

Integrated stress response inhibitor reverses sex-dependent behavioral and cell-specific deficits after mild repetitive head trauma

Karen Krukowski PhD^{1,2*}, Amber Nolan MD PhD^{1,2,3*}, Elma S. Frias^{1,2,4}, Katherine Grue^{1,2}, McKenna Becker^{1,2}, Gonzalo Ureta PhD⁵, Luz Delgado PhD⁵, Sebastian Bernales PhD⁵, Vikaas S. Sohal MD PhD⁶, Peter Walter PhD^{7,8}, Susanna Rosi PhD^{1,2,9,10,11#}

¹ Department of Physical Therapy and Rehabilitation Science, University of California, San Francisco, CA, USA.

² Brain and Spinal Injury Center, University of California, San Francisco, CA, USA.

³ Department of Pathology, University of California, San Francisco, CA, USA.

⁴ Department of Biomedical Sciences, University of California, San Francisco, CA, USA.

⁵ Fundación Ciencia & Vida, Santiago, Chile

⁶ Department of Psychiatry, University of California, San Francisco, CA, USA.

⁷ Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA

⁸ Howard Hughes Medical Institute, University of California, San Francisco, CA, USA

⁹ Department of Neurological Surgery, University of California, San Francisco, CA, USA.

¹⁰ Weill Institute for Neuroscience, University of California San Francisco, CA, USA.

¹¹ Kavli Institute of Fundamental Neuroscience, University of California, San Francisco, CA

* Equal contribution to first authorship

Corresponding author, Tel: 415-206-3708, Address: 1001 Potrero Ave, Zuckerberg San Francisco General Hospital, Building#1 room 101, San Francisco, CA 94110, Email address: susanna.rosi@ucsf.edu

Running Title: ISR inhibitor reverses repetitive trauma deficits

Author Contact Information:

Karen Krukowski, PhD, Postdoctoral Fellow

Zuckerberg San Francisco General Hospital, 1001 Potrero Ave

Building#1 room 101

San Francisco, CA 94110

Phone: 415-206-3708, Fax: 415-206-3948

Email: karen.krukowski@ucsf.edu

Amber Nolan, MD PhD, Research Fellow and Clinical Instructor

Department of Pathology, University of California San Francisco

505 Parnassus Ave, Rm. M545

San Francisco, CA 94143

Phone: 415-476-5236, Fax: 415-476-4666

Email: amber.nolan2@ucsf.edu

Elma Frias, Graduate Student

Zuckerberg San Francisco General Hospital, 1001 Potrero Ave

Building#1 room 101

San Francisco, CA 94110

Phone: 415-206-3708, Fax: 415-206-3948

Email: elma.frias@ucsf.edu

Katherine Grue, Staff Research Associate

Zuckerberg San Francisco General Hospital, 1001 Potrero Ave

Building#1 room 101

San Francisco, CA 94110

Phone: 415-206-3708, Fax: 415-206-3948

Email: katherine.grue@ucsf.edu

McKenna Becker, Staff Research Associate

Zuckerberg San Francisco General Hospital, 1001 Potrero Ave

Building#1 room 101

San Francisco, CA 94110

Phone: 415-206-3708, Fax: 415-206-3948

Email: mckenna.becker@ucsf.edu

Gonzalo Ureta, PhD, Research Scientist

Zañartu 1482, Santiago, Chile

Phone: +56 2 22382062

Email: g.ureta.diaz@gmail.com

Luz Delgado, PhD, Research Scientist

Zañartu 1482, Santiago, Chile

Phone: +56 2 22382086

Email: delgado.luz@gmail.com

Sebastián Bernales, PhD, Principal Investigator

Zañartu 1482, Santiago, Chile

Phone: +56 2 22382062

Email: sbernales@gmail.com

Vikaas S. Sohal, MD PhD, Associate Professor

UCSF School of Medicine, Department of Psychiatry

675 Nelson Rising Lane

San Francisco CA 94158

Phone: 415-502-7377, Fax: 415-476-7320

Email: Vikaas.Sohal@ucsf.edu

Peter Walter, PhD, Professor

Box 2200, UCSF

San Francisco, CA 94143

Phone: 415-476-5017

Email: peter@walterlab.ucsf.edu

Susanna Rosi, PhD, Professor

Zuckerberg San Francisco General Hospital, 1001 Potrero Ave

Building#1 room 101

San Francisco, CA 94110

Phone: 415-206-3708, Fax: 415-206-3948

Email: susanna.rosi@ucsf.edu

ABSTRACT

Mild repetitive traumatic brain injury (rTBI) induces chronic behavioral and cognitive alterations and increases the risk for dementia. Currently there are no therapeutic strategies to prevent or mitigate chronic deficits associated with rTBI. We previously developed an animal model of rTBI that recapitulates some of the cognitive and behavioral deficits observed in humans. Here we report that rTBI results in an increase in risk-taking behavior in male but not female mice. This behavioral phenotype is associated with chronic activation of the integrated stress response and cell-specific synaptic alterations in the type A subtype of layer V pyramidal neurons in the medial prefrontal cortex (mPFC). Strikingly, by briefly treating animals weeks after injuries with ISRIB, a selective inhibitor of the integrated stress response (ISR), we (i) relieve ISR activation (ii) reverse the increased risk-taking behavioral phenotype and (iii) restore cell-specific synaptic function in the affected mice. Our results indicate that targeting the ISR even at late time points after injury can permanently reverse behavioral changes. As such, pharmacological inhibition of the ISR emerges as a promising avenue to combat rTBI-induced behavioral dysfunction.

key words: repetitive mild traumatic brain injury, integrated stress response, risk-taking behavior, synaptic function, prefrontal cortex

INTRODUCTION

Traumatic brain injury (TBI) is a growing health problem. Approximately 5 million Americans are living with a TBI related disability¹⁻⁴, and even mild TBI can elicit chronic cognitive dysfunction and functional limitation^{2, 3, 5}. Furthermore, a prior TBI increases the risk for additional TBI⁶⁻⁸, making it imperative to understand the potential compounding effects of repetitive trauma. Rising concern for repetitive head trauma has led to an increase in studies of concussive and sub-concussive injuries (reviewed in^{9, 10}). While there is still debate as to the severity of symptoms, the majority of studies report poorer cognitive and behavioral outcomes in individuals following multiple mild head traumas⁹⁻¹¹. Specifically, increased risk-taking behavior and disinhibited symptoms are often reported by individuals who have suffered from mild single or repetitive traumatic brain injury (rTBI)¹²⁻¹⁷. Despite the increased interest in understanding the long-term consequences of rTBI, little is known about the underlying cellular and functional correlates responsible for these maladaptive behavioral and cognitive changes. With no identified mechanisms, there are no pharmacological treatment options for individuals suffering from rTBI-induced deficits.

The integrated stress response (ISR) is a universal cellular pathway that serves to cope with different cellular stressors¹⁸ and it is induced after a single TBI¹⁹⁻²¹. In mammals, activation of the ISR modulates protein translation through the phosphorylation of a single serine on the α subunit of the translation initiation factor eIF2, which leads to a reduction in global protein translation^{22, 23}. A select number of proteins are up-regulated with ISR activation including the β -site APP-cleaving enzyme 1 (BACE1)²⁴⁻²⁷ and activating transcription factor 4 (ATF4)^{28, 29}. Chronic ISR activation creates maladaptive cellular and functional changes including cognitive decline^{19, 30-32}. It is not known if mild rTBI induces ISR activation and if it plays a role in behavioral changes and the associated neuronal dysfunction in the prefrontal cortex. Recently, we discovered a potent non-toxic ISR inhibitor (ISRIB)³³⁻³⁵ that reverses cognitive deficits resulting from single impact TBI. When administered weeks after injury, ISRIB treatment reversed memory deficits in two distinct focal and diffuse TBI rodent models¹⁹. Furthermore, ISRIB reversed trauma-induced suppression in hippocampal

long-term potentiation. These findings lay the foundation for targeting the ISR for reversal of trauma-induced behavioral changes in the prefrontal cortex.

We recently developed a preclinical rodent model to investigate cognitive and behavioral outcomes associated with rTBI³⁶. In this model, animals receive a mild impact to the head daily for five days. When we investigated behavioral (risk-taking and social behaviors) and cognitive performance (episodic and working memory), we found behavioral impairments in animals that received five mild impacts. This is in line with other studies of repetitive TBI in rodent models³⁷⁻³⁹ and corresponds with patient reported outcomes following rTBI¹²⁻¹⁵. Our previous studies did not measure significant changes in the intrinsic excitability of layer V (L5) pyramidal neurons in the medial prefrontal cortex (mPFC), a brain region vital for integration of these complex behaviors⁴⁰⁻⁴². In the current study, we evaluate excitatory synaptic input in L5 pyramidal neurons and explicitly assess function by neuronal subtype, as divergent patterns of synaptic input have been reported in L5 pyramidal neuron subtypes⁴³. Specifically, we analyze the two subtypes of L5 pyramidal neurons that project subcortically (“type A”) or callosally to other cortical locations (“type B”)⁴³. Finally, we investigate if ISR activation plays a role in these deficits and if ISRIB treatment can reverse behavioral and cellular changes associated with rTBI.

METHODS

Animals. All experiments were conducted in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco (AN170302). Male and female C57B6/J wild-type (WT) mice were received from Jackson Laboratories. Male animals were 8-10 weeks of age at the time of surgeries. Female animals were 10 weeks of age at the time of surgery to ensure full sexual maturity. Animal shipments were received at least one week prior to start of experimentation to allow animals to habituate to the new surroundings. Mice were group housed in environmentally controlled conditions with a reverse light cycle (12:12 h light: dark cycle at 21 ± 1 °C; ~50% humidity) and provided food and water ad libitum.

Surgical Procedure. All animals were randomly assigned to each TBI or sham surgery groups. Animals were anesthetized and maintained at 2-2.5% isoflurane.

Sham with sutures: Sham with sutures was performed as previously described³⁷. Briefly, animals were secured to a stereotaxic frame with nontraumatic ear bars. A midline incision was made to expose the skull and the scalp was sutured.

Sham with no sutures: Sham without sutures was performed as previously described³⁶.

Repetitive mild TBI: rTBI animals underwent multiple mild closed head injuries using the CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) device as previously reported^{36, 38, 39}. Briefly, mice were placed supinely into an angled holding platform to ensure their head was level with the piston target hole and that the impact was centered at the midline dorsal convexity of the skull, targeting bregma. A nose cone delivering isoflurane was removed just prior to impact. The impact was administered with a velocity range of ~3.9-4.5 m/s, resulting in an impact energy of 0.5 J from the 5 mm, 50 g piston. rTBI animals received an injury once per day for 5 days with ~ 24 hour interval in between impacts.

Focal TBI: Controlled Cortical Impact (CCI). CCI surgery was performed as previously described^{19, 37, 40}. Briefly, animals were secured to a stereotaxic frame with nontraumatic ear bars. A midline incision was made to expose the skull followed by a 3.5-mm diameter craniectomy, a removal of part of the skull, using an electric microdrill. The coordinates of the craniectomy were: anteroposterior, -2.00 mm and mediolateral, +2.00 mm with respect to bregma. Any animal that experienced excessive bleeding due to disruption of the dura was removed from the study. After the craniectomy, the contusion was induced using a 3-mm convex tip attached to an electromagnetic impactor (Leica). The contusion depth was set to 0.95 mm from dura with a velocity of 4.0 m/s sustained for 300 ms. Following impact the scalp was sutured. These injury parameters were chosen to target, but not penetrate, the hippocampus.

Concussive TBI: Closed Head Injury. CHI surgery was performed as previously described¹⁹. Briefly, animals were secured to a stereotaxic frame with nontraumatic ear bars and the

head of the animal was supported with foam before injury. Contusion was induced using a 5-mm convex tip attached to an electromagnetic impactor (Leica) at the following coordinates: anteroposterior, -1.50 mm and mediolateral, 0 mm with respect to bregma. The contusion was produced with an impact depth of 1 mm from the surface of the skull with a velocity of 5.0 m/s sustained for 300 ms. Any animals that had a fractured skull after injury were excluded from the study. Following impact the scalp was sutured.

The approximate isoflurane exposure for each procedure is as follows: ~20-25 minutes for CCI and CHI, ~15-25 minutes for rTBI (3-5 minutes per exposure, and 5 exposures total), ~20 minutes for sham with sutures, ~15-25 minutes for sham without sutures (3-5 minutes per exposure, and 5 exposures total). After all surgeries, the animal recovered in an incubation chamber set to 37 °C. Animals were returned to their home cage after showing normal walking and grooming behavior. All animals fully recovered from the surgical procedures as exhibited by normal behavior and weight maintenance monitored throughout the duration of the experiments.

Drug Administration. ISRIB solution was made by dissolving 5 mg ISRIB in 1 mL

dimethyl sulfoxide (DMSO) (PanReac AppliChem, 191954.1611). The solution was gently heated in a 40 °C waterbath and vortexed every 30 s until the solution became clear. Next 1 mL of Tween 80 (Sigma Aldrich, P8074) was added, solution was gently heated in a 40 °C waterbath and vortexed every 30 s until the solution became clear. Next, 10 ml of polyethylene glycol 400 (PEG400) (PanReac AppliChem, 142436.1611), solution was added gently heated in a 40 °C waterbath and vortexed every 30 s until the solution became clear. Finally, 36.5 ml of 5% dextrose (Hospira, RL-3040) was added. The solution was kept at room temperature throughout the experiment. Each solution was used for injections up to 7 d maximum. The vehicle solution consisted of the same chemical composition and concentration (DMSO, Tween 80, PEG400 and 5% dextrose). Stock ISRIB solution was at 0.1 mg/ml, but injections were at 2.5 mg/kg. We previously reported that a dose of 2.5 mg/kg reversed trauma-induced cognitive decline¹⁹ and improved memory function in healthy animals³³. Additionally, we identified that a minimal dosing paradigm (3-4 treatments) was sufficient for learning and memory rescue¹⁹.

Risk-taking Behavioral test. For all behavioral assays the experimenters were blinded to surgery and drug intervention. Prior to behavioral analysis animals were inspected for gross motor impairments. Animals were inspected for suture healing, whisker loss, limb immobility (included grip strength) and eye occlusions. If animals displayed any of these impairments, they were eliminated from the study. Behavioral tests were recorded and scored using a video tracking and analysis setup (Ethovision XT 8.5, Noldus Information Technology). If tracking was unsuccessful, videos were scored by two individuals blinded to surgery and treatment.

Risk-taking behavioral phenotype was evaluated using the Elevated Plus Maze (EPM) between 21-31 days (~one-month) post injury (counted from the day of the first injury) as previously described³⁶. The EPM consists of two exposed, open arms (35 cm) opposite each other and two enclosed arms (30.5 cm) also across from each other. The four arms are attached to a center platform (4.5 cm square) and the entire maze are elevated 40 cm off the floor. Bright white lights illuminated both ends of the open arm. Mice were placed individually onto the center of the maze and allowed to explore the maze for 5 minutes and their activity was recorded. The maze was cleaned with 70% ethanol between animals. Risk-taking behavior was measured by changes in time spent in the open arms + center.

No difference in time spent exploring the open arms was observed when comparing the sham animals +/- sutures and animals +/- vehicle injections (**Supplemental Table 1**) therefore sham groups were combined for analysis. No differences in time spent exploring the open arms were observed when comparing the rTBI +/- vehicle injections (**Supplemental Table 2**) thus rTBI groups were combined for analysis. Finally, no differences were observed when comparing animals that were exposed to the EPM once or twice (with three months in between exposures, **Supplemental Table 3**).

Western Blot Analysis.

At the end of the behavioral test, animals were sacrificed and the hemibrains were extracted and snap frozen on dry-ice and then stored at -80°C. Hemibrains were used for protein analysis as preliminary analysis found insufficient protein amounts (ATF4, BACE-1) in isolated brain regions. The frozen samples were then homogenized with a T 10 basic

10

ULTRA-TURRAX (IKA) in ice-cold buffer lysis (Cell Signaling 9803) and protease and phosphatase inhibitors (Roche). Lysates were sonicated for 3 min and centrifuged at 13,000 rpm for 20 minutes at 4°C. Protein concentration in supernatants was determined using BCA Protein Assay Kit (Pierce). Equal amount of proteins (30 µg) was loaded on SDS-PAGE gels. Proteins were transferred onto 0.2 µm PVDF membranes (BioRad) and probed with primary antibodies diluted in Tris-buffered saline supplemented with 0.1% Tween 20 and 3% bovine serum albumin.

Phospho-eIF2α (Cell Signaling), eIF2α (Cell signaling), BACE-1 (Cell Signaling), ATF4 (Abcam) and β-actin (Sigma-Aldrich) antibodies were used as primary antibodies. HRP-conjugated secondary antibodies (Rockland) were employed to detect immune-reactive bands using enhanced chemiluminescence (ECL Western Blotting Substrate, Pierce) according to the manufacturer instructions. Quantification of protein bands was done by densitometry using ImageJ software.

Phospho-eIF2α was normalized to total eIF2 protein levels. ATF4 and BACE-1 levels were normalized to β-actin expression and fold change is calculated as the levels relative to the expression in sham + vehicle-treated derived samples.

Electrophysiology.

Coronal brain slices (250 µm) including the medial prefrontal cortex were prepared from mice that underwent rTBI or a sham procedure, 35-60 days prior, as well as mice that underwent rTBI and received an ISRIB injection at ~30 days post injury. In order to conserve mice, a sham group that received an ISRIB injection was not included, as there was no difference in EPM behavior at 1 month after injury between sham mice alone compared to sham+ISRIB mice. Mice were anesthetized with Euthazol (0.1 ml/25 g, Virbac, Fort Worth, TX, NDC-051311-050-01), and transcardially perfused with an ice-cold sucrose solution containing (in mM): 210 sucrose, 1.25 NaH₂PO₄, 25 NaHCO₃, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 7 dextrose, 1.3 ascorbic acid, 3 sodium pyruvate (bubbled with 95% O₂-5% CO₂, pH 7.4) (see **Supplemental Table 4** for reagent information). Mice were then decapitated, and the brain was isolated in the same sucrose solution, and cut on a slicing vibratome (Leica, VT1200S, Leica Microsystems, Wetzlar, Germany). Slices were incubated in a holding solution (composed of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂, 10 dextrose, 1.3 ascorbic acid, 3 sodium pyruvate, bubbled with 95%

O₂-5% CO₂, pH 7.4) at 36°C for 30 minutes and then at room temperature for at least 30 minutes until recording.

Whole cell recordings were obtained from these slices in a submersion chamber with a heated (32–34°C) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 10 dextrose (bubbled with 95% O₂/5% CO₂, pH 7.4). Patch pipettes (3–6MΩ) were manufactured from filamented borosilicate glass capillaries (Sutter Instruments, Novato, CA, BF100-58-10) and filled with an intracellular solution containing (in mM): 135 KGlucuronate, 5 KCl, 10 HEPES, 4 NaCl, 4 MgATP, 0.3 Na₃GTP, 7 2K-phosphocreatine, and 1-2% biocytin. Layer V pyramidal neurons were identified using infrared microscopy with a 40× water-immersion objective (Olympus, Burlingame, CA). Recordings were made using a Multiclamp 700B (Molecular Devices, San Jose, CA) amplifier, which was connected to the computer with a Digidata 1440A ADC (Molecular Devices, San Jose, CA), and recorded at a sampling rate of 20k Hz with pClamp software (Molecular Devices, San Jose, CA). We did not correct for the junction potential, but access resistance and pipette capacitance were appropriately compensated before each recording.

The passive membrane and active action potential spiking characteristics were assessed by injection of a series of hyperpolarizing and depolarizing current steps with a duration of 250 msec from –250 pA to 700 pA (in increments of 50 pA). The resting membrane potential was the measured voltage of the cell 5 minutes after obtaining whole cell configuration without current injection; this value thus represents the resting potential of the cell after internal dialysis with the intracellular solution reported above. A holding current was then applied to maintain the neuron at ~-67 mV before/after current injections. The membrane resistance was determined from the steady-state voltage reached during the -50 pA current injection. The time constant was the time required to reach 63% of the maximum change in voltage for the -50 pA current injection. Action potential parameters including the half width, threshold, amplitude and spike afterhyperpolarization (AHP) were measured from the current injection that was 100 pA above the first current injection that elicited spiking. Action potential times were detected by recording the time at which the positive slope of the membrane potential crossed 0 mV. From the action potential times, the instantaneous frequency for each action potential

was determined (1/inter spike interval). Action potential rate as a function of current injection was examined by plotting the first instantaneous action potential frequency versus current injection amplitude. The F/I slope was then determined from the best linear fit of the positive values of this plot. The action potential or spike threshold was defined as the voltage at which the third derivative of V (d^3V/dt) was maximal just prior to the action potential peak. The action potential (AP) amplitude was calculated by measuring the voltage difference between the peak voltage of the action potential and the spike threshold. The half width of the action potential was determined as the duration of the action potential at half the amplitude. The spike AHP was the voltage difference between the AP threshold and the minimum voltage prior to the next action potential. The rising and falling slopes of the action potential were the maximum of the first derivative of V between the threshold to the peak amplitude, and the peak amplitude to the minimum voltage prior to the next AP, respectively. The adaptation index of each cell was the ratio of the last over the first instantaneous firing frequency, calculated at 250 pA above the current step that first elicited spiking.

Subthreshold properties indicative of the degree of I_H current were assessed with two parameters: 1) the voltage sag- defined as the change in voltage between the maximum hyperpolarization and the steady state potential for the -200 pA current injection; 2) the rebound afterdepolarization (ADP)- defined as the change in voltage between the maximum depolarization and the steady state potential achieved immediately after ending the -200 pA current injection. Finally, the rebound afterhyperpolarization was measured as the change in voltage between the maximum hyperpolarization and the steady state potential achieved immediately after ending the +250 pA current injection. The sum of these three variables was used as previously described to categorize pyramidal neurons as type A (> 6,5 mv) or type B (<6.5 mV)⁴¹.

To measure the spontaneous excitatory postsynaptic currents (sEPSCs), cells were recorded in voltage clamp at a holding potential of -75 mV for 4 minutes, a holding potential that should have little inhibitory component given the reversal potential of chloride with these solutions. Analysis of sEPSCs was performed using a template matching algorithm in ClampFit 10.7 (Molecular Devices, San Jose, CA). The template was created using recordings from multiple pyramidal cells and included several hundred synaptic

events. Access resistance (Ra) was monitored during recordings, and recordings were terminated if Ra exceeded 30 megaohms. Only stable recordings (< 50 pA baseline change) with a low baseline noise (< 8 pA root mean square) were included. The first 250 synaptic events or all the events measured in the 4 min interval from each cell were included for analysis. The median frequency and amplitude for these events was calculated for each cell to compare between groups.

Statistical Analysis.

All data were analyzed with GraphPad Prism 8 statistical software. Statistical significance between groups for most variables was determined using a two-tailed *t*-test, one-way ANOVA, or two-way ANOVA. If data were outside of the normal distribution as indicated by an alpha < 0.01 for both D'Agostino-Pearson omnibus and Shapiro-Wilk tests, a nonparametric test was used, specifically a Kruskal-Wallis test was used for the electrophysiology parameters. The firing responses to increasing current injections were analyzed as a repeated measures two way ANOVA. P values below 0.05 were considered significant.

RESULTS

Repetitive mild traumatic brain injury increases risk-taking behaviors in males but not female cohorts.

We previously reported that five mild repetitive head traumas (rTBI) resulted in increased risk-taking behaviors in male rodents³⁶. Here we wanted to determine if the changes in risk-taking behavior measured after rTBI are also observed after other commonly used single injury models and if female mice are equally impaired. We compared trauma-induced behavioral deficits between: (i) five rTBI³⁶, (ii) a single mild concussive closed head injury (CHI)¹⁹, (iii) a moderate, focal injury model-controlled cortical impact (CCI)^{19, 37, 40}, and (iv) sham surgery in male and female mice. We evaluated changes in risk-taking behavior approximately one-month post injury with the elevated plus maze (EPM) testing paradigm, **Figure 1A**. In the EPM, animals are positioned in the center of a four-arm arena and allowed to freely explore the maze composed of two dark arms and two brightly illuminated arms for five minutes. Healthy rodents explore all the arms but spend the majority of time in the closed arms⁴². Increased time spent exploring the open arms

denotes increased risk-taking behavior. When comparing different trauma models we found that only male mice that received rTBI spent significantly more time in the open arms compared to sham control animals (**Figure 1B**). None of the injury models evaluated impacted risk-taking behavior in the female cohorts (**Figure 1C**). When comparing male and female rTBI mice, we measured a significant sex-dependent injury effect; furthermore, we did not observe differences in the time-spent in the open arms when comparing male and female sham animals. These results demonstrate that rTBI results in increased risk-taking behavior in male but not in female mice. Since no deficits were observed in female mice, we focused on rTBI-induced cellular changes in male mice.

The integrated stress response is activated at one-month after rTBI.

Aberrant activation of the integrated stress response (ISR) has been measured acutely and chronically in different models of TBI¹⁹⁻²¹. Here, we investigated if mild rTBI induces chronic activation of the ISR by quantifying p-eIF2 α , ATF4, and BACE-1 levels in hemibrain lysates ~one-month post injury (**Supplemental Figure 1**). When investigating the impact of rTBI and ISRIB on p-eIF2 α protein expression we measured a significant injury ($p < 0.001$) and ISRIB effect ($p < 0.01$) (**Figure 2A**). Downstream modulators of ISR activation, ATF4 and BACE-1 were significantly increased when comparing sham and rTBI male mice (**Figure 2B,C**). Importantly, ISRIB administration reversed rTBI-induced ISR activation and downstream molecules to levels comparable to sham animals (**Figure 2**). These data indicate that rTBI triggers a persistent activation of the ISR and that ISRIB can relieve this activation.

The small-molecule Integrated Stress Response Inhibitor, ISRIB, reverses rTBI mediated increased risk-taking behavior.

We previously demonstrated that ISR inhibition with the small molecule ISRIB can fully reverse trauma-induced hippocampal-dependent learning and memory deficits¹⁹. Here, we investigated if ISR inhibition can reverse the increased risk-taking behavior. Male mice received five mild head injuries or sham surgery. We then assessed risk-taking behavior one-month post injury with the EPM (**Figure 3A**). The evening before EPM (day 30), animals received intraperitoneal injections of ISRIB (2.5 mg/kg) or vehicle (**Figure 3A**).

Quantification of the time spent in the open arms was indistinguishable when comparing rTBI + ISRIB and sham groups (**Figure 3B**). Notably, a single injection of ISRIB was sufficient to reverse the rTBI-induced risk-taking phenotype.

Next, we investigated if ISRIB could permanently reverse rTBI-induced risk-taking behavior. Animals received two additional doses of ISRIB, one immediately after EPM (day 31) and one the next day (day 32, **Figure 3A**- three doses in total¹⁹). At ~three months (100 days) post injury, we measured risk-taking behavior. No additional doses of ISRIB were administered following the initial three injections (**Figure 3A**). Once again, rTBI + vehicle animals displayed significant increases in time spent in the open arms of the EPM (**Figure 3C**). Importantly, the three ISRIB injections administered ~70 days prior reversed long-term rTBI-induced risk-taking behavior (**Figure 3C**). ISRIB administration also reduced time spent in the open arms in sham animals. These data demonstrate that a pulse administration of ISRIB, one month after injury, can completely reverse the long-lasting risk-taking behavior phenotype following rTBI.

ISRIB treatment restores cell-specific vulnerability in type A layer V pyramidal neurons of the medial prefrontal cortex after rTBI.

The mPFC is a brain region implicated in complex behaviors, including contextual response and risk-taking behavior⁴³. To investigate the neurophysiological correlates of risk-taking behavior after rTBI and ISRIB treatment, we evaluated the intrinsic and synaptic properties of layer V pyramidal neurons in the mPFC, the primary output of the microcircuit. Layer V pyramidal neurons include at least two subtypes, denoted type A and type B, characterized by their morphology, intrinsic ion channel composition and axonal projections to primarily subcortical or other cortical regions, respectively⁴¹ (**Figures 4A and 5A**).

We distinguished these layer V neuronal subtypes by their electrophysiological properties (with prominence of the I_h -induced parameters in type A neurons including the sag and rebound in response to hyperpolarization, as well as the afterhyperpolarization following depolarization), as previously reported⁴¹. We detected no effect of trauma on the intrinsic excitability of either type A or type B neurons, similar to our prior results that grouped layer V neurons together³⁶ (**Supplemental Figures 2 and 3, Supplemental Tables 5 and 6**).

Conversely, we found a cell-specific change in synaptic input after rTBI. Specifically, type A neurons demonstrated a significant increase in the frequency of spontaneous excitatory synaptic currents (sEPSC) after rTBI (**Figure 4B,C**) without changes in the amplitude of sEPSCs (**Figure 4B,D**). Strikingly, a single treatment with ISRIB ~one month after rTBI reversed this synaptic hyperexcitability, decreasing the frequency of synaptic input to sham levels (**Figure 4B,C**). Excitatory synaptic frequency and amplitude did not change after rTBI or with ISRIB treatment in type B neurons (**Figure 5**).

DISCUSSION

An estimated three million traumatic brain injuries (TBIs) are reported annually in the US. 70-90% of the reported cases are considered mild⁴⁴ and anywhere from 10-40% of these individuals will develop long-term behavioral and cognitive impairments⁴⁵⁻⁴⁷. Often deficits may not initially be observed after a single TBI, however additional head injuries can have cumulating effects including both increased susceptibility to future injury as well as behavioral and cognitive deficits⁶⁻⁸. There are no clinically available therapeutics to treat or even ameliorate the devastating effects of mild repetitive TBI (rTBI). In this study we found that male mice which received a mild TBI daily for five days (5 total) displayed increased risk-taking behavior measured 1 and 3 months later. This behavior was specific to both type of trauma and sex of the animal. We detected no increase in risk-taking behavior in male mice exposed to other trauma models including a single focal injury and a single concussive injury without rotational acceleration. Furthermore, female mice exposed to an identical rTBI paradigm did not display increased risk-taking behavior. This persistent behavioral phenotype corroborates the long-term maladaptive behavioral effects of rTBI also observed in humans. At the cellular and functional level, we found that the risk-taking behavior was associated with increased activation of the integrated stress response (ISR) and cell-specific synaptic alterations in the type A subtype of layer V pyramidal neurons of the mPFC. Importantly, targeting the ISR using the small molecule inhibitor ISRIB reversed rTBI-induced risk-taking behavior and the associated cell-specific deficits in synaptic function. Thus, we have identified for the first time the effectivity of ISRIB in reversing chronic behavioral deficits after rTBI along with cell-specific synaptic dysfunction in the mPFC.

In the general population, men are 40% more likely to suffer from a TBI^{48, 49}. However, studies of female athletes have found that women suffer more concussions than men when playing the same sport⁵⁰, making understanding of sex-dimorphic injury responses critical for a comprehensive appreciation of rTBI pathology. Furthermore, a recent review suggested that in ~93% of preclinical TBI studies biological sex as a variable was ignored⁵¹. Here we identified a different behavioral response to rTBI (5 midline impacts) in male and female mice. Whereas multiple mild traumas resulted in significant increased risk-taking behavior in males, female rodents did not develop this maladaptive behavioral response. To our knowledge, this is the first study to investigate sex-dimorphic responses at a chronic time point after repetitive TBI. Only one other group has investigated sex differences acutely after a unilateral rTBI (3 total impacts) — in adolescent rats and measured increased anxiety-like behavior in both male and female rats^{52, 53}. The behavioral endpoint, number of impacts or placement of the impact could account for the differences in our readouts. Other studies of repetitive head injury replicated this increase in risk-taking phenotype in male mice⁵⁴⁻⁵⁷; however, sex differences were not evaluated in these previous reports.

Young female athletes are reported to have increased rates of concussion and more neurologic symptoms that last longer compared to male counterparts⁵⁸⁻⁶⁰. However, the frequency of global disability measured in females after work-related concussion was lower when compared to male cohorts⁵⁸. Importantly, superior executive function measured with the Wisconsin Card Sorting Test was identified in females compared to males with TBI after controlling for education, ethnicity, and injury severity⁶¹. These studies suggest that much more research is needed to identify factors affecting vulnerability or resilience to TBI, especially rTBI, in women. Likewise, the physiology underlying the sex-dimorphic response to rTBI that we have identified here is unclear. The biophysics of injury to the mPFC might be different in female mice given their smaller head size⁶². Notably, the volume of the mPFC exhibits sexual dimorphism in humans⁶³ and inhibitory neuronal subtypes show striking differences in composition by sex in some brain regions⁶⁴. These differences might contribute to increased vulnerability to repetitive brain injury in male mice, however, the exact mechanism remains to be determined.

The ISR is a universal pathway that responds to a wide array of cellular stressors. The central regulatory step of the ISR is the phosphorylation of the α -subunit of eIF2^{18, 65}. Phosphorylation of eIF2 α leads to inhibition of general protein synthesis^{22, 23}, with translation of a few, select proteins including ATF4^{28, 29} and BACE-1²⁴⁻²⁷. Previously, our group and others have identified that both focal and diffuse head injuries induce phosphorylation of eIF2 α in the brain chronically after injury¹⁹⁻²¹. Brief interference with this pathway using the small-molecule inhibitor ISRIB at chronic time points completely rescued trauma-induced hippocampal-dependent learning and memory deficits¹⁹. In this study we demonstrate that rTBI results in chronic activation of the ISR, and ISRIB administration at ~one month post injury significantly reduced ATF4 and BACE-1 protein levels to those comparable to sham. Furthermore, ISRIB treatment completely reversed rTBI-induced risk-taking behavior and this rescue was long-lasting. Interestingly, while ISRIB treatment had no effect in sham animals acutely after treatment, we measured a long-term effect in the elevated plus maze assay at the three month time point, similar to our previous reports that ISRIB treatment can improve performance in naïve and sham rodents^{19, 33-35}. Our previous studies have focused on hippocampal-dependent memory, but risk-taking behavior has been associated with PFC function^{43, 68}. Taken together these studies suggest that ISR intervention strategies can restore and modify not only cognitive, but also behavioral changes controlled by diverse brain regions.

The mPFC integrates complex sensory information and modulates behavioral response⁶⁹; as such, deficits in mPFC function have been linked to cognitive and behavioral dysfunction, including risk-taking behavior^{43, 68}. Here, we demonstrated that an increase in the frequency of excitatory synaptic input specifically occurs in the type A layer V pyramidal neuron subtype at chronic time points after mild rTBI, associated with risk-taking behavior. This is the first study to examine mPFC function after repetitive TBI, and only one other study has evaluated synaptic input in the mPFC after a single moderate TBI. Smith *et al.* identified a prominent increase in the frequency of spontaneous and miniature excitatory postsynaptic currents and a decrease in the amplitude of inhibitory input in layer 2/3 pyramidal neurons ~1 week after a single lateral fluid percussion injury which was associated with deficits in working memory⁷⁰. In contrast, layer V neurons did not

show changes in synaptic input and risk-taking behavior was not evaluated. The discrepancy between our studies could be due to several factors including a different (and not repetitive) model of injury, the more subacute rather than chronic timing of their studies, and/or in particular, the fact that they did not specifically evaluate layer V neuronal subtypes. Subtype-specific vulnerability of type A or subcortically projecting L5 pyramidal neurons has been identified in other animal models with behavioral deficits, including models of autism⁷¹, suggesting that changes in synaptic input to these neurons might also directly affect mPFC-mediated behaviors after TBI. Properties of synaptic input to type A neurons in the mPFC have been hypothesized to be specifically responsive to focal strong input in the dendritic tree, ultimately providing a salient signal to subcortical structures⁴¹. Thus, altered synaptic properties in type A neurons after repetitive injury might be responsible for lack of appreciation of danger or risk in the animal.

Not only did we identify subtype-specific vulnerability in layer V after TBI, we notably found that this synaptic hyperexcitability could be rescued by a single injection of ISRIB at 1 month after injury. ISRIB has been found to exert activity-dependent changes in synaptic function including blocking mGluR-mediated LTD in the ventral tegmental area (VTA) *in vitro*⁷² and enhancing cocaine-induced LTP in the VTA after *in vivo* injection⁷³. In addition, ISRIB application *in vitro* has rescued TBI-induced deficits in LTP in the hippocampus¹⁹. Here, we expand the range of ISRIB treatment effect to include not only activity-dependent synaptic regulation, but also a rescue of cell-specific vulnerability in synaptic hyperexcitability. These intriguing findings suggest that cell-specific vulnerability, a common feature in neurodegenerative disease and now identified in rTBI, might be specifically addressed with inhibiting cellular stress.

In summary, we demonstrated that rTBI leads to a sex-dependent increase in long-lasting risk-taking behavior in male mice associated with synaptic hyperexcitability specifically in type A L5 pyramidal neurons of the mPFC. For the first time we identified that inhibition of the integrated stress response with ISRIB rescues a mPFC-dependent behavioral deficit and the associated cell-specific vulnerability. Overall these results suggest that the ISR pathway may represent a key target in maladaptive circuit and cognitive functions after injury.

ACKNOWLEDGMENTS

This work was supported by NIH NIA grants R01AG056770 (SR), the generous support of the Roger's family to SR and PW, the Weill Innovation Award to SR and PW, Calico Life Sciences to PW and "Programa de Apoyo a Centros con Financiamiento Basal AFB 170004" to SB at Fundacion Ciencia & Vida. PW is an Investigator of the Howard Hughes Medical Institute. KK was supported by an NRSA post-doctoral fellowship from the NIA F32AG054126. EF is supported by the National Institute for General Medicine (NIGMS) Initiative for Maximizing Student Development (R25GM056847) and the National Science Foundation (NSF) Graduate Fellowship Program. AN is supported by the National Center for Advanced Translational Sciences at NIH (UCSF-CTSI Grant Number TL1 TR001871) and the NIH/NINDS (K08NS114170).

AUTHOR DISCLOSURE STATEMENT: P.W. is an inventor on U.S. Patent 9708247 held by the Regents of the University of California that describes ISRIB and its analogs. Rights to the invention have been licensed by UCSF to Calico.

REFERENCES

1. Smith, D.H., Johnson, V.E. and Stewart, W. (2013). Chronic neuropathologies of single and repetitive TBI: substrates of dementia? *Nat Rev Neurol* 9, 211-221.
2. DeKosky, S.T., Ikonomic, M.D. and Gandy, S. (2010). Traumatic brain injury--football, warfare, and long-term effects. *N Engl J Med* 363, 1293-1296.
3. Engberg, A.W. and Teasdale, T.W. (2004). Psychosocial outcome following traumatic brain injury in adults: a long-term population-based follow-up. *Brain Inj* 18, 533-545.
4. Sullivan, P., Petitti, D. and Barbaccia, J. (1987). Head trauma and age of onset of dementia of the Alzheimer type. *JAMA* 257, 2289-2290.
5. Nelson, L.D., Temkin, N.R., Dikmen, S., Barber, J., Giacino, J.T., Yuh, E., Levin, H.S., McCrea, M.A., Stein, M.B., Mukherjee, P., Okonkwo, D.O., Diaz-Arrastia, R., Manley, G.T., and the, T.-T.B.I.I., Adeoye, O., Badjatia, N., Boase, K., Bodien, Y., Bullock, M.R., Chesnut, R., Corrigan, J.D., Crawford, K., Mis, Duhaime, A.C., Ellenbogen, R., Feeser, V.R., Ferguson, A., Foreman, B., Gardner, R., Gaudette, E., Gonzalez, L., Gopinath, S., Gullapalli, R., Hemphill, J.C., Hotz, G., Jain, S., Korley, F., Kramer, J., Kreitzer, N., Lindsell, C., Machamer, J., Madden, C., Martin, A., McAllister, T., Merchant, R., Noel, F., Palacios, E., Perl, D., Puccio, A., Rabinowitz, M., Robertson, C.S., Rosand, J., Sander, A., Satris, G., Schnyer, D., Seabury, S., Sherer, M., Taylor, S., Toga, A., Valadka, A., Vassar, M.J., Vespa, P., Wang, K., Yue, J.K. and Zafonte, R. (2019). Recovery After Mild Traumatic Brain Injury in Patients Presenting to US Level I Trauma Centers: A Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI) Study. *JAMA Neurol*.
6. Lasry, O., Liu, E.Y., Powell, G.A., Ruel-Laliberte, J., Marcoux, J. and Buckeridge, D.L. (2017). Epidemiology of recurrent traumatic brain injury in the general population: A systematic review. *Neurology* 89, 2198-2209.
7. Belanger, H.G., Spiegel, E. and Vanderploeg, R.D. (2010). Neuropsychological performance following a history of multiple self-reported concussions: a meta-analysis. *J Int Neuropsychol Soc* 16, 262-267.
8. Geddes, J.F., Vowles, G.H., Nicoll, J.A. and Revesz, T. (1999). Neuronal cytoskeletal changes are an early consequence of repetitive head injury. *Acta Neuropathol* 98, 171-178.

9. Manley, G., Gardner, A.J., Schneider, K.J., Guskiewicz, K.M., Bailes, J., Cantu, R.C., Castellani, R.J., Turner, M., Jordan, B.D., Randolph, C., Dvorak, J., Hayden, K.A., Tator, C.H., McCrory, P. and Iverson, G.L. (2017). A systematic review of potential long-term effects of sport-related concussion. *Br J Sports Med* 51, 969-977.
10. McAllister, T. and McCrea, M. (2017). Long-Term Cognitive and Neuropsychiatric Consequences of Repetitive Concussion and Head-Impact Exposure. *J Athl Train* 52, 309-317.
11. Castile, L., Collins, C.L., Mcllvain, N.M. and Comstock, R.D. (2012). The epidemiology of new versus recurrent sports concussions among high school athletes, 2005-2010. *Br J Sports Med* 46, 603-610.
12. McKee, A.C., Cantu, R.C., Nowinski, C.J., Hedley-Whyte, E.T., Gavett, B.E., Budson, A.E., Santini, V.E., Lee, H.S., Kubilus, C.A. and Stern, R.A. (2009). Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. *J Neuropathol Exp Neurol* 68, 709-735.
13. Stern, R.A., Daneshvar, D.H., Baugh, C.M., Seichepine, D.R., Montenigro, P.H., Riley, D.O., Fritts, N.G., Stamm, J.M., Robbins, C.A., McHale, L., Simkin, I., Stein, T.D., Alvarez, V.E., Goldstein, L.E., Budson, A.E., Kowall, N.W., Nowinski, C.J., Cantu, R.C. and McKee, A.C. (2013). Clinical presentation of chronic traumatic encephalopathy. *Neurology* 81, 1122-1129.
14. Mez, J., Daneshvar, D.H., Kiernan, P.T., Abdolmohammadi, B., Alvarez, V.E., Huber, B.R., Alosco, M.L., Solomon, T.M., Nowinski, C.J., McHale, L., Cormier, K.A., Kubilus, C.A., Martin, B.M., Murphy, L., Baugh, C.M., Montenigro, P.H., Chaisson, C.E., Tripodis, Y., Kowall, N.W., Weuve, J., McClean, M.D., Cantu, R.C., Goldstein, L.E., Katz, D.I., Stern, R.A., Stein, T.D. and McKee, A.C. (2017). Clinicopathological Evaluation of Chronic Traumatic Encephalopathy in Players of American Football. *JAMA* 318, 360-370.
15. Juengst, S.B., Kumar, R.G., Arenth, P.M. and Wagner, A.K. (2014). Exploratory associations with tumor necrosis factor-alpha, disinhibition and suicidal endorsement after traumatic brain injury. *Brain Behav Immun* 41, 134-143.

16. Polinder, S., Clossen, M.C., Real, R.G.L., Covic, A., Gorbunova, A., Voormolen, D.C., Master, C.L., Haagsma, J.A., Diaz-Arrastia, R. and von Steinbuechel, N. (2018). A Multidimensional Approach to Post-concussion Symptoms in Mild Traumatic Brain Injury. *Front Neurol* 9, 1113.
17. Tellier, A., Marshall, S.C., Wilson, K.G., Smith, A., Perugini, M. and Stiell, I.G. (2009). The heterogeneity of mild traumatic brain injury: Where do we stand? *Brain Inj* 23, 879-887.
18. Donnelly, N., Gorman, A.M., Gupta, S. and Samali, A. (2013). The eIF2alpha kinases: their structures and functions. *Cell Mol Life Sci* 70, 3493-3511.
19. Chou, A., Krukowski, K., Jopson, T., Zhu, P.J., Costa-Mattioli, M., Walter, P. and Rosi, S. (2017). Inhibition of the integrated stress response reverses cognitive deficits after traumatic brain injury. *Proc Natl Acad Sci U S A* 114, E6420-E6426.
20. Dash, P.K., Hylin, M.J., Hood, K.N., Orsi, S.A., Zhao, J., Redell, J.B., Tsvetkov, A.S. and Moore, A.N. (2015). Inhibition of Eukaryotic Initiation Factor 2 Alpha Phosphatase Reduces Tissue Damage and Improves Learning and Memory after Experimental Traumatic Brain Injury. *J Neurotrauma* 32, 1608-1620.
21. Rubovitch, V., Barak, S., Rachmany, L., Goldstein, R.B., Zilberstein, Y. and Pick, C.G. (2015). The neuroprotective effect of salubrinal in a mouse model of traumatic brain injury. *Neuromolecular Med* 17, 58-70.
22. Hinnebusch, A.G., Ivanov, I.P. and Sonenberg, N. (2016). Translational control by 5'-untranslated regions of eukaryotic mRNAs. *Science* 352, 1413-1416.
23. Sonenberg, N. and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136, 731-745.
24. De Pietri Tonelli, D., Mihailovich, M., Di Cesare, A., Codazzi, F., Grohovaz, F. and Zacchetti, D. (2004). Translational regulation of BACE-1 expression in neuronal and non-neuronal cells. *Nucleic Acids Res* 32, 1808-1817.
25. Lammich, S., Schobel, S., Zimmer, A.K., Lichtenthaler, S.F. and Haass, C. (2004). Expression of the Alzheimer protease BACE1 is suppressed via its 5'-untranslated region. *EMBO Rep* 5, 620-625.
26. Mihailovich, M., Thermann, R., Grohovaz, F., Hentze, M.W. and Zacchetti, D. (2007). Complex translational regulation of BACE1 involves upstream AUGs and stimulatory elements within the 5' untranslated region. *Nucleic Acids Res* 35, 2975-2985.

27. O'Connor, T., Sadleir, K.R., Maus, E., Velliquette, R.A., Zhao, J., Cole, S.L., Eimer, W.A., Hitt, B., Bembinster, L.A., Lammich, S., Lichtenthaler, S.F., Hebert, S.S., De Strooper, B., Haass, C., Bennett, D.A. and Vassar, R. (2008). Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. *Neuron* 60, 988-1009.
28. Chen, A., Muzzio, I.A., Malleret, G., Bartsch, D., Verbitsky, M., Pavlidis, P., Yonan, A.L., Vronskaya, S., Grody, M.B., Cepeda, I., Gilliam, T.C. and Kandel, E.R. (2003). Inducible enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. *Neuron* 39, 655-669.
29. Pasini, S., Corona, C., Liu, J., Greene, L.A. and Shelanski, M.L. (2015). Specific downregulation of hippocampal ATF4 reveals a necessary role in synaptic plasticity and memory. *Cell Rep* 11, 183-191.
30. Costa-Mattioli, M., Gobert, D., Stern, E., Gamache, K., Colina, R., Cuello, C., Sossin, W., Kaufman, R., Pelletier, J., Rosenblum, K., Krnjevic, K., Lacaille, J.C., Nader, K. and Sonenberg, N. (2007). eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. *Cell* 129, 195-206.
31. Batista, G., Johnson, J.L., Dominguez, E., Costa-Mattioli, M. and Pena, J.L. (2016). Translational control of auditory imprinting and structural plasticity by eIF2alpha. *Elife* 5.
32. Jiang, Z., Belforte, J.E., Lu, Y., Yabe, Y., Pickel, J., Smith, C.B., Je, H.S., Lu, B. and Nakazawa, K. (2010). eIF2alpha Phosphorylation-dependent translation in CA1 pyramidal cells impairs hippocampal memory consolidation without affecting general translation. *J Neurosci* 30, 2582-2594.
33. Sidrauski, C., Acosta-Alvear, D., Khoutorsky, A., Vedantham, P., Hearn, B.R., Li, H., Gamache, K., Gallagher, C.M., Ang, K.K., Wilson, C., Okreglak, V., Ashkenazi, A., Hann, B., Nader, K., Arkin, M.R., Renslo, A.R., Sonenberg, N. and Walter, P. (2013). Pharmacological brake-release of mRNA translation enhances cognitive memory. *Elife* 2, e00498.
34. Sidrauski, C., McGeachy, A.M., Ingolia, N.T. and Walter, P. (2015). The small molecule ISRIB reverses the effects of eIF2alpha phosphorylation on translation and stress granule assembly. *Elife* 4.

35. Sidrauski, C., Tsai, J.C., Kampmann, M., Hearn, B.R., Vedantham, P., Jaishankar, P., Sokabe, M., Mendez, A.S., Newton, B.W., Tang, E.L., Verschuere, E., Johnson, J.R., Krogan, N.J., Fraser, C.S., Weissman, J.S., Renslo, A.R. and Walter, P. (2015). Pharmacological dimerization and activation of the exchange factor eIF2B antagonizes the integrated stress response. *Elife* 4, e07314.
36. Nolan, A., Hennessy, E., Krukowski, K., Guglielmetti, C., Chaumeil, M.M., Sohal, V.S. and Rosi, S. (2018). Repeated Mild Head Injury Leads to Wide-Ranging Deficits in Higher-Order Cognitive Functions Associated with the Prefrontal Cortex. *J Neurotrauma* 35, 2425-2434.
37. Krukowski, K., Chou, A., Feng, X., Tiret, B., Paladini, M.S., Riparip, L.K., Chaumeil, M.M., Lemere, C. and Rosi, S. (2018). Traumatic Brain Injury in Aged Mice Induces Chronic Microglia Activation, Synapse Loss, and Complement-Dependent Memory Deficits. *Int J Mol Sci* 19.
38. Namjoshi, D.R., Cheng, W.H., McInnes, K.A., Martens, K.M., Carr, M., Wilkinson, A., Fan, J., Robert, J., Hayat, A., Cripton, P.A. and Wellington, C.L. (2014). Merging pathology with biomechanics using CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration): a novel, surgery-free model of traumatic brain injury. *Mol Neurodegener* 9, 55.
39. Namjoshi, D.R., Cheng, W.H., Bashir, A., Wilkinson, A., Stukas, S., Martens, K.M., Whyte, T., Abebe, Z.A., McInnes, K.A., Cripton, P.A. and Wellington, C.L. (2017). Defining the biomechanical and biological threshold of murine mild traumatic brain injury using CHIMERA (Closed Head Impact Model of Engineered Rotational Acceleration). *Exp Neurol* 292, 80-91.
40. Chou, A., Krukowski, K., Morganti, J.M., Riparip, L.K. and Rosi, S. (2018). Persistent Infiltration and Impaired Response of Peripherally-Derived Monocytes after Traumatic Brain Injury in the Aged Brain. *Int J Mol Sci* 19.
41. Lee, A.T., Gee, S.M., Vogt, D., Patel, T., Rubenstein, J.L. and Sohal, V.S. (2014). Pyramidal neurons in prefrontal cortex receive subtype-specific forms of excitation and inhibition. *Neuron* 81, 61-68.
42. Lister, R.G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* 92, 180-185.

43. Bogg, T., Fukunaga, R., Finn, P.R. and Brown, J.W. (2012). Cognitive control links alcohol use, trait disinhibition, and reduced cognitive capacity: Evidence for medial prefrontal cortex dysregulation during reward-seeking behavior. *Drug Alcohol Depend* 122, 112-118.
44. Cassidy, J.D., Carroll, L.J., Peloso, P.M., Borg, J., von Holst, H., Holm, L., Kraus, J., Coronado, V.G. and Injury, W.H.O.C.C.T.F.o.M.T.B. (2004). Incidence, risk factors and prevention of mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. *J Rehabil Med*, 28-60.
45. Binder, L.M., Rohling, M.L. and Larrabee, G.J. (1997). A review of mild head trauma. Part I: Meta-analytic review of neuropsychological studies. *J Clin Exp Neuropsychol* 19, 421-431.
46. Willer, B. and Leddy, J.J. (2006). Management of concussion and post-concussion syndrome. *Curr Treat Options Neurol* 8, 415-426.
47. Mittenberg, W., Canyock, E.M., Condit, D. and Patton, C. (2001). Treatment of post-concussion syndrome following mild head injury. *J Clin Exp Neuropsychol* 23, 829-836.
48. Faul, M. and Coronado, V. (2015). Epidemiology of traumatic brain injury. *Handb Clin Neurol* 127, 3-13.
49. Coronado, V.G., McGuire, L.C., Sarmiento, K., Bell, J., Lionbarger, M.R., Jones, C.D., Geller, A.I., Khoury, N. and Xu, L. (2012). Trends in Traumatic Brain Injury in the U.S. and the public health response: 1995-2009. *J Safety Res* 43, 299-307.
50. Covassin, T., Moran, R. and Elbin, R.J. (2016). Sex Differences in Reported Concussion Injury Rates and Time Loss From Participation: An Update of the National Collegiate Athletic Association Injury Surveillance Program From 2004-2005 Through 2008-2009. *J Athl Train* 51, 189-194.
51. Spani, C.B., Braun, D.J. and Van Eldik, L.J. (2018). Sex-related responses after traumatic brain injury: Considerations for preclinical modeling. *Front Neuroendocrinol* 50, 52-66.
52. Yamakawa, G.R., Lengkeek, C., Salberg, S., Spanswick, S.C. and Mychasiuk, R. (2017). Behavioral and pathophysiological outcomes associated with caffeine consumption and repetitive mild traumatic brain injury (RmTBI) in adolescent rats. *PLoS One* 12, e0187218.
53. Wright, D.K., O'Brien, T.J., Shultz, S.R. and Mychasiuk, R. (2017). Sex matters: repetitive mild traumatic brain injury in adolescent rats. *Ann Clin Transl Neurol* 4, 640-654.

54. Mouzon, B.C., Bachmeier, C., Ferro, A., Ojo, J.O., Crynen, G., Acker, C.M., Davies, P., Mullan, M., Stewart, W. and Crawford, F. (2014). Chronic neuropathological and neurobehavioral changes in a repetitive mild traumatic brain injury model. *Ann Neurol* 75, 241-254.
55. Petraglia, A.L., Plog, B.A., Dayawansa, S., Chen, M., Dashnaw, M.L., Czerniecka, K., Walker, C.T., Viterise, T., Hyrien, O., Iliff, J.J., Deane, R., Nedergaard, M. and Huang, J.H. (2014). The spectrum of neurobehavioral sequelae after repetitive mild traumatic brain injury: a novel mouse model of chronic traumatic encephalopathy. *J Neurotrauma* 31, 1211-1224.
56. Kondo, A., Shahpasand, K., Mannix, R., Qiu, J., Moncaster, J., Chen, C.H., Yao, Y., Lin, Y.M., Driver, J.A., Sun, Y., Wei, S., Luo, M.L., Albayram, O., Huang, P., Rotenberg, A., Ryo, A., Goldstein, L.E., Pascual-Leone, A., McKee, A.C., Meehan, W., Zhou, X.Z. and Lu, K.P. (2015). Antibody against early driver of neurodegeneration cis P-tau blocks brain injury and tauopathy. *Nature* 523, 431-436.
57. Mouzon, B.C., Bachmeier, C., Ojo, J.O., Acker, C.M., Ferguson, S., Paris, D., Ait-Ghezala, G., Crynen, G., Davies, P., Mullan, M., Stewart, W. and Crawford, F. (2018). Lifelong behavioral and neuropathological consequences of repetitive mild traumatic brain injury. *Ann Clin Transl Neurol* 5, 64-80.
58. Mollayeva, T., El-Khechen-Richandi, G. and Colantonio, A. (2018). Sex & gender considerations in concussion research. *Concussion* 3, CNC51.
59. Covassin, T., Schatz, P. and Swanik, C.B. (2007). Sex differences in neuropsychological function and post-concussion symptoms of concussed collegiate athletes. *Neurosurgery* 61, 345-350; discussion 350-341.
60. Hatskevich, C.W., Itkis, M.L. and Maloletnev, V.I. (1992). Off-line methods for detection and correction of EEG artefacts of various origin. *Int J Psychophysiol* 12, 179-185.
61. Niemeier, J.P., Marwitz, J.H., Leshner, K., Walker, W.C. and Bushnik, T. (2007). Gender differences in executive functions following traumatic brain injury. *Neuropsychol Rehabil* 17, 293-313.
62. Caccese, J.B., Buckley, T.A., Tierney, R.T., Arbogast, K.B., Rose, W.C., Glutting, J.J. and Kaminski, T.W. (2018). Head and neck size and neck strength predict linear and rotational acceleration during purposeful soccer heading. *Sports Biomech* 17, 462-476.

63. Ruigrok, A.N., Salimi-Khorshidi, G., Lai, M.C., Baron-Cohen, S., Lombardo, M.V., Tait, R.J. and Suckling, J. (2014). A meta-analysis of sex differences in human brain structure. *Neurosci Biobehav Rev* 39, 34-50.
64. Kim, Y., Yang, G.R., Pradhan, K., Venkataraju, K.U., Bota, M., Garcia Del Molino, L.C., Fitzgerald, G., Ram, K., He, M., Levine, J.M., Mitra, P., Huang, Z.J., Wang, X.J. and Osten, P. (2017). Brain-wide Maps Reveal Stereotyped Cell-Type-Based Cortical Architecture and Subcortical Sexual Dimorphism. *Cell* 171, 456-469 e422.
65. Ron, D. and Harding, H.P. (2012). Protein-folding homeostasis in the endoplasmic reticulum and nutritional regulation. *Cold Spring Harb Perspect Biol* 4.
66. Tsai, J.C., Miller-Vedam, L.E., Anand, A.A., Jaishankar, P., Nguyen, H.C., Renslo, A.R., Frost, A. and Walter, P. (2018). Structure of the nucleotide exchange factor eIF2B reveals mechanism of memory-enhancing molecule. *Science* 359.
67. Zyryanova, A.F., Weis, F., Faille, A., Alard, A.A., Crespillo-Casado, A., Sekine, Y., Harding, H.P., Allen, F., Parts, L., Fromont, C., Fischer, P.M., Warren, A.J. and Ron, D. (2018). Binding of ISRIB reveals a regulatory site in the nucleotide exchange factor eIF2B. *Science* 359, 1533-1536.
68. St Onge, J.R. and Floresco, S.B. (2010). Prefrontal cortical contribution to risk-based decision making. *Cereb Cortex* 20, 1816-1828.
69. Riga, D., Matos, M.R., Glas, A., Smit, A.B., Spijker, S. and Van den Oever, M.C. (2014). Optogenetic dissection of medial prefrontal cortex circuitry. *Front Syst Neurosci* 8, 230.
70. Smith, C.J., Xiong, G., Elkind, J.A., Putnam, B. and Cohen, A.S. (2015). Brain Injury Impairs Working Memory and Prefrontal Circuit Function. *Front Neurol* 6, 240.
71. Brumback, A.C., Ellwood, I.T., Kjaerby, C., Iafrati, J., Robinson, S., Lee, A.T., Patel, T., Nagaraj, S., Davatolhagh, F. and Sohal, V.S. (2018). Identifying specific prefrontal neurons that contribute to autism-associated abnormalities in physiology and social behavior. *Mol Psychiatry* 23, 2078-2089.
72. Di Prisco, G.V., Huang, W., Buffington, S.A., Hsu, C.C., Bonnen, P.E., Placzek, A.N., Sidrauski, C., Krnjevic, K., Kaufman, R.J., Walter, P. and Costa-Mattioli, M. (2014). Translational control of mGluR-dependent long-term depression and object-place learning by eIF2alpha. *Nat Neurosci* 17, 1073-1082.

73. Placzek, A.N., Prisco, G.V., Khatiwada, S., Sgritta, M., Huang, W., Krnjevic, K., Kaufman, R.J., Dani, J.A., Walter, P. and Costa-Mattioli, M. (2016). eIF2alpha-mediated translational control regulates the persistence of cocaine-induced LTP in midbrain dopamine neurons. *Elife* 5.

FIGURE LEGENDS.

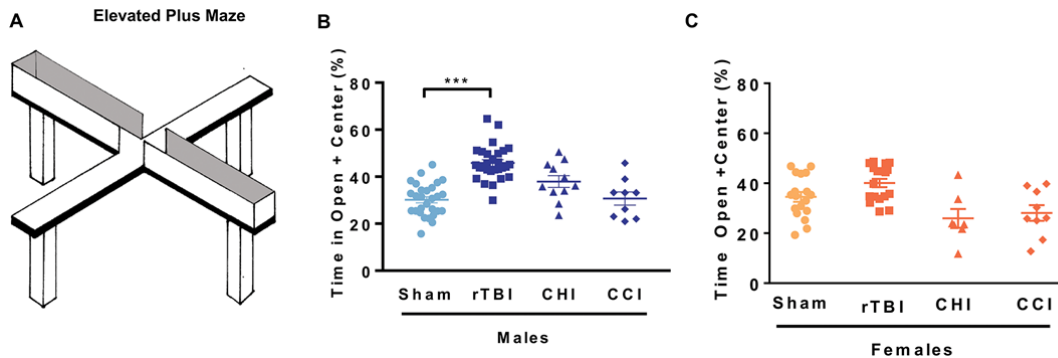


Figure 1. Repetitive mild traumatic brain injury increases risk-taking behaviors in males but not female cohorts. Risk-taking behavior was measured in the elevated plus maze by increased time spent in the open arms (including center space). Comparison of three rodent trauma models found that risk-taking behavior was only measured following mild repetitive trauma (rTBI) in male cohorts. **(A)** Schematic of the elevated plus maze. **(B)** Male mice were exposed to either (1) mild repetitive trauma (rTBI) (2) mild concussive injury-closed head injury (CHI) (3) moderate focal injury- controlled cortical impact (CCI) or (4) sham surgery and risk-taking behavior was measured 30 days post injury. Significant increases in time spent in the open arm were measured following rTBI. **(C)** Female mice were exposed to either (1) rTBI (2) CHI (3) CCI or (4) sham surgery and risk-taking behavior was measured one-month post injury. No differences in risk taking behavior were measured between groups. Two-way ANOVA measured significant injury ($p < 0.001$), sex effects ($p < 0.001$) and interaction ($p < 0.001$). Tukey post hoc analysis reveal group differences. Individual animal scores represented in dots, lines depict group mean and SEM.

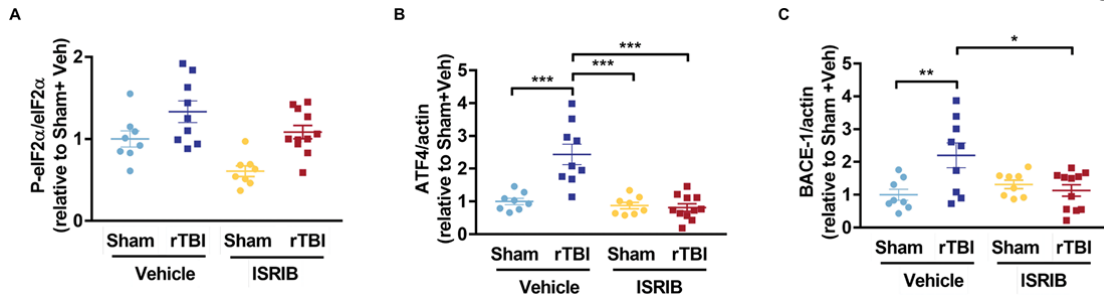


Figure 2. Integrated stress response activation one-month after rTBI. The impact of rTBI on ISR activation was investigated by western blot analysis of hemibrain lysates. **(A)** Mild rTBI induced increases in p-eIF2 α protein levels one-month post injury. p-eIF2 α protein levels normalized to total eIF2 α and standardized to sham vehicle group. Two-way ANOVA revealed a significant injury ($p < 0.001$) and ISRIB effect ($p < 0.01$). rTBI significantly increased **(B)** ATF4 and **(C)** BACE-1 protein levels. ISRIB treatment reversed rTBI-induced increases. **(B)** Two-way ANOVA revealed a significant injury ($p < 0.001$), ISRIB ($p < 0.001$) and ISRIB x injury effect ($p < 0.001$). Tukey post-hoc analysis revealed differences between groups. **(C)** Two-way ANOVA revealed a significant injury ($p < 0.05$) and ISRIB x injury effect ($p < 0.01$). Tukey post-hoc analysis revealed differences between groups. Individual animal scores represented in dots, lines depict group mean and SEM.

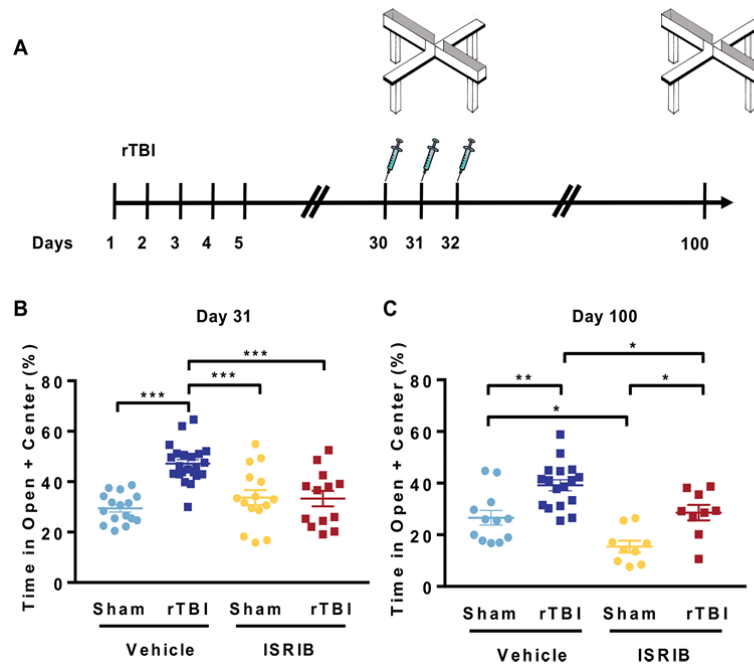


Figure 3. Small molecule inhibitor, ISRIB, reverses rTBI mediated risk-taking behavior. (A) Experimental Design. Elevated Plus maze was performed at ~31 and 100 days post injury (dpi). ISRIB (2.5 mg/kg intraperitoneal) was administered on days 30, 31 and 32 dpi. **(B)** A single ISRIB dose (2.5 mg/kg intraperitoneal) reversed trauma-induced risk-taking behavior at one-month post-surgery. Two-way ANOVA measures significant injury ($p < 0.001$), ISRIB ($p < 0.05$) and ISRIB x injury effect ($p < 0.001$). Tukey post-hoc analysis revealed differences between groups. **(C)** Permanent changes in risk-taking behavior were observed at ~3 months (100 days) post injury. Three injections of ISRIB (70 days prior) complete reversed trauma-induced behavioral changes. Two-way ANOVA measures significant injury ($p < 0.001$) and ISRIB ($p < 0.001$) effect. Tukey post-hoc analysis revealed differences between groups. Individual animal scores represented in dots, lines depict group mean and SEM.

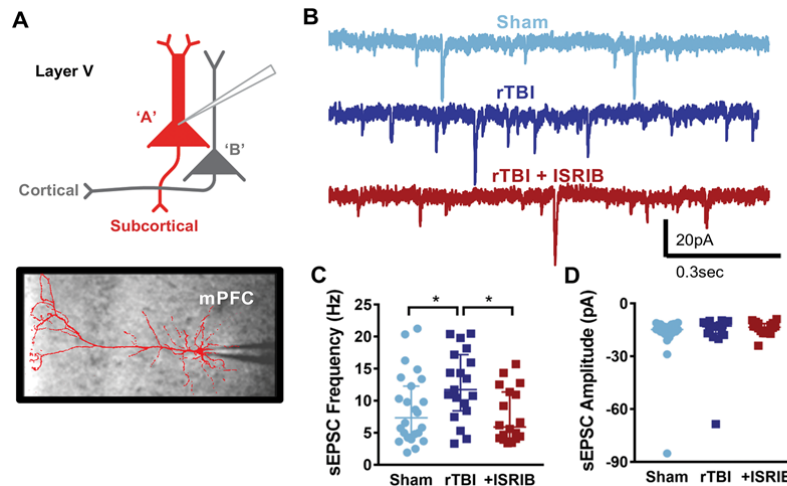


Figure 4. Cell-specific vulnerability to mild repetitive TBI is observed in the prefrontal cortex with an increase in the frequency of synaptic input in type A layer V pyramidal neurons that is restored after ISRIB treatment. (A) Depiction of layer V pyramidal neurons indicating type A being recorded along with representative image of a filled type A pyramidal neuron overlaying image of patch pipette in the mPFC. (B) Representative whole cell voltage-clamp recordings showing spontaneous excitatory postsynaptic currents (sEPSC) from layer V type A pyramidal neurons from sham mice (light blue), rTBI mice (dark blue), and rTBI mice treated with ISRIB (red). (C) The sEPSC frequency is increased in the rTBI neurons compared to both sham and rTBI+ISRIB groups ($p=0.028$ for group effect; Kruskal-Wallis test, $*p<0.05$ for post hoc tests controlling for false discovery rate). (D) The sEPSC amplitude is not significantly different between groups ($p=0.469$ for group effect; Kruskal-Wallis test). The median frequency or amplitude for each neuron is represented with a symbol; solid lines indicate the median \pm 95% CI ($n=24, 19, 18$ sham, rTBI, rTBI+ISRIB neurons respectively from 11 (sham), 7 (rTBI) and 9 (rTBI+ISRIB) animals/group with 1-4 neurons recorded per animal).

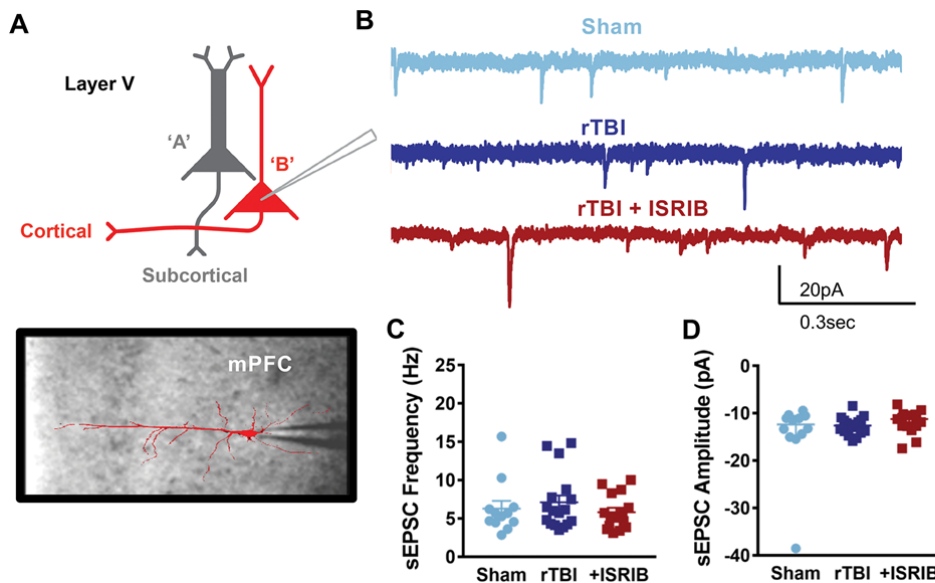


Figure 5. Synaptic input in type B layer V pyramidal neurons in the prefrontal cortex is not affected by mild repetitive TBI or ISRIB treatment. (A) Depiction of layer V pyramidal neurons indicating type B being recorded along with representative image of a filled type B pyramidal neuron overlaying image of patch pipette in the mPFC. (B) Representative whole cell voltage-clamp recordings showing spontaneous excitatory postsynaptic currents (sEPSC) from layer V type B pyramidal neurons from sham mice (light blue), rTBI mice (dark blue), and rTBI mice treated with ISRIB (red). (C) The sEPSC frequency is not significantly different between groups ($p=0.664$ for group effect; Kruskal-Wallis test). (D) The sEPSC amplitude is not significantly different between groups ($p=0.401$ for group effect; Kruskal-Wallis test). The median frequency or amplitude for each neuron is represented with a symbol; solid lines indicate the median \pm 95% CI ($n=12, 17, 15$ sham, rTBI, rTBI+ISRIB neurons respectively from 7 (sham), 5 (rTBI) and 8 (rTBI+ISRIB) animals/group with 1-4 neurons recorded per animal).