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Acute restraint stress enhances hippocampal endocannabinoid function via glucocorticoid receptor activation

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Abstract

Exposure to behavioural stress normally triggers a complex, multilevel response of the hypothalamic–pituitary–adrenal (HPA) axis that helps maintain homeostatic balance. Although the endocannabinoid (eCB) system (ECS) is sensitive to chronic stress, few studies have directly addressed its response to acute stress. Here we show that acute restraint stress enhances eCB-dependent modulation of GABA release measured by whole-cell voltage clamp of inhibitory postsynaptic currents (IPSCs) in rat hippocampal CA1 pyramidal cells *in vitro*. Both Ca²⁺-dependent, eCB-mediated depolarization-induced suppression of inhibition (DSI), and muscarinic cholinergic receptor (mAChR)-mediated eCB mobilization are enhanced following acute stress exposure. DSI enhancement is dependent on the activation of glucocorticoid receptors (GRs) and is mimicked by both *in vivo* and *in vitro* corticosterone treatment. This effect does not appear to involve cyclooxygenase-2 (COX-2), an enzyme that can degrade eCBs; however, treatment of hippocampal slices with the L-type calcium (Ca²⁺) channel inhibitor, nifedipine, reverses while an agonist of these channels mimics the effect of *in vivo* stress. Finally, we find that acute stress produces a delayed (by 30 min) increase in the hippocampal content of 2-arachidonoylglycerol, the eCB responsible for DSI. These results support the hypothesis that the ECS is a biochemical effector of glucocorticoids in the brain, linking stress with changes in synaptic strength.

Keywords

2-arachidonoylglycerol, COX-2, DSI, GABA inhibition, IPSC, muscarinic receptor

Introduction

Stress activates the hypothalamic–pituitary–adrenal (HPA) axis and induces the release of glucocorticoid hormones that exert widespread effects on the brain and periphery (McEwen, 2008). Appropriate regulation of the HPA axis is critical for health and survival, and several limbic brain structures, including the hippocampus, are involved in the integration of HPA hormonal responses (Herman et al., 2005). The hippocampus has an abundance of glucocorticoid receptors (GRs) and is exquisitely sensitive to glucocorticoids (De Kloet et al., 1998). Exposure to stress reduces the expression of long-term potentiation and concurrently enhances the expression of long-term depression (LTD), which are neural correlates of mnemonic processes (Kim et al., 1996; Chaouloff et al., 2007; Wong et al., 2007). Stress and glucocorticoids probably modulate synaptic plasticity in the hippocampus via changes in postsynaptic ionotropic glutamate receptor trafficking (Kim et al., 1996; Wong et al., 2007; Groc et al., 2008), although changes in presynaptic transmitter release also occur (Karst et al., 2005).

Stress and glucocorticoids could also modulate plasticity within the hippocampus by recruiting the endocannabinoid system (ECS). In the central nervous system (CNS), the ECS comprises the eCB receptor (CB1R), the endocannabinoids (eCBs) *N*-arachidonylethanolamine (anandamide,

AEA) and 2-arachidonoylglycerol (2-AG), and enzymes that synthesize, transport and hydrolyze the eCBs. Activation of CB1Rs on presynaptic terminals by 2-AG can modulate synaptic transmission by regulating both GABA and glutamate release (Kano et al., 2009). Stimulation of 2-AG synthesis by increased diacylglycerol lipase alpha (DGL α) activity (Gao et al., 2010; Tanimura et al., 2010) follows postsynaptic depolarization and/or metabotropic receptor activation.

Stress and/or glucocorticoids increase eCB mobilization, and support for the hypothesis that the ECS is an important effector of glucocorticoid receptor (GR) activation in the

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brain is accumulating (Hill and McEwen, 2010). Exposure to footshock increases both 2-AG and AEA levels in the periaqueductal gray (Hohmann et al., 2005) and bath application of glucocorticoids increases eCB production and CB1R-mediated inhibition of glutamate release in hypothalamic slices (Di et al., 2003, 2005). The rapid behavioural effects of glucocorticoids and/or stress on sexual behaviour and emotional memory depend on CB1R activation (Coddington et al., 2007; Campolongo et al., 2009). Taken together, these studies suggest that glucocorticoids can affect behaviour via the ECS in limbic brain regions. Our hypothesis is that the ECS is a general effector of GR actions on signalling in the CNS. We tested this hypothesis in the hippocampus, a brain region with a high density of CB1R and well-characterized eCB regulation of GABAergic signalling. We used the Ca^{2+} -dependent, eCB-mediated suppression of inhibition called depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku et al., 2001; Wilson et al., 2001) as an assay of ECS function. We find that exposure of rats to a single episode of restraint causes GR-dependent potentiation of the ECS in CA1 pyramidal cells, and increases hippocampal tissue content of 2-AG. The effect of stress on DSI was blocked by an L-type Ca^{2+} channel inhibitor, and mimicked by an L-channel agonist, suggesting that glucocorticoids enhance Ca^{2+} -dependent 2-AG mobilization.

Methods

Subjects

All animal handling procedures were approved by the University of Maryland School of Medicine IACUC (specific approval for behavioural and brain slice studies; approval #0609001), and by the Animal Care Committee of the University of British Columbia (specific approval for behavioural and eCB measurement studies; approval # A09-0220). Male Sprague Dawley rats were housed in groups of three. Rats were 5–6 weeks old (Charles River Lab., Wilmington, Massachusetts, USA) in the electrophysiological studies and 9–10 weeks old in the biochemical studies (Charles River, Montreal, Canada). Animal rooms were maintained at 21°C on a reversed 12 h light/dark cycle. All rats were given ad libitum access to food and water. Subjects were randomly assigned to either control or acute stress groups. Acute restraint stress was induced by putting the rat into a Plexiglas cylindrical restrainer (Kent Scientific Corp., Torrington, Connecticut, USA) for 30 min.

Drugs

ω -Agatoxin was purchased from Ascent Scientific Ltd (Bristol, UK). All other drugs were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) or Tocris (Ellisville, Missouri, USA). Drugs used for bath application or pretreatment were first dissolved in DMSO (LY 341495, LY 225910, corticosterone); ethanol (nifedipine, FPL 64176, SR 141716A); or water (atropine, meloxicam, carbachol), then added to the bathing saline at the desired final concentrations (the ratios of final concentration to stock concentration were from 1:1000 to 1:10,000). In the electrophysiology studies,

RU 38486 in pure DMSO (20 mg/mL) was injected subcutaneously 30 min before acute stress (Stress + RU) or 90 min before control slice preparation (Con + RU) at a dose of 20 mg/kg. In the biochemical studies, RU 38486 (20 mg/kg) was injected subcutaneously in 1:1 saline and propylene glycol vehicle. In some studies, corticosterone (CORT; 10 mg/kg) was dissolved in 1:1 saline and propylene glycol and was injected subcutaneously 1 h before decapitation. DMSO and 1:1 saline and propylene glycol were injected subcutaneously into naïve animals 90 min and 1 h before decapitation, respectively, to serve as vehicle control to each group. In all cases the injection volume was 1 mL/kg.

Hippocampal slice preparation

Rats were killed by decapitation after heavy sedation with isoflurane. Hippocampal slices were prepared from the stressed groups either immediately (≤ 5 min, Stress-immed) or 30 min after removal of the animal from the restrainer (Stress-30 min). Hippocampal slices were prepared between 09:00 and 11:00, which is during the active (dark) phase. Hippocampi were isolated and sectioned into 400- μm -thick slices in ice-cold saline using a Leica VT 1200S Vibratome (Leica Microsystems Inc., Bannockburn, Illinois, USA). The slices were maintained at room temperature for over 1 h in an interface holding chamber in a humidified atmosphere saturated with 95% O_2 /5% CO_2 . The recording chamber (Warner Instr., Hamden, Connecticut, USA) warmed the submerged slices, and experiments were performed at $30 \pm 1^\circ\text{C}$.

Electrophysiology

Whole-cell voltage-clamp recordings of CA1 pyramidal cells were made using the 'blind' patch method. Electrode resistances in the bath were 3–5 M Ω . During experiments, series resistance was checked by -2 mV hyperpolarizing voltage steps, and if it exceeded 35 M Ω , increased by > 15%, or if current baselines were unstable, data were discarded. Cell membrane potentials were held at -70 mV. IPSCs were elicited by 100 μs extracellular stimuli (eIPSCs) delivered with concentric bipolar stimulating electrodes (David Kopf Instruments, Tujunga, California, USA) placed in the stratum (s.) radiatum between CA3 and CA1, 0.5–1 mm away from the recording site. The eIPSCs were evoked at 4-s intervals. Data were collected using an Axopatch 1C amplifier (Molecular Devices, Sunnyvale, California, USA), filtered at 2 kHz, and digitized at 5 kHz using a Digidata 1200 and pClamp 8 software. Representative continuous traces were collected on a WINDAQ Data Q DI-710 (DATAQ Instr., Inc., Akron, Ohio, USA) and are used for illustrative purposes only.

The extracellular recording solution contained in mM: 120 NaCl, 3 KCl, 2.5 CaCl_2 , 2 MgSO_4 , 1 NaH_2PO_4 , 25 NaHCO_3 , and 20 glucose, and was bubbled with 95% O_2 , 5% CO_2 (pH 7.4) at 30°C. The solution flowed continuously through the recording chamber at a rate of ~ 0.6 mL/min. Ionotropic glutamate responses were blocked with 20 μM AP-5 plus 10 μM NBQX. All slices were pretreated with 300 nM agatoxin for ≥ 30 min (usually ≥ 1 h) before being transferred to the recording chamber. ω -Agatoxin IVA (agatoxin) is irreversible within the timeframe of our experiments (Wheeler et al.,

1994), hence P/Q channels remained inhibited despite the absence of agatoxin in the perfusion solution. Whole-cell pipettes contained (in mM) 90 CsCH₃SO₃, 50 CsCl, 10 HEPES, 0.2 BAPTA, 0.3 Tris-GTP, 2 Mg-ATP, 1 MgCl₂ and 5 QX-314 (pH 7.25). Cells were voltage-clamped at -70 mV and eCB-dependent eIPSC suppression (DSI) was induced by 0.5-, 1-, 2-, or 3-s voltage steps to 0 mV.

Data analysis

Both the magnitude and duration of DSI were analyzed. The DSI magnitude was determined by the reduction from the mean amplitude of five successive eIPSCs evoked just before the depolarizing pulse was given to the pyramidal cell (baseline), compared to the mean amplitude of four successive eIPSCs taken just after the pulse (DSI period). The reduction ratio of these values (1-DSI period/baseline) was multiplied by 100%. A DSI value of 0% means that the eIPSC was not reduced by depolarization and a value of 100% means it was abolished. Whenever possible, three DSI trials were averaged to obtain mean DSI in a given condition, but at least two trials were used in every case. In some experiments (Figures 1A1 and C, Figure 3 and Supplemental Figure 1), the mean magnitude of DSI induced by a 2-s step in the control group (either naïve, vehicle-treated or tested prior to drug application) was normalized to 100% and all other values were expressed relative to this value. The decay time constant (τ_d) of DSI, which was obtained from single exponential fits of the envelope of the peak amplitudes of the eIPSCs during recovery from DSI, was used to calculate DSI duration. Changes of DSI magnitude were also assessed in some experiments by approximating the integrals of the area under the envelope of the eIPSC amplitudes from the point of maximal IPSC suppression throughout recovery of DSI to baseline. These DSI integrals were obtained by subtracting the amplitude of the normalized eIPSC from the baseline at each time point and multiplying this value by interstimulus interval, i.e. (100-normalized eIPSC%)*4 (example shown in Figure 2C). The mean of the last five eIPSCs of each DSI trial was taken as baseline, and all the recovery responses in this trial were normalized ($\times 100\%$) to this mean.

Hippocampal lipid extraction and eCB analysis

All rats for this study were injected subcutaneously with RU 38486 (20 mg/kg) or an equivalent amount of propylene glycol/saline vehicle; those rats subjected to restraint stress were placed into restrainers 30 min after injection. Three groups of animals were compared: non-stressed (returned to home cage after injection and killed 60 min later); killed immediately following 30 min restraint stress (Stress-imm); and killed 30 min following the cessation of restraint stress (Stress-30 min). For all groups, brains were removed following rapid decapitation and the hippocampus was rapidly dissected free, frozen in dry ice within 5 min of decapitation, and stored at -80°C until analysis. The hippocampus was subjected to a lipid extraction process described previously (Hill et al., 2009). Samples were weighed and placed into borosilicate glass culture tubes containing 2 mL of acetonitrile with 84 pmol of [²H₈]AEA and 186 pmol of

[²H₈]2-AG. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at -20°C to precipitate proteins, then centrifuged at 1,500 $\times g$ to remove particulates. The supernatants were removed to a new glass tube and evaporated to dryness under N₂ gas. The samples were re-suspended in 300 μ L of methanol to recapture any lipids adhering to the glass tube and dried again under N₂ gas. Dried extracts were suspended in 20 μ L of methanol and stored at -80°C until analysis. The contents of the two primary eCBs, AEA and 2-AG, were determined using isotope-dilution, liquid chromatography-mass spectrometry (Patel et al., 2005).

Statistical analyses

Data analyses were done using Clampfit 8 (Axon Instruments) or Prism (GraphPad) and graphs were drawn in SigmaPlot 8.0 or in Prism. Two-way analyses of variance (ANOVA, performed by SigmaStat 3.0 or Prism) were used to compare groups when stimulation duration and in vivo or in vitro treatment are the main effects; when in vitro treatments were compared in groups treated differently in vivo; and eCB content in the hippocampus. Bonferroni's corrected *t* tests were used to compare groups if significant main effects or interactions were identified by the ANOVA. When only a single stimulation condition was used, one-way ANOVA was used for group comparisons of physiological data followed by Student-Newman-Keuls tests for multiple comparisons. Paired *t* tests were used for single comparisons within groups, and unpaired *t* tests were used for comparisons between two groups under the same conditions. The significance level for all tests was $p < 0.05$ (*), except for the K-S test, for which $p \leq 0.01$. Group means \pm SEMs are shown in all graphs.

Results

Acute stress prolongs DSI in hippocampal CA1 pyramidal cells

There are two sources of perisomatic GABA_A-mediated eIPSCs in hippocampal CA1 pyramidal cells, the cholecystokinin-containing (CCK) and parvalbumin-containing (PV) interneurons (Somogyi and Klausberger, 2005; Freund and Katona, 2007). Of the two, CB1Rs are only on the CCK cells (Freund et al., 2003), which release GABA via Ca²⁺ influx through N-type (ω -conotoxin GVIA-sensitive) voltage-gated Ca²⁺ channels (VGCCs). The PV cells release GABA via P/Q-type (agatoxin-sensitive) VGCCs. To isolate the eCB-sensitive eIPSCs from the insensitive ones, agatoxin pretreatment was used to prevent occurrence of eIPSCs from PV cells (see Methods), and whole-cell recordings were made in acute hippocampal slices.

Depolarizing voltage-clamp command pulses delivered to pyramidal cells generate the Ca²⁺-dependent suppression of inhibition called DSI (see Alger, 2002, for a review), which is mediated by eCB release (Kano et al., 2009). The magnitude of DSI is dependent on the depolarization duration (Lenz et al., 1998). We used four durations (0.5 s, 1 s, 2 s, 3 s) to vary DSI magnitude and obtain a sensitive assay of eCB function (Figure 1A1). Recordings were made in slices from

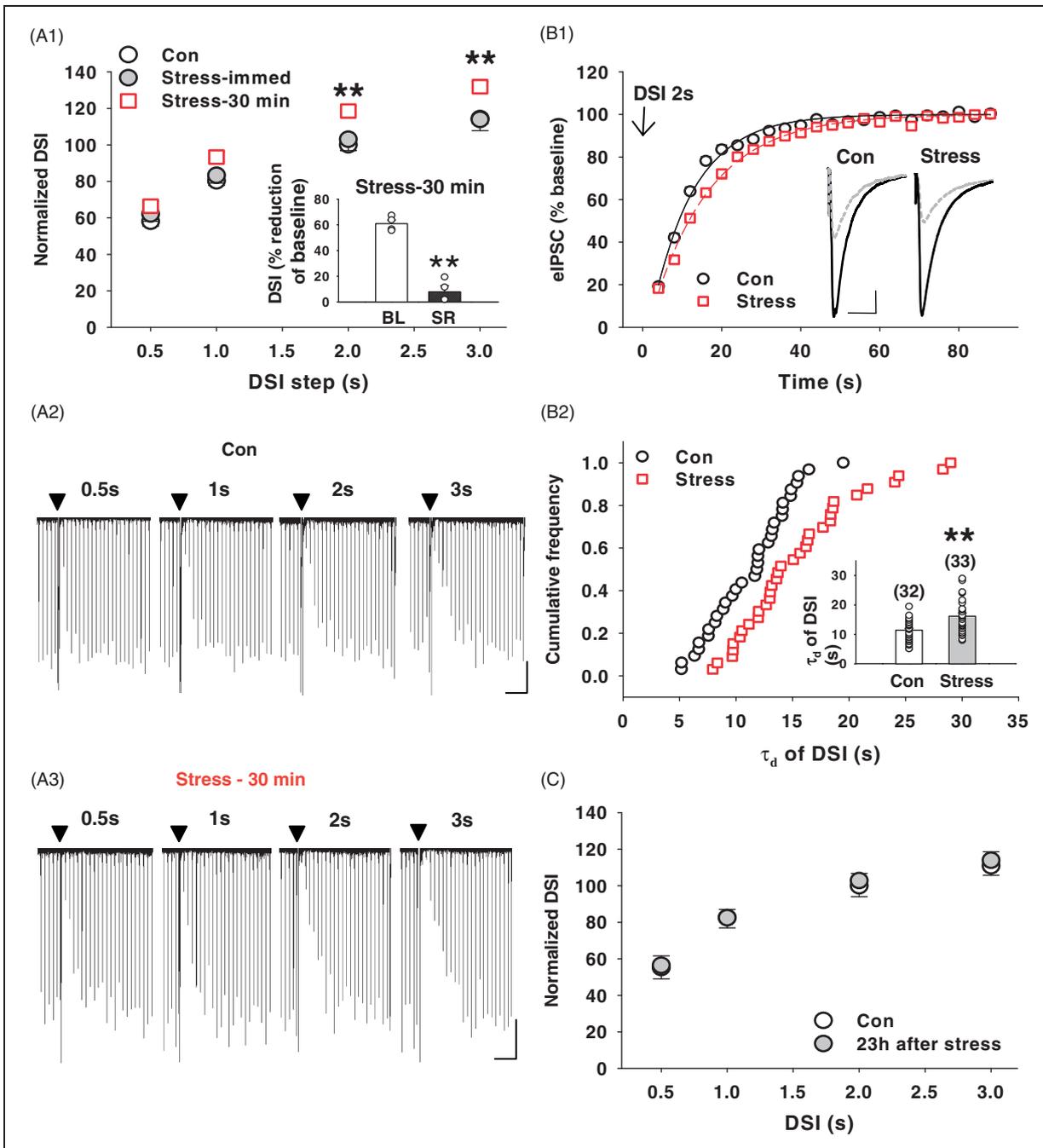


Figure 1. Acute stress enhances hippocampal DSI. (A1) The magnitudes of depolarization-induced suppression of inhibition (DSI) induced by 2-s and 3-s DSI steps were enhanced over control levels (Con) by acute stress, if slices were prepared 30 min after the animal's removal from the restrainer (Stress-30 min), but not if they were prepared immediately after removal (Stress-immed, $n = 11$ cells) $^{*}p < 0.05$, Stress-30 min ($n = 32$ cells) vs Con ($n = 33$ cells). (Cells from acutely stressed animals studied 30 min after exposure to restraint are referred to as 'stressed' cells.) Insert shows that the DSI in Stress-30 min group was blocked by the cannabinoid receptor (CB1R) antagonist, SR 141716A ('SR') ($^{*}p < 0.01$, BL vs SR, $n =$ four cells). (A2 and A3) Representative whole-cell voltage clamp of DSI trials measured from a control and a stressed cell. Each downward deflection represents an evoked inhibitory postsynaptic current (eIPSC), which is compressed at this slow time scale. At the points indicated by the black triangles, a single voltage step from the holding potential of -70 mV to 0 mV was given for the indicated duration. DSI is the period after the voltage step where the eIPSCs are reduced and then gradually recover to their baseline amplitudes prior to the voltage step. Cal. bar: 20 s, 200 pA. (B1) Time course of mean DSI induced by a 2-s DSI step from control (Con, $n = 32$) and stressed (Stress, $n = 33$) cells; the voltage step ended at time 0. Inner traces were taken before (solid line, average of five consecutive eIPSCs just before depolarization) and after (dotted line, average of four consecutive eIPSCs just after depolarization) 2-s DSI step, control and stressed cells respectively. Cal. bar: 50 ms, 200 pA. Both datasets were fit with single exponential functions, solid black or dashed red lines, which were used to determine the time constants of decay, τ_d , of DSI for the groups. (B2) Frequency distribution of τ_d following 2-s DSI steps, determined from individual control and stressed cells. Inner histogram shows the group data for τ_d . Each circle represents one cell. $^{**}p < 0.01$, Stress vs Con. (C) The magnitude of DSI recovers to control level 23 h after acute stress. A group of animals was untreated (Con, $n = 17$) and DSI was measured from slices as usual (2-s step). Another group was subjected to the usual acute stress protocol and then returned to their home cages for 23 h before slices were made ($n = 20$). There were no differences in the DSI measured from the two groups.

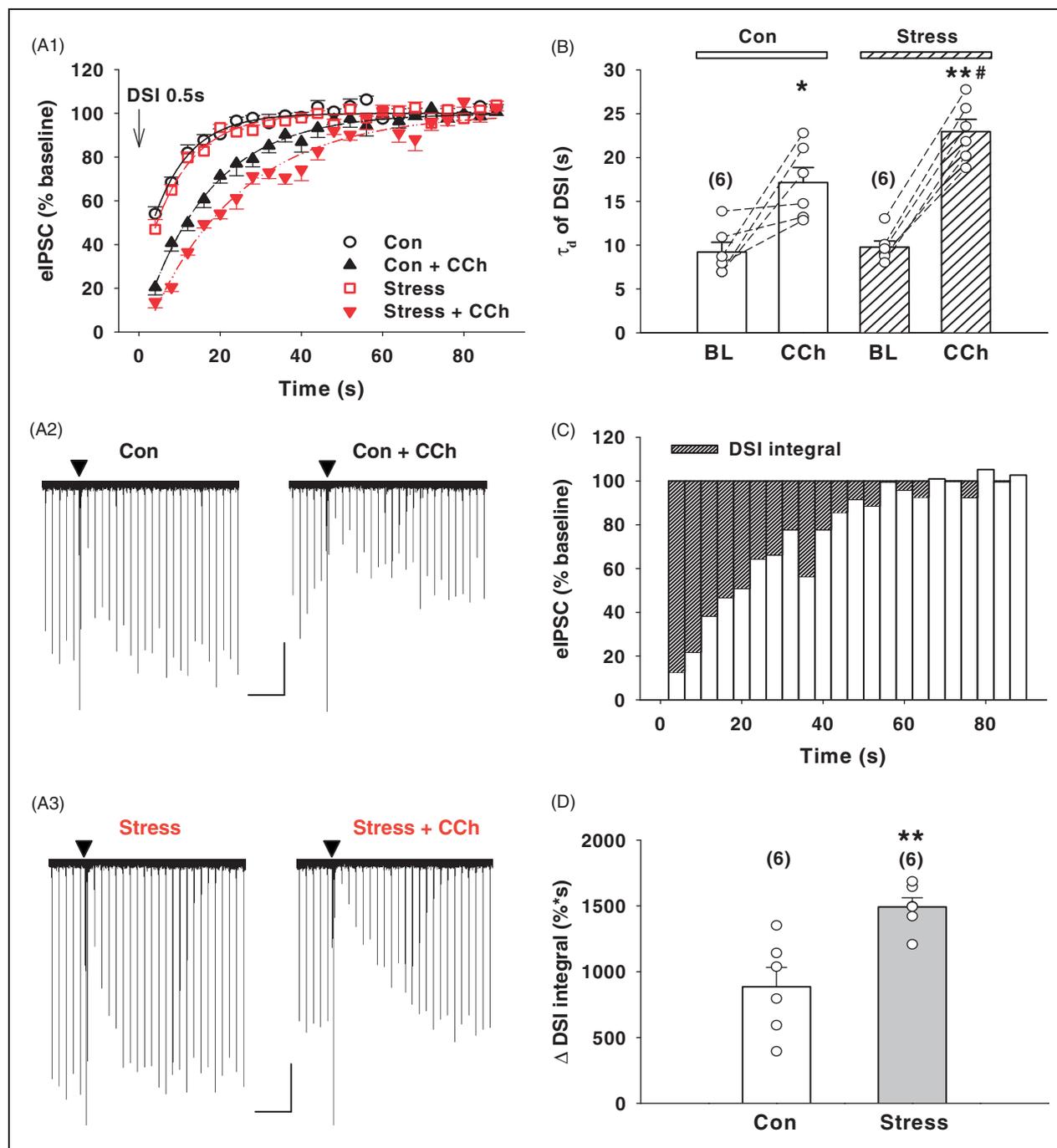


Figure 2. Acute stress increases carbachol-induced enhancement of depolarization-induced suppression of inhibition (DSI). (A1) Time course of mean DSI, 0.5-s step, from control (Con, $n = six$) and stressed cells (Stress, $n = six$) before (open symbols) and after (filled symbols) 0.5 μM carbachol (CCh) application. (A2 and A3) Representative traces from a control and a stressed cell respectively. Cal. bar 20 s, 200 pA. (B) Group data, DSI decay time constant τ_d from control (open bars) and stressed cells (striped bars) before (baseline, BL) and after CCh application (CCh). Each circle represents one cell. (C) Illustration of the estimated DSI integral, 0.5-s DSI step, obtained with data from the stressed cell shown in A3 after CCh application. The dark bars represent the integral of the area below 100%. (D) The difference of the DSI integral (Δ DSI integral (%*s)) after CCh application between control and stressed cells. Each circle represents one cell. ** $p < 0.01$ Stress vs Con.

control (Con, $n = 32$ cells from 27 rats) and two groups of stressed animals: one in which the slices were harvested within 5 min after the end of the stress period (Stress-immed, $n = 11$ cells from five rats) and a second in which the slices were

harvested 30 min after the end of the stress period (Stress-30 min, $n = 33$ cells from 21 rats). Two-way ANOVA of these results indicates a significant effect of in vivo treatment on the magnitude of DSI ($F_{2,218} = 10$; $p = 0.0001$) and a

significant interaction between *in vivo* treatment and duration of depolarization ($F_{6,218}=2.4$; $p=0.02$). Post hoc tests revealed significant differences between the control and Stress-30 min groups at depolarization durations of 2 and 3 s (Figure 1A1). However, there were no significant differences in DSI magnitude in slices from control and Stress-immersed groups at any depolarization duration. Thus, DSI enhancement was observed only when the slices were prepared 30 min after the end of the restraint period, and not when they were prepared immediately. DSI observed in the Stress-30 min group was dependent on CB1R activation, as it was abolished by bath application of the CB1R antagonist, SR141716 (SR; 10 μ M; Figure 1A1 inset, $n=4$ cells; 3-s depolarizing step in Stress-30 min group: DSI was $60.9 \pm 2.8\%$ before and $7.8 \pm 4.8\%$ in the presence of SR, $p < 0.001$, paired t test). Figures 1A2 and 1A3 illustrate representative traces obtained with different DSI steps from a control cell and from a Stress-30 min cell respectively. We used the Stress-30 min protocol ('stressed' rats) to test the effect of stress in all subsequent experiments.

Stress exposure also altered DSI duration. Figure 1B1 shows the eIPSC recovery following a 2-s DSI step in slices from non-stressed and stressed groups. The time constants of decay (τ_d) of the single exponential fits to the eIPSC recovery from peak DSI back to baseline were calculated and compared. The cumulative frequency distributions of τ_d showed a significant rightward shift to more prolonged DSI values in the stressed group (Figure 1B2, K-S test, $p < 0.01$) and the mean τ_d of DSI increased from 11.3 ± 0.6 s (Con, $n=32$) to 15.6 ± 0.9 s (Stress, $n=33$) ($p < 0.001$, t test, Figure 1B2 inset).

We examined the duration of DSI enhancement by a single episode of acute stress. After the termination of restraint stress, rats were put back to their home cages and the slices were prepared 23 h later. Cells from a new group of unstressed rats served as controls and DSI was measured at depolarization durations of 0.5, 1, 2 and 3 s (Figure 1C; 23 h after stress, $n=20$ cells from six rats and Con, $n=17$ cells from five rats). Two-way ANOVA of these data demonstrated that *in vivo* treatment did not significantly affect DSI duration ($F_{1,140}=0.24$; $p=0.63$); thus DSI in the 23-h delay group was indistinguishable from DSI in never-stressed, control animals.

Acute stress increases mAChR-amplification of DSI

Although DSI itself is a purely Ca^{2+} -dependent form of eCB mobilization, it can be amplified by activating muscarinic cholinergic receptors (mAChR) with the cholinergic agonist, carbachol (CCh) (Kim et al., 2002; Ohno-Shosaku et al., 2003). To determine if the synergistic effect of Ca^{2+} and mAChR activation on eCB mobilization was also enhanced by stress, we used a 0.5-s depolarization together with CCh application (0.5 μ M, 4 min), a protocol that markedly enhances and prolongs DSI (Kim et al., 2002). Brief depolarization steps were used in these experiments to maximize the opportunity to observe an increase in DSI, and to preclude the possibility of missing an enhancement because of a ceiling effect, which could occur if maximal DSI was induced with longer depolarizing steps. The τ_d of DSI in slices from control

($n=6$ cells from five rats) and stressed ($n=6$ cells from six rats) rats was determined before (BL) and after (CCh) the application of CCh *in vitro* (Figures 2A1 and 2B; representative traces in Figures 2A2 and 2A3). Two-way ANOVA of the τ_d values demonstrated significant effects of both CCh application ($F_{1,20}=69$, $p < 0.0001$) and stress exposure ($F_{1,20}=6.3$, $p < 0.05$) and a nearly significant interaction ($F_{1,20}=4.1$, $p=0.056$). Thus, CCh significantly prolonged DSI in cells from both control and stressed rats, and tended to have a greater effect on DSI τ_d in the slices from stressed rats. To determine if the CCh effect was indeed greater in stressed animals, we also tested the DSI integral, which encompasses changes in both peak amplitude and duration of DSI (see Methods). Figure 2C shows how the integral is defined using the cell illustrated in Figure 2A3 after the addition of CCh. The CCh-induced increase in the DSI integral (Δ DSI integral) was significantly larger in stressed cells than in control cells (Figure 2D, $p < 0.004$ using t test). Hence, in addition to enhancing ECS actions per se, stress also increases the coupling between mAChRs and the ECS.

The glucocorticoid receptor mediates the prolongation of DSI by stress

Many effects of stress in the CNS occur because the increase in circulating corticosteroid (CORT) concentrations is enough to fully activate CNS GRs (Chrousos and Kino, 2007; McEwen, 2008). We tested three predictions of the hypothesis that activation of GR by CORT is responsible for the restraint stress-induced increase in eCB signalling. The first prediction was that inhibition of GRs *in vivo* would prevent the enhancement of DSI by stress. To test this, we pretreated control and stressed rats *in vivo* with RU 38486 (20 mg/kg), an antagonist of GR. An additional non-stressed group was pretreated with DMSO, the vehicle for RU 38486 in these studies. Vehicle treatment had no effect on DSI responses in slices from non-stressed rats (control data in Figure 1C were used for comparison; $F_{1,80}=0.01$, $p=0.9$). Two-way ANOVA revealed that there is no main effect of *in vivo* treatment on DSI magnitude among the three treatment groups (RU 38486 pretreated, non-stressed (Con + RU, $n=6$ cells from five rats) and RU 38486 pretreated, stressed (Stress + RU, $n=14$ cells from 10 rats) and non-stressed rats pretreated with DMSO (Con + Vehicle, $n=5$ cells from three rats) (Figure 3A; $F_{2,66}=0.35$, $p=0.71$). All DSI values were normalized to the 2-s DSI value of the Con + Vehicle group. Thus, GR activation is required for the effects of stress to increase hippocampal DSI.

The second prediction was that elevation of CORT *in vivo* would enhance eCB responses in unstressed rats. To test this, rats were injected subcutaneously with 10 mg/kg CORT (CORT s.c., $n=9$ cells from five rats) or propylene glycol/saline vehicle (Vehicle s.c., $n=13$ cells from five rats) 60 min prior to the harvest of hippocampal slices and determination of DSI (Figure 3B). Injection of the propylene glycol/saline vehicle did not significantly affect DSI compared to non-injected controls (control data in Figure 1C were used for comparison; $F_{1,112}=1.5$, $p=0.2$). Two-way ANOVA demonstrated a significant effect of CORT injection on DSI

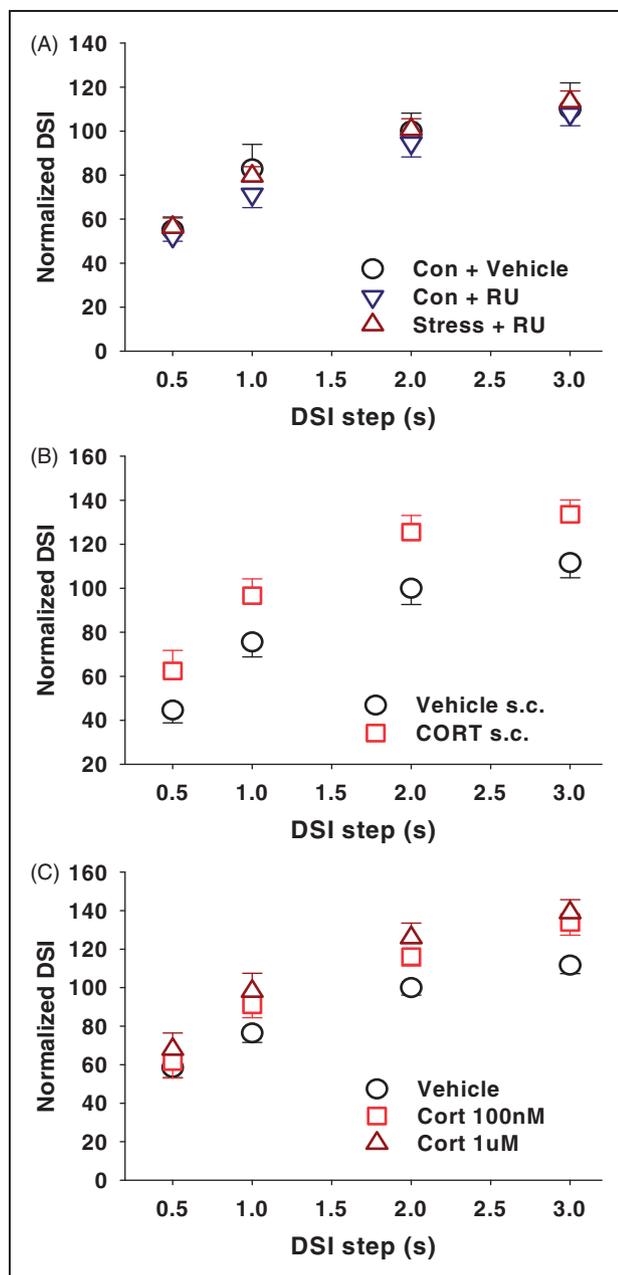


Figure 3. Glucocorticoid receptor (GR) activation mediates the enhancement of depolarization-induced suppression of inhibition (DSI) by restraint stress. (A) In vivo pretreatment with the GR antagonist RU 38486 (RU, 20 mg/kg), prior to exposure to restraint stress (Stress plus RU, $n = 14$) prevented the enhancement of DSI (Con plus RU, $n = 9$ and Con plus Vehicle, $n = 5$). (B) Subcutaneous injection of corticosterone (10 mg/kg CORT, s.c., $n = 9$) or vehicle ($n = 13$) 1 h before slice preparation mimicked the stress effect on DSI magnitude. (C) Comparison of the effects of in vitro incubation with CORT (100 nM and 1 μ M, $n = 8$ and 9 respectively), or vehicle ($n = 13$) on the magnitude of DSI. See text for statistical comparisons.

($F_{1,60} = 5$, $p = 0.036$). The effect of CORT was to increase DSI, mimicking the effects of stress.

A third prediction of the hypothesis is that application of CORT in vitro to hippocampal slices from naïve rats will enhance DSI. To test this prediction, slices from untreated rats were exposed for 20 min to CORT, which was then washed out for at least 1 h before electrophysiological recordings were started (protocol modified from Verkuyl et al., 2005). Two concentrations of CORT, 100 nM ($n = 8$ cells from five rats) and 1 μ M ($n = 9$ cells from seven rats) were compared to vehicle ($n = 14$ cells from 13 rats). DSI was determined at four depolarization times (Figure 3C). Two-way ANOVA demonstrated that CORT treatment has a significant effect on DSI ($F_{2,81} = 44$, $p = 0.02$). Thus, in addition to stress exposure and CORT injection, exposure of hippocampal slices to CORT also enhanced DSI.

Acute stress enhances mGluR-dependent hippocampal LTD via a GR-dependent mechanism (Chaouloff et al., 2007), and other transmitter systems, including mGluRs, cholecystokinin receptor (CCK2) and mAChRs can stimulate the ECS (Kano et al., 2009). To determine if acute stress enhances DSI directly by stimulating the ECS, or indirectly by stimulating another neurotransmitter system, we used a cocktail of antagonists. The cocktail included 100 μ M LY 341495 (broad spectrum mGluR antagonist at this dose), 2 μ M LY 225910 (CCK₂ receptor antagonist) and 2 μ M atropine (mAChR antagonist). After collecting baseline DSI data, we applied the cocktail for 15 min and tested DSI again. DSI enhancement was observed in slices from stressed animals in the presence of the cocktail (Supplemental Figure 1, $n = 8$ cells from seven rats, see legend for details).

COX-2 inhibition does not mediate the effects of stress on DSI

In the hypothalamus, glucocorticoids can shift arachidonic acid metabolism toward eCB synthesis by inhibiting cyclooxygenase-2 (COX-2) (Malcher-Lopes et al., 2008). Both 2-AG and AEA are substrates for COX-2 (Yu et al., 1997; Kozak et al., 2000), and inhibition of COX-2 enhances DSI in the hippocampus (Kim and Alger, 2004; Hashimoto et al., 2007). These earlier findings suggest the hypothesis that stress and CORT enhance DSI via inhibition of COX-2. If this hypothesis is correct, then enhancement of DSI by a pharmacological inhibitor of COX-2 will be occluded in slices from stressed animals. To test this prediction, a selective COX-2 inhibitor, meloxicam (30 μ M), was bath-applied for 15–20 min prior to the induction of DSI. Meloxicam (Mel) prolonged DSI equally in hippocampal slices from both control (Figure 4A, $n = 7$ cells from seven rats) and stressed rats (Figure 4B, $n = 8$ cells from seven rats). The effects of meloxicam on mean τ_d in slices from control and stressed rats were compared using two-way ANOVA (Figure 4C). The effects of both stress exposure ($F_{1,26} = 12.4$, $p = 0.0016$) and in vitro treatment with meloxicam ($F_{1,26} = 27$, $p < 0.0001$) were highly significant; however, the interaction was not ($F_{1,26} = 0.07$, $p = 0.8$), indicating no difference in the effect of meloxicam between slices from stressed and control rats. Meloxicam also caused similar increases in the DSI integrals above their baseline values in control (Δ DSI integral of 435.8 ± 101.9 %·s) and stressed cells (Δ DSI integral of

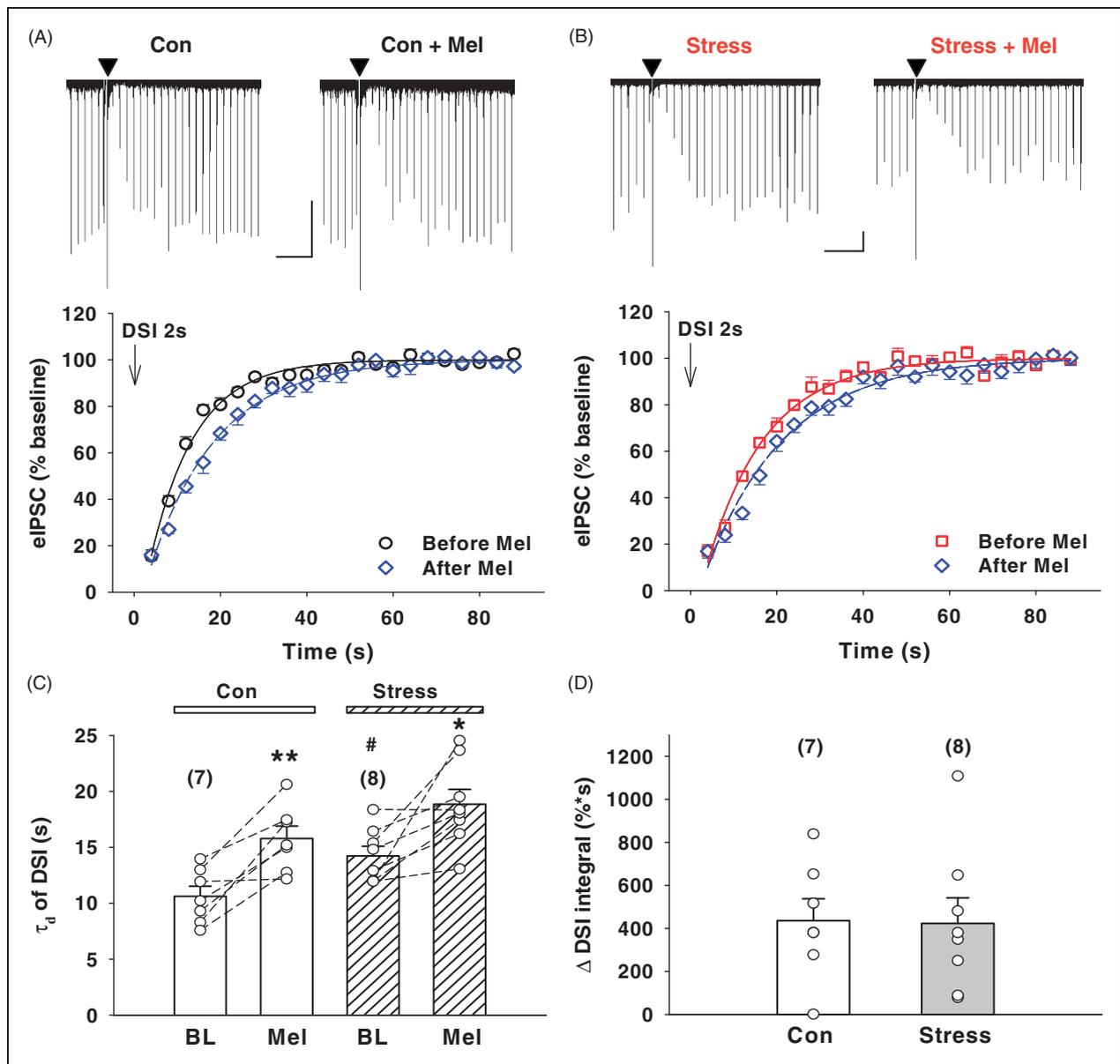


Figure 4. A cyclooxygenase 2 (COX-2) inhibitor does not occlude the effect of stress on depolarization-induced suppression of inhibition (DSI). (A and B) Bath-application of the COX-2 inhibitor, meloxicam (Mel), 30 μ M, prolonged DSI (2-s step) duration in control (A, $n =$ seven) and stressed cells (B, $n =$ eight). Representative traces from a control (A) and a stressed (B) cell are shown above. Cal. bar: 20s, 200pA. (C) Group data τ_d ; each circle represents one cell. Meloxicam enhances τ_d in control, ** $p < 0.01$, and stressed cells * $p < 0.05$; Stress BL τ_d is greater than control BL τ_d ; # $p < 0.05$. (D) Meloxicam increased Δ DSI integral similarly in control and stressed cells. Each circle represents one cell.

423.3 \pm 118.9 %*s), $p > 0.02$, t test; Figure 4D). Taken together, these data indicate that inhibition of COX-2 is not the mechanism involved in the effects of stress to increase DSI.

Ca²⁺ influx through L-type VGCCs is required for the stress-induced increase in DSI

CORT increases the amplitude of whole-cell VGCCs in hippocampal CA1 pyramidal cells (Kerr et al., 1992;

Joels et al., 2003). Acute stress and glucocorticoid treatment increase Ca^{2+} influx through L-type Ca^{2+} channels (L-channels) (Chameau et al., 2007). Although the induction of DSI is mainly dependent on N-channel current, L-channel current can also contribute under certain circumstances (Pitler and Alger, 1992; Lenz et al., 1998; Ohno-Shosaku et al., 2007). To test the possibility that L-channels are involved in the effect of stress on DSI, we bath applied the L-channel blocker, nifedipine (Nif, 10 μ M). Nifedipine did not significantly affect the duration of DSI induced by a 2-s

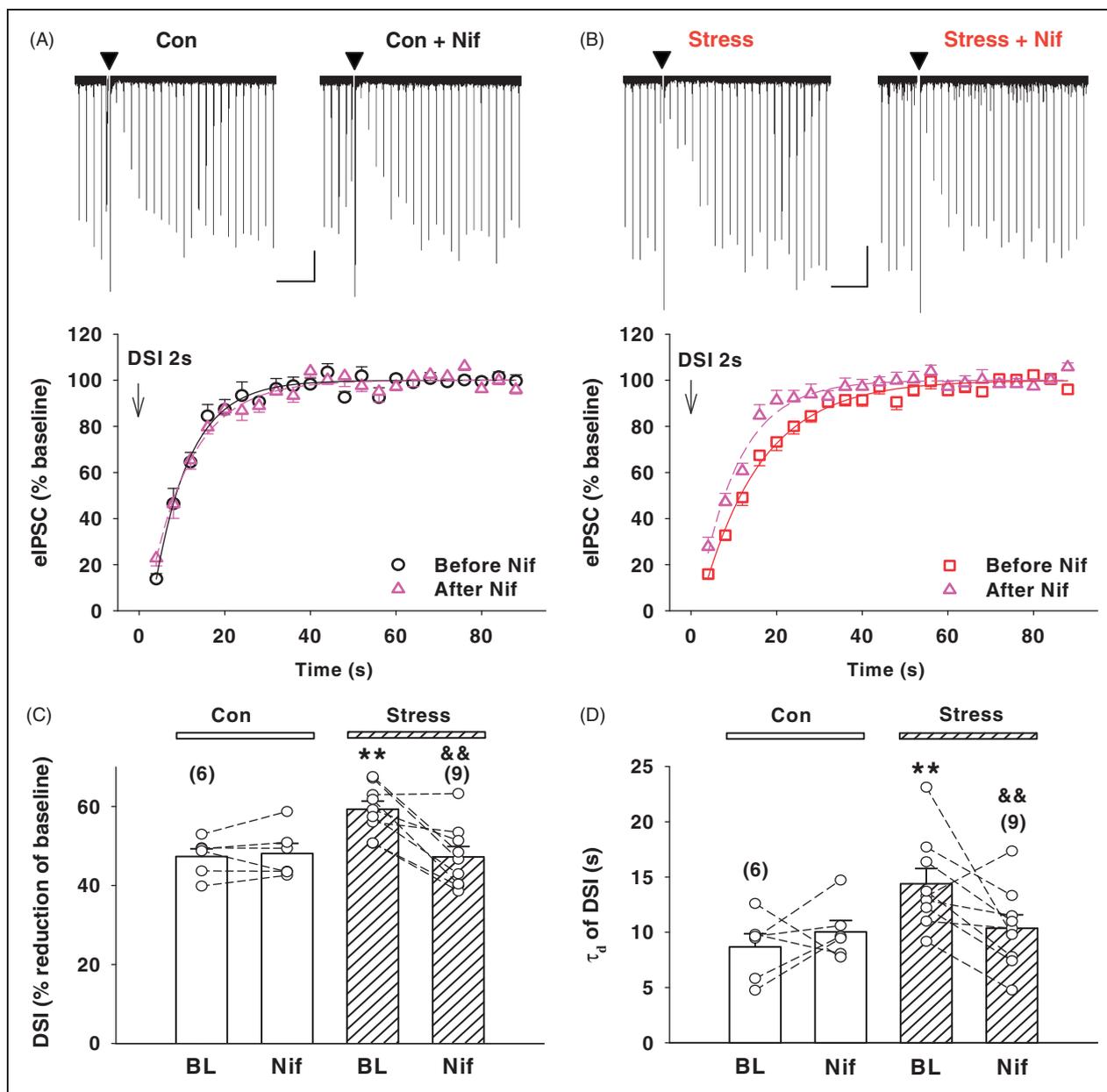


Figure 5. L-channel Ca^{2+} current mediates the stress effect on depolarization-induced suppression of inhibition (DSI). (A and B) Bath-application of the L-channel blocker, nifedipine (Nif, $10 \mu\text{M}$), did not affect DSI duration in control cells (A, $n = \text{six}$), but it decreased DSI duration in stressed cells (B, $n = 9$). Representative traces from a control (A) and a stressed (B) cell are shown above. Cal. bar: 20s, 200pA. (C) Group data, %DSI; each circle represents one cell. Nif reduced DSI in stressed cells, but not in control cells; $*p < 0.05$. (D) Group data, DSI τ_d ; each circle represents one cell. Nif reduced τ_d in stressed cells, but not in control cells, $*p < 0.05$.

depolarization in hippocampal slices from untreated rats (Figure 5A, $n = \text{six}$ cells from six rats), whereas it significantly decreased DSI duration in hippocampal slices from rats exposed to stress (Figure 5B, $n = \text{nine}$ cells from nine rats). Percent DSI was compared in slices from control and stressed rats before and after the addition of nifedipine (Figure 5C). Two-way ANOVA demonstrated that in vitro nifedipine treatment produced a significant effect on DSI ($F_{1,13} = 11$, $p = 0.006$), whereas stress did not have a significant main effect ($F_{1,13} = 3.4$, $p = 0.09$). There was a significant

interaction between stress and in vitro treatment ($F_{1,13} = 14$, $p = 0.002$). Comparisons using Bonferroni's post hoc tests indicated that nifedipine decreased DSI in cells from stressed rats ($p < 0.001$), but not in cells from unstressed rats. Nifedipine also affected the prolongation of DSI τ_d (Figure 5D) Two-way ANOVA demonstrated that neither stress ($F_{1,13} = 4.49$, $p = 0.054$) nor nifedipine treatment ($F_{1,13} = 1.29$, $p = 0.277$) had significant main effects on τ_d . However, there was a significant interaction ($F_{1,13} = 5.25$, $p = 0.039$) and Bonferroni's post hoc tests indicated that

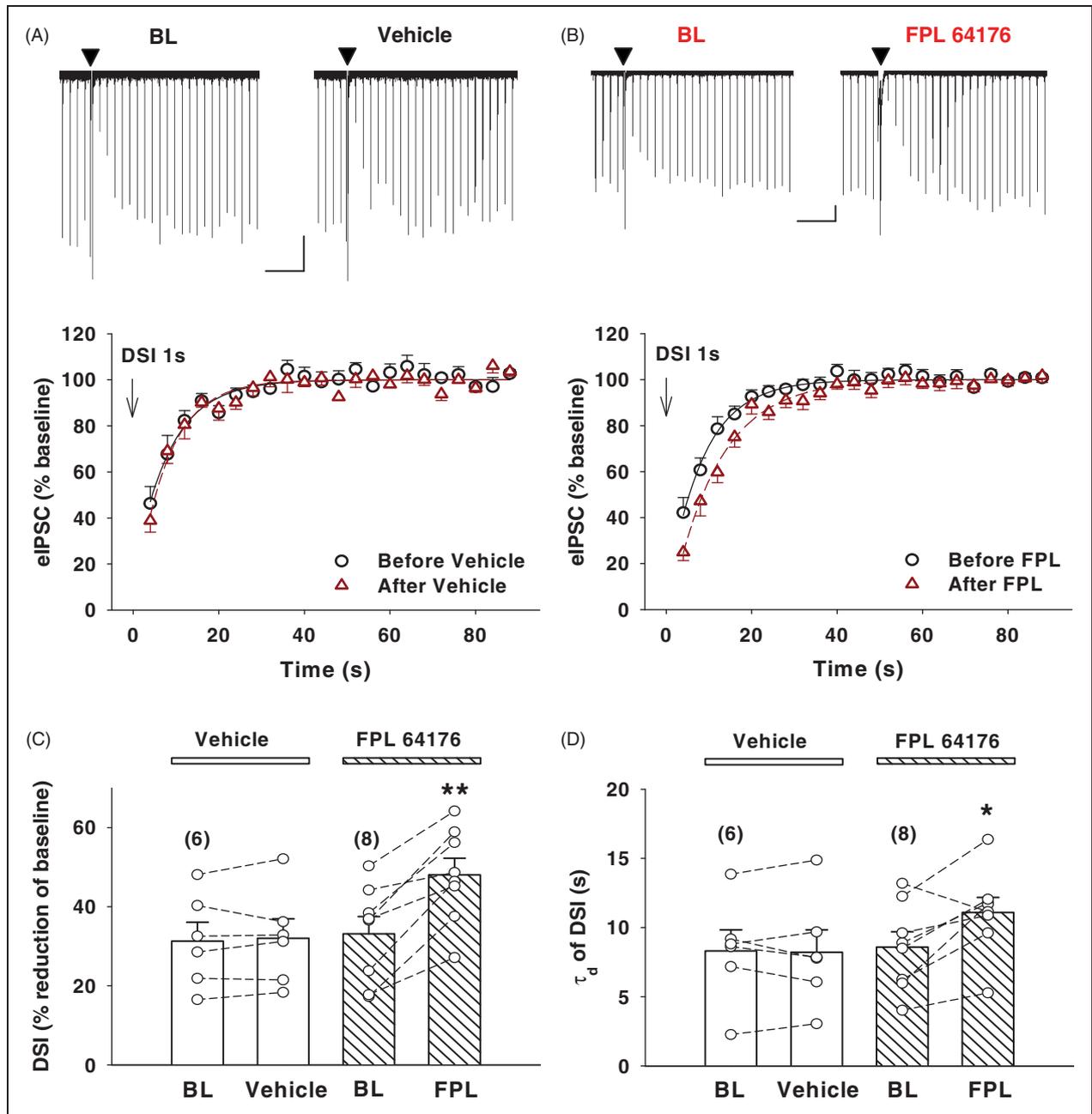


Figure 6. L-channel activation increases responses to postsynaptic depolarization. (A) Bath-application of vehicle (ethanol, 1:5,000) did not affect the responses of control cells to 1-s postsynaptic depolarization. Cal. bar: 20s, 200pA. (B) Bath-application of L-channel activator, FPL 64176 (FPL, 5 μ M or 10 μ M) increased depolarization-induced suppression of inhibition (DSI) responses. Cal. bar: 20s, 200pA. Representative traces from a vehicle-treated (A) or an FPL 64176 treated (B) cell are shown above. (C) Group data, %DSI; each circle represents one cell. FPL 64176 enhanced DSI ** $p < 0.01$. (D) Group data, DSI τ_d ; each circle represents one cell. FPL 64176 enhanced DSI τ_d , * $p < 0.05$.

nifedipine significantly ($p < 0.05$) decreased τ_d in cells from stressed rats, but not in control cells. Finally, as a direct test of the hypothesis that an increase in L-channel activity can enhance DSI, we bath-applied FPL 64176 (5 μ M or 10 μ M; $n =$ eight cells from eight rats), an agonist of L-channels, or its vehicle ($n =$ six cells from three rats), to control slices and used a 1-s DSI step to avoid the possibility that a ceiling effect might occur with longer step durations. FPL

64176 increased both DSI magnitude (from $33.2 \pm 4.3\%$ to $48 \pm 4.2\%$, $p < 0.001$, paired t test,) and duration (from $8.6 \pm 1.1\%$ to $11.1 \pm 1.1\%$, $p < 0.02$, paired t test) (examples and grouped data shown in Figures 6A and B; statistical tests done on data in Figures 6C and D), whereas treatment of the slices with vehicle had no effect. Taken together, these results support the hypothesis that stress enhances hippocampal DSI by recruiting Ca^{2+} influx through L-type Ca^{2+} channels.

Acute stress increases the 2-AG content of the hippocampus

In the studies described thus far, we have used DSI as a bioassay of the effects of stress on the ECS. If acute restraint potentiates activation of the ECS, a possible mechanism would be stress-induced increases in synaptic 2-AG. To test this prediction, we measured eCBs in bulk tissue contents within the hippocampus following stress exposure. There was a significant interaction between stress exposure and RU 38486 treatment ($F_{2,36} = 5.44$; $p < 0.01$), with post hoc analysis revealing that, compared to control, rats exposed to 30 min of restraint stress exhibited a significant increase in hippocampal 2-AG content ($p < 0.001$; Stress-immersed vs Con, Figure 7A) and the 2-AG content was still increased 30 min after stress ($p < 0.05$, Stress-30 min vs Con). The effect of stress on 2-AG content was prevented by in vivo RU 38486 pretreatment (Figure 7A). Interestingly, there was also a significant interaction between stress and RU 38486 treatment with respect to tissue levels of the other major eCB AEA ($F_{2,36} = 4.23$, $p < 0.05$), such that in the Stress-immersed group, stress exposure significantly reduced hippocampal AEA compared to control ($p < 0.01$, Figure 7B). AEA content recovered to control concentrations within 30 min after stress exposure ($p > 0.05$, Stress-30 min vs Con, Figure 7B). The reduction of AEA was also reversed by RU 38486 pretreatment (Figure 7B), suggesting a role of GR activation in this change as well. These data suggest the hypothesis that GR activation during stress results in increased 2-AG content and a concomitant facilitation of DSI in the hippocampus.

Discussion

We find that acute exposure to restraint stress enhances eCB-mediated signalling in the hippocampus of male rats. It is unknown whether this effect occurs in female rats. The magnitude and duration of Ca^{2+} -dependent eCB mobilization (DSI) and its enhancement by mAChR activation are increased in hippocampal slices. Focusing on DSI, we found that the effect of stress took time to develop, not being evident until 30 min after the restraint period ended. DSI enhancement was blocked by the GR antagonist, RU 38486, and mimicked by in vivo or in vitro CORT treatment. The effects of stress on ECS in the hippocampus are likely to be mediated by increases in circulating CORT as they were blocked by a GR antagonist and mimicked by CORT administration both in vivo and in vitro. The cellular mechanism altered by stress has not been fully determined, but $[Ca^{2+}]_i$ is critical for both DSI and mAChR enhancement of DSI (Kano et al., 2009), and, using pharmacological tools, we found that L-type Ca^{2+} channels may be the proximate downstream effectors of stress on the ECS. These results are consistent with the report of Chameau et al. (2007) that stress rapidly upregulates L-channels. Restraint stress also increased the 2-AG content in the hippocampus, as expected if DSI is mediated by 2-AG (Gao et al., 2010; Tanimura et al., 2010).

Several factors could contribute to the stress- and CORT-induced changes in DSI, including the amount of eCB

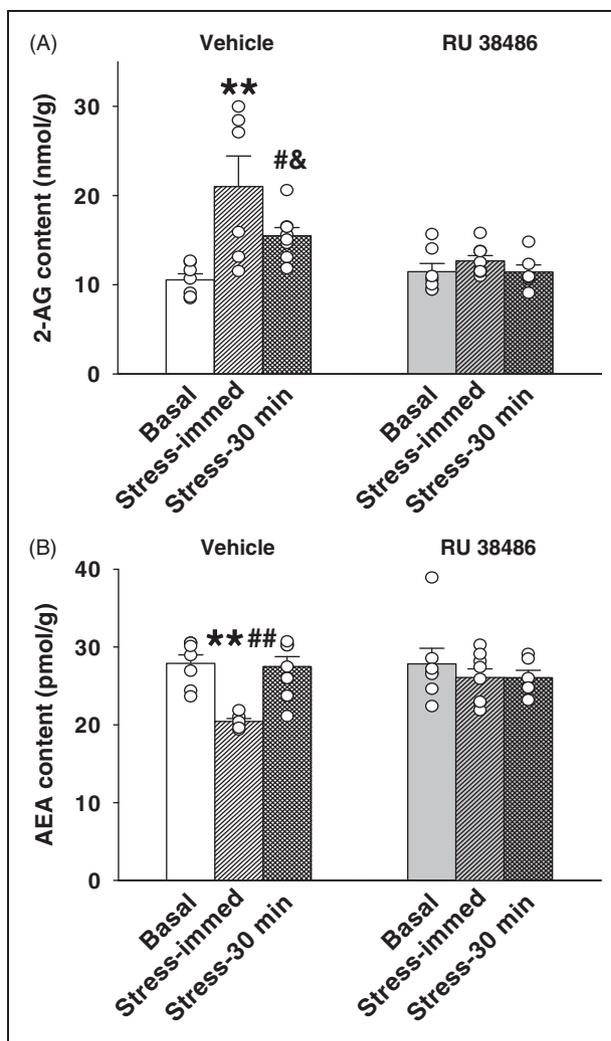


Figure 7. Acute stress enhances the content of 2-arachidonoylglycerol (2-AG) via glucocorticoid receptor (GR) activation. (A) Acute stress enhanced the content of 2-AG in hippocampus, and this was prevented by in vivo RU 38486 pretreatment. Stress increased 2-AG content over basal levels ($n = seven$) both in the Stress-immersed ($**p < 0.01$, $n = six$) and Stress-30 min ($^{\#}p < 0.05$, $n = eight$) conditions. 2-AG content was greater in the Stress-immersed than the Stress-30 min group ($^{\&}p < 0.05$). (B) Acute stress transiently decreased the content of anandamide (AEA), which was prevented by in vivo RU 38486 pretreatment. AEA in the Stress-immersed group differed from basal AEA content ($**p < 0.01$), and the Stress-30 min group differed from the Stress-immersed group given in vivo vehicle treatment ($^{\#\#}p < 0.01$), but not if RU 38486 had been given.

available (owing to changes in synthesis, release, uptake or degradation) or the sensitivity or number of CB1R. Changes in CB1Rs occur after chronic unpredictable stress (Hill et al., 2005, 2008b; Reich et al., 2009) or prolonged glucocorticoid treatment (Hill et al., 2008a). In striatum, the sensitivity of GABA synapses to eCB is impaired following one social defeat stress exposure and is abolished by exposures over 3 or 7 days (Rossi et al., 2008). However, CORT administration for shorter periods (i.e. 1 day) does not affect CB1R agonist

binding parameters in the hippocampus, suggesting that longer lasting increases of CORT are required for the regulation of CB1R expression. Indeed, upregulation of CB1R cannot fully explain our results, as increased CB1R expression would have affected all eCB-mediated responses, yet significant DSI enhancement was not seen with all DSI-inducing voltage steps. An effect of stress on CB1R signalling downstream from the receptor is not ruled out by our results.

An alternative explanation is that stress and CORT enhance DSI by increasing eCB mobilization. Hippocampal 2-AG content is significantly increased following stress, which is consistent with an increase in 2-AG mobilization, and with earlier reports that footshock and glucocorticoid application stimulate eCB biosynthesis (both AEA and 2-AG) in the periaqueductal gray matter and hypothalamus respectively (Di et al., 2003; Hohmann et al., 2005; Malcher-Lopes et al., 2006). The acute effect of stress on tissue 2-AG could be brain region-specific, as a single exposure to restraint increases 2-AG content in the medial prefrontal cortex in a temporal and GR-dependent fashion (Hill et al., 2011) that is similar to what we find in hippocampus, but does not increase 2-AG content in the amygdala (Hill et al., 2009). Injection of 20 mg/kg CORT in rats does not significantly affect hippocampal 2-AG tissue contents measured 24 h later (Hill et al., 2008a), and our data obtained now show that the effect of stress on DSI does not persist for 23 h either. However, the relationship of bulk measurements of 2-AG to cellular 2-AG mediated signalling processes is not clear. Recent studies with mutant mice lacking functional DGL α (Gao et al., 2010; Tanimura et al., 2010) reveal that a significant fraction of total 2-AG measured in different brain areas is independent of DGL α , and, in fact, much of the DGL α -dependent 2-AG could be unrelated to rapid physiological signalling (Gao et al., 2010). Thus, measured increases in the tissue content of 2-AG may not be directly related to the 2-AG that is available for signalling through CB1Rs. These considerations could account in part for the disparity between the bulk increase in 2-AG that is present immediately after cessation of the restraint stress period (Figure 7), and the physiologically measured increase in DSI, which is not detectable until 30 min later (Figure 1A1). The proposed existence of multiple functionally distinct 'pools' of 2-AG or DGL α creates further complexity in relating bulk measurements of 2-AG with specific CB1R-mediated phenomena (Edwards et al., 2006; Zhang et al., 2011; Alger and Kim, 2011). Finally, stress could interact with the ECS via more than one mechanism.

Stress-enhanced DSI was not affected by inhibiting receptors, such as mGluRs, mAChRs and CCK $_2$ Rs, that are affected by stress (Beck et al., 1996; Venero and Borrell, 1999) and that also mediate eCB generation in the hippocampus, suggesting that these receptors are not part of the underlying mechanism. COX-2 hydrolyzes 2-AG in hippocampus, and COX-2 inhibition prolongs DSI (Kim and Alger, 2004; Hashimoto-dani et al., 2007). In the hypothalamus, glucocorticoids may shift arachidonic acid metabolism toward eCB synthesis via COX-2 inhibition (Malcher-Lopes et al., 2008). However, we found that a COX-2 inhibitor prolonged DSI equally in stressed and control slices, arguing against an involvement of COX-2 in stress-enhanced eCB function. We noted that even when DSI itself was not enhanced by stress (i.e.

when the DSI step was only 0.5 or 1.0 s), the ability of CCh to enhance DSI was amplified. This could represent a non-linear interaction between the Ca $^{2+}$ - and GPCR-pathways, an additional action of stress on the GPCR pathway, or an increase Ca $^{2+}$ -sensitivity of the eCB mobilization mechanisms.

Corticosterone, which is released in high concentrations after stress, can easily pass the blood-brain barrier and bind to intracellular receptors in the brain (De Kloet et al., 2005). Activation of the relatively low-affinity GRs will be initiated, as the higher affinity mineralocorticoid receptors will already be substantially occupied by normally circulating corticosteroid levels. The activation of GRs increases the amplitude of L-currents in the hippocampal CA1 region (Chameau et al., 2007). L-channels exist on CA1 pyramidal cells in high densities (Westenbroek et al., 1990) and contribute significantly to the total somatic Ca $^{2+}$ current measured in these cells. Although the principal carriers of Ca $^{2+}$ current for hippocampal eCB mobilization are N-channels, L-channel influx can contribute to DSI (Pitler and Alger, 1992; Lenz et al., 1998; Ohno-Shosaku et al., 2007). Our results can best be explained by a glucocorticoid-mediated increase in L-channel Ca $^{2+}$ influx into the pyramidal cells, and consequent increase in eCB mobilization.

Di et al. (2003) were the first to report that GR activation affects eCB synaptic signalling. They found that bath-application of CORT resulted in rapid suppression of glutamate release from terminals innervating parvocellular secretory neurons of the paraventricular nucleus in the hypothalamus. The effect of CORT required CB1R engagement, leading the authors to hypothesize that CORT recruited the ECS to produce synaptic plasticity at this synapse. These investigators showed further that the effect of CORT was consistent with a non-genomic mechanism such that it was membrane-delineated and required G protein activation (Di et al., 2003). Although the ECS is also involved in the effects that we observe, several differences between our findings and those of the Tasker group suggest that the cellular mechanisms involved are likely to be different in the two systems. First, the effect of stress on DSI in the hippocampus is not seen immediately after stress offset, when circulating CORT concentrations are at a peak (Diorio et al., 1993) but is seen 30 min later. In the hypothalamus, CORT had an immediate effect on glutamate release. Second, the effect of stress in the hippocampus requires L-type Ca $^{2+}$ channels; the increase in Ca $^{2+}$ currents mediated by glucocorticoids is a genomic effect that depends on protein synthesis (Karst et al., 1994). Thus, the mechanism in the hippocampus may involve genomic GR signalling, as opposed to the non-genomic mechanism of the hypothalamus.

Our biochemical data are consistent with other evidence that 2-AG, and not AEA, is the eCB for DSI in the hippocampus (Kim and Alger, 2004; Hashimoto-dani et al., 2008; Gao et al., 2010; Tanimura et al., 2010). In fact, we also observed a significant, although transient, decrease in AEA content caused by stress. This is consistent with previous reports of stress-induced reduction in AEA in other brain regions (Patel et al., 2005; Rademacher et al., 2008; Hill et al., 2009). Although the linkage is unclear, recent studies show significant reductions in AEA in DGL knock-out mice (Gao et al., 2010; Tanimura et al., 2010). Our data show an inverse, rather than a direct, relationship between AEA and 2-AG, nevertheless, it

may be interesting that the AEA and 2-AG systems seem to be altered by common forms of behavioural experience.

As increases in eCB actions decrease GABA release, the present data may help account for previous reports of reductions in GABA levels within the hippocampus following acute stress exposure (Harvey et al., 2004; Briones-Aranda et al., 2005). Patel et al. (2009) recently reported that, in the amygdala, repeated restraint episodes are required to amplify DSI and increase 2-AG tissue content, hence the hippocampus may be more sensitive in this regard. Stress-induced mobilization of 2-AG could be a general mediator of stress-induced modulation of synaptic transmitter release, and it will be interesting to learn if the ECS is affected by acute restraint stress in other areas.

In conclusion, we show that hippocampal ECS is potentiated by acute stress and GR activation. These findings are consonant with recent behavioural findings that acute stress and/or glucocorticoids modify emotional and motivated behaviours via increased eCB signalling (Coddington et al., 2007; Campolongo et al., 2009). In contrast, prolonged exposure to stress and/or glucocorticoids decreases CB1R expression and eCB levels (Hill et al., 2005, 2008a, 2008b; Reich et al., 2009), suggesting that the relationship between stress and the ECS is biphasic. This is reminiscent of other responses to stress that also exhibit a biphasic response, such as immune functioning, which is facilitated by acute stress, but which is subject to immunosuppression under chronic stress conditions (Dhabhar, 2003). This shift may reflect the burden of 'allostatic load'; the loss of ECS function during chronic stress could contribute to allostatic load and to the development of disease states (McEwen, 2004). Stress-induced mobilization of eCB signalling could represent an adaptive response to acute stress, which would help maintain emotional and behavioural flexibility in the face of aversive stimuli, while the loss of eCB signalling following chronic stress could contribute to the development of stress-related psychiatric disorders, such as depression and anxiety disorders (Gorzalka et al., 2008; Lutz, 2009).

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