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.

Introduction

Exposure to chronic stress is a major risk factor for a variety of psychiatric illnesses that share common symptoms including low mood, anhedonia, helplessness, and impaired physiological functioning. These include major depressive disorder (MDD), bipolar disorder (BPD), anxiety disorders, schizophrenia, and post-traumatic stress disorder (PTSD), which together account for one-third of global years lived with disability (Vigo et al., 2016). Although antidepressant drugs (ADs) have demonstrated utility for mood enhancement and neuroprotective uses across stress-related illnesses, current first-line monoaminergic ADs require several rounds of treatment to achieve remission in a majority of patients, necessitating lengthy delays until remission (Gaynes et al., 2009; Holtzheimer and Mayberg, 2011). Meanwhile, the likelihood of achieving or sustaining remission is negatively associated with an increasing duration of depressive episodes and an increasing number of treatment courses (Okuda et al., 2010; Ghio et al., 2015; Berwian et al., 2016). Ultimately, even multiple treatments fail to adequately reduce symptoms in one-third of patients (Rush et al., 2006). Given the challenges of treating chronic and recurrent stress-related disorders, the development of prophylactic treatments targeting pathologies underlying symptom emergence may represent a promising avenue to increase stress resilience in vulnerable subjects or maintain response in remitted patients.

Collective evidence indicates altered excitatory glutamatergic (Glu) neurotransmission resides among pathological deficits associated with stress-related illnesses. Elevated circulating Glu levels and cortical hyperactivity were observed in MDD and PTSD patients, and altered expression or function of Glu receptors was identified in postmortem subjects (Sanacora et al., 2008)). Changes in Glu neuron structure and function are exacerbated by deficits within the tripartite synapse extending to inhibitory gamma-aminobutyric acid (GABA) interneurons (Fee et al., 2017) and homeostatic glial cells such as astrocytes (Rajkowska and Stockmeier, 2013; Sanacora and Banasr, 2013), which tightly regulate excitatory neurotransmission. Preclinical studies demonstrated that stress induces Glu release in corticolimbic brain regions, including the prefrontal cortex (PFC), hippocampus (HPC), and amygdala

(Bagley and Moghaddam, 1997; Musazzi et al., 2010). Acute stress-induced Glu release can have adaptive neuroprotective effects through activating synaptic signaling cascades coupling to, for example, induction of brain-derived neurotrophic factor (BDNF) expression (Duman and Monteggia, 2006). Conversely, high or sustained Glu levels after chronic stress may confer excitotoxic effects via extrasynaptic signaling pathways (Hardingham and Bading, 2010; Pál, 2018) that are 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).

Riluzole is a glutamate-modulating medication that was originally developed as an anticonvulsant and is currently FDA-approved for amyotrophic lateral sclerosis. Evidence of neuroprotective effects of riluzole in preclinical studies led to ongoing investigations of riluzole treatment as a potential AD candidate (Fumagalli et al., 2008; Yoshizumi et al., 2012). However, clinical evidence for the therapeutic utility of riluzole in stress-related illnesses is mixed. Early open-label studies in MDD patients found promising response, remission, and tolerability comparable to monoaminergic ADs from riluzole monotherapy (Zarate et al., 2004, 2005; Brennan et al., 2010) and exceeding those rates as an adjunctive treatment to monoaminergic ADs (Sanacora et al., 2007). A later randomized, double-blind, placebo-controlled (RDBPC) trial found that riluzole + citalopram combination therapy achieved greater response and earlier onset compared to placebo (Salardini et al., 2016). However, follow-up RDBPCs found no effect from riluzole monotherapy in patients with BPD (Park et al., 2017) or from riluzole as an adjunct to monoaminergic ADs in patients with treatment-resistant depression (Mathew et al., 2017). In patients with schizophrenia, riluzole used adjunctively to risperidone improved intractable negative symptoms (Farokhnia et al., 2014). Although positive effects and strong tolerability were found, the therapeutic efficacy of riluzole in psychiatric disease generally (de Boer et al., 2019) or depressive disorders specifically (Yao et al., 2020) was not confirmed by recent meta-analyses of RDBPCs. The variability in these outcomes ultimately raises questions about whether riluzole has value for targeting specific

symptom dimensions of chronic stress-related illnesses, or whether riluzole therapy could be improved with alternative treatment strategies.

The neuroprotective vs. neurotoxic effects of Glu signaling rely on complex interactions with several receptor systems. Glu release is first initiated by voltage-gated sodium and calcium channels that mediate axon depolarization and synaptic release, respectively. Released Glu binds to post-synaptic ionotropic amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPAR) and kainate receptors located synaptically, ionotropic N-methyl-D-aspartate (NMDAR) receptors located synaptically and extrasynaptically, and metabotropic glutamate receptors (mGluRs) including presynaptic type II mGluR ($mGlu_{2/3}$) (Pittenger et al., 2008; Murrough et al., 2017). Regulation of Glu signaling is mediated via presynaptic inhibition by GABA interneurons, homeostatic Glu recycling via excitatory amino acid transporters (EAATs) on glial cells (and neurons), and negative feedback regulation via presynaptic $mGlu_{2/3}$ receptors. Thus, whereas stress-related illnesses involve net excitotoxicity from elevated Glu levels activating extrasynaptic vs. synaptic NMDARs (and exacerbated by decreased EAAT and GABA regulatory control), modulation of these pathways may represent a viable therapeutic strategy.

Evidence from preclinical studies suggests that riluzole possesses potential as a neuro-protective, antidepressant, and anti-stress agent. Riluzole has widespread mechanisms which may contribute to these effects, including inhibition of Glu signaling both pre- (i.e., direct and indirect inhibition of voltage-gated sodium and calcium channels) and post-synaptically (i.e., antagonism of NMDARs, kainate receptors, and AMPARs), increasing Glu re-uptake and recycling via glial cell transporters, and promoting a net increase in the ratio of synaptic to extrasynaptic signaling that promotes neurotrophic factor expression (Pittenger et al., 2008). Indeed, in chronic stress- or glucocorticoid-exposed rodents, chronic riluzole administration reversed and attenuated PFC and HPC deficits in structural and functional markers of synaptic Glu signaling and metabolism, increased BDNF levels, and conferred antidepressant-like behavioral effects (Gourley et al., 2009; Banasr et al., 2010). These results indicate that riluzole shares neurotrophic effects

with conventional ADs and modulation of Glu signaling with rapid-acting ADs, such as the NR2B-containing NMDAR antagonist ketamine (Deutschenbaur et al., 2016).

Whereas antidepressant efficacy has been less consistent in humans, it may be that the robust Glu-modulating properties of riluzole are more appropriate for targeting altered Glu homeostasis earlier in the pathogenesis of chronic stress-related disorders. Indeed, chronic preventative administration of monoaminergic ADs blocked acute stress-induced Glu release and prevented the effects of chronic stress on corticolimbic remodeling and depressive-like behaviors, which are collectively linked to excessive Glu activity (Musazzi et al., 2011). Moreover, lithium maintenance therapy may increase resilience in clinical MDD and BPD patients, and has been demonstrated in rodents to prevent chronic stress-induced HPC remodeling when administered prophylactically (Wood et al., 2004; Sanacora et al., 2012). Therefore, the use of prophylactic riluzole to regulate Glu homeostasis and therefore prevent chronic stress-related pathogenesis may be valuable for individuals who are at high risk for developing stress-related disorders. These use cases could include partially remitted or treatment discontinuing MDD or BPD patients that are at high risk for relapse (Judd, 1997; Mueller et al., 1999; Nierenberg et al., 2010; Iovieno et al., 2011) or individuals exposed to acute trauma that are at high risk for developing PTSD (Roque, 2015).

In summary, preclinical and clinical findings indicate that riluzole may have therapeutic utility for stress-related illnesses. However, it is unclear whether specific treatment protocols or symptom dimensions are most appropriate for its use. Rodent chronic stress paradigms are well-validated tools that recapitulate biological features of stress-related disorders (e.g., elevated corticosterone, corticolimbic neuronal atrophy/hypertrophy) together with behavioral phenotypes that reflect human symptom dimensions, including anxiety-, anhedonia-, helplessness- and depressive-like behavior, and physiological deficits (Willner, 2005a; Mineur et al., 2006; Monteiro et al., 2015). In this study, we aimed to determine whether riluzole confers enhanced stress resilience when administered prophylactically, i.e., concurrent with chronic stress, with regards to the prevention of 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., 2012), 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. Animals were initially group housed (4/cage). 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 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 by the experimenter.

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 the 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 EPM 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 (s) 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. Fluid deprivation was then repeated (14h), and water solution intake (mL) was measured over 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 were 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 are 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 (**Figure 1a**). Fur coat deterioration was tracked weekly (**Figure 1a**). In the LA assessment, distance travelled in a home cage-like environment was not significantly affected by stress ($F_{1,36}$=.25; p=.87), drug treatment ($F_{1,36}$=.67; p=.18), or stress*drug interaction ($F_{1,36}$=3.02; p=.09), confirming that subsequent tests were not biased by alterations in ambulatory movement (*not shown*).

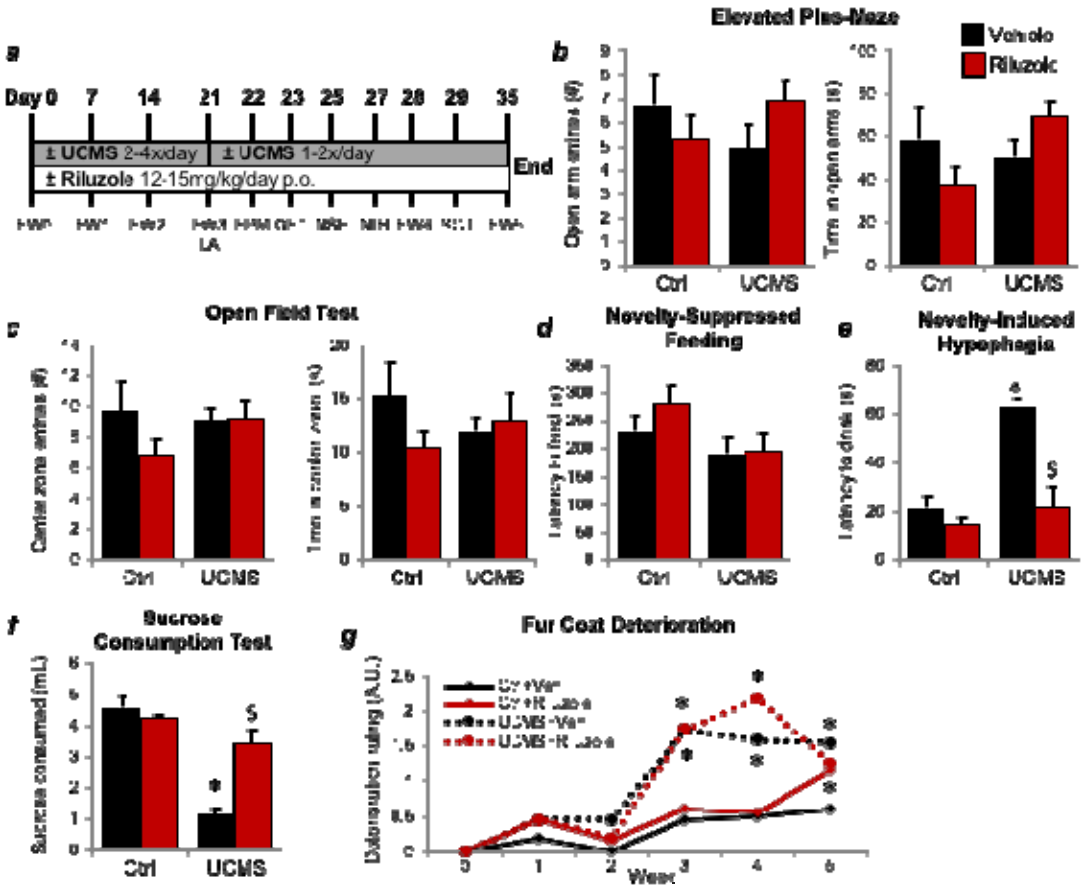

Figure 1. Riluzole prevents the emergence of anhedonia-like behaviors induced by unpredictable chronic mild stress (UCMS). (*a*) study design for behavioral analysis of Fur/Weight (FW0-5), Locomotor Activity (LA), Elevated-Plus Maze (EPM), Open Field Test (OFT), Novelty-Suppressed Feeding (NSF), Novelty-Induced 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<.05 UCMS+Veh vs. Ctrl+Veh; $$p$<.05 UCMS+Riluzole vs. UCMS+Veh.

In the EPM, two-way ANOVA of open arm entries revealed no main effect of stress ($F_{1,36}$=.009; p=.92), drug ($F_{1,36}$=.67; p=.18), or a stress*drug interaction ($F_{1,36}$=2.68; p=.11; **Figure 1b**). For time spent in the open arms, two-way ANOVA revealed a significant stress*drug interaction ($F_{1,36}$=4.49; p=.041; **Figure 1b**); 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 (**Figure 1c**).

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=.061; **Figure 1d**). 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=.011). 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 approach the milk reward in a novel environment (**Figure 1e**). *Post-hoc* analysis revealed that this was driven by an anxiogenic 3-fold increase in latency to drink induced by UCMS (p<.001) that was prevented by riluzole (p<.001). 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 appetitive or locomotor changes.

In the SCT, two-way ANOVA revealed a significant main effect of stress ($F_{1,36}$=35.45; p<0.001), drug treatment ($F_{1,36}$=6.14; p=0.018), and stress*drug interaction ($F_{1,36}$=11.80; p<0.01) for 1hr sucrose consumption following overnight deprivation (**Figure 1f**). *Post-hoc* analysis revealed a significant decrease in sucrose consumption (p<.001) that was prevented by riluzole (p<.001). 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<.05), 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.

Repeated-measures ANOVA of weekly fur coat state assessments from weeks 0 to 5 of UCMS/daily handling revealed a significant stress*drug*time interaction ($F_{1,5}$= 5.023; p=0.002), wherein UCMS induced persistent fur coat deterioration starting in week 3 that was significant for each following week (ps<.01; **Figure 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 effect of stress ($F_{1,36}$=4.04; p=.052) and a significant stress*drug interaction ($F_{1,36}$=5.66; p=.023; **Figure 2a**). UCMS induced an elevation in anxiety-like behavior (p<.05) that was prevented by riluzole (p<.05). Z-anhedonia (reflecting NIH, SCT) was significantly affected by stress ($F_{1,36}$=42.58; p<.001), drug treatment ($F_{1,36}$=21.42; p<.001), and their interaction ($F_{1,36}$=18.96; p<.001), wherein UCMS induced an elevation in anhedonia-like behavior (p<.001) that was prevented by riluzole (p<.001; **Figure 2b**). Summary of behavioral emotionality via analysis of Z-emotionality scores (Z-anxiety, Z-anhedonia, fur coat state) revealed significant effects of stress ($F_{1,36}$=30.24; p<.001) and a

stress*drug interaction ($F_{1,36}$=23.32; p<.001; **Figure 2c**). Overall, behavioral emotionality was significantly elevated by UCMS (p<.001) and prevented by riluzole (p<.01).

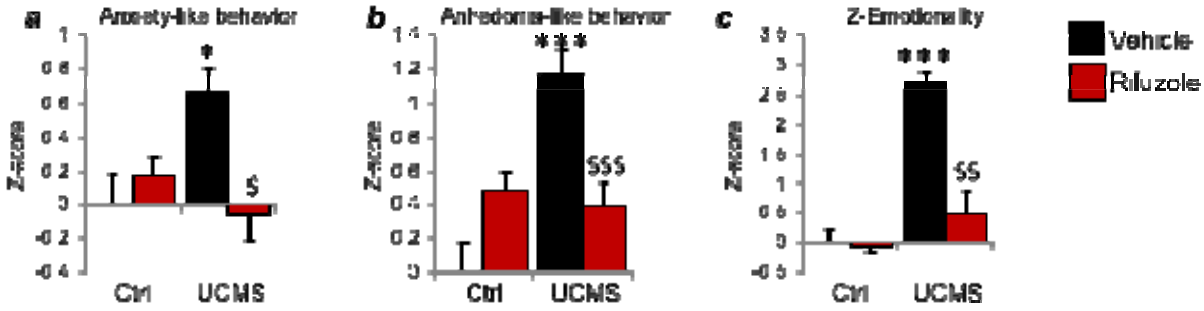

Figure 2. Z-score normalization reveals that riluzole prevents the emergence of anxiety- and anhedonia-like behaviors, and emotionality-like behavior induced by unpredictable chronic mild stress (UCMS). (*a*) Z-anxiety scores (reflecting elevated-plus maze, open field test, novelty-suppressed feeding, and novelty-induced hypophagia behavioral performances). (*b*) Z-anhedonia scores (reflecting novelty-induced hypophagia, sucrose consumption test performances). (*c*) Z-emotionality (reflecting all previous plus fur coat deterioration). (n=10/group) ***p<.001, *p<.05 UCMS+Veh vs. Ctrl+Veh; $$$$p$<.001, $$$p$<.01, $$p$<.05 UCMS+Riluzole vs. UCMS+Veh.

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 (**Figure 3a**). 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<.001), wherein compared to vehicle, riluzole decreased the number of escape failures (**Figure 3b**).

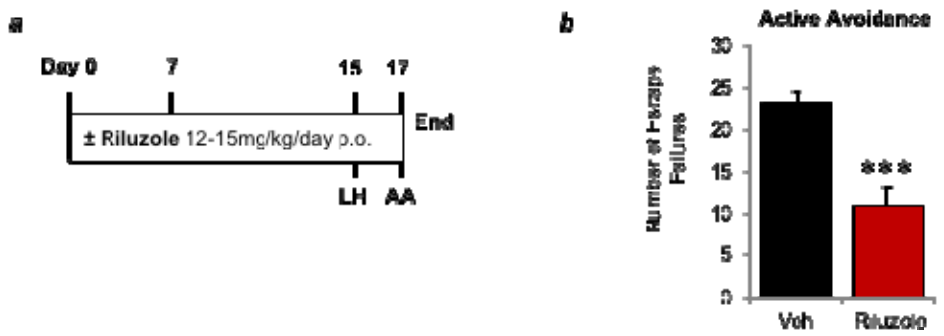

Figure 3. Riluzole blocks the development of learned helplessness (LH). (a) Study design for chronic preventative riluzole treatment prior to learned helplessness conditioning including 60 inescapable footshocks with active avoidance (AA) testing 2 days later. (b) Number of escape failures in the active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

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 (Mineur et al., 2006; Hill et al., 2012; Nollet et al., 2013). 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, 2005b). 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., 2008), 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 (Mathew et al., 2010, 2017; Ibrahim et al., 2012; Park et al., 2017; 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., 2008). 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-Semba 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; Seney, M., Tripp, A., McCune, S., Lewis, D., Sibille, 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., K.M., and M.B., conducted all experiments, and analyzed data. C.F. 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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