Prophylactic efficacy of riluzole against anxiety- and depressive-like behaviors in two rodent stress models

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Significance Statement (150 words):

Riluzole is a glutamate-modulating drug with mixed evidence of efficacy in clinical studies of patients with stress-related disorders. Based on evidence suggesting that glutamatergic dysfunction contributes to the pathogenesis of stress-related disorders, we investigated whether prophylactic treatment with riluzole could prevent the onset of behavioral deficits induced by unpredictable chronic mild stress or learned helplessness in mice. Riluzole effectively prevented the emergence of anxiety-, anhedonia-like behavior, and overall behavioral emotionality in mice exposed to unpredictable chronic mild stress. In a separate cohort, riluzole blocked the emergence of helplessness-like behavior as measured in an active avoidance paradigm. These results support testing riluzole as a prophylactic treatment strategy in stress-related illnesses such as major depressive disorder or post-traumatic stress disorder.

Running title: Prophylactic riluzole and stress resilience

Abstract

Background: Chronic stress-related illnesses, such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD), share symptomatology, including anxiety, anhedonia, and helplessness. Across disorders, neurotoxic dysregulated glutamate (Glu) signaling may underlie symptom emergence. Current first-line antidepressant drugs (ADs), which do not directly target Glu signaling, fail to provide adequate benefit for many patients and are associated with high relapse rates. Riluzole modulates glutamatergic neurotransmission by increasing metabolic cycling and modulating signal transduction. Clinical studies exploring riluzole's efficacy in stress-related disorders have provided varied results. However, the utility of riluzole for treating specific symptom dimensions or as a prophylactic treatment has not been comprehensively assessed.

Methods: We investigated whether chronic prophylactic riluzole (~12-15/kg/day p.o.) could prevent the emergence of behavioral deficits induced by unpredictable chronic mild stress (UCMS) in mice. We assessed i) anxiety-like behavior using the elevated-plus maze, open field test, and novelty-suppressed feeding, ii) mixed anxiety/anhedonia-like behavior in the novelty-induced hypophagia test and, iii) anhedonia-like behavior using the sucrose consumption test. Z-scoring summarized changes across tests measuring similar outcomes. In a separate learned helplessness (LH) cohort, we investigated whether chronic preventative riluzole treatment could block the development of helplessness-like behavior.

Results: UCMS induced an elevation in anxiety-, anhedonia-like behavior, and overall behavioral emotionality that was blocked by prophylactic riluzole. In the LH cohort, preventative riluzole blocked the development of helplessness-like behavior.

Conclusion: This study supports the utility of riluzole as a prophylactic medication, and potential relapse-preventing treatment targeting anhedonia, anxiety, and helplessness symptoms associated with stress-related disorders.

Keywords: Riluzole, Chronic Stress, Prophylactic, Depression, Antidepressants

Introduction

Chronic stress is the primary risk factor of various psychiatry illnesses including major depressive disorder (MDD), bipolar disorder (BPD), schizophrenia, and post-traumatic stress disorder (PTSD) (Kim et al., 2007; Yang et al., 2015; McEwen and Akil, 2020). These mental health illnesses share common symptoms including low mood, anhedonia and helplessness (APA, 2014). Together these illnesses account for one-third of global years of productivity loss due to disability (Vigo et al., 2016). This number has substantially increased in last year's due do to the global COVID-19 pandemic (Ettman et al., 2020; Xiong et al., 2020). A first-line treatment approach for mental illnesses involves selective serotonin reuptake inhibitors (SSRIs) (Garnock-Jones and McCormack, 2010; Clevenger et al., 2018; Huang et al., 2020). Indeed, antidepressant drugs (ADs) have demonstrated utility for mood enhancement and neuroprotection across stress-related illnesses, but require several rounds of treatment to achieve remission in a majority of patients, thus lengthy delays until remission (Gaynes et al., 2009; Holmes et al., 2017a). Moreover, in PTSD only 59% of individuals receiving SSRI treatment responded after fourteen weeks (Stein et al., 2006). Other pharmacotherapies for stress-related illnesses include selective noradrenergic reuptake inhibitors, tricyclic antidepressants and anti-adrenergic agents (Henry et al., 2007; Banzi et al., 2015; Tran et al., 2021). Unfortunately, even multiple treatments fail to adequately reduce symptoms in one-third of the MDD patients (Rush et al., 2006). The limited therapeutic efficacy of these drugs in standalone, in combination or with psychotherapy, point to the need for more effective treatment therapies or potential strategies to prevent the onset of mental illnesses.

Each individual respond differently to stressful life events (Kessler, 1997; Sokratous et al., 2013; Kessler et al., 2018; McEwen and Akil, 2020). Some individuals show resiliency i.e. ability to adapt to the stressful situations and others are susceptible and develop pathological states such as MDD or PTSD. Similar resiliency and susceptibility to stress exposure can be found in chronic stress rodent models (Sillivan et al., 2017; Peña et al., 2019; Colucci et al., 2020; Torrisi et al., 2021). Preclinical studies have focused on leveraging these individual differences by developing pharmaceuticals with prophylactic properties to prevent the maladaptive response to stress and the associated symptom emergence (Horn et al., 2016;

Howlett and Stein, 2016; Bernardini et al., 2017; McGowan et al., 2017). Several drugs were investigated for potential prophylactic effects. It was shown that fluoxetine treatment has little to no prophylactic efficacy in rodent stress models (Brachman et al 2016). However, pretreatment with serotonin receptor 4 agonists or ketamine showed effective prophylactic properties (Chen et al., 2020). Specifically, pretreatment with ketamine the glutamate (Glu) *N*-methyl-D-aspartate (NMDA) receptor antagonist was shown to attenuate fear response in the contextual fear conditioning (McGowan et al., 2017) and prevent induction of depressive-like behavior in the chronic social-defeat stress, learned helplessness (LH), and chronic corticosterone models (Amat et al., 2016; Brachman et al., 2016; Soumier et al., 2016; Mastrodonato et al., 2020). Theoretically, the use of prophylactic drugs could prevent chronic stress-related pathogenesis in individuals and be beneficial in clinical settings, in particular in MDD or BPD patients who discontinued their treatment and are at high risk for relapse (Judd, 1997; Mueller et al., 1999; Nierenberg et al., 2010; Iovieno et al., 2011). Other population that could benefit from prophylactic treatment include individuals exposed to acute trauma and who are at high risk for developing PTSD (Roque, 2015). This is accordance with findings of the literature suggesting that some treatments such as lithium maintenance therapy can increase resilience in clinical MDD and BPD patients (Sanacora et al., 2012).

Extensive evidence from both preclinical and clinical studies indicate abnormalities of glutamatergic neurotransmission or glutamatergic dysfunction in the development of psychiatric disorders (Sanacora et al., 2012; Amat et al., 2016; Henter et al., 2018; Li et al., 2019; Jiang et al., 2020). Specifically, elevated circulating Glu levels and altered expression/function of Glu receptors have been reported in MDD and PTSD patients, and postmortem subjects (Sanacora et al., 2008; Feyissa et al., 2009; Holmes et al., 2017b; Duman et al., 2019). Similar glutamatergic dysfunctions are found in rodent models of chronic stress (Popoli et al., 2011). Specifically, stress exposure induced Glu release in corticolimbic brain regions, including the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (Bagley and Moghaddam, 1997; Musazzi et al., 2010). Chronic stress-induced elevated or sustained Glu levels causes excitotoxic effects via enhanced extrasynaptic signaling activity (Hardingham and Bading, 2010; Popoli et al., 2011; Pál, 2018).

These alterations were linked to maladaptive changes to the structure and function of neurons (Musazzi et al., 2015; Lener et al., 2017) and glial cells (Banasr et al., 2010; Sanacora and Banasr, 2013; Mayegowda and Thomas, 2019; Codeluppi et al., 2021). Homeostatic glial cells such as astrocytes play a pivotal role in regulating stress-induced glutamatergic dysfunction (Popoli et al., 2011; Rajkowska and Stockmeier, 2013; Sanacora and Banasr, 2013; Abbink et al., 2019) as are accountable for over 80% of Glu reuptake, Glu synthesis and metabolism (Chen et al., 2014; Baek et al., 2019; Fullana et al., 2020). Drugs that enhance Glu clearance may prevent the deleterious effects of excess (Grant et al., 2010; Fontana, 2015; Peterson and Binder, 2019; Wilkie et al., 2021).

Riluzole, a FDA-approved drug for amyotrophic lateral sclerosis provides neuroprotection by enhancing synaptic reuptake of elevated glutamate *via.* glutamate transporters present on the astrocytes thus, increase the ratio of synaptic to extra-synaptic signaling which further promotes neurotrophic factor expression (Fumagalli et al., 2008; Yoshizumi et al., 2012; Pittenger et al., 2008). Indeed, in chronic stress- or glucocorticoid-exposed rodents, chronic riluzole reversed depressive-like behavior (Gourley et al., 2009; Banasr et al., 2010). Further, chronic riluzole administration attenuated the structural and functional deficits in PFC and HPC as indicated by markers of synaptic Glu signaling and metabolism, and increased levels of brain derived neurotropic factor (BDNF) (Gourley et al., 2009; Banasr et al., 2010). Riluzole shares neurotrophic effects with conventional antidepressants and modulation of Glu signaling with rapid-acting anti-depressants, such as the NR2B-containing NMDAR antagonist ketamine (Deutschenbaur et al., 2016). Moreover, early open-label studies in MDD patients reported riluzole as monotherapy and an adjunctive treatment to monoaminergic antidepressants exhibited better response, remission, and tolerability comparable to monoaminergic antidepressants (Zarate et al., 2004, 2005; Brennan et al., 2010; Sanacora et al., 2007). Further, in another randomized, double-blind, placebo-controlled (RDBPC) clinical trial, MDD patients showed significantly greater response and greater speed to riluzole and citalopram combination treatment when compared to placebo groups (Salardini et al., 2016). However it is important to mention that the antidepressant efficacy of riluzole is not consistently found across clinical studies (Zarate et al., 2004, 2005; Sanacora et al., 2007; Brennan et al., 2010; Spangler et al., 2020). This discrepancy may be

due to difference in study design, patient history (i.e. length, severity, prior drug administration) or the timing of treatment intervention in relation to the illness.

Recent studies in rodent models of other pathologies such as spinal cord injury (Shimizu et al 2018) or autoimmune encepthalomyelitis (Rotolo et al., 2021) suggest that riluzole treatment might be more effective at reducing behavioral and cellular deficits associated with these models when administered prophylactically. Thus, in this study we investigated if riluzole confers enhanced stress resilience when administered prophylactically, i.e., concurrent with unpredictable chronic mild stress (UCMS) specifically assessing anxiety-, anhedonia-like behaviors, and overall behavioral emotionality. In a separate cohort, we also investigated whether chronic preventative riluzole treatment could block the development of learned helplessness, a core feature of PTSD and other mood disorders. Given that chronic stress and learned helplessness models also recapitulate the glutamatergic tripartite synapse deficits observed in humans with stress-related illnesses (Popoli et al., 2011), we predicted that the Glu-modulating drug riluzole would prevent the emergence of anxiety-, anhedonia-, and helplessness-like behavior.

Methods

Animals

Eight week-old male C57BL/6 mice (Jackson Laboratory, Saint Constant, QC) were housed under a 12h light/dark cycle at constant temperature (20-23°C) with *ad libitum* access to food and water, except when subjected to light disturbances or food/water deprivation during unpredictable chronic mild stress (UCMS) or learned helplessness (LH) procedures. Following 2-week facility habituation, animals were single-housed and randomly assigned to 1 of 4 treatment groups: control+vehicle, control+riluzole, UCMS+vehicle, or UCMS+riluzole (n=10/group). A separate cohort of 5-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were habituated to the facility under group-housed conditions throughout the experiment. LH mice were randomly assigned to 1 of 2 treatment groups: LH+vehicle or LH+riluzole (n=10/group). All testing was performed during the animal's light cycle and in accordance with Institutional and Canadian Council on Animal Care (CCAC) guidelines.

Drug Administration

To increase palatability of oral drug treatment, riluzole was diluted into 0.3% (v/v) saccharin (Sigma Aldrich, St. Louis, MO) in drinking water at a concentration of 60ug/mL, translating to ~12-15mg/kg/day p.o. for mice weighing 22-30g and drinking 6-8mL/day, following a previous study (Gourley et al., 2009). Riluzole was dissolved by stirring in room temperature saccharin solution. Solutions were changed every 48-72hrs. Mice not treated with riluzole consumed saccharin alone (vehicle). To assess the prophylactic efficacy of riluzole in UCMS, drug administration commenced on the same day as stressors. To assess preventative efficacy in LH, riluzole commenced 2 weeks prior to the inescapable footshock session. Both cohorts were maintained on riluzole throughout the duration of the experiments, except when fluid deprivation was required for the sucrose and water consumption tests.

Unpredictable Chronic Mild Stress (UCMS) Procedure

UCMS mice were subjected to 5 weeks of randomized stressors (2-4/day) and maintained for 2 weeks during testing (1-2/day) based on previously validated methods (Nikolova et al., 2018; Prevot et al., 2019; Fee et al., 2020). Stressors included: forced bath (~2cm of water in rat cage for 15min), wet bedding (30min-1h), aversive predator odor (20min-1h exposure to fox urine), light cycle reversal or disruption, social stress (rotate mice between cages within drug groups), tilted cage (45^0 tilt up to 2h), reduced space (dividers in cages up to 2h), acute restraint (15-30min in 50mL falcon tube with air/tail holes), bedding change (replacing soiled bedding with clean bedding), bedding or nestlet removal (up to 2h). Control animals underwent regular handling.

Behavioral Tests

For the UCMS cohort, behavioral testing commenced in week 3. Tests assessed locomotor activity (LA), anxiety-like behaviors in the elevated plus-maze (EPM), open field test (OFT) and novelty-suppressed feeding (NSF) tests, anhedonia-like behavior in the sucrose consumption test (SCT), or both in the novelty-induced hypophagia test (NIH). Physiological deficits were tracked with weekly fur coat assessment during

5 weeks of UCMS exposure and drug treatment. Testing order was randomized and experimenters were blinded to the treatment history.

Locomotor Activity (LA): On day 21, LA was measured for 20min in a regular housing cage (26x15x12cm) using an aerial-mounted video camera. ANY-maze tracking software (Stoelting Co., Wood Dale, IL) extracted distance traveled. LA was assessed to exclude the possibility that changes observed in other behavioral tests were due to bias introduced by treatment-related alterations in ambulatory movement.

Elevated Plus Maze (EPM): On day 22, animal behavior was assessed in an plus shaped maze consisting of 4 white Plexiglas arms, two open arms (27x5cm) and two enclosed arms (27x5x15cm) situated 55cm high with similar arms faced opposite each other. Animals freely explored the EPM for 10min in a 20 lux room. An aerial-mounted camera/ANY-maze software measured the time spent (s) and number of entries in open and closed arms.

Open Field Test (OFT): On day 23, behavior was assessed in an open field (70x70x33cm) placed on the floor. An aerial-mounted camera/ANY-maze software divided the field into 3 concentric square zones of equal area, and measured time spent and number of entries into the innermost zone (40x40cm).

Novelty-suppressed Feeding (NSF): On day 25, following 16h food deprivation, animals were introduced to a novel arena (62x31x48cm) containing a single food pellet. An experimenter blind to treatment conditions measured latency to feed on the pellet during a 12min period. As a control for differences in appetitive drive, latency to feed was then measured in the animal's home cage during a 6min period.

Novelty-Induced Hypophagia (NIH): On days 25-26, animals were habituated to a daily liquid reward (1mL of 1/3 v/v sweetened condensed milk). On day 26, latency to drink the reward was measured in the home cage as a control for potential treatment effects on appetite or activity. The following day, the animals were moved to a new cage (26x15x12cm) in a brightly lit (500-600 lux) room and the latency to drink the milk was measured over a 6min trial as a measure for hyponeophagia.

Sucrose Consumption Test (SCT): On days 27-28, home cage water bottles were switched to sucrose solution (1%; Sigma, St. Louis, MO) ± riluzole. Animals were habituated to sucrose for 48h and then fluid deprived overnight (~14h). On day 29, sucrose intake (mL) was measured over a 1h test. After the SCT, animals were returned to regular solutions (saccharine±riluzole) for 24h. The same test was repeated the next day i.e. fluid deprivation (14h) and water intake (1h) to control for potential treatment effects on fluid intake.

Fur Coat Assessment: Animals' coat state was assessed on 7 anatomical areas by attributing a score of 0, 0.5, or 1 for each, from maintained to unkempt. Coat assessment was scored by an experimenter blind to treatment conditions every week for 5 weeks of UMCS.

Learned Helplessness (LH) Procedure

A separate cohort of animals was habituated to a shuttle box (Med Associates, St. Albans, VT) for 5min and underwent a session consisting of 60 randomized, unpredictable, and inescapable footshocks (0.35 mA) administered over ~1h. Two days after the footshock session, mice were tested in the active avoidance (AA) paradigm. Mice underwent 30 trials of randomized footshocks (0.35 mA). Each trial lasted a maximum of 1min and included a minimum of 5sec before each footshock. Then, the door separating 2-halves of the chamber was opened (2sec before footshock initiation) and remained open for the duration of the shock. The first 5 trials required one chamber crossing (FR1) to terminate the footshock, whereas the next 25 trials required two crossings (FR2). The AA session lasted ~30min, during which the number of escape failures was recorded.

Statistical analysis

Data was analyzed using SPSS (IBM, NY). Based on validated methods (Guilloux et al., 2011; Piantadosi et al., 2016), parameters were z-scored and averaged across tests reflecting anxiety-like behavior (EPM, OFT, NSF, NIH), anhedonia-like behavior (NIH, SCT), or overall emotionality (all previous + fur coat state in the last week). Normalized z-scores integrate behavioral tests measuring similar phenotypes to increase

the consistency and reliability of these characteristically inconsistent tests (Guilloux et al., 2011; Piantadosi et al., 2016; Nikolova et al., 2018). Behavioral data were analyzed with two-way (stress x drug) analysis of variance (ANOVA), followed by Bonferroni-corrected *post-hoc* comparisons. Weekly coat state measures were analyzed using repeated-measures ANOVA. AA escape failures were analyzed using *student's t-test* comparing data between vehicle and riluzole groups. Data is presented as mean ± standard error of the mean (SEM).

Results

Riluzole prevents the emergence of UCMS-induced anhedonia-like behaviors

We first sought to determine whether chronic prophylactic riluzole treatment (i.e., administered concurrent with UCMS) could block the emergence of behavioral deficits induced by UCMS. Mice were subjected to 3 weeks daily handling (control) or UCMS ± vehicle or riluzole and tested for 2 weeks, including for LA and in the OFT, EPM, NSF, NIH, and SCT (**Fig 1*a***). Fur coat deterioration was tracked weekly (**Fig 1*a***). In the LA assessment, distance travelled in a home cage-like environment was not significantly affected by stress ($F_{1,36}$=0.025; p=0.87), drug treatment ($F_{1,36}$=0.183; p=0.67), or stress*drug interaction ($F_{1,36}$=3.02; p=0.09), confirming that subsequent tests were not biased by alterations in ambulatory movement (*not shown*).

In the EPM, two-way ANOVA of open arm entries revealed no main effect of stress ($F_{1,36}$=0.009; p=0.92), drug ($F_{1,36}$=0.084; p=0.77), or a stress*drug interaction ($F_{1,36}$=2.68; p=0.11; **Fig 1*b***). For time spent in the open arms, two-way ANOVA revealed a significant stress*drug interaction ($F_{1,36}$=4.49; p=0.041; **Fig 1*b***); however, group-wise differences did not survive *post-hoc* correction.

In the OFT, no significant differences were found for the effects of stress, drug treatment, or their interaction on the number of entries or time spent in the center zone (**Fig 1*c***).

In the NSF, two-way ANOVA revealed a trend towards a main effect of stress on latency to feed in the novel environment ($F_{1,36}$=3.71; p=0.061; **Fig 1*d***). However, this may have been confounded by an UCMS-induced increase in appetitive drive, since feeding latency in the home cage control test was significantly reduced for both stress groups ($F_{1,36}$=12.68; p=0.0011) (CTR+vehicle: 71.7±10.9; CTR+riluzole: 77.1±8.3; UCMS+vehicle: 49.3±6; UCMS+riluzole: 42.4±5.5). The UCMS paradigm includes brief food deprivation experiences which may bias animals towards increased motivation for feeding after longer deprivation periods such as with the NSF. Indeed, in the NIH, animals were not food-deprived and underwent a similar assessment for reward approach. In the NIH, two-way ANOVA detected a significant main effect of stress ($F_{1,36}$=19.29; p<0.001), drug treatment ($F_{1,36}$=19.13; p<0.001), and a stress*drug interaction ($F_{1,36}$=10.15; p=0.003) for latency to drink the milk in a novel environment (**Fig 1*e***). *Post-hoc* analysis revealed that this was driven by an anxiogenic 3-fold increase in latency to drink induced by UCMS (p<0.0001) that was prevented by riluzole (p<0.0001). Home cage latency was not affected by stress, drug treatment, or their interaction, suggesting that novel environment differences in approach latency were not due to appetite (CTR+vehicle: 23.2±4.8; CTR+riluzole: 29.2±5.2; UCMS+vehicle: 27.4±11.2; UCMS+riluzole: 46.1±9.4).

In the SCT, two-way ANOVA revealed a significant main effect of stress ($F_{1,36}$=39.61; p<0.0001), drug treatment ($F_{1,36}$=7.880; p=0.008), and stress*drug interaction ($F_{1,36}$=14.76; p=0.0005) for 1hr sucrose consumption following overnight deprivation (**Fig 1*f***). *Post-hoc* analysis revealed a significant decrease in sucrose consumption (p<0.0001) that was prevented by riluzole (p=0.0002). After 24hrs recovery, mice were again deprived and measured for water consumption under identical conditions. Although we detected a main effect of stress on water consumption ($F_{1,36}$=5.89; p=0.02), reflecting reduced water intake among UCMS-exposed animals, group-wise differences did not survive *post-hoc* analyses, confirming that sucrose consumption differences were reliably due to stress and/or drug treatment effects (CTR+vehicle: 1.6±0.2; CTR+riluzole: 1.5±0.1; UCMS+vehicle: 1.1±0.1; UCMS+riluzole: 0.9±0.2).

Repeated-measures ANOVA of weekly fur coat state assessments from week 0 to 5 revealed significant main effect of stress ($F_{1,36}$= 125.46; p<0.0001), time ($F_{5,180}$= 111.3; p<0.0001), stress*time interaction ($F_{5,180}$= 32.03; p<0.0001) and stress*drug*time interaction ($F_{5,180}$= 5.023; p=0.002), but no main effect of drug and no drug*stress or drug*time interactions. *Post-hoc* analysis showed that UCMS induced persistent fur coat deterioration starting on week 3 which was also significant for each following week (p<0.0001; Fig 1g) and not modified by riluzole treatment.

Z-score normalization confirms that riluzole prevents the emergence of anxiety- and anhedonia-like behavior, and composite emotionality-like behavior in UCMS-exposed mice

One issue with rodent chronic stress paradigms and classical testing methods, noted by us and others, is insufficient reliability to consistently measure depressive-like features across tests, cohorts, and between labs (Andreatini and Bacellar, 2000; Wahlsten et al., 2006; Prevot et al., 2019). To address this, we employed multiple tests assessing common dimensional phenotypes (anxiety- and anhedonia-like behavior, and emotionality) and analyzed both individual test parameters and normalized z-scores averaged across tests, based on validated methods (Guilloux et al., 2011).

Two-way ANOVA of normalized Z-anxiety scores (reflecting EPM, OFT, NSF, NIH) revealed a marginally significant stress*drug interaction ($F_{1,36}$=3.996; p=0.0532; **Fig 2*a***) with no main effect of stress or drug. However the post hoc analysis did not find group differences. Z-anhedonia (reflecting NIH, SCT) was significantly affected by stress ($F_{1,36}$=36.74; p<0.001), drug treatment ($F_{1,36}$=5.899; p=0.0203), and their interaction ($F_{1,36}$=9.032; p=0048), wherein UCMS induced an elevation in anhedonia-like behavior (p<0.0001) that was prevented by riluzole (p=0.0029; **Fig 2*b***). Summary of behavioral emotionality via analysis of Z-emotionality scores (Z-anxiety and Z-anhedonia) revealed significant effects of stress ($F_{1,36}$=11.93; p=0.0014), of drug ($F_{1,36}$=2.68; p=0.0109) and a stress*drug interaction ($F_{1,36}$=11.46; p=0.0017; **Fig 2*c***). Overall, behavioral emotionality was significantly elevated by UCMS (p=0.0001) and prevented by riluzole (p=0.0065).

Riluzole prevents the development of learned helplessness

We next investigated whether preventative riluzole treatment could block the development of helplessness-like behavior in the active avoidance test. A separate cohort of mice (n=10/group) were administered riluzole or vehicle for 2 weeks and submitted to unpredictable inescapable footshocks as part of the LH protocol (**Fig 3*a***). In the AA paradigm, *t-test* analysis revealed a significant effect of drug treatment on the number of escape failures (t=4.55, df=18; p=0.0001), wherein compared to vehicle, riluzole decreased the number of escape failures (**Fig 3*b***).

Discussion

This report provides evidence that chronic prophylactic riluzole treatment can increase stress resilience, as evidenced by the prevention of specific behavioral deficits induced by unpredictable or uncontrollable stress exposure. Animals exposed to UCMS exhibited intra-test deficits on mixed anxiety-/anhedonia-like behavior that were prevented by chronic prophylactic riluzole treatment (i.e., administered concurrently with stress and testing) and physiological deficits that were not modulated by treatment. Dimensional z-scores, confirmed this prophylactic efficacy since riluzole prevented significant elevations in anxiety-, anhedonia-like behavior, and overall behavioral emotionality induced by UCMS. In a separate cohort of mice, chronic preventative riluzole treatment (i.e., 2 weeks prior and during LH) blocked the development of helplessness-like behavior in an AA test. These findings suggest that the Glu-modulating drug riluzole may have utility as a prophylactic therapy enhancing stress resilience and protecting against the development of anxiety, anhedonia, and helplessness, common symptoms of stress-related illnesses such as MDD, BPD, and PTSD.

UCMS is a frequently-used behavioral paradigm due to its face and construct validity for recapitulating behavioral and biological alterations related to human stress-related illnesses, and predictive validity for detecting anxiolytic and antidepressant effects (Jesberger and Richardson, 1988; Mineur et al., 2006; Hill et al., 2012). In the current study, UCMS did not induce significant behavioral changes in the

EPM, OFT, or NSF. However, Z-score normalization of anxiety-like behavior, reflecting EPM, OFT, NSF, and NIH parameters, revealed an overall anxiogenic effect of UCMS. This test-specific behavioural variability is unsurprising as it has been noted using UCMS by our lab (Maluach et al., 2017; Nikolova et al., 2018; Prevot et al., 2019; Fee et al., 2020) and others (Ramos, 2008; Willner, 2017), whereas top-down dimension reduction via Z-scoring consistently increases the sensitivity and reliability of these same tests (Guilloux et al., 2011). Although this approach may improve the quality of conclusions about preclinical behavior changes, it is also important to mention that repeated testing is itself a source of variability (Voikar et al., 2004). In contrast, we found that UCMS induced robust anhedonia-like behaviors that were significantly prevented by riluzole at the test (NIH, SCT) and z-score level, consistent with evidence that rodent UCMS paradigms more reliably induce reward-related deficits (Willner, 2005). UCMS also induced physiological deficits on coat state deterioration that were unaffected by riluzole, consistent with evidence that fur coat quality tracks the induction of stress-related deficits, but does not respond to most antidepressant treatments (Prevot et al., 2019; Fee et al., 2020). Finally, in consideration of the potential sedative effects of high doses of riluzole (Pittenger et al., 2008a), we confirmed that behavioral outcomes were not influenced by locomotor changes, in agreement with past studies using similar dosing regimens (Banasr et al., 2010).

The LH paradigm models the inescapable and uncontrollable aspects of naturalistic stressors (Maier, 1984) and shows predictive validity for classical AD activity (Zazpe et al., 2007). The acute/intense LH stressor may be more closely related to PTSD given that it reproduces aspects of avoidance, anxiety, and hyperarousal (Schöner et al., 2017). Indeed, although distinct, escape failures in the active avoidance paradigm reflect helplessness-like behavior that partially correlates with other symptom-like dimensions, including sucrose-related anhedonia (Landgraf et al., 2015). Here, we identified for the first time that chronic preventative riluzole treatment significantly reduced the number of escape failures in the active avoidance test, therefore blocking the development of learned helplessness in mice. This finding raises the possibility of new therapeutic uses for riluzole, including as a preventative or prophylactic treatment in

individuals experiencing not only chronic stress, but also those subjected to severe acute stressors with high likelihood to develop helplessness. This goal may be consistent with the results of several small studies indicating that prophylactic treatments targeting arousal and fear responses associated with acute stress (e.g., propranolol or hydrocortisone) can effectively prevent PTSD emergence (Roque, 2015).

In the current study, prophylactic riluzole treatment prevented the emergence of anxiety-, anhedonia-, helplessness-like behavior, and overall behavioral emotionality induced by UCMS or LH paradigms in mice. Despite differences in treatment regimen, these findings are consistent with a study by Banasr et al., wherein chronic riluzole administered in the last 3 of 5-weeks UCMS (plus AA testing) in rats, reversed anhedonia- and helplessness-like behavior (Banasr et al., 2010). In non-stressed mice, chronic riluzole induced antidepressant-like improvement in the forced swim test and reward-related incentive disengagement test (Gourley et al., 2012). Acute or sub-chronic riluzole also reversed the expression of behavioral emotionality in olfactory bulbectomized rats, a model for hypercortisolemia known to impact Glu markers (Takahashi et al., 2011). Collectively, animal studies using classical chronic AD dosing regimens indicated antidepressant-like efficacy of riluzole, paralleling the early success observed in open label clinical studies (Zarate et al., 2004, 2005; Sanacora et al., 2007; Brennan et al., 2010). However, later (and larger) clinical RDBPCs found limited efficacy of riluzole monotherapy or adjunctive therapy to monoaminergic ADs or ketamine (Pittenger et al., 2008b; Mathew et al., 2010, 2017; Ibrahim et al., 2012; Yao et al., 2020). Our findings of enhanced stress resilience from prophylactic treatment may instead suggest that riluzole's Glu-modulating mechanisms are well-suited for currently remitted MDD or BPD patients who are at high risk for relapse. Indeed, following monoaminergic AD treatment, a large majority (~90%) of remitted patients have residual symptoms that may increase their vulnerability to stress and disrupt normal functioning (Nierenberg et al., 2010; Iovieno et al., 2011). Further, monoaminergic AD discontinuation is associated with high one-year relapse rates (~50-90%) (Judd, 1997; Mueller et al., 1999). It is also possible that mixed efficacy of riluzole in clinical studies is due to variance in treatment protocol or patient characteristics. For example, extension of an 8-week open-label riluzole trial to 12 weeks

achieved response in one-fourth of non-responders (Sakurai et al., 2019). Riluzole may also be useful in patients with specific biomarkers given that lower pre-treatment BDNF predicted treatment response in humans (Wilkinson et al., 2018) and riluzole increased BDNF exposure in rodents chronically exposed to exogenous glucocorticoids (Gourley et al., 2012).

Although the current study is limited by not investigating cellular mediators of potential AD-like effects of riluzole, other studies demonstrate that chronic stress paradigms reliably induce Glu and GABA tripartite synaptic deficits that contribute to the expression of depressive-like behaviors in rodents and parallel clinical findings (Sanacora et al., 2008; Rajkowska and Stockmeier, 2013; Fee et al., 2017). Riluzole is an attractive AD or adjunct candidate drug due to its multifaceted actions that stabilize Glu activity, including by inhibiting pre-synaptic hyperactivity through voltage-gated sodium and calcium channel blockade, inhibiting pre- and post-synaptic Glu receptors, and increasing Glu metabolism (clearance, uptake, and recycling) via glial cell uptake (Pittenger et al., 2008a). These changes are expected to counter excessive Glu signaling that biases towards neurotoxic extrasynaptic NMDAR pathways, and is exacerbated by reduced regulation due to GABAergic (Banasr et al., 2017; Fee et al., 2017) and glial deficits (Choudary et al., 2005; Banasr and Duman, 2008). Indeed, whereas excessive Glu signaling is associated with corticolimbic remodeling, for example with PFC and HPC atrophy (McEwen et al., 2016), riluzole reversed the effect of excitotoxic PFC lesions on decreased neuronal density (Risterucci et al., 2006). Parallel pathological mechanisms involving Glu excitotoxicity have been noted in neurodegenerative disorders (Hardingham and Bading, 2010), drawing parallels to chronic stress-related disorders recently considered as mild neurodegenerative conditions (Sanacora et al., 2012; Duman, 2014). Interestingly, riluzole reversed or prevented cognitive decline in aged rodents, and increased dendritic complexity and expression of glial EAAT in the HPC (Pereira et al., 2014, 2017). In a mouse model of Alzheimer's disease, riluzole enhanced cognition, reduced amyloid beta pathology, and reversed alterations in NMDAR subunit expression (Okamoto et al., 2018). Collectively, these findings implicate the modulation of Glu

homeostasis, and subsequently neurotrophic and neuroprotective effects, in the therapeutic actions of riluzole.

The neuroprotective changes associated with riluzole are corroborated by evidence that riluzole increases BDNF expression in neurons and astrocytes (Mizuta et al., 2001; Katoh et al., 2002). Another main neuroprotective mechanism of riluzole seems to occur through induction of glial cell Glu metabolism, given that riluzole reversed chronic stress and glucocorticoid-induced reductions in astrocytic structural and functional markers (e.g., GFAP, GLT-1, and GLAST), increased BDNF expression, and conferred antidepressant-like behavioral changes in rodents (Fumagalli et al., 2008; Banasr et al., 2010; Gourley et al., 2012; Yoshizumi et al., 2012).

Consistent with existing evidence of antidepressant activity in preclinical and some clinical studies, we demonstrated that riluzole could prevent or block the emergence of specific behaviors related to symptom dimensions of stress-related illnesses, including anxiety-, anhedonia-, helplessness-like behavior, and overall behavioral emotionality. A final limitation of the current study was the inclusion of male mice only. Given that MDD is more prevalent among females (World Health Organization, 2017), and postmortem studies identify more severe synaptic deficits in females (Behan et al., 2011; Seney et al., 2013; Halstead et al., 2015), future studies should investigate whether riluzole has sex-dependent properties. Although these findings did not conclusively investigate cellular mediators of antidepressant mechanisms, this study provides a promising starting point for the use of riluzole for enhancing stress resilience through prophylactic or preventative treatment regimens.

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C.F., Y.B., K.M., and M.B., conducted all experiments, and analyzed data. C.F., Y.B., S.C., and M.B. wrote the manuscript. E.S., R.B., V.C., and G.S., provided scientific advice on study designs and reviewed the manuscript.

Conflicts of Interest Disclosure

G.S. has received consulting fees from Allergan, Alkermes, AstraZeneca, Avanier, Axsome Therapeutics, Pharmaceuticals, Biohaven Pharmaceuticals, Bristol-Myers Squibb, Clexio Biosciences, EMA Wellness, Epiodyne, Intra-Cellular Therapies, Janssen, Lundbeck, Merck & Co., Minerva pharmaceuticals, Navitor, Neurocrine biosciences, NeruoRx, Novartis, Noven Pharmaceuticals, Otsuka, Perception Neuroscience, Praxis Therapeutics, Sage Pharmaceuticals, Servier Pharmaceuticals, Taisho Pharmaceuticals, Teva, Valeant, and Vistagen therapeutics over the last 36 months. He has also received additional research contracts from AstraZeneca, Bristol-Myers Squibb, Eli Lilly, Johnson & Johnson, Hoffman La-Roche, Merck & Co., and Usona over the last 36 months. Free medication was provided to GS for an NIH-sponsored study by Sanofi-Aventis. In addition, he holds shares in BioHaven Pharmaceuticals Holding Company and is a co-inventor on a patent 'Glutamate agents in the treatment of mental disorders' (Patent number: 8778979), and a U.S. Provisional Patent Application No. 047162-7177P1 (00754) filed on August 20, 2018 by Yale University Office of Cooperative Research OCR 7451 US01. RMB and VC are employees and stockholders of Biohaven Pharmaceuticals. R.M.B. and V.C. are employees of Biohaven Pharmaceuticals.

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Figure legends:

Figure 1. Riluzole prevents the emergence of anhedonia-like behaviors induced by unpredictable chronic mild stress (UCMS). (**A**) study design for behavioral analysis of Fur/Weight (FW0-5), Locomotor Activity (LA), Elevated-Plus Maze (EPM), Open Field Test (OFT), Novelty-Suppressed Feeding (NSF), Novelty-Induced Hypophagia (NIH), and Sucrose Consumption Test (SCT) beginning on day 21 of Control (Ctrl)/UCMS ± Vehicle (Veh)/Riluzole 13.2mg/kg/day p.o. (n = 10/group). (**B**) Number of open arm entries (left) and time spent in open arms in the EPM. (**C**) Number of center zone entries and time spent in the center zone in the OFT. (**D**) Novel environment latency to feed in the NSF. (**E**) Novel environment latency to drink a milk reward in the NIH. (**F**) Sucrose consumed in the SCT. (**G**) Weekly fur coat deterioration ratings. ****p<0.0001 UCMS + Veh vs. Ctrl + Veh; $^{\$\$\$\$}p$<0.0001, $^{\$\$}p$<0.001 UCMS + Riluzole vs. UCMS + Veh.

Figure 2. Z-score normalization reveals that riluzole prevents the emergence of anxiety- and anhedonia-like behaviors, and emotionality-like behavior induced by unpredictable chronic mild stress (UCMS). (**A**) Z-anxiety scores (reflecting elevated-plus maze, open field test, novelty-suppressed feeding, and novelty-induced hypophagia behavioral performances). (**B**) Z-anhedonia scores (reflecting novelty-induced hypophagia, sucrose consumption test performances). (**C**) Z-emotionality (reflecting all z-score). (n=10/group) ****p<0.0001, ***p<0.001 UCMS + Veh vs. Ctrl+Veh; $^{\$\$\$}p$<.001, $^{\$\$}p$<.01 UCMS + Riluzole vs. UCMS + Veh.

Figure 3. Riluzole blocks the development of learned helplessness (LH). (**A**) Study design for chronic preventative riluzole treatment prior to learned helplessness conditioning including 60 inescapable footshocks with active avoidance (AA) testing 2 days later. (**B**) Number of escape failures in the active avoidance session (n=10/group). ***p<.001 Riluzole vs. Vehicle (Veh).

Figure 1

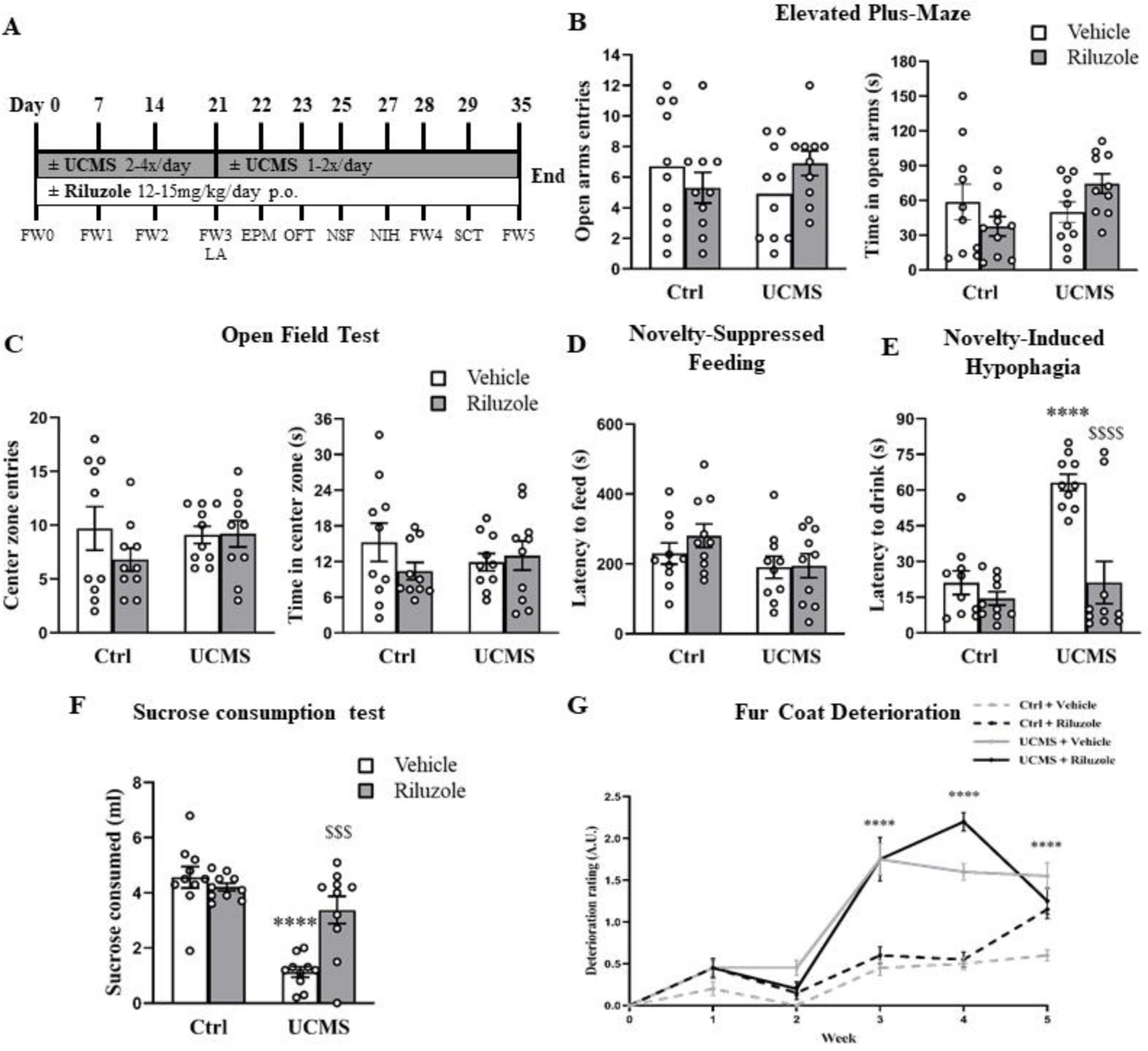

Figure 2

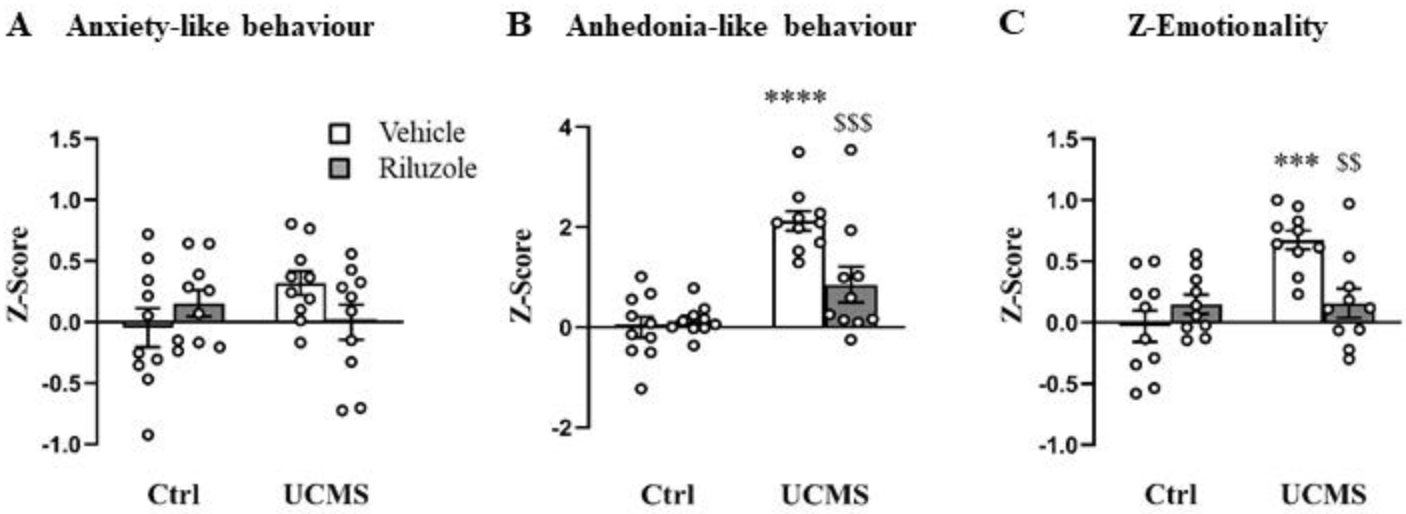

Figure 3