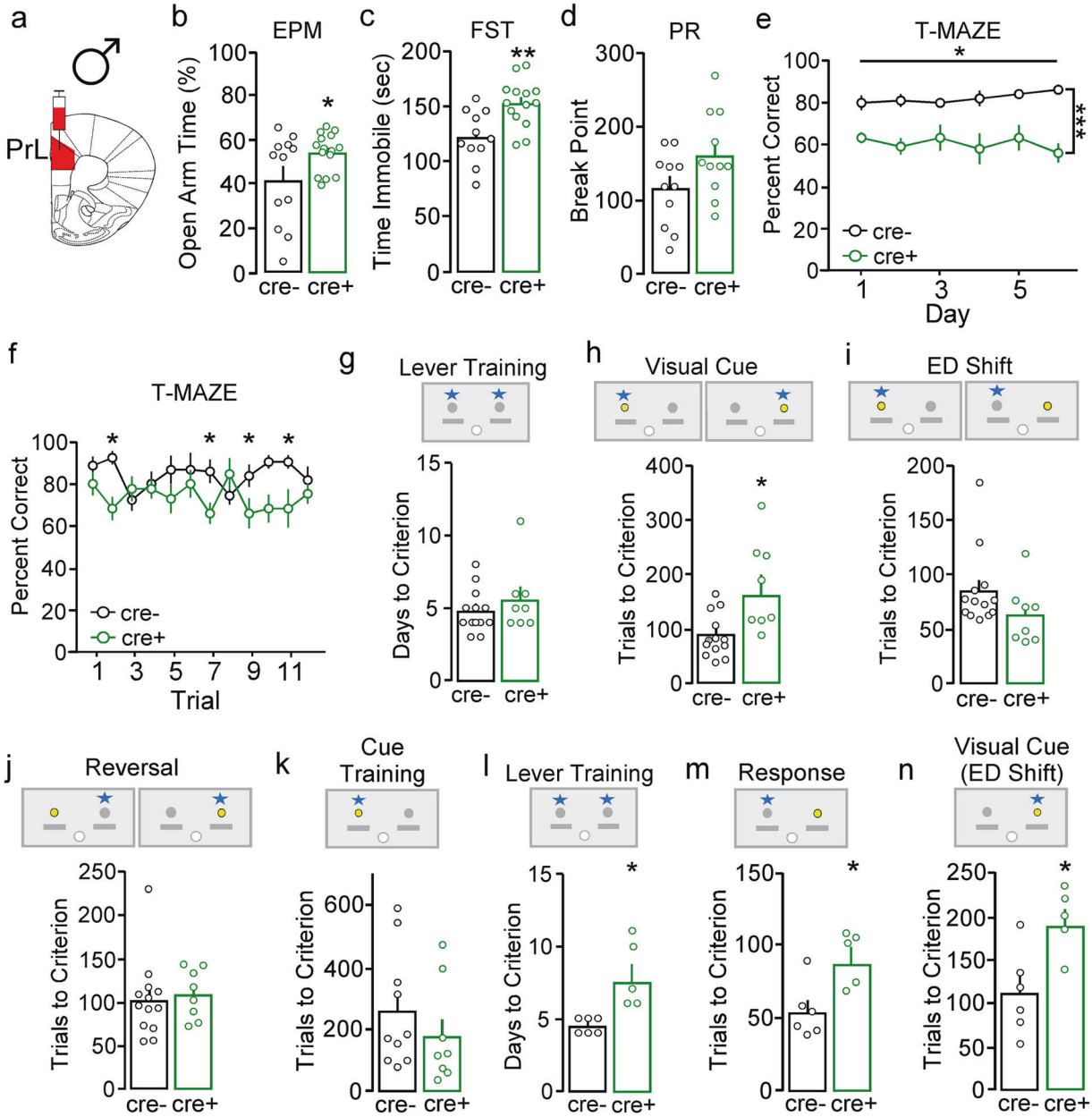

Fig. 2 Impact of PrL pyramidal neuron GIRK knockout on affect-related behavior, working memory, and cognitive flexibility in males. **a** Schematic targeting of infusion to the PrL in male mice. **b** Percent time in the open arm of the EPM was increased in cre+ male mice compared to cre- controls. **c** Total time (s) spent immobile during the FST was increased in cre+ compared to cre- mice. **d** Breakpoint during a PR test was similar in cre+ males versus cre- controls. **e** During the forced alternation T-maze paradigm, a main effect of virus was found, with cre+ male mice having reduced percent correct choice. **f** Comparison of trials across days showed that Cre+ male mice performed significantly worse than cre- during trials 2, 7, 9, and 11. **g**–**n** Attentional set-shifting paradigm. **g** There was no difference between cre- and cre+ male mice in the number of days to reach lever training criterion. **h** During the visual cue test, cre+ mice took significantly more trials to reach criterion compared to cre- mice. **i** During the ED Shift test, cre- and cre+ male mice took similar number of trials to reach criterion. **j** During the reversal test, cre- and cre+ mice took similar trials to reach criterion. **k** Cre+ and cre- male mice did not differ in trials to reach criterion for visual cue training (**l**) Using an alternative response-to-cue paradigm, where the visual cue test is the ED shift, Cre+ male mice took significantly more days to reach lever training criterion. **m** Cre+ mice required greater number of trials to reach criterion during a response test. **n** Cre+ mice also took more trials to reach criterion during the subsequent ED Shift visual cue test. *$p < 0.05$, **$p < 0.01$. Star in schematics denotes correct response, yellow circle denotes cue, gray rectangles denote left and right levers.

visual cue simply due to attentional deficits to the cue or due to visual acuity.

As mice were initially lever trained in a pseudorandom fashion to press either the left or the right lever when it was presented (i.e., only attend to levers), it is possible that the addition of a visual cue rule was acting as an attentional shift (i.e., ignore previous lever presentation order and attend to visual cue) which may have resulted in the unexpected deficits during the visual cue test in male cre+ mice. To address this, additional cohorts underwent lever training during which cre+ male mice took longer to obtain criterion compared to controls ($t_{(8)} = -3.06$, $p = 0.02$; Fig. 2l). Notably, this significance appears largely driven by the lack of variability in days to reach criterion in cre- mice. After lever training, mice received a response test (lever opposite of bias

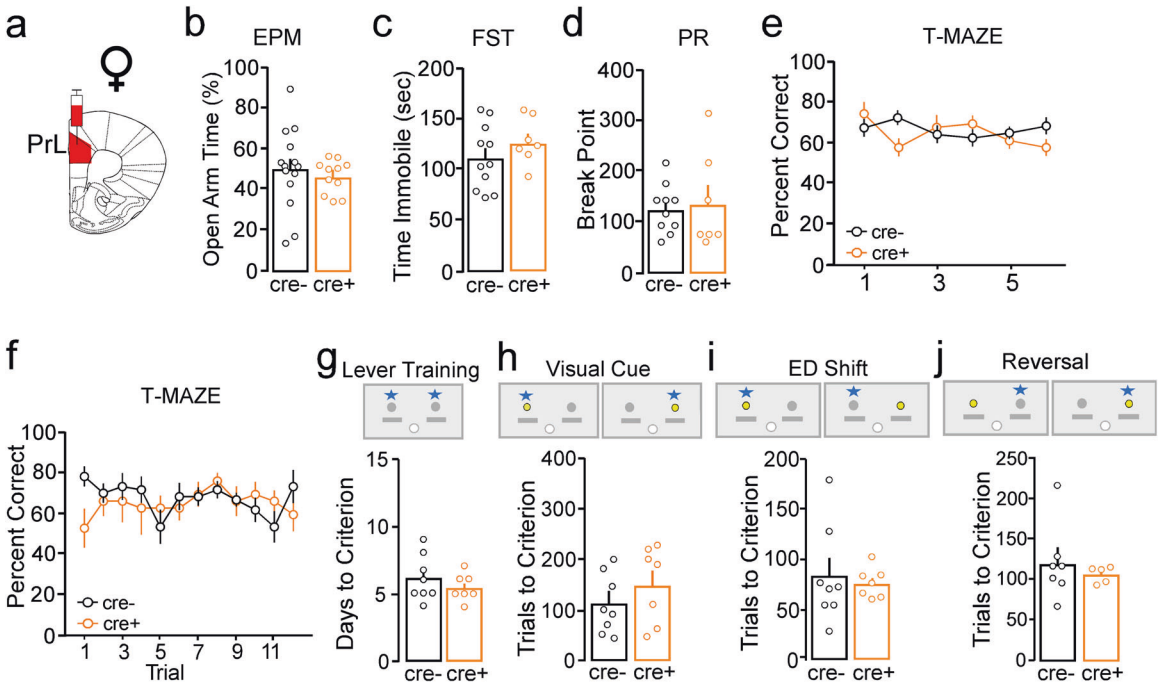

Fig. 3 a. Impact of PrL pyramidal neuron GIRK knockout on affect-related behavior, working memory, and cognitive flexibility in females. a Schematic targeting infusion to the PrL in female mice. **b** Percent open arm time in the EPM was similar in female cre+ and cre- female mice**. c** Total time spent immobile during the FST was similar between cre+ and cre- female mice. **d** During a progressive ratio (PR) test of motivation, breakpoint for number of responses to receive liquid reward did not differ in cre+ and cre- females. **e** Cre- and cre+ female mice did not differ on the percent correct choice (out of 12) for each day in a t-maze spontaneous alternation task. **f** Cre+ and cre- female mice did not differ on percent correct for each trial when averaged across days. Attentional set-shifting paradigm. Cre+ and cre- female mice took a similar number of days to reach lever training criterion (**g**), as well as trials to reach criterion during the visual cue test (**h**), the ED Shift (**i**), and the reversal test (**j**). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

is correct) and a subsequent visual cue test. During the response test, cre+ mice took significantly more trials ($t_{(8)} = -2.55$, $p = 0.03$; Fig. 2m) and errors ($t_{(8)} = -3.78$, $p = 0.01$; Supplementary 3b) and had similar omissions ($t_{(8)} = -2.05$, $p = 0.08$; Supplementary Table 3). During the visual cue set-shift, cre+ mice also required a greater number of trials ($t_{(8)} = -2.67$, $p = 0.03$; Fig. 2n) but did not significantly differ in errors ($t_{(8)} = -2.06$, $p = 0.07$; Supplementary 3c) or omissions ($t_{(8)} = -1.06$, $p = 0.32$; Supplementary Table 3) to reach criterion compared to cre- mice. These findings combined with a lack of difference to acquire a visual cue training indicate that loss of GIRK channel activity alters PrL function (i.e., cognitive flexibility) in a manner distinct from lesions.

Influence of PrL pyramidal neuron GIRK knockout on female performance in EPM, FST, and progressive ratio

Intrinsic sex differences in mPFC physiology and function may have translational significance regarding resilience or susceptibility to pathological disorders [72–74]. Past studies have identified sex-dependent differences in GIRK-dependent signaling in adolescence [42], therefore we next examined whether loss of PrL pyramidal GIRK1 (Fig. 3a) signaling similarly impacts female behavior.

Unexpectedly, cre+ and cre- females showed no difference in open arm time ($t_{(23)} = 0.67$, $p = 0.51$; Fig. 3b) or center entries ($t_{(23)} = 0.20$, $p = 0.84$; data not shown) during the EPM. There was no difference in the time spent immobile in the FST ($t_{(16)} = -1.12$, $p = 0.28$; Fig. 3c) however cre+ female mice did have significantly more immobile episodes compared to cre- mice ($t_{(16)} = 2.61$, $p = 0.02$; data not shown). There were also no differences in the number of active lever responses ($t_{(15)} = 0.35$, $p = 0.73$; data not shown) or break point ($U = 31.00$, $p = 0.73$; Fig. 3d) during a progressive ratio test (side-by-side comparisons of male vs female data can be found in Supplementary 6a–c).

Working memory in PrL pyramidal neuron GIRK knockout female mice

Using the same forced alternation T-maze paradigm to assess working memory as was used in males, there were no differences in the percent correct for each day comparing cre- and cre+ female mice (condition: $F_{(1,13)} = 0.40$, $p = 0.54$; day: $F_{(5,65)} = 0.83$, $p = 0.53$; day by condition: $F_{(5,65)} = 2.12$, $p = 0.07$; Fig. 3e). When each individual trial was collapsed across days and the percent correct taken for each trial there was also no main effect of condition ($F_{(1,13)} = 0.30$, $p = 0.59$), trial ($F_{(11,143)} = 0.65$, $p = 0.79$), or a day by condition interaction ($F_{(11,143)} = 1.08$, $p = 0.38$; Fig. 3f). These data together indicate that PrL pyramidal neuron GIRK1-dependent signaling does not play a direct role on working memory in female mice, at least within the forced alternation T-maze paradigm (side-by-side comparisons of male vs female data can be found in Supplementary 6d).

Impact of PrL pyramidal neuron GIRK knockout on cognitive flexibility in female mice

No differences were observed between cre+ and cre- females across any measure related to ASST, including days to reach lever training criterion ($t_{(13)} = 0.94$, $p = 0.36$; Fig. 3k), or number of trials (visual cue: $t_{(13)} = -1.21$, $p = 0.25$; ED shift: $t_{(13)} = 0.44$, $p = 0.67$; reversal: $t_{(10)} = -0.63$, $p = 0.54$; Fig. 3k–n), errors (visual cue: ($t_{(13)} = -1.04$, $p = 0.32$; ED shift: $t_{(13)} = 0.38$, $p = 0.71$; reversal: $t_{(10)} = 0.48$, $p = 0.64$; Supplementary 4a–c), or omissions (visual cue: $t_{(13)} = -0.06$, $p = 0.96$; ED shift: $t_{(13)} = -0.08$, $p = 0.94$; reversal: $t_{(10)} = 0.10$, $p = 0.93$; Supplementary Table 4) to reach criterion. Unlike male PrL GIRK1 knockout mice, who showed an unexpected deficit during the visual cue test, females show no deficits during testing and therefore further attention set-shift tests to understand why this deficit arises during the visual cue

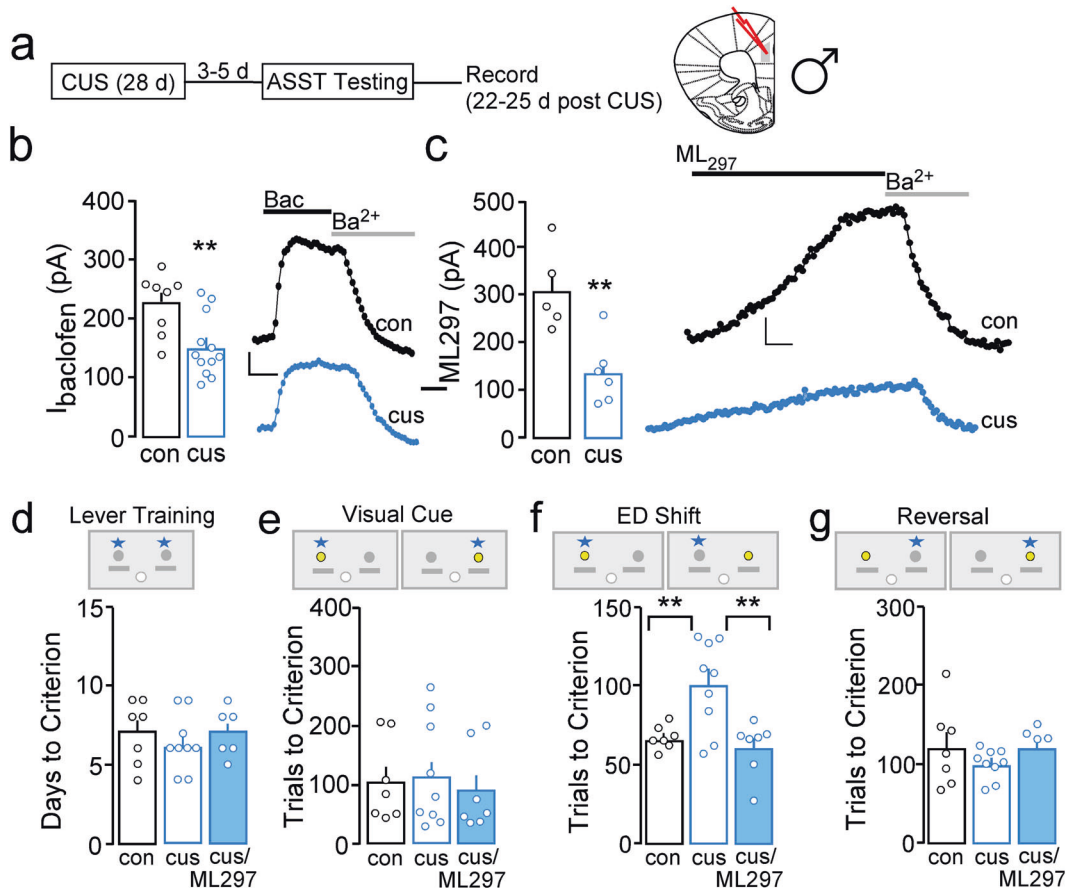

Fig. 4 Role of PrL GIRK-dependent signaling in chronic stress-induced cognitive flexibility deficits in male mice. a Timeline of CUS, behavioral testing, and electrophysiology recordings. **b** $I_{baclofen}$ in PrL L5/6 was reduced in CUS exposed male mice compared to CUS naïve (control). **c** I_{ML297} was significantly reduced in CUS male mice compared to control. **d** There were no differences between the three groups of male mice in days to reach lever training criterion. **e** No differences were observed in trials to reach criterion during the visual cue test. **f** During the ED Shift, CUS male mice required more trials than control mice and compared to CUS that received ML297 whereas control mice and CUS mice that received ML297 did not differ. **g** No difference was observed in trials to reach criterion for the reversal test. $^{**}p < 0.01$. Scale bars: 50 pA,100 s.

(i.e., is it acting as an attentional shift or do they have general cue attention deficits) were performed. These data indicate that although $GABA_B$-GIRK signaling in PrL pyramidal neurons is present and suppressed by viral targeting in females, it does not appear to influence PrL function as it relates to operant-based cognitive flexibility in females (side-by-side comparisons of male vs female data can be found in Supplementary 6e–h).

Role of PrL GIRK-dependent signaling in chronic stress-induced cognitive flexibility deficits in male mice

Stress-induced mPFC dysfunction is thought to reflect excitation: inhibition imbalances, however the mechanism through which this occurs has yet to be identified [75]. Using our recently established mouse model of CUS shown to promote disruptions in affective behavior [60], we next asked whether this model promotes changes in PrL L5/6 pyramidal $GABA_B$-GIRK signaling and deficits in cognitive flexibility in male mice (Fig. 4a). Approximately 3 weeks following 4 weeks of CUS exposure, there was a significant reduction in $I_{Baclofen}$ ($t_{(18)} = 3.04$, $p = 0.007$; control $n = 8/N = 6$; CUS $n = 12/N = 9$; Fig. 4b) and I_{ML297} ($t_{(9)} = 3.67$, $p = 0.005$; control $n = 5/N = 3$; CUS $n = 6/N = 4$; Fig. 4c) compared to naïve male controls.

Initial cohorts assessing cognitive flexibility using ASST in males indicated that CUS exposure impaired performance in the ED Shift. To determine if augmenting GIRK1 signaling restored deficits, subsequent groups were divided into three conditions: stress naïve (con), CUS, or CUS injected with ML297 prior to the ED Shift (CUS/ML297). No differences were observed across condition for days to reach lever training criterion ($F_{(2,\ 20)} = 0.39$, $p = 0.68$; Fig. 4d) or trials ($F_{(2,\ 20)} = 0.14$, $p = 0.87$; Fig. 4e), errors ($F_{(2,\ 20)} = 0.63$, $p = 0.54$; Supplementary Fig. 5a) or omissions ($H_{(2)} = 0.84$, $p = 0.66$; Supplementary Table 5) during the visual cue test. During the ED test, there was a significant difference in trials ($F_{(2,20)} = 8.94$, $p = 0.002$; Fig. 4f), but not errors ($F_{(2,20)} = 1.89$, $p = 0.18$; Supplementary 5b), to reach criterion. Post-hoc comparisons showed CUS male mice required more trials than control ($p = 0.003$) and CUS/ML297 ($p = 0.004$), while control and CUS/ML297 did not differ ($p = 0.77$). A significant difference in omissions was detected ($F_{(2,20)} = 7.93$, $p = 0.003$; CUS/ML297 mice had higher omissions compared to control ($p = 0.007$) and CUS mice without ML297 ($p = 0.002$; Supplemental Table 5). During reversal testing, CUS/ML297 who had received ML297 the day prior, CUS, and control mice showed no differences in trials ($F_{(2,19)} = 1.01$, $p = 0.38$; Fig. 4g), errors ($F_{(2,19)} = 0.80$, $p = 0.46$; Supplementary 5c), or omissions ($F_{(2,19)} = 0.57$, $p = 0.58$; Supplementary Table 5) to criterion. Together, these findings indicate that CUS suppresses PrL L5/6 pyramidal neuron $GABA_B$-GIRK signaling with a corresponding deficit in cognitive flexibility that can be rescued by systemic activation of these channels.

Role of chronic stress on GIRK1-signaling and cognitive flexibility in female mice

The established CUS model results in reductions in $I_{Baclofen}$ and I_{ML297} evoked-current in L5/6 PrL pyramidal neurons from male

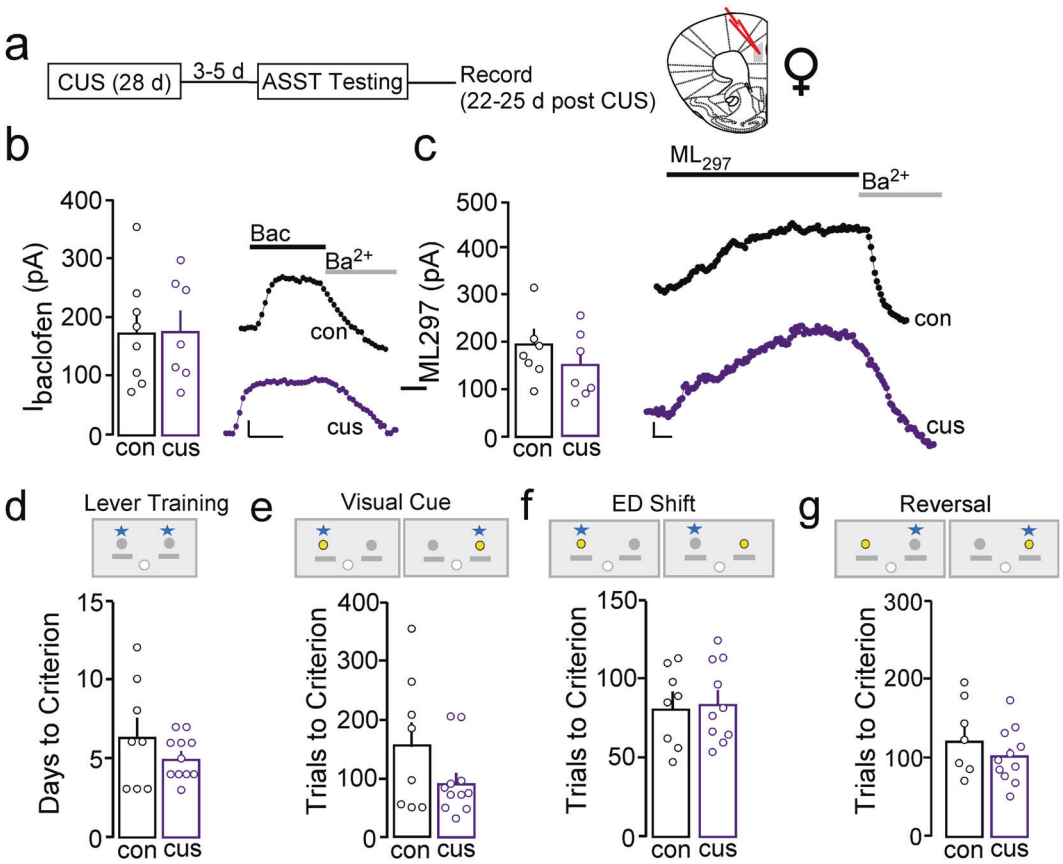

Fig. 5 Role of chronic stress on GIRK1-signaling and cognitive flexibility in female mice. a Timeline of CUS, behavioral testing, and electrophysiology recordings. **b** $I_{baclofen}$ in PrL L5/6 was similar in CUS exposed female mice compared to CUS naïve (control). **c**. I_{ML297} was similar in CUS female mice compared to control. **d** There were no differences between the two groups of female mice in days to reach lever training criterion. **e** No differences were observed in trials to reach criterion during the visual cue test. **f** During the ED Shift, CUS female mice similar trials compared to CUS. **g** No difference was observed in trials to reach criterion for the reversal test.

mice, thus, we also exposed female mice to the same CUS paradigm and determined if it impacted $GABA_B$-GIRK signaling (Fig. 5a). Unlike males, pyramidal neurons from female mice exposed to the stress paradigm do not show altered baclofen-evoked current ($t_{(13)} = -0.07$, $p = 0.94$; Fig. 5b) or ML297-evoked current ($t_{(12)} = 0.97$, $p = 0.35$; Fig. 5c) indicating that this stress paradigm does not result in reductions in PrL L5/6 pyramidal neuron $GABA_B$-GIRK signaling in female mice. Similar to males, chronic stress exposure did not alter the number of days to reach lever training criterion compared to controls ($t_{(17)} = 1.14$, $p = 0.27$; Fig. 5d) nor were there differences in trials $t_{(17)} = 1.61$, $p = 0.13$; Fig. 5e), errors $t_{(17)} = 1.62$, $p = 0.12$; Supplementary 5a), or omissions ($t_{(17)} = 1.22$, $p = 0.24$; Supplementary Table 6) to reach the visual cue criterion. Unlike males, females that were exposed to CUS required a similar number of trials ($t_{(16)} = -0.16$, $p = 0.88$; Fig. 5f) and errors ($t_{(16)} = -0.17$, $p = 0.87$; Supplementary 5b) to reach criterion during the ED shift and also had similar number of omissions compared to controls ($U = 38.50$, $p = 0.88$; Supplementary Table 6). There were also no differences in trials ($t_{(16)} = 0.72$, $p = 0.48$; Fig. 5g), errors ($t_{(16)} = 1.12$, $p = 0.28$; Supplementary 5c), or omissions ($U = 27.50$, $p = 0.31$; Supplementary Table 6) to reach criterion during the reversal test (side-by-side comparisons of male vs female data can be found in Supplementary 7).

DISCUSSION

Impact of GIRK1 ablation on affect and cognition in males

Imbalances in PFC cellular excitation:inhibition can give rise to abnormalities in both affect and cognition and represents a unifying substrate for shared pathology across neuropsychiatric disorders. Our past work has shown that PrL pyramidal neuron excitability is regulated by GIRK channels, however, the contribution of PrL GIRK channels on regulation of affect and cognition, and whether disruption in PrL GIRK signaling contributes to stress-related maladaptive behavior has never been examined. Constitutive knockout of GIRK1 or GIRK2 reduces anxiety-like behavior [52, 53], whereas global reductions in GIRK channel activity promote depression-resistant phenotypes [76] and increase motivation for appetitive rewards [52]. Here, we found that loss of PrL pyramidal neuron GIRK channels increases EPM open arm time, increases FST time immobile, and does not alter motivation for an appetitive reward. Together these findings indicate that PrL GIRK1-dependent signaling is a primary effector of the behaviors observed in male mice, and that the resulting increase in PrL pyramidal neuron excitation following loss of GIRK1 signaling alters neuronal processing necessary for normal behavioral responses in these tests (e.g., less time in an EPM open arm) [77–81].

We find that PrL GIRK1 ablation produced a consistent reduction in working memory in male mice as assessed using a forced alternation model. Assessment of more complex processes using the ASST showed an impaired performance in cre+ during the visual cue test in males, while not altering performance during the ED shift. These findings were unexpected as PrL lesions have previously shown to impair performance in the ED shift, but insufficient to disrupt performance in a cue-based discriminative test [14, 34]. It is important to note, however, that reduction in GIRK1-signaling and disorganized mPFC activity, both of which we

show is evident in GIRK1 knockout mice, is inherently different than PrL lesioning (and therefore lack of activity). Given that loss of GIRK channel activity did not alter the ability to acquire a visual cue-based learning task, it is unlikely that this deficit reflects general attention to a cue or reduced visual acuity [82]. Rather, loss of GIRK1 alters PrL function in a manner where the addition of a visual cue contingency not present during lever training is sufficient to act as an attentional shift. In support, experiments involving response-to-cue shifts showed that cre+ male mice also performed worse when the contingency was changed from lever training to response (attend to one lever only), and that these impairments persisted during a subsequent response-to-visual cue ED Shift. Cognitive processes associated with working memory are reliant, although not exclusively, on coordinated PrL activity [83–86] therefore it is likely that increased/disorganized firing following loss of PrL pyramidal neuron GIRK underlies the observed deficits in working memory and cognitive flexibility.

PrL PYR GIRK1 knockout and CUS exposure in females

Sex differences in susceptibility to anxiety, mood, and other neuropsychiatric disorders have been established in humans [87–90]. Therefore, the influence that mPFC physiology and function has on behavior in both males and females has significant translational value for identifying both susceptibility and treatment options. We replicated findings showing that $GABA_B$-GIRK signaling is present in female PrL pyramidal neurons [42] and similar to males, a reduction in $GABA_B$-GIRK signaling results in decreased threshold to fire an action potential and a leftward shift in action potential firing at higher current injections. Unlike males, ablation did not impact affect, working memory, or cognitive flexibility. Our past work has shown that $GABA_BR$-dependent GIRK currents were greater in PrL (but not infralimbic) pyramidal neurons in adolescent (P30-40) males compared to their female counterparts, however this difference was no longer present in young adult mice (P60-70) [42]. As mice underwent viral surgeries at ~P63-70, it is unlikely that intrinsic sex differences in GIRK1 signaling impacted viral manipulations. This is further supported by findings that agonist-induced GIRK1-specific currents were reduced compared to controls to a similar degree in females and males.

The lack of effect in females also does not appear to reflect the nature of the behavioral measures chosen, as multiple tasks known to be regulated by the PrL were not impacted. Moreover, it does not likely reflect a reduced role of the female PrL in regulating these behaviors, as our recent findings highlight PrL pyramidal neuron dysfunction in females as the underlying mechanism responsible for opioid-induced deficits in cognitive flexibility using the set-shifting task [91]. Rather, data point towards a difference in the functional role GIRK channels play in male and female pyramidal neurons, thus highlighting the possibility of fundamental sex differences in the physiology underlying prefrontal mediated behaviors (although see below). To our knowledge, this is the first study to examine the role of GIRK signaling in females selectively in mPFC pyramidal neurons. It is possible that while GIRK knockdown had similar efficacy on ex vivo pyramidal neuron physiology, other factors that vary across sex may impact the results of the negative behaviors observed in females including compensatory changes that are not evident in males or sexual dimorphisms related to brain size [92] that confer distinctions in viral distribution to adjoining regions.

The lack of change in behavioral flexibility following CUS in females may reflect resiliency to stress, as others have shown chronic stress does not influence or enhances performance in females in a variety of tasks, including behavioral flexibility [93–101]. Females also have attenuated mPFC dendritic and plasticity changes following chronic stress compared to males [102–104]. The lack of CUS effects in females may reflect the type of stress exposure chosen. Rather than unpredictable stress models (e.g., restraint, forced swim) which have been optimized for male rodents, social stressors have increased ethological validity for female rodents [105] and social stress has been shown to impair reversal learning during a set-shift task in females [106, 107] and alter mPFC plasticity in female rats [108]. Importantly, studies showing set-shift task deficits in females following stress exposure used a task that is not operant-based, but rather employs odor and digging medium as cues [17, 109], which may have different capacities to identify stress-induced cognitive changes.

Impact of CUS on $GABA_B$-GIRK signaling and cognitive flexibility in males

Similar to cognitive inflexibility in humans [9, 110, 111], exposure to CUS impairs performance in the ED Shift test in male mice. Deficits in flexibility aligned with a reduction in $GABA_B$-GIRK signaling, highlighting a role in CUS-induced PrL dysfunction. Unlike GIRK1 knockouts, deficits in flexibility following CUS were specific to the ED Shift, as has previously been shown following chronic stress in male rats [101, 112]. Similar to PrL lesion studies [18, 34], CUS exposure may impair ED Shift performance rather than producing deficits in the visual cue test as it likely has more global effects than just reducing PrL pyramidal neuron GIRK1-dependent signaling [113]. As converging lines of evidence show that exposure to unpredictable stressors negatively impacts cognitive control in humans and rodents [9, 110, 111, 114, 115], the ability of systemic ML297, a GIRK1-selective agonist, to rescue this deficit has significant impact on future therapeutics aimed at treating cognition-related deficits produced by stress. Collectively, we highlight the importance of GIRK-dependent signaling in male, but not female, PrL pyramidal neurons in the regulation of both affect and cognition and demonstrate that PrL GIRK channels in males are targets of stress.

There are several limitations to the study. First, it is important to note that the collection of female data for both GIRK and stress-related studies were performed at a later date, however, all groups of animals were run with proper controls in parallel. As direct comparison of sex may confer false positive or negative data, findings were presented separately. Thus, while our data provide a proper assessment of the impact of GIRK knockout and stress in females and males alone, without a direct statistical comparison conference of sex differences in the functional role GIRK channels play should be considered with caution. Second, although the locus of ML297 effects were not identified, systemic ML297 can rescue stress-induced cognitive deficits in males and therefore demonstrates therapeutic potential. It is possible that the rescue of deficits is due to augmentation of residual GIRK1 signaling in PrL pyramidal neurons, and that this is sufficient to restore optimal neuronal activity within this region. It is also possible that the effectiveness of ML297 reflects activation of GIRK1 and thus inhibition of other cortical structures that drive behavioral rigidity. While we demonstrate that loss of GIRK1 increases output of PrL pyramidal neurons, it is unknown how viral- and stress-related elevations in activity alter an often reciprocally and/or collaterally connected mPFC microcircuitry. It is also not clear whether behavioral deficits in males are disproportionally driven by reduced GIRK signaling and increased output of pyramidal neuron subpopulations based on the downstream target. These questions have important implications as both increased (disorganized) activity as well as mPFC hypoactivity have been highlighted in neuropsychiatric disease states.

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AUTHOR CONTRIBUTIONS

All authors have contributed to this work in a meaningful way. EMA and MCH designed the work, acquired, analyzed, and interpreted the work, and wrote the manuscript. SL designed the work and acquired, analyzed, and interpreted the work. BW, AE, SD, KO, acquired, analyzed, and interpreted the work. EH and KW designed and interpreted the work. All authors helped with drafting and revision of the work and have given approval for this work to be published.

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