Central vasopressin V1A receptor blockade alters patterns of cellular activation and prevents glucocorticoid habituation to repeated restraint stress exposure

Megan Gray*, Leyla Innala and Victor Viau
Neuroscience Program, Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada

Abstract
Our previous experiments implicated a role for the arginine vasopressin (AVP) V1A receptor subtype in mediating the normal decline (habituation) of hypothalamic–pituitary–adrenal (HPA) axis responses to repeated restraint exposure. To explore pathways mediating the endogenous effects of central AVP on stress HPA axis habituation, here we compared cellular (Fos) and hormone responses in male rats receiving chronic icv infusion of vehicle or a V1A receptor antagonist that began 7 d before stress testing, continued through the duration of acute and repeat restraint exposure. As a group, rats with V1A antagonism displayed a modest reduction in ACTH habituation, whereas the decline in corticosterone was completely prevented. V1A antagonized rats also showed reduced evidence of habituated Fos responses in the paraventricular nucleus of the hypothalamus, medial amygdala, and within the anterior division of the bed nucleus of the stria terminalis. Based on these cellular and neuroendocrine responses, we then examined whether repeated restraint is reflected by changes in V1A receptor binding. Relative to stress naïve animals, repeatedly exposed rats showed lower levels of V1A binding in the dentate gyrus of the hippocampus, thalamus and central amygdala, but higher levels in the septum and anterior BST. Taken together, these findings suggest that AVP may act within multiple targets to regulate the normal decline in stress-induced drive to the HPA axis, and that this may involve the net of V1A receptor stimulatory and inhibitory influences on neuroendocrine habituation.

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Introduction
The hypothalamic–pituitary–adrenal (HPA) axis is a neuroendocrine system that involves the sequential release of different classes of hormones from the hypothalamus to the adrenals. Governed by the paraventricular nucleus of the hypothalamus (PVN), this system ultimately regulates the synthesis and release of glucocorticoid steroid hormones that are critical for meeting the metabolic demands of stress. When exposure to the same (homotypic) stimulus is repeated in a predictable manner, equally important for survival is the effective attenuation or habituation of glucocorticoid responses (Grissom and Bhatnagar, 2009). This process is thought to be adaptive as it minimizes potential detrimental effects of excessive glucocorticoid exposure, but maintains HPA responses to new or unanticipated challenges (Dallman, 2003).

Several lines of evidence suggest that arginine vasopressin (AVP)-producing cells of extrahypothalamic origin may be important contributors to the process of neuroendocrine habituation. Normal declines in HPA axis responses to repeated restraint are associated with increases in AVP expression within the posterior bed nuclei of the stria terminalis (BST) and medial amygdala (Gray et al., 2010). Connectivity studies support a direct pathway from the posterior BST to neuroendocrine neurons of the PVN (Bingham et al., 2011). Moreover, the posterior BST and medial amygdala issue prominent projections to multiple afferent mediators of the HPA axis, including the septum, central amygdala, hippocampus and cortex. V1A receptors follow the distribution of these projections to suggest that AVP of extrahypothalamic origin may reach out to several cortical, subcortical and limbic-related pathways to regulate adaptive neuroendocrine responses (Ring, 2005). Consistent with this...
possibility, injections of a V1A receptor antagonist into the vicinity of the PVN and/or the dorsomedial hypothalamus (DMH) potentiate corticosterone responses to osmotic challenge and social defeat (Wotjak et al., 1996, 2002). We recently compared the propensities for V1A receptor antagonism delivered via the intraventricular route to alter ACTH and corticosterone responses to acute and repeated restraint (Gray et al., 2012). V1A receptor antagonism reduced the decline in HPA output responses to repeated restraint exposure, produced by sustained elevations in hormone responses on the last, but not on the first day of restraint exposure. We interpreted this restricted influence of V1A receptor antagonism to reflect a shift towards enhanced central AVP utilization and/or receptor activation.

In the present study we first analyzed the number of cells recruited to express Fos protein in response to acute and repeated restraint exposure in rats with or without continuous icv V1A antagonism. In addition to the PVN, forebrain regions chosen for analysis were based on their functional connectivity with the HPA axis, tendency for showing repeated stress-induced decrements in Fos induction, and expression of V1A receptors. In the second experiment we employed an autoradiographic approach to determine whether changes in V1A binding could explain the shift in HPA responses to V1A antagonism.

Method

Animals and treatment

Adult male Sprague-Dawley rats (Charles River, Canada) were used, starting from 54 to 55 d old for icv surgeries (experiment 1) and 62 d old for receptor binding (experiment 2). Rats were pair housed under controlled temperature (23±2°C) and lighting conditions (12:12 h light:dark cycle, lights on at 07:00 hours) with food and water available ad libitum. All protocols were approved by the University of British Columbia Animal Care Committee. Restraint stress was performed in the morning (09:00–12:00 hours) in Plexiglass restrainers for 3 h each day for five consecutive days. Controls (unstressed animals) were comparably handled, but never restrained.

Experiment 1. Repeated restraint and V1A receptor antagonism: Stress-induced Fos and endocrine responses

Rats were anesthetized under gaseous isoflurane and implanted with Alzet osmotic micropumps (model 2004, USA), designed to deliver into the right lateral ventricle either vehicle (saline) or d(CH2)5Tyr(Me)AVP (10 mg/d, 0.25 μl/h, Sigma V2255), using the following stereotaxic coordinates: anteroposterior, −0.40 mm from bregma; mediolateral, ±1.50 mm; dorsoventral, −4.50 mm from skull. Based on our previous studies (Gray et al., 2012), the dosage used for this selective AVP V1A receptor antagonist (Laszlo et al., 1991) produces a reliable effect to impede endocrine responses to repeated restraint exposure. All animals received continuous infusion of vehicle or drug for 7 d before restraint exposure that continued throughout the duration of the testing period. This treatment schedule was to ensure adequate drug delivery, provide sufficient time for surgical recovery, as well as to avoid disturbing animals during stress testing. The V1A antagonist has been shown to exert maximal behavioral and physiological effects during the first 7 h, modest at 12 h, and abolished from 24–48 h of administration (Ferris and Potegal, 1988). Based on this profile, and because continuous drug infusions were initiated 1 wk in advance of stress testing, it is unlikely that the 4 d discrepancy in treatment between acutely and repeatedly restrained animals (8 and 12 d from drug onset, respectively) introduced any more of a differential or additive effect. Nonetheless, this discrepancy conforms to the blood data derived from cohorts of animals repeatedly sampled on day 1 and day 5 of restraint exposure. Evans Blue dye (50 μl, 0.02%) was injected into indwelling cannula in advance of perfusion to assess cannula patency. Animals showing evidence of improper placement or blocked cannula were removed from analysis. Final analyses included vehicle (n=24) and drug (n=32) total treatment group sizes. Subsets of tissue from vehicle (n=6) and drug treated (n=8) animals were obtained at 90 min of restraint onset or under basal conditions on the first and last day of stress testing.

Tissue processing

Rats were anesthetized for perfusion using a lethal dose of chloral hydrate (700 mg/kg) and sequentially perfused with 0.9% saline (5 min) and 4% paraformaldehyde (20 min) at a flow rate of 20–25 ml/min. Brains were post-fixed for 4 h, and cryoprotected in 15% sucrose in 0.1 M potassium phosphate buffer overnight at 4°C. Five one-in-five series of coronal sections (30 μm) were collected in antifreeze (30% ethylene glycol, 20% glycerol in 0.05 M sterile KPBS) and stored at −20°C until processing.

Fos immunohistochemistry

Restraint-responsive neurons were localized using Fos protein as a marker of cellular activation using a primary antiserum (1:40000) raised against amino acids 4–17 of the human Fos protein (Ab-5, Calbiochem). Immunolocalization was performed using a conventional nickel intensified, avidin-biotin-immunoperoxidase (Vectastain Elite ABC kit; Vector Laboratories) procedure (Williamson and Viatu, 2008). Total cell number estimates were generated by counting bilaterally the number of Fos-positive cells through each region of interest, averaged by dividing the total number of cell counts by slice number, corrected for double counting errors (Guillery, 2002), and multiplying this product by a factor of five to account for slice frequency (one-in-five sections). Cross-sectional
area measurements in PVN, amygdalae and hippocampus (Pham et al., 2003) revealed no reliable effects of restraint and/or treatment to alter the volume of these regions. Numbers of sections analyzed per region of interest included: three stions for hippocampus, cortex, anterior and posterior divisions of the BST, medial preoptic and suprachiasmatic nuclei, PVN subregions and septum; four stions for the dorsomedial hypothalamus and posterior paraventricular thalamus; and six stions for medial and central amygdala. Parceling of the rat brain and discrete subpopulations of the PVN followed the mapping of Fos-ir as defined by the morphological features provided by Thionin staining of adjacent tissue series, as previously described (Viau and Sawchenko, 2002; Gray et al., 2010). Boundaries defining other regions were likewise drawn from adjacent Thionin stained series, in addition to assessing patterns of Fos labeling. Cell counts for each were standardized using uniformly sized, rectangular regions of interest (VanElzakker et al., 2008), whose locations were matched between animals using the same rostral-caudal coordinates. Light-level images were captured using a Retiga 1300 CCD digital camera (Q-imaging, Canada), analyzed using Macintosh OS X-driven, Open Lab Image Improvement, version 3.0.9 (Quorum Technologies, Canada), exported to Adobe Photoshop v.10.0 (San Jose, CA, USA), where standard methods were used for final image assembly.

Blood sampling

Hormone levels were determined from a subset of rats employed for Fos-based analysis, limited to those with repeated tail bleeds on both day 1 and day 5 of restraint testing (vehicle, n=6; drug, n=8). Blood samples (300 ul) obtained via tail vein nick were collected in ice-chilled tubes containing aprotinin and EDTA, acquired from individual animals immediately following home cage removal (0 min), and at 30, 60, and 90 min from restraint onset. Plasma obtained by centrifugation (10000 r/min for 20 min at 4 °C) was stored at −20 °C.

Radioimmunoassay

Plasma hormone concentrations were measured using commercial RIA kits (MP Biomedicals, OH) with [125I] as tracer, as previously described (Gray et al., 2010). The intra- and inter-assay coefficients of variation for assays ranged from 1–7% and 2–13%, respectively. The standard curve ED50 for ACTH and corticosterone was 89.12 pg/ml and 160.1 ng/ml, with detection limits of 8.1 pg/ml and 6.72 ng/ml, respectively.

Experiment 2. Repeated restraint and V1A receptor binding

Receptor autoradiography

The thrust of this aim was to determine if repeated restraint is associated with steady-state changes in V1A receptor binding. Thus, to meet this goal and to mitigate potential changes in V1A receptor activation and occupancy evoked by acute stimulus exposure, tissues were obtained 24 h after the last of five daily exposures of 3 h restraint (n=6) and compared to unstressed controls (n=6). Following decapitation, brains were removed, frozen in −50 °C isopentane, and stored at −80 °C. Multiple series of 20 um thick sections were stored at −80 °C until the time of assay and processing for V1A binding, as previously described (Francis et al., 2002). For total binding, sections were incubated in a 50 nm Tris-HCl (pH 7.4) solution containing 50 pm of the V1A antagonist, [125]I-lin-vasopressin (Perkin Elmer). Non-specific binding was determined by incubating alternate sections with radioactive ligand and 2 μM of unlabeled (d(CH2)5I, Tyr(Me)2,Arg8)-vasopressin (Tocris). Following 1 h incubation, sections were rinsed, air-dried, and apposed to BioMaxMR film (Amersham) alongside [125]I microscale standards for 2–4 d to optimize signal detection. Captured images were analyzed using NIH Image J v.1.38 software to generate optical density levels, converted to binding activity units (uCi/g) according to the [125]I microscale standard curve.

Statistical analyses

Three-way mixed (between treatment, within subject) design ANOVAs were used to analyze hormone responses using day (acute, repeat) and time as repeated measures. To gauge relative effects of treatment on stress HPA axis habituation, total hormone (area under the curve) responses were calculated for the first and last day of exposure, analyzed using two-way ANOVAs (between treatment, within subject). Grouped data for Fos-based studies were compared using three-way ANOVAs for stress × time interaction for ACTH and corticosterone (panels C, D) responses during acute and repeat restraint are shown. Three-way analysis indicated significant effects of stress (F1,12=119.5; p<0.0001) and time (F3,36=51.1; p<0.0001), and a significant interaction between stress and time (F3,36=28.9; p<0.0001) for ACTH; whereas a significant three-way interaction between treatment, stress and time (F3,36=4.6; p=0.0076) was revealed for corticosterone. Post-hoc analyses of the stress × time interaction for ACTH and corticosterone confirmed reduced hormone responses in the repeated

Results

Effects of V1A receptor antagonism on stress-induced endocrine responses

In Fig. 1, time courses for ACTH (panels A, B) and corticosterone (panels C, D) responses during acute and repeat restraint are shown. Three-way analysis indicated significant effects of stress (F1,12=119.5; p<0.0001) and time (F3,36=51.1; p<0.0001), and a significant interaction between stress and time (F3,36=28.9; p<0.0001) for ACTH; whereas a significant three-way interaction between treatment, stress and time (F3,36=4.6; p=0.0076) was revealed for corticosterone. Post-hoc analyses of the stress × time interaction for ACTH and corticosterone confirmed reduced hormone responses in the repeated
restraint condition. Post-hoc analyses of the three-way interaction for corticosterone confirmed smaller responses on the last day of restraint in vehicle, but no change for V1A antagonist infused animals (Fig. 1d). Based on area under curve analysis discussed below, a priori comparisons of hormone concentrations confirmed higher plasma ACTH (time 60 min; Fig. 1b) and corticosterone (time 30 and 60 min; Fig. 1d) levels in V1A antagonized compared to control animals on the last day of restraint.

Analyses of total (area under curve) hormone responses between the first and last day of restraint suggested only a modest effect of V1A antagonism to reduce ACTH habituation (Fig. 1e) relative to control animals. Thus, there was a tendency for an interaction between stress and treatment ($F_{1,12}=3.7; p=0.07$), but no significant effect of treatment ($F_{1,12}=1.7; p=0.21$). For corticosterone, there was a main effect of treatment ($F_{1,12}=15.0; p=0.002$) and a significant interaction between stress and treatment ($F_{1,12}=14.15; p=0.027$). Post-hoc analysis confirmed a significant decline in total corticosterone responses in control animals, but not in those treated with the V1A antagonist (Fig. 1f).

**Effects of V1A receptor antagonism on stress-induced activation of the PVN and HPA axis regulating cell groups**

**Characterization of restraint-responsive PVN neurons**

Three-way analyses ($F_{1,48}$) revealed significant main effects of stress ($p’s<0.0001$) and reliable interactions between day and stress ($p’s \leq 0.008$) in all subregions of the PVN. Significant main effects of day were also revealed for all subregions of the PVN ($p’s<0.002$), except for posterior magnocellular (PM) neurosecretory neurons ($p=0.117$). Post-hoc analyses of the day x stress interaction indicated significant decrements ($p’s<0.05$) in Fos-ir in the repeated restraint condition in all subregions of the PVN (Fig. 2). There was a tendency for an interaction between stress and treatment ($F_{1,48}=2.8; p=0.09$) and a significant effect of treatment ($F_{1,48}=4.50; p=0.04$) for the medial parvocellular dorsal (mpd) part, but not for the remainder of the PVN ($p’s \geq 0.638$ in all cases). For the mpd PVN, post-hoc analyses confirmed significantly lower Fos responses during repeated restraint in control animals than those treated with the V1A antagonist (Fig. 2c).
Limbic forebrain: Central and medial extended amygdala

The central and medial amygdala nuclei exert marked effects on basal and stress HPA activity, but show little to no direct connections with hypophysiotropic neurons of the PVN. As with the hippocampus and various cortical regions, amygdalar influences on the HPA axis are mediated through multiple hypothalamic relays, in addition to their respective rostral extensions within the bed nucleus of the stria terminalis (Herman et al., 2005). Initial qualitative assessments of Fos responses within the anterior division of the BST, preferentially innervated by the central amygdala (Bienkowski and Rinaman, 2013), revealed cells occupying the dorsomedial subdivision to show the most reliable increases from control levels (Fig. 3a). Fos levels were low to moderately high within oval and fusiform nuclei under basal conditions, respectively, but showed no discernible changes from baseline during restraint. For the dorsomedial subdivision, there were significant main effects of day ($F_{1,48}=8.4; p=0.006$) and stress ($F_{1,48}=66.0; p<0.0001$), and a significant interaction between stress and treatment ($F_{1,48}=4.8; p=0.034$). Post-hoc analyses of this interaction confirmed a repeated stress-induced decrement in Fos-ir responses in control animals (Fig. 3a, b), but not in those treated with the V1A antagonist (Fig. 3c). For the central nucleus of the amygdala, there was a main effect of stress ($F_{1,48}=14.8; p<0.0001$), but no main effects of V1A antagonism and treatment interactions. Thus, from basal levels that were already moderately high, there...
was a detectable decline in Fos cell numbers in the repeated restraint condition (Fig. 4a).

For the medial amygdala, initial assessments indicated that the dominant site of Fos induction occurred within the posterodorsal part, with fewer cells detected and/or least reliable induction within the anteroventral part of the nucleus. There were significant main effects of day ($F_{1,48}=5.5; p=0.024$) and stress ($F_{1,48}=92.7; p<0.0001$), and a significant three-way interaction between stress, day, and treatment ($F_{1,48}=4.3; p=0.044$). Post-hoc analyses of this interaction confirmed a repeated stress-induced decrement in Fos-ir responses in control animals, but not in those treated with the V1A antagonist (Fig. 4b). Preferentially innervated by the medial amygdala (Dong et al., 2001), the dominant site for Fos induction within the posterior division of the BST was biased towards its ventral part, including the interfascicular and transverse nuclei. For these cells groups, there were significant main effects of day ($F_{1,48}=5.5; p=0.023$) and stress ($F_{1,48}=28.0; p<0.0001$), and a significant interaction between day and stress ($F_{1,48}=9.0; p=0.004$). Post-hoc analyses of this interaction confirmed significant decrements ($p's<0.05$) in Fos-ir in the repeated restraint condition regardless of treatment status (Fig. 4c).

**Hippocampus and select relays**

**Hippocampal subfields and dentate gyrus.** Initial assessments revealed moderately high numbers of Fos-ir cells in CA3 and low to undetectable levels in the CA1 subfield of the hippocampus in unstressed controls. Implicated as a source of influence of hippocampal outflow to the hypothalamus, only the CA1 showed reliable increases from basal levels. For this hippocampal subregion, there was a significant main effect of stress ($F_{1,48}=13.9; p=0.001$), and a reliable interaction between stress and treatment ($F_{1,48}=6.9; p=0.012$). Post-hoc analyses attributed this interaction to significant increments in Fos-ir during acute and

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**Fig. 3.** Representative brightfield photomicrographs to show Fos-ir staining at 90 min of acute and repeat restraint exposure in the anterior BST, dorsomedial subdivision (3A and 3B, respectively) and in the ventrolateral septum (3C and 3D, respectively) of control animals receiving vehicle. Scale bar=250 μm (applies to A–D). Relative strength of Fos induction under acute and repeat restraint conditions in the anterior BST (3E) and septum (3F). Mean±s.e.m. number (No.) of Fos-ir neurons at 90 min of restraint exposure in animals receiving vehicle or V1A antagonist infusions. *$p<0.05$ vs. acute counterpart; †$p<0.05$ vs. acute, vehicle-treated ($n=6–8$/group).
repeated restraint in vehicle controls, but not in V1A antagonized animals. (Fig. 4e). For the dentate gyrus, there was a main effect of stress ($F_{1,48}=14.8; p<0.0001$), attributed to a significant decline in Fos cell numbers in the repeated restraint condition, regardless of drug treatment (data not shown).

**Dorsomedial hypothalamic and septal nuclei.** Both the dorsomedial hypothalamic nucleus (DMH) and septum represent important intermediaries for descending hippocampal influences on the HPA axis (Myers et al., 2013). Both of these nuclei showed reliable increments from basal levels, with Fos induction dominating the ventrolateral part of the septum (Fig. 3c). For the DMH, there were significant main effects of day ($F_{1,48}=6.8; p=0.012$) and stress ($F_{1,48}=337.4; p<0.0001$), a tendency for a main effect of treatment ($F_{1,48}=3.8; p=0.059$), but a reliable interaction between stress and treatment ($F_{1,48}=4.5; p=0.039$). Post-hoc analyses of this interaction in the DMH confirmed a repeated stress-induced decrement in Fos-ir responses in vehicle controls, but not in V1A antagonized animals (Fig. 4f). This interaction, however, was actually attributed to relatively higher levels of Fos induction in control animals in the acute restraint condition compared to V1A antagonized animals.

**Limbic sensory-related pathways**

As previously proposed, alterations in the activity of sensory systems may very well explain the global decrements in Fos induction that occur in response to mild homotypic stress (Girotti et al., 2006). Thus, we next surveyed patterns of cellular activation within sensory-related structures most intimately involved with the limbic system. This included the posterior paraventricular nucleus of the thalamus (pPVT), which is targeted by the amygdala and frontal cortex and implicated in the development of stress habituation to repeated homotypic stressors (Jaferi and Bhatnagar, 2006). We also focused on the entorhinal and perirhinal cortex, which collectively provide the bulk of processed sensory information to the hippocampus (VanElzakker et al., 2008). For the pPVT, there were significant main effects of day ($F_{1,48}=41.5; p<0.0001$) and stress ($F_{1,48}=140.9; p<0.0001$), a reliable interaction between day and stress ($F_{1,48}=44.9; p<0.0001$), but no effect of treatment ($F_{1,48}=0.1; p=0.92$) or treatment interactions ($p’s>0.66$). Post-hoc analyses of the day × stress interaction confirmed significant decreases in Fos-ir in the repeated restraint condition, regardless of drug treatment (Fig. 4g). For the entorhinal cortex, initial qualitative assessments revealed reliable increments from basal control levels within the inner layer-5, but not within the outer layer-2, representing output and input elements of the hippocampal formation, respectively. For the inner layer of the entorhinal cortex (Fig. 4d), there was a significant main effect of stress ($F_{1,48}=18.6; p<0.0001$), but no significant effects of day ($F_{1,48}=0.3; p<0.25$), treatment...
(F\(_{1,48}=0.1; \ p=0.91\) or treatment interactions (p’s\(\geq 0.68\). For the perirhinal cortex (Fig. 4h), there were significant effects of stress (F\(_{3,48}=59.8; \ p<0.0001\) and day (F\(_{1,48}=12.6; \ p=0.0009\), and a reliable interaction between day and stress (F\(_{3,48}=14.2; \ p<0.0005\), but no effect of treatment (F\(_{1,48}=0.02; \ p<0.89\) or reliable treatment interactions (p’s\(\geq 0.44\). Post-hoc analyses confirmed significant decrements in Fos-ir for the perirhinal cortex in the repeated restraint condition, regardless of drug treatment (Fig. 4h).

Repeated restraint and V1A receptor binding

Consistent with previous surveys, specific labeling was most apparent within the dentate gyrus of the hippocampus, central amygdala, anterior BST, thalamus, and suprachiasmatic nucleus (Fig. 5). Relative to unstressed controls, in repeatedly restrained animals V1A binding was decreased in the dentate gyrus (p = 0.017), anteroventral and ventrolateral thalamus (p = 0.007), and in the caudal part of the CeA where CRH expressing neurons are amassed (p = 0.023). In contrast, V1A binding was increased in the anterolateral subdivision of the anterior BST (p = 0.039) and ventrolateral zone of the lateral septum (p = 0.016). There were no reliable group differences for the SCN (p = 0.08) and the dorsolateral zone of the lateral septum (p = 0.08).

Discussion

Relative to vehicle controls, V1A receptor antagonism increased HPA axis output during repeat, but not acute restraint exposure, to suggest that habituated neuroendocrine responses rely on changes in V1A receptor signaling pathways. Repeated restraint was associated with region-specific decreases, as well as increases in V1A receptor binding. Thus, the process of stress HPA axis habituation may very well enlist both increments and decrements in AVP neuromodulation within multiple brain regions. V1A receptor antagonized rats showed reduced evidence of habituated Fos responses within preautonomic (mpv, dp) compartments of the PVN following repeated restraint, but to comparable levels in vehicle and V1A antagonized animals. This does not preclude an involvement of other AVP containing or V1A sensitive projections that may access autonomic centers of brainstem and spinal cord (Kalsbeek et al., 2002; Ring, 2005; Ulrich-Lai and Herman, 2009). Thus, central AVP may ultimately alter HPA output through putitious dependent and independent routes that have yet to be fully characterized on phenotypic and connectional grounds.

V1A antagonism prevented the development of the habituated HPA responses, but not the acute responses, to suggest that the process of habituation involves a shift towards enhanced V1A receptor utilization. Increments in V1A receptor binding in the anterior BST and in the ventrolateral part of the septum could account for this restricted influence of V1A receptor antagonism, as both of these structures have been identified as PVN-projecting, as well as to provide stimulatory and inhibitory influences, respectively, on stress-induced ACTH release (Herman et al., 2004; Choi et al., 2007). Repeated restraint, however, was also associated with a decrease in V1A receptor binding within the dentate gyrus of the hippocampus, thalamus and central amygdala. It is plausible to consider that repeated activation of these regions could result in a down-regulation of V1A receptor expression. If the process of stress habituation requires a decrease in AVP signaling in these regions, this could explain why there was no effect of V1A antagonism to alter patterns of cellular habituation in the dentate gyrus and central amygdala. Taken together, our findings leave open the possibility that both increases and decreases in endogenous AVP signaling may ultimately fine-tune the magnitude of the HPA response to repeated stress exposure. Elsewhere, continuous icv infusions of AVP induce sensitized motor and AVP receptor signaling responses (Poulin et al., 1995), independent of changes in V1A receptor availability. Thus, our survey of changes in receptor binding likely understimates the substrate responsible for mediating the central effects of AVP on neuroendocrine adaptation.

Animals exposed to repeated restraint and continuous V1A receptor antagonism continued to show decreases in cellular activation in several brain regions. This would imply that habituation is not a unitary process and that only a select complement of V1A receptor expressing nuclei may be uniquely responsible for mediating the normal decline in HPA axis responses. Nonetheless, the effect of V1A antagonism to decrease cellular habituation in the PVN was paralleled by similar decrements in the anterior BST and medial amygdala. Based on previous connectivity experiments, these structures may function cooperatively to mediate the endogenous effects of central AVP and/or V1A receptors on neuroendocrine adaptation. Recent evidence has identified the dorsomedial...
part of the BST as representing an important point of convergence for limbic-related influences on the HPA axis, including those originating from prefrontal cortex and hippocampus (Radley and Sawchenko, 2011). V1A receptor binding was increased in the anterior BST after repeated restraint, implicating this region as an important nodal point for sensitized HPA responses to central AVP.

Electrical stimulation and lesion studies reflect a stimulatory influence of the medial amygdala to increase HPA axis responses (Dayas et al., 1999), although this has never been studied in the context of homotypic stress. Available connectivity data suggests that this influence could be mediated via disynaptic relays within the posterior BST; however, we found no effect of treatment to alter Fos responses within this part of the BST. The medial amygdala receives input from a variety of brain regions implicated in HPA axis control, as well as reciprocal AVP-containing projections to these very same structures, including the septum, BST, amygdala, hippocampus, and cortex. Thus, the nature by which AVP expressing neurons in the medial amygdala respond to repeated restraint, let alone how downstream changes in V1A receptor gating of these responses regulate the process of HPA axis habituation, remains elusive. The posterior

Fig. 5. Representative autoradiographs to show V1A receptor distribution through the rostrocaudal extent (top to bottom panels) of forebrain regions sampled. Sections were processed for total $^{[125]}$I lin-AVP binding (a–e) and in the presence of unlabeled ligand for non-specific binding (f). Regions identified as showing reliable labeling included the lateral septum (a), anterolateral part of the anterior BST (b), suprachiasmatic nucleus (c), the central nucleus of the amygdala (d), anteroventral and ventral anterolateral thalamus (d and e), and lower and upper limbs of the dentate gyrus (e). Mean±S.E.M. V1A receptor binding (g) to show repeated restraint-induced decreases in V1A receptors in dentate gyrus (DG), thalamus (Thal) and central amygdala (CeA); increases in anterior BST and in the ventrolateral zone of the lateral septum (SEP), and no significant change in the suprachiasmatic nucleus (SCN). *p<0.05 vs. unstressed (n=6/group).
BST and MeA issue AVP-containing projections to a vast array of brain regions, including various cortico-limbic, hypothalamic, midbrain and brainstem nuclei. Brain regions identified as showing changes in V1A receptor binding in the present study might also figure prominently in other components of the stress response network, including those mediating behavioral, autonomic, immune, and other neuroendocrine responses.

The remaining drug effect to alter Fos activation in the repeated restraint condition was revealed for the CA1 region of the hippocampus, a source of outflow to the hypothalamus. In contrast to other regions examined, repeated restraint was associated with an increase in the number of Fos responding neurons in the CA1 region, and V1A receptor antagonism blocked this increment in cellular activation. The functional and anatomical nature by which this finding relates to stress HPA axis habituation remains unclear (Fevurly and Spencer, 2004). However, it is reasonable to propose a local influence of AVP in the hippocampus to coordinate processes of memory consolidation and neuroendocrine responses associated with anticipated or predictable stimuli.

It is important to note that the experimental design used in the current studies did not permit us to discriminate whether V1A receptor antagonism modulates the development vs. the expression of stress response habituation. Such an examination is warranted, based on previous studies, to suggest that the substrate mediating acute HPA axis responses is distinct from those mediating adaptive neuroendocrine responses to repeated stimulus exposure (Cole et al., 2000; Weinberg et al., 2010). Within the realm of these possibilities, we identified a drug effect to decrease Fos activation in the septum and DMH that was restricted to acute restraint. Taken together with the increase in V1A receptor binding in the septum, these findings imply that AVP may operate on both the development and expression of stress HPA habituation.

Emerging evidence indicates that individual variations in central AVP release may be a predisposing factor underlying post-traumatic stress disorder and depression (Neumann and Landgraf, 2012). As previously proposed (Maier and Watkins, 2010), stressors that are controllable protect an individual from elevations in glucocorticoids, but from a learning perspective may also promote the expression of different types of coping styles in response to unanticipated challenges. Habituation to repeated stress is a critical component of healthy coping, and maladaptive behavioral coping styles and impaired expression of stress HPA axis habituation have been proposed as leading causes for different types of mood disorders. Worthy of pursuit is whether disrupted neuroendocrine habituation (as with V1A antagonism) renders animals less flexible to adapt to different environmental conditions. Fundamental for understanding individual vulnerability to stress-related disorders, our current findings provide several new frameworks in this regard.

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Statement of Interest

None.

References


