Corticosterone mediates the synaptic and behavioral effects of chronic stress at rat hippocampal temporoammonic synapses

Mark D. Kvarta1,3,4, Keighly E. Bradbrook5, Hannah M. Dantrassy5, Aileen M. Bailey5, and Scott M. Thompson1,2,3,6

Departments of Physiology1 and Psychiatry2, Programs in Neuroscience and Membrane Biology3, Medical Scientist Training Program4, University of Maryland School of Medicine, Baltimore, MD 21201

5Department of Psychology, Saint Mary's College of Maryland, St. Mary's City, 20686

6To whom correspondence should be addressed:

655 W. Baltimore Street, Bressler Research Building 5-007, Baltimore MD 21201

E-mail: sthom003@umaryland.edu

Tel: (410) 706-5817

Fax: (410) 706-8341

Abbreviated title: Corticosterone mediates synaptic effects of stress

Abstract word count: 250

Number of figures: 7
Abstract - Chronic stress is thought to impart risk for depression via alterations in brain structure and function, but contributions of specific mediators in generating these changes remain unclear. We test the hypothesis that stress-induced increases in corticosterone (CORT), the primary rodent glucocorticoid, are the key mediator of stress-induced depressive-like behavioral changes and synaptic dysfunction in the rat hippocampus. In rats, we correlated changes in cognitive and affective behavioral tasks—spatial memory consolidation, anhedonia, and neohypophagia—with impaired excitatory strength at temporoammonic-CA1 (TA-CA1) synapses; an archetypical stress-sensitive excitatory synapse. We tested whether elevated CORT was sufficient and necessary to generate a depressive-like behavioral phenotype and decreased excitatory signaling observed at TA-CA1 after chronic unpredictable stress (CUS). Chronic CORT administration induced an anhedonia-like behavioral state and neohypophagic behavior. Like CUS, chronic, but not acute, CORT generated an impaired synaptic phenotype characterized by reduced AMPAR-mediated excitation at TA-CA1 synapses, decreased GluA1 protein expression, and altered 5-HT1B-R-mediated potentiation. Repeatedly blunting stress-induced increases of CORT during CUS with the CORT synthesis inhibitor metyrapone (MET) prevented these stress-induced neurobehavioral changes. MET also prevented the CUS-induced impairment of spatial memory consolidation. We conclude that corticosterone is sufficient and necessary to mediate glutamatergic dysfunction underlying stress-induced synaptic and behavioral phenotypes. Our results indicate that chronic excessive glucocorticoids cause specific synaptic deficits in the hippocampus, a major center for cognitive and emotional processing, that accompany stress-induced behavioral dysfunction. Maintaining excitatory strength at stress-sensitive synapses at key loci throughout cortico-mesolimbic reward circuitry appears critical for maintaining normal cognitive and emotional behavior.
Keywords: AMPA receptor, anhedonia, depression, metyrapone, synaptic strength

Introduction

Depression afflicts up to 20% of the population and has the greatest impact of all biomedical diseases on disability in the United States (Flint and Kendler, 2014). It is a leading risk factor for the estimated one million deaths by suicide per year worldwide. Current treatments for depression are effective in only a subset of patients and act slowly, complicating treatment (Nestler et al., 2002; Gaynes and Warden, 2009). Depression results from an interaction between environmental and genetic factors (Kessler, 1997; Flint and Kendler, 2014). Stress is one environmental trigger that increases the likelihood of, or even precipitates, depressive episodes (Billings et al., 1983; Holsboer, 2000; Anacker et al., 2011). Chronic stress leads to changes in brain structure and neuronal function in many brain areas thereby causing the diverse cognitive and behavioral symptoms of depression [e.g. (Watanabe et al., 1992; Berton and Nestler, 2006)]. Marked morphological, functional, and volumetric brain changes correlate with stress load, depressive episode duration, and response to antidepressants (Sheline, 2000; Sheline et al., 2003; Koolschijn et al., 2009; Lorenzetti et al., 2009). Two of the more robust changes are dendritic atrophy and spine loss in the PFC and hippocampus (Christoffel et al., 2011). These two cortical areas send glutamatergic projections to reward areas, including the nucleus accumbens (Russo and Nestler, 2013), where activity is positively correlated with cortico-mesolimbic connectivity (Downar et al., 2014). Conversely, aberrant function in these same regions is a pathophysiological feature of a core symptom of depression, anhedonia (Lim et al., 2012; Thompson et al., 2015). Understanding how chronic stress causes these changes is of utmost importance for understanding the etiology of depression and designing preventative and treatment strategies.
In animal models, chronic stress triggers depressive-like changes in motivated reward behaviors, such as anhedonia, as well as synaptic and neuronal dysfunction. Chronic, but not acute, antidepressants restore both normal behavior and synaptic function in stressed animals [e.g. (Fales et al., 2009)]. In the rodent hippocampus, chronic stress decreases dendritic spine size and number, and decreases GluA1 mRNA in pyramidal cells, particularly in the most distal apical dendrites (Magariños and McEwen, 1995a; Sousa et al., 2000; Schmidt et al., 2010). At these synapses, formed by inputs from the entorhinal cortex via the temporoammonic (TA) pathway (Steward and Scoville, 1976), chronic stress decreases AMPAR-mediated signaling and alters serotonin-mediated potentiation of excitatory transmission (Cai et al., 2013; Kallarackal et al., 2013; Fischell et al., 2015). This stress-induced synaptic phenotype is accompanied by impaired consolidation of spatial memories (Kallarackal et al., 2013), a function of TA-CA1 synapses (Remondes and Schuman, 2004). Taken together with stress-induced decreases in AMPAR-mediated excitation in the prefrontal cortex (Yuen et al., 2012) and nucleus accumbens (Lim et al., 2012), stress may promote depressive symptoms by weakening excitatory synaptic transmission at multiple specific sites in cortico-mesolimbic reward circuitry (Thompson et al., 2015).

How does chronic stress alter behavior and synaptic structure and function? Chronic stress activates the hypothalamic-pituitary-adrenal (HPA) axis and causes elevation of corticosteroids and other stress hormones (Pitman et al., 1988). HPA dysregulation is particularly prevalent in depressed patients with melancholia (up to 90%), as identified using modified criteria relying centrally on anhedonia (Taylor and Fink, 2008). Chronic administration of exogenous corticosterone (CORT), the principal corticosteroid in rodents, produces behavioral changes resembling those seen after chronic unpredictable stress (CUS), a validated and widely-
used intervention to generate a depressive-like behavioral profile of decreased reactivity to
rewarding stimuli (Willner et al., 1987; Willner, 2005; Gourley and Taylor, 2009).

We hypothesized that repeated stress-induced elevations of CORT are necessary and sufficient to cause not only the behavioral changes following CUS, but also the associated synaptic dysfunction. To test this hypothesis, we chronically administered exogenous CORT to unstressed rats. In separate experiments, we administered the CORT-synthesis inhibitor metyrapone during CUS to blunt peak stress-induced increases in CORT. In addition to behavioral assays for anhedonia and hyponeophagia (a preference for neophobic behavior over feeding motivation in an ethologically relevant novelty-suppressed feeding task), we examined several neurobiological consequences of chronic stress at the synaptic level. We demonstrate that chronic elevations of CORT are necessary and sufficient for decreased GluA1 expression and TA-CA1 excitation, aberrant serotonin-mediated plasticity, anhedonic and hyponeophagic behavior, and impaired spatial memory consolidation induced by chronic stress.

**Materials and Methods**

All protocols were submitted to and approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

**Subjects:** Male Sprague-Dawley rats (Harlan Laboratories, 3-4 weeks old at start) kept on a regular 12 hour light/dark cycle were group housed. All rats sacrificed for in vitro electrophysiology and molecular biology experiments were 7-8 weeks old and were previously tested for sucrose preference and novelty-suppressed feeding. In the water maze experiment, rats obtained from Charles River were trained in the water maze at 3-4 weeks old. They were tested for memory consolidation at 7-8 weeks, and were tested weekly for sucrose preference.
Corticosterone administration: CORT was dissolved in tap water (50 μg/ml) and administered in water bottles so as to elevate plasma CORT levels in a manner tied to diurnal activity cycles (Gourley and Taylor, 2009). This dose induces several depressive-like behaviors (reduced sucrose intake and impaired forced swim performance) over several weeks, paralleling chronic stress models. After a one-night baseline sucrose preference test, rats were randomized to receive either the CORT solution or regular tap water ad libitum for 3-4 weeks. Over the course of the paradigm, rats were exposed to an average dose of 7.1mg/kg/day.

CUS and metyrapone (MET) administration: Rats were exposed to 2 stressors per light cycle for 3-4 weeks, as described previously (Willner et al., 1992; Cai et al., 2013). Stressors included restraint (30 minutes), strobe light (30 minutes), forced swim in cold water (5 minutes), cage rotation (3 hours), cage tilt (3 hours), white noise (3 hours), and overnight food deprivation. Rats were randomized to receive either MET (50mg/kg i.p.) or vehicle (VEH)(60% sterile saline and 40% PEG), prior to each stressor. Another cohort of rats received injections of either MET or VEH without exposure to stress. These animals were given injections on the same schedule as those exposed to CUS.

Sucrose preference test: While singly housed for a single dark cycle, control and treated rats were presented with two identical bottles placed on the cage top, containing either tap water or a dilute sucrose solution for 16 hours. Rats were familiarized with the task once with 2% sucrose, then tested with 1% for subsequent tests including baseline and final readouts. Tests lasted from 2 hours before the dark cycle until two hours after. Single housing during this period is potentially a stressor. Data are quantified as sucrose solution consumed as a percent of total fluid consumption.
**Novelty-suppressed feeding (NSF) test:** Rat chow pellets were placed in the center of a brightly lit arena in a dark room, as described previously (Santarelli et al., 2003; Dulawa, 2009; Cai et al., 2013). Control and treated rats were placed in the corner of the arena and latency to feed was measured. After one bite, the rat was returned to the familiar home cage to feed *ad libitum* for 5 minutes, to ensure sufficient hunger (all consumed >0.05g). No group tested fed significantly more or less than any other groups, demonstrating no major effect on appetite overall (p>0.05, One-way ANOVA). Rats that did not feed in the arena were assigned the maximum time allowed. This data was therefore treated as ordinal. The chamber was cleaned between rats with 70% ethanol and a 0.01% sodium hypochlorite solution diluted from household bleach. In the chronic CORT experiment (Figure 1), rats were first food deprived for 24 hours to instigate feeding behavior, and the maximum time was 400 seconds. In the CUS±MET experiment (Figure 4), rats were food deprived for 16 hours (as part of the CUS paradigm) and the maximum time was 600 seconds. Food deprivation preceding the task is potentially a stressor.

**Acute slice electrophysiology:** Standard methods were used to prepare 400µm thick transverse slices from middle and ventral hippocampus. Dissection and recording were performed in artificial cerebrospinal fluid (ACSF) containing: 120mM NaCl, 3 mM KCl, 1.0 mM NaH2PO4, 1.5 mM MgSO4*7H2O, 2.5 mM CaCl2, 25 mM NaHCO3 and 20mM glucose, and was bubbled with carbogen (95%O2/5%CO2). Slices were then transferred to a submersion-type recording chamber and perfused at 20–22 °C (flow rate = 0.5–2ml/min). Picrotoxin (100µM) and CGP52432 (2µM) were included to block GABA_A and GABA_B receptors, respectively. The DG and CA3 regions were removed via microdissection to prevent retrograde excitation of CA3 and anterograde activation of area CA1.
Synaptic currents at TA-CA1 synapses are most accurately assayed using local extracellular recordings of local field excitatory postsynaptic potentials (fEPSPs), because they are electrotonically remote from CA1 cell somata. Recording pipettes (3-5MΩ) containing ACSF were placed in *stratum lacunosum-moleculare* (SLM). fEPSPs were amplified 1000x, filtered at 3kHz, and digitized at 10kHz. Concentric bipolar tungsten electrodes were placed >500µm from the stimulating electrodes in SLM to stimulate TA afferents (10µs stimuli, 0.05Hz). The stimulus intensity was set to result in 0.1-0.3mV fEPSPs.

AMPA:NMDA ratios were recorded and quantified as in our previous work (Kallarackal et al., 2013), in Mg^{2+}-free ACSF. Six to ten consecutive responses were averaged with fiber volley (FV) amplitudes nearest to 0.2mV, which is in the linear range of responses to varying stimulus intensity (Kallarackal et al., 2013). The fEPSP slope in the initial rising phase was calculated over a 2ms window, 2-5 ms after initiation, for AMPAR-mediated responses. DNQX (50 µM, Tocris) was then added to the ACSF perfusion for 15 minutes and the slope of the response was calculated over 3-5ms, at 5-10ms after its initiation, for quantification of NMDAR-mediated responses. APV (80µM, Sigma-Aldrich, St. Louis, MO) was used to verify NMDAR-mediated responses. When multiple recordings were made from slices from a single animal, average AMPA:NMDA values in that animal were calculated to avoid the potential confound of nested data.

*Western blotting:* SLM or *stratum pyramidale* (SP) tissue punches (1 mm diameter) were taken from CA1 in hippocampal slices on a glass slide resting on dry ice, and deposited in standard lysis buffer (protease and phosphatase inhibitor cocktail, Sigma-Aldrich). Protein quantification for each sample was performed using a standard Bradford Assay (Coomassie reagent, Thermo Scientific, Rockford, IL). Antibodies used were rabbit anti-GluA1 (0.5 µg/ml;
Chemicon), anti-glucocorticoid receptor (GR, 1:1000; Millipore), and anti-β–actin (1:5000; Cell Signaling Technology), and an HRP-linked anti-rabbit IgG secondary antibody (1:1000; Cell Signaling Technology). Expression is quantified as signal intensity normalized to β-actin. Densitometry may non-linearly transform data, so we analyzed Western Blots as ordinal, with non-parametric statistics.

*Corticosterone quantification:* Blood samples were collected from tail veins of MET or vehicle-treated rats during restraint stress. Samples were collected in EDTA microtubes (Greiner, Bio-One North America Inc., Monroe, NC). After centrifugation, plasma CORT was quantified by radioimmunoassay (University of Virginia Ligand Core, Charlottesville, VA). For fecal pellets, enzyme-linked immunosorbent assays were performed. Briefly, each rat was singly housed in a clean cage during the dark cycle. Two hours after light cycle onset, 2-4 fecal pellets were collected and pooled for each rat. CORT was extracted with ethanol. After ethanol evaporation, extracts were reconstituted with methanol and diluted 1:20 for ELISA analysis alongside CORT standards (Assaypro, Inc., St. Charles, MO). Fecal CORT reflects an integrated measure of several hours of prior serum corticosteroids. Stress-induced CORT in feces is observed within 6-12 hours after stress (Bamberg et al., 2001; Harper and Austad, 2012). This method is minimally invasive and is less susceptible to ultradian and circadian interference than single time points.

*Morris Water Maze:* Rats were trained, as described previously (Remondes and Schuman, 2004; Kallarackal et al., 2013). Rats received 10 blocks of training (4 trials/block) over 6 days, with the platform in a fixed location. One day after training, rats completed a probe trial, with path length and latency to target measured. They were then subjected to CUS for 3
weeks, and administered MET or VEH, as above, then left unstressed for 1 week. On day 28
after training, long-term consolidation was tested with a probe trial.

Statistics: Data are presented as mean ± SEM. Statistics were calculated using SPSS
(IBM, Armonk, NY) and Graphpad (Graphpad Software, Inc., La Jolla, CA). All data tested with
parametric tests (t-tests for 2 groups, ANOVA for >2 with Bonferroni post-hocs) were normally
distributed and homoscedastic. Non-parametric statistics were used for ordinal data (NSF, blots).

Results

Is elevated CORT sufficient to generate the neurobiological correlates of chronic stress?

We first tested whether chronic exogenous administration of CORT for 21 days would
mimic the effects of CUS in causing reduced sucrose preference and neohypophagia in
unstressed animals. Chronic CORT reduced sucrose preference (Fig. 1A; group*time interaction
F(1,54)=8.461, n=57, p=0.005 2x2 mixed ANOVA, p<0.005 vs. all other groups), similar to
previous work which reported reduced sucrose intake (Gourley et al., 2008). ELISA analysis of
CORT levels from fecal pellets demonstrated that chronic CORT treatment significantly elevates
the total amount of CORT exposure (Fig. 1B, n=21, p=0.038 Student’s t-test). The total amount
of liquid consumed did not differ by group, suggesting no effect of CORT treatment on water
balance. Similarly, after 2 weeks of treatment, CORT-treated animals exhibited a significantly
increased latency to feed in the NSF test (Fig. 1C, n=11, p=0.007, Mann-Whitney U). All control
rats fed within the allotted time limit, while 2 of 6 CORT-treated rats never fed (Fig. 1D). The
body weight of rats treated chronically with CORT was not different than controls (Fig. 1E,
n=25, p=0.36 Student’s t-test). Together with no difference in food consumed in the home cage
at the conclusion of the NSF test (n=11, p=0.6), we infer that the increased latency to feed is not
driven by a change in overall appetite. These results demonstrate that chronic CORT treatment is sufficient to induce anhedonia and neohypophagia.

CUS causes a decrease in the AMPAR-mediated component of excitatory postsynaptic fEPSPs at TA-CA1 synapses (Kallarackal et al., 2013). As with CUS, chronic CORT reduced AMPA:NMDA ratios (Fig. 2A,B; p=0.010, n=18 slices, Student’s t-test). AMPAR strength was also reduced significantly following CORT administration when normalized to the fiber volley (FV), a measure of the number of axons activated (Fig. 2C, n=18, p=0.042, t-test). There was no difference in NMDAR:FV responses (Fig. 2C, n=17 p=0.4, t-test). Western blots revealed a ~31% reduction in GluA1 protein levels in SLM from CORT-treated animals, compared to SLM in untreated animals (p=0.009, n=19 samples, Mann-Whitney U test). Chronic CORT also downregulated GR protein levels in CA1 stratum pyramidale (n=8, p=0.01, Mann-Whitney U test), as observed following CUS (Sapolsky et al., 1984; Herman et al., 1995). Thus, chronic administration of CORT in unstressed animals was sufficient to cause deficits in TA-CA1 excitatory synapses that are comparable to those produced by CUS (Kallarackal et al., 2013).

We compared these results to the effects of acute CORT administration (Joëls, 2006). In contrast to CUS and chronic CORT, acute (~16 hours) CORT increased AMPA:NMDA ratios at TA-CA1 synapses (Fig. 2A,B right, n=11, p=0.046 Student’s t-test). Acute and chronic CORT elevations thus exert opposing actions on excitatory strength at TA-CA1 synapses.

Endogenous serotonin and the 5-HT1B-selective agonist anpirtoline potentiate TA-CA1 synapses as a result of CaMKII-mediated phosphorylation of GluA1 (Cai et al., 2013). In brain slices from animals subjected to chronic stress, 5-HT1B-R-mediated potentiation becomes greater in magnitude and irreversible (Cai et al., 2013). In slices from control rats, anpirtoline (50μM) induced an average potentiation of fEPSP slope to 125±8% of the baseline, which reversed to
95±11% of baseline after one hour of washout. In slices from chronic CORT rats, TA-CA1 fEPSPs increased to a maximum of 210±32%, and remained at 196±35% after washout (Fig. 3, n=18, interaction effect F(2,32)=6.87, p<0.05 2x3 mixed ANOVA, p<0.05 Bonferroni *post-hoc* CORT peak and washout vs. CORT baseline and all control). Therefore, chronic CORT was sufficient to alter TA-CA1 response to serotonergic modulation from reversible to persistent, as occurs after CUS.

Taken together, these results show that chronic CORT elevation is sufficient to mimic the both the behavioral effects of CUS and the corresponding neurophysiological and molecular changes in excitatory synaptic transmission at TA-CA1 synapses. Elevated CORT alone is thus sufficient to mimic the behavioral and synaptic consequences of chronic stress in this model.

*Is elevated CORT necessary to generate the neurobiological correlates of chronic stress?*

CUS causes a chronic elevation of average CORT levels and repeated spikes in CORT levels in response to each individual stressor (Sapolsky et al., 1984; Magariños and McEwen, 1995a), as part of a complex constellation of concerted neuromodulator interactions [reviewed in (Joëls and Baram, 2009)]. HPA axis responses to unpredictable or varied stress do not habituate even after 3 weeks [e.g. fig. 1 in (Magariños and McEwen, 1995a), reviewed in (Herman, 2013)]. We predicted that if chronic stress-induced elevations in CORT are *necessary* for the changes triggered by CUS, then partially inhibiting CORT synthesis prior to each stressor with metyrapone (MET, 50mg/kg i.p.), an inhibitor of the adrenocortical enzyme 11β-hydroxylase (Holsboer, 2000), would prevent the CUS-induced synaptic and behavioral deficits. During CUS, we tested whether MET attenuated the increase in basal CORT caused by stress-induced dysregulation of the HPA axis (Johnson and Yamamoto, 2009). CORT was extracted from fecal
pellets collected overnight, reflecting CORT levels integrated over a day of stress. The CUS-
induced increase in CORT, observed in vehicle (VEH) treated rats, was prevented by MET as
measured by ELISA of fecal pellet extractions (Fig. 4A, 2x2 ANOVA interaction F(1,37)=4.45,
p=0.04, p<0.05 Tukey’s HSD post-hoc for CUS+VEH vs. all other groups). In unprotected t-
tests, MET treatment alone reduced fecal CORT compared to all other groups (p<0.02).
Furthermore, MET blunted peak plasma CORT during an individual restraint stressor (Fig. 4A,
n=11, p=0.0008 Student’s t-test), as measured by radioimmunoassay.

Unlike VEH-treated rats subjected to CUS, MET-treated rats subjected to CUS
(CUS+MET) maintained a high sucrose preference that was not different from the sucrose
preference of unstressed MET- or VEH-treated (CUS+VEH) rats (Fig. 4B, n=36, F(1,20)=18.6,
4x2 mixed ANOVA p<0.001, p<0.05 CUS+VEH vs. baseline and all other groups). In the NSF
test, CUS+MET rats exhibited a shorter latency to feed than CUS+VEH rats and were not
different from unstressed VEH or MET rats (Fig. 4C, Kruskal-Wallis H=9.52, p=.0231 n=33,
CUS+VEH U-test post-hoc p<0.05 vs. all others). Fewer CUS+VEH rats fed within the allotted
time limit than any other group (Fig. 4D p<0.05, Fisher’s exact test). We found a main effect of
CUS on body weight (F(1,33)=43.21, p<0.0001), but no effect of MET (F(1,33)=0.02, p=0.89;
Fig. 4E), consistent with previous reports of lack of role of CORT in the weight loss associated
with chronic stress (Magariños and McEwen, 1995b). Together with no difference in food
consumed in the home cage at the conclusion of the NSF test (n=19, p=0.4, not shown), we infer
that the increased latency to feed is not driven by a change in overall appetite. In summary,
limiting stress-induced increases in CORT prevented the stress-induction of anhedonia and
neohypophagia.

We predicted that MET would also prevent the changes observed at TA-CA1 synapses
after CUS. AMPA:NMDA ratios were significantly lower in slices from CUS+VEH rats compared to CUS+MET and unstressed MET and VEH rats (Fig. 5A,B, 2x2 ANOVA main effect of CUS (F(1,54)=14.71, p=0.0003) and MET (F(1,54)=11.93, p=0.0011, MET*CUS interaction F(1,54)=11.84, p=0.0011. p<0.01 Tukey’s HSD post-hocs, n=57). AMPA:FV responses were also lower in slices from CUS+VEH than CUS+MET rats and both non-CUS groups (Fig. 5C, main effect of MET F(1,56)=7.43, p=0.0085), while NMDAR:FV responses were not significantly different (p>0.05 for both CUS and MET main effects). TA-CA1 AMPA:NMDA strength correlated significantly with hedonic state, measured by sucrose preference (Fig. 5B, right. R=0.52, t(29)=3.293, p=0.002).

GluA1 expression in SLM decreased by 25% in tissue from CUS+VEH rats as compared to CUS+MET rats (Fig. 5D, n=14, p=0.049, Mann-Whitney U test). GR expression was also lower in SP of CUS+VEH rats, compared to CUS+MET (n=8, p=0.02, Mann-Whitney U test).

Finally, anpirtolone potentiated TA-CA1 fEPSP slope to 155±5% of baseline after 60 minutes in slices from vehicle-injected CUS rats, peaked at 199.5±14% and it remained elevated (166±11%) after washout (Fig. 6, n=32, mixed ANOVA interaction effect F(2,54)=7.95, p=0.009. p<0.05 Tukey’s HSD post-hoc for CUS+VEH peak and washout vs. CUS+VEH baseline, and vs. all CUS+MET). In slices from CUS+MET rats, in contrast, TA-CA1 fEPSP potentiation peaked at 149±12% and reversed to 106±27% after washout. MET thus prevented the exaggerated, persistent response to anpirtolone at TA-CA1 synapses observed normally after CUS.

Taken together, these results show that blunting the elevation of CORT produced by the CUS stressors is sufficient to prevent both the behavioral effects of CUS and the corresponding neurophysiological and molecular changes in excitatory synaptic transmission at TA-CA1
Does MET prevent a CUS-induced TA-CA1 synapse-specific cognitive deficit?

TA-CA1 synapses are required for long-term consolidation of spatial memory (Remondes and Schuman, 2004), and CUS impairs this function (Kallarackal et al., 2013). Impaired spatial memory has been described in depressed humans (Gould et al., 2007). We asked whether blunting CUS-induced elevation of CORT with MET treatment during CUS would prevent the normal CUS-induced loss of consolidation in a spatially cued version of the Morris water maze. After naïve rats were trained to learn the location of a hidden platform, confirmed with an initial 24 hour probe trial, they were randomly divided into unstressed control groups and CUS+MET, CUS+VEH, using the same procedures described above. Twenty-eight days after training, path length and latency to target were measured and compared to the initial probe trial (Fig. 7., 3x2 mixed ANOVA stress*MET interaction for path length F(2,18)=3.22 p=0.06, and latency F(2,18)=2.96 p=0.077). Unstressed control rats performed as well as they did in the initial probe trial (Fig. 7, n=7, p>0.05 unprotected paired t-test), indicating successful consolidation. CUS+VEH rats, in contrast, required a significantly longer latency to reach the platform location (t(6)=3.42, p=0.014, n=7) and traveled a significantly longer path to reach the target (t(6)=3.10, p=0.02), compared to their previous probe trial, indicating that CUS in this group disrupted memory consolidation. However, latency (p>0.05, n=7) and path to target were similar in CUS+MET rats to their baseline responses (p>0.05), indicating successful memory consolidation despite CUS exposure.

Because MET treatment prevented the effects of CUS on three separate behaviors (anhedonia, neohypophagia and impaired spatial memory consolidation) and the
neurophysiological and molecular deficits of excitatory synaptic transmission at a key stress-sensitive synapse, we conclude that chronically elevated CORT is a necessary mediator of the adverse effects of chronic stress at this pathway.

Discussion

Physical and psychosocial stressors are significant risk factors for depression, in addition to anxiety and other mood-disorders. Stress affects the hippocampus, a critical region for cognitive processing, mood, and other complex behaviors that are altered in depression, as well as synapses in the prefrontal cortex and nucleus accumbens, two other regions involved in processing reward. Potential mediators of stress include endocannabinoids (Gray et al., 2013), corticotropin-releasing hormone (Chen et al., 2012) and other neuropeptides, opioids such as dynorphin (Mague et al., 2003; McLaughlin et al., 2003), monoamines such as serotonin and norepinephrine, glutamate (Palucha and Pilc, 2005), and glucocorticoids (Joëls and Baram, 2009). The stress-sensitive TA-CA1 synapse provides a tractable locus at which to examine the neurobiological effects of the stress response and better understand how chronic stress causes altered behavioral phenotypes by changing synapses and circuits. Generalizing the current results from TA-CA1 synapses, we conclude that chronic CORT elevation is both sufficient and necessary to induce the chronic stress phenotype at molecular, synaptic, and behavioral levels.

CORT is necessary and sufficient to mediate the synaptic effects of chronic stress:

Chronic CORT administration induces depressive-like behaviors, including anhedonia and increased helplessness, that are reversed by chronic antidepressants (Gourley and Taylor, 2009). We replicated these previous behavioral results and further observed that chronic CORT
induced hyponeophagia in the NSF task, an ethologically relevant choice between feeding motivation and neophobic behavior. These tests are sensitive to chronic, but not acute, SSRI administration (Dulawa et al., 2004). MET treatment significantly reduced the peak CORT response to a stressor and the average CORT levels during CUS. Because CORT administration mimicked, and MET treatment prevented, anhedonia-like and neophobic behaviors in our experiments, we conclude that stress-induced CORT elevation is sufficient and necessary to generate these behavioral changes in the CUS model.

Adolescence and early adulthood present vulnerable time windows to stress that may increase the likelihood of cognitive impairment or psychiatric disease (Eiland and Romeo, 2013; Holder and Blaustein, 2014). The rats we tested were no older than P60 when they were sacrificed for \textit{in vitro} studies. It is possible that chronic stress or CORT may be affecting puberty trajectory or testosterone levels (Almeida et al., 2000; Pervanidou and Chrousos, 2012). Further studies would be necessary to determine whether this has any effect on mood-related behavior and TA-CA1 physiology.

Chronic CORT was also sufficient to decrease AMPAR-mediated excitation and GluA1 subunit expression at TA-CA1 synapses in unstressed animals. The effect was specific for AMPARs, as there was no accompanying change in NMDAR-mediated fEPSPs. Conversely, MET administration during CUS prevented this dysfunction, illustrating that chronic stress-induced elevations of CORT are \textit{necessary} for these effects. A decreased AMPA:NMDA ratio at these synapses is consistent with results from synapses in the PFC and NAc (Lim et al., 2012; Yuen et al., 2012), and we suggest that CORT elevation during chronic stress is a potential mediator at those synapses, as well. We suggest further that weakening of synaptic excitation at multiple synapses in cortico-mesolimbic reward circuits contributes to depressive-like signs after
chronic stress. Corresponding restoration of synaptic strength at these stress-sensitive synapses is a critical mechanism of antidepressant action in these models (Popoli et al., 2012; Tizabi et al., 2012; Kallarackal et al., 2013; Musazzi et al., 2013).

Chronic stress also alters serotonin-induced potentiation at TA-CA1 synapses, both qualitatively and quantitatively (Cai et al., 2013). Mediated by 5-HT1B Rs and phosphorylation of GluA1, serotoninergic modulation co-varies with depressive-like behavior and changes in AMPAR function. Conversely, chronic antidepressant treatment restores normal serotonin responses at TA-CA1 synapses in conjunction with restoration of normal behavior and synaptic strength. In the present study, the aberrant stress-induced anpirtoline response was mimicked by chronic CORT treatment alone, and prevented by MET treatment in rats subjected to CUS.

TA-CA1 synapses are required for spatial memory consolidation, and impaired spatial memory has been described in humans (Gould et al., 2007). The ability of MET treatment to preserve TA-CA1 synaptic strength and memory consolidation in rats subjected to CUS demonstrates that CORT-induced changes in synaptic strength likely underlie this stress-induced cognitive deficit.

Structurally, chronic restraint stress reduces the number of spines in CA1 cell distal apical dendrites (Pawlak et al., 2005; Maras et al., 2014). Functionally, chronic stress and CORT administration impair LTP at proximal CA1 synapses in some studies (Alfarez et al., 2003), but not all [for review see (Joëls et al., 2012)]. Chronic stress or chronic CORT cause atrophy of terminal segments of CA1 dendrites (Sousa et al., 2000), which are more vulnerable to stress than more proximal synapses, but are often overlooked in stress-related studies. A history of chronic CORT persistently simplifies CA1 basal dendritic arbors, illustrating that lasting effects on structural organization may contribute to anhedonia via impaired processing of
reward-related contextual stimuli (Gourley et al., 2013). Loss of distal dendrites and TA-CA1 synapses may contribute significantly to the atrophy observed after chronic stress in rodents and contribute to the hippocampal volume loss in severe human depression.

*Acute vs. chronic effects of CORT*

In contrast to the impairment of AMPAR-mediated transmission produced by chronic CORT (~weeks), acute CORT (~hrs) increased transmission at TA-CA1 synapses (Fig. 2A,B), consistent with previous observations at Schaffer collateral synapses (Karst et al., 2005). This short-term effect of CORT to increase CA1 excitability is consistent with the well-established dichotomy between acute and chronic stress and with the transient mood-boosting effects of steroid treatment (Joëls, 2006). *Acute* stress increases plasticity in short time windows via non-genomic MR (Karst and Joëls, 2005; Wiegert et al., 2006; Olijslagers et al., 2008), promoting stress-salient memories. In contrast, *chronic* stress dampens plasticity via GR-mediated genetic regulation (Joëls, 2008). We suggest that the synaptic consequences of chronic CORT elevation described here are likely to be mediated by chronic, excess GR activation.

*Glucocorticoids in human disease*

There is compelling evidence of a causal link between altered HPA function and a large subset of severely depressed patients, particularly with regard to anhedonia. Depression is common in patients with primary abnormalities of GC production and patients receiving exogenous GCs (Quarton et al., 1955; Jeffcoate et al., 1979). HPA dysregulation is correlated with severity of symptoms and is common (~80% of patients) in treatment-resistant depression (Anacker et al., 2011). The correlation is even greater in melancholia patients (up to 90%),
identified using modified criteria relying primarily on anhedonia (Taylor and Fink, 2008).
Abnormal HPA function is also correlated with a nine-fold increase in suicide risk (Coryell and
Schlesser, 2001). HPA dysregulation may be both a causative factor and a consequence of
depression.

The dose of MET used here produced effects that are similar to those produced by
clinically relevant doses in humans, effectively halving circulating corticosteroids (O’Dwyer et
al., 1995). In stressed rats, MET treatment preserved the normal response of TA-CA1 synapses
to serotonergic modulation, suggesting that MET treatment protected the serotonin-innervated
excitatory synapses upon which SSRIs act (Sigalas et al., 2012; Cai et al., 2013). MET also
prevented normal stress-induced downregulation of GRs, suggesting protection from HPA
dysregulation. There is clinical evidence that MET enhances antidepressant response (Sigalas et
al., 2012; McAllister-Williams et al., 2013) and is effective as a stand-alone therapy in
hypercortisolemic patients with depressive symptoms (Jeffcoate et al., 1979). Our results suggest
several mechanisms by which treatment with MET should oppose pro-depressive changes caused
by stress, suggesting potential efficacy as a clinical add-on, particularly for depressed patients
with HPA or GC abnormalities.

In conclusion, we have demonstrated that glucocorticoids play a critical role as mediators
of the synaptic effects of chronic stress, as well as behavioral changes in rats, which are similar
to the mood and cognitive changes characterizing human depression, strengthening the
hypothesis that excitatory synaptic dysfunction contributes to the symptoms of depression
(Kruger et al., 2010; Joëls et al., 2012; Popoli et al., 2012; Duman, 2014; Fischell et al., 2015;
Thompson et al., 2015). That is, pathological chronic elevation of glucocorticoids causes
depressive-like behavioral changes because it weakens specific subsets of synapses in cortico-
mesolimbic circuits, such as the hippocampus, PFC (Duman and Aghajanian, 2012; Yuen et al., 2012), and ventral striatum (Lim et al., 2012), thereby resulting in the anhedonia that is a prominent feature of human depression. Conventional antidepressants like SSRIs, fast-acting antidepressants like ketamine, and non-pharmacological treatment like electroconvulsive therapy all act to oppose the deleterious actions of chronically elevated GCs on excitatory synapses (Berman et al., 2000; Tizabi et al., 2012; Kallarackal et al., 2013; Zarate et al., 2013). Protecting and restoring excitatory strength at stress-sensitive synapses at key loci throughout the reward systems appears critical for maintaining normal cognitive and emotional behavior.

Acknowledgments: We would like to thank Leelah Jaberi, Kaitlin Gaylor, Jennifer Marsh, and Pamela Gorgei for technical assistance, Drs. Bruce Krueger and Matthew Trudeau for access to imaging equipment and software, and Dr. Tara LeGates, Adam Van Dyke, and Dr. Thomas Blanpied and his laboratory for helpful comments and criticisms.

Funding and disclosure: Supported by R01MH086828 (SMT) and T32NS063391 (MDK). The authors declare no competing financial interests.

References


M.D. Kvarta et al


Figure Captions

Figure 1: Chronic corticosterone (CORT) administration mimics the depressive-like behavioral effects of chronic stress. Rats received either CORT in their drinking water or tap water alone, and were tested in the sucrose preference and novelty-suppressed feeding tests after 4 weeks. a, Chronic CORT administration caused a significant reduction in sucrose preference, inducing an anhedonia-like phenotype (group*time 2x2 mixed ANOVA \[F(1,54)=8.461 \text{ (p=0.005)}\] *p<0.05 vs. all other groups Bonferroni post-hoc). b, ELISA of fecal pellets collected from rats being treated with chronic CORT demonstrate a significant increase in CORT levels compared to control (n=21 individual samples, p=0.038, Student’s t-test) c, Chronic CORT elevation caused a significantly increased latency to feed in the novelty-suppressed feeding task, relative to controls (n=11, *p=0.007 Mann-Whitney U test). d, A survival plot illustrating the proportion of rats that remained unfed vs. time in the novelty–suppressed feeding task. e, Body weights for rats treated chronically with CORT revealed no difference compared to controls (n=25, p=0.36, t-test)

Figure 2: Chronic CORT elevation decreases AMPAR-mediated signaling at TA-CA1 synapses by decreasing GluA1 expression. a, Example TA-CA1 fEPSPs recorded in SLM in Mg$^{2+}$-free ACSF before (black) and after (gray) DNQX wash-in, exemplifying
representative AMPAR- and NMDAR-mediated responses, respectively. TA-CA1 fEPSPs from chronic CORT-treated rats exhibited a marked reduction in AMPAR-mediated component relative to control, while TA-CA1 fEPSPs in slices from rats treated acutely with CORT were increased. AMPA:NMDA ratios were calculated as the slope of the black AMPAR-mediated fEPSP divided by the slope of the gray NMDAR-mediated fEPSP. **b,** Quantification of group data for experiments from **a,** in which comparisons of the slope of each response was measured for responses generating a fiber volley (FV) of ~0.2mV. Chronic CORT treatment significantly reduced AMPA:NMDA ratios compared to controls (p=0.010, n=18, Student’s t-test), while acute CORT treatment significantly increased AMPA:NMDA (p=0.046, n=11, Student’s t-test). **c,** Chronic CORT also reduced AMPAR-mediated signaling when normalized to FV compared to control (p=0.042), but not NMDAR-mediated signaling. **d,** *Left:* Chronic CORT decreased GluA1 protein expression in SLM, where TA-CA1 synapses are formed (p=0.009, n=19, Mann-Whitney U). *Right:* In *stratum pyramidale,* where pyramidal cell bodies reside, GR protein expression is also decreased (n=8, p=0.01, Mann-Whitney U).

**Figure 3: Chronic CORT elevation quantitatively and qualitatively alters the potentiation of TA-CA1 fEPSPs by the 5-HT_{1B}R agonist anpirtoline.** **a,** Example TA-CA1 fEPSPs demonstrating the reversible effect of anpirtoline (50µM) on fEPSPs in slices from control rats (left) and the persistent potentiation in slices from chronic CORT-treated rats (right). Traces taken from the time-points indicated in the graph below. **b,** Group data illustrating the difference in time-course of 5-HT_{1B}R-mediated potentiation in slices from control (gray symbols) and CORT-treated (black symbols) rats. Data are normalized to the 10 minutes prior to the beginning of anpirtoline application. **c,** Comparison of TA-CA1 fEPSPs at
baseline, peak of potentiation, and after washout. Anpirtoline generated a significantly
greater peak response in hippocampal slices from chronic CORT-treated rats. This
potentiation persisted through washout. Mixed ANOVA interaction F(2,32)=6.87, p=0.03.
*p<0.05 Bonferroni post-hoc vs. baseline CORT and all control.

**Figure 4:** MET prevents stress-induced increases in CORT during CUS and generation of
anhedonia-like and neophobic behaviors. **a,** *Left:* To determine if MET was effective in
reducing time averaged stress-induced CORT levels, fecal pellets were collected overnight
from singly-housed rats after a typical day of stressors and compared to values in age-
matched unstressed rats. CUS significantly elevated CORT in vehicle- but not MET-treated
rats (2x2 ANOVA interaction F(1,37)=4.45, p=0.04 *p<0.05 Tukey’s HSD post-hoc vs. all
other groups). In unprotected t-tests, MET treatment alone reduced fecal CORT compared
to all other groups (†p<0.02) *Right:* Rats undergoing CUS received either vehicle or MET
treatment prior to restraint stress, and blood samples were taken from their tail veins 10-15
minutes after placing them in restraint tubes. Plasma CORT was quantified via
radioimmunoassay. MET significantly reduced plasma CORT levels induced by the stressor
(*p=0.0008, n=11, Student’s t-test). **b,** CUS decreased sucrose preference when rats were
pretreated with vehicle, but not MET, prior to each stressor. Daily vehicle or MET
injections without stress had no effect. Mixed ANOVA 3-way interaction F(1,30)=4.841
(p=0.036), *p<0.05 vs all other groups Bonferroni post-hoc. **c,** MET pretreatment
significantly decreased the latency to feed in the NSF task in CUS rats vs. vehicle-
treatment, to levels not significantly different than unstressed vehicle or MET groups
(Kruskal Wallis H = 9.52, p=.0231 n=33; *p<0.05 vs all others, U-test) **d,** Survival plot of
NSF task in vehicle- and MET-treated rats unstressed or subjected to CUS. 8/10 CUS+MET
and 2/9 CUS+vehicle fed within 10 minutes (*p=0.023, Fisher’s exact test). 5/6 and 8/10 of unstressed vehicle- and MET- treated rats fed, respectively. e, Body weights of stressed and unstressed rats administered VEH or MET reveal a main effect of CUS on weight (F(1,33)=43.21, **p<0.0001; 2x2 ANOVA), but no main effect of MET (F(1,33)=0.02, p=0.89).

**Figure 5: MET prevents CUS-induced decreases in AMPAR-mediated TA-CA1 signaling and GluA1 expression.** a, Example TA-CA1 fEPSPs recorded in SLM in Mg$^{2+}$-free ACSF before (black) and after (gray) DNQX wash-in, exemplifying an AMPAR-mediated and NMDAR-mediated trace, respectively. b, TA-CA1 fEPSPs from MET-treated rats exhibited a higher AMPAR-mediated signaling component relative to vehicle-treated CUS rats, measured by AMPA:NMDA ratio (Main effect of CUS F(1,54)=14.71, p=0.0003; main effect of MET F(1,54)=11.93, p=0.0011; MET*CUS interaction effect (F(1,54)=11.84, p=0.0011). The CUS+VEH group was different from all others (**p<0.01, Tukey’s HSD) Right: TA-CA1 AMPA:NMDA strength correlated significantly with hedonic state, measured by sucrose preference (R=0.52, t(29)=3.293, p=0.0026). c, The AMPAR-mediated excitatory component at TA-CA1 was also significantly higher in slices from MET-treated rats when normalized instead to FV (*-Main effect of MET F(1,56)=7.43, p=0.0085). NMDAR:FV was not different (p>0.05 for both CUS and MET main effects). d, SLM punches revealed MET treatment prevented the CUS-induced decrease in GluA1 protein expression observed in the vehicle group (n=14, p=0.049 Mann-Whitney U). SP punches revealed that MET also preventing GR downregulation (p=0.02, Mann-Whitney U).
Figure 6: MET treatment prevents stress-induced changes in the potentiation of TA-CA1 fEPSPs by the 5-HT$_{1B}$R agonist anpirtoline. a, Example TA-CA1 fEPSPs demonstrating the effect of anpirtoline (50µM) on slices from vehicle- (left) and MET-treated CUS rats (right). Left: Anpirtoline (red, 2) elicited a robust potentiation from baseline (black, 1), that persisted after washout (gray, 3) in slices from vehicle-treated CUS rats. Right: After MET treatment, anpirtoline elicited a more modest potentiation of TA-CA1 fEPSPs that was reversed upon washout. b, Group data and time course of 5-HT$_{1B}$R-mediated potentiation. Data are normalized to the 10 minutes prior to the beginning of anpirtoline application. c, Comparison of TA-CA1 fEPSPs at baseline, peak of potentiation, and after washout. Anpirtoline generated a significantly greater peak TA-CA1 response in slices from vehicle-treated rats, and it persisted during washout, whereas a reversible, modest potentiation was elicited in slices from CUS rats treated with MET, as in unstressed controls. Repeated measures mixed ANOVA interaction effect F(2,54)=7.95, p=0.0009. *p<0.05 Tukey’s HSD post-hoc vs. baseline vehicle and from all MET timepoints.

Figure 7: MET treatment prevents stress-induced impairment of spatial memory consolidation. Rats were trained to find the location of a hidden platform in a water maze in 10 blocks over 6 days. Twenty-four hrs after the 10$^\text{th}$ block, they were tested in a probe trial (black) to ensure they had learned the platform location. They were then randomized to control and CUS groups. CUS-subjected rats received either a MET or VEH injection prior to each stressor, while control rats were left undisturbed. After 28 days, rats were tested for spatial memory consolidation in another probe trial (“consolidation”-white) by comparing their performance to their initial probe trial (“24 Hr probe”-black). a, Example diagrams of consolidation trial, with start (open circle), finish (closed circle) and path (connecting line)
for rats from each group. **b, Path length to platform location (left, 3x2 mixed ANOVA F(2,18)=3.22, p=0.06)** and latency to platform location (right, F(2,18)=2.96 p=0.077) for each group. Control, unstressed rats performed as well after 28 days as after the initial probe trial (p>0.05 for both path length and latency). CUS+VEH rats performed significantly worse after the 28 day consolidation period, with significantly greater latency (t(6)=3.42, p=0.014) and path length (t(6)=3.10, p=0.02) to platform location, indicating impaired memory consolidation with unprotected paired t-tests. CUS+MET rats successfully consolidated, with no significant difference in latency or path length between trials (p>0.05, n=6 per group).
Unstressed

SPT  CORT  NSF  SPT
(4 weeks)

Slice preparation

A

Sucrose preference
(% sucrose/total)

Baseline  4 weeks

Control  Chronic CORT

B

Fecal CORT (ng/g)

Control  Chronic CORT

C

Latency to feed (s)

Control  Chronic CORT

D

% Unfed

Latency to feed (s)

Control  Chronic CORT

E

Body weight (g)

Control  CORT

FIGURE 1
FIGURE 3

Unstressed

Control

Chronic CORT

A

B

C

Anpirtoline 50 μM

fEPSP slope (% baseline)

Time (minutes)

Baseline Peak Wash

fEPSP slope (% baseline)

0 50 100 150 200 250 300

-20 0 20 40 60 80 100 120

0.1 mV

5 ms

0

100

150

200

250

300

1

2

3

1

2

3

CORT

Control

* *
FIGURE 4

A

**Fecal CORT (ng/g)**

- VEH
- MET
- CUS+VEH
- CUS+MET

- **Plasma CORT (ng/mL)**

- CUS+VEH
- CUS+MET

B

**% Sucrose**

- Baseline
- 4 Weeks

C

**Latency to feed (s)**

- VEH
- MET
- CUS+VEH
- CUS+MET

D

**% Unfed**

- CUS+Vehicle
- CUS+MET
- Vehicle
- MET

E

**Body Weight (g)**

- VEH
- MET
- CUS+VEH
- CUS+MET
FIGURE 5

A. Comparison of AMPA:NMDA ratios between different treatment groups: VEH, MET, CUS+VEH, and CUS+MET. The graph shows a significant effect of CUS+MET compared to CUS+VEH, as indicated by the asterisks.

B. Scatter plot and linear regression analysis of Sucrose Preference (% vs. AMPA:NMDA) for different treatment groups. A Pearson's r of 0.52 is observed, indicating a moderate positive correlation.

C. Bar graph depicting the response: FV ratio for AMPA:FV and NMDA:FV across groups VEH, MET, CUS+VEH, and CUS+MET. The response ratio for AMPA:FV is significantly higher in CUS+MET compared to CUS+VEH, as indicated by the asterisk. The response ratio for NMDA:FV is not significantly different among groups (ns).

D. Histograms showing the relative expression of GluA1 and GR compared to β-actin. The relative expression of GluA1 is significantly increased in CUS+MET compared to CUS+VEH, as indicated by the asterisk. The relative expression of GR is also increased in CUS+MET compared to CUS+VEH, but the change is not statistically significant (ns).
**FIGURE 7**

**A**

- **Unstressed**
- **CUS + VEH**
- **CUS + MET**

**B**

- Path length (m)
  - Control: ns
  - CUS: *
  - CUS + MET: ns

- Latency to Platform (s)
  - Control: ns
  - CUS+VEH: *
  - CUS + MET: ns

**Legend:**
- 24Hr Probe
- Consolidation

- **Training**
  - 24 hr probe
  - 28 day consolidation trial

- **CUS (+VEH or MET)**
  - (3 weeks)
  - (1 week)

- **Unstressed**

- **Control CUS CUS + MET**