Psychogenic Stress Activates C-Fos in Nucleus Accumbens-Projecting Neurons of the Hippocampal Ventral Subiculum

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Abstract

Background: The ventral subiculum is known to be activated by the presentation of novel stressors. It has been hypothesized that neuronal ensembles at the ventral aspect of the hippocampal formation are involved in context-dependent processing and can guide the learning of appropriate action selections in response to threatening contexts. Artificial activation of the ventral subiculum can excite medium spiny neurons of the nucleus accumbens and can increase the excitability of mesolimbic dopamine neurons via a polysynaptic pathway through the basal ganglia. However, it remains unknown whether this circuit can be activated by aversive experience, and if so, whether ventral subiculum engages nucleus accumbens monosynaptically.

Methods: To address this, the retrograde tracer fluorogold was used in rats to label neurons projecting to the caudomedial nucleus accumbens. One to 2 weeks later, the same rats were exposed to psychogenic stress (i.e., acute restraint in a novel test room) or served as nonhandled controls, followed by dual immunocytochemical localization of retrogradely transported tracer and nuclear Fos.

Results: Compared with controls, rats exposed to psychogenic stress displayed more fluorogold-positive ventral subiculum neurons that were double-labeled for Fos.

Conclusion: This study establishes that the direct pathway from ventral subiculum to the caudomedial nucleus accumbens is activated by stressful experience.

Keywords: stress, restraint, subiculum, accumbens, striatum

Introduction

The ventral hippocampal formation (vHPC) of the rodent, corresponding to the anterior aspect in primates, is known to be recruited by aversive stressors or by arousing stimuli more broadly (Fanselow and Dong, 2010). The vHPC may be recruited during stressors to facilitate associative learning between the arousing context and adaptive coping strategies. It has been hypothesized that neuronal ensembles at the vHPC are involved in contextual representation, and the vHPC is heavily connected to regions involved in action selection, via its CA1 and subiculum (vSub) output regions. As suggested by Hasselmo (2008), both the context representations and stress responsivity of the vHPC may be related to its considerably large place fields (Kjelstrup et al., 2008), which may more succinctly capture contextual information and be more relevant for behaviors...
Sigificance Statement

This study establishes that the direct pathway from ventral subiculum (vSub) to the caudomedial nucleus accumbens (NAc) is activated by stressful experience. Given the role of vSub and NAc in contextual representation and action selection, respectively, plasticity at this synapse during the initial exposure to a stressor is likely to shape responses to subsequent presentations of the same threatening conditions. However, while the vSub-to-NAc pathway may facilitate stress coping, hyperactivity of this pathway has been implicated in animal models of psychosis. Thus, the findings presented here identify the vSub-NAc pathway as a substrate upon which stress and psychosis may cross-sensitize.

Materials and Methods

Animals

Experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (300–350 g; Harlan Laboratories) were housed individually in stainless-steel cages in a controlled environment (20–22°C, 12-hour light-dark cycle; lights off at 7:00 pm) with ad libitum access to water and chow.

Iontophoretic Tracer Delivery

We used the same iontophoretic method and parameters described previously (Bienkowski and Rinaman, 2011). Briefly, rats were anesthetized by isoflurane inhalation (1–3% in oxygen) and secured in a stereotaxic frame. An incision was made in the scalp, and small holes were drilled bilaterally in the skull to expose the cortical surface overlying NAc. A micropipette, back-filled with a 1–2% solution of FG (Fluorochrome), was lowered into the caudomedial NAc using stereotaxic coordinates (+1.5 from bregma, ±1.0 lateral, -6.0 ventral) and a 0.5-μA retaining current. FG was iontophotically ejected for 5 minutes (7-second pulses of positive current, 5 μA). Ejections were performed bilaterally in all animals except for one rat in each experimental condition to assess the uptake of tracer into the contralateral hemisphere. The micropipette tip was left in place for 5 minutes after ejection and then withdrawn. The incision sites were closed with stainless-steel clips. Rats were injected with 1 mg of analgesic (Ketofen, s.c.) and returned to their home cages after regaining consciousness and full mobility.

Restraint Stress Exposure

After 7 to 14 days postsurgery, 6 FG-injected rats were exposed to 30 minutes of restraint stress (restraint rats) in a clear plastic cylindrical tube (Kent Scientific Corporation), and 5 FG-injected rats served as unrestrained controls (control rats). Restraint rats were transported to an adjacent room, put into the restrainer, and then left undisturbed in the restrainer within the transport cage for 30 minutes. Rats were then returned to their home cage for an additional 60 minutes to ensure maximal stress-induced neural Fos expression, which generally peaks 60 minutes after treatment-induced neural stimulation (Kovacs, 1998). Rats were then deeply anesthetized and perfused with fixative (see Perfusion and Histology). Control rats were not handled for at least 24 hours before perfusion. All manipulations were performed during the light phase of the rat's diurnal cycle.

Perfusion and Histology

Rats were transthoracally perfused as previously described (Bienkowski and Rinaman, 2011). Fixed brains were removed from the skull, postfixied overnight at 4°C, and then cryoprotected in 20% sucrose solution for 24 to 72 hours. Coronal 35-μm-thick tissue sections were cut using a freezing microtome, with sections collected sequentially into 6 adjacent series and stored in cryopreservant solution (Watson et al., 1986).
Fos Immunocytochemistry

One set of sections from each rat was removed from storage and rinsed in buffer (0.1 M sodium phosphate, pH 7.4). Tissue sections were initially processed for immunoperoxidase localization of Fos protein using a rabbit polyclonal antiserum (1:500,000; provided by Dr. Philip Larsen, Denmark), diluted in buffer containing 0.3% Triton-X100 and 1% normal donkey serum. The specificity of this antibody for Fos has been reported (Rinaman et al., 1997). After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:500) and Vectastain Elite ABC reagents (Vector Laboratories) and reacted with nickel sulfate-intensified diaminobenzidine to generate a blue-black reaction product in the nuclei of Fos-positive cells.

FG Immunocytochemistry

Following Fos immunocytochemistry, sections were rinsed in buffer and incubated overnight in rabbit anti-FG antiserum (1:30,000; Chemicon International) diluted in buffer containing 0.3% Triton x100 and 1% normal donkey serum. After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:500) and Vectastain Elite reagents followed by a nonintensified diaminobenzidine-hydrogen peroxide reaction to produce brown immunoprecipitate localizing the FG neural tracer delivery site and retrogradely labeled neurons. Immunostained tissue sections were then rinsed in buffer, mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

Quantification of FG and Fos Labeling within the vSub

Quantification was performed by an experimenter who was blind to the experimental groups. A neuron was counted as FG-positive if it contained brown cytoplasmic immunoreactivity and had a visible nucleus. A neuron was considered Fos-positive if it contained blue-black nuclear immunolabeling, regardless of intensity. Neurons fulfilling both criteria were considered double-labeled. For each subject, all sections (12–15) containing FG-positive neurons within the region of interest (ROI) comprising the ventral Cornu Ammonis and vSub (rostro-caudal level -4.2 to -7.4, relative to bregma) were analyzed bilaterally for double-labeled neurons. In one representative restraint case and one representative control case, all Fos-positive and all FG-positive neurons were counted.

Statistical Analysis

A 2-way ANOVA was used with experimental treatment (restraint vs. control) and hemisphere (left vs. right) as independent variables. Posthoc testing was performed using the Holm-Sidak method.

RESULTS

Iontophoretic FG Injection Sites

FG was successfully iontophoresed in all 10 rats. Iontophoretic tracer delivery sites produced spherical tracer deposits localized within the caudo-medial portion of NAc, medial to the anterior commissure, and ventral to the lateral ventricle (Figure 1E). Most tracer delivery sites encompassed parts of NAc shell, with little or no FG labeling present within the adjacent medial and lateral septum. The tracer deposits were distributed rostro-caudally from +2.0 to +1.0 mm from bregma.

Distribution of FG Labeled Neurons in the Hippocampal Formation

Retrogradely labeled hippocampal neurons were observed almost exclusively within vSub and the adjacent ventral portion of the CA1; the entorhinal and piriform cortices were also moderately labelled. The brown FG immunoreaction product labelled the somata as well as large apical dendrites of neurons in the pyramidal cell layer in the most densely labeled portions of vSub. In cases where iontophoretic tracer deposits were made into the more caudal portion of NAc, the densest retrograde labeling occurred in the distal portion of vSub, with little or no labeling in CA1 (Supplemental Figure 1B). On the other hand, more rostral FG deposits led to retrograde transport that was more sparsely distributed between the proximal vSub and CA1 (Supplemental Figure 1C). In cases in which the tracer was delivered unilaterally (n = 2), little to no FG labeling was observed in the contralateral hippocampal formation. Additionally, no differences in hippocampal FG labeling were observed between
cases where FG iontophoresic deposits were concentrated in the shell vs in the core of the NAc.

**Restraint Stress Induces Fos Expression in NAc-Projecting vSub Neurons**

Significantly more double-labeled neurons were present within the specified ROI (i.e., ventral cornu Ammonis and vSub) in restraint cases compared with controls (Figure 1A-D). ANOVA revealed a main effect of experimental group (restraint 4.3 ± 1.2; control 0.2 ± 0.1 double-labeled cells per section, P < .01; Figure 2A), but no effect of hemisphere. Double-labeled neurons were most densely concentrated in vSub, approximately at bregma level -6.0 mm (Figure 2B).

To estimate the proportion of NAc-projecting neurons expressing Fos and the proportion of Fos-expressing neurons projecting to the NAc, we counted the total number of Fos-positive and FG-positive neurons within the ROI in one representative control case and one representative restraint case. In the restraint case, 1100 neurons were Fos-positive, 2776 were FG-positive, and 102 were double labeled, accounting for 9.3% of the Fos-positive and 3.7% of the FG-positive neurons. In the control case, 119 neurons were Fos-positive, 1500 were FG-positive, and 9 were double labeled, accounting for 7.6% of the Fos-positive and 0.6% of the FG-positive neurons.

**Discussion**

The hippocampus is known to be strongly activated following a variety of stressors. In this study, Fos expression was induced throughout vSub in restrained animals, but only sparsely in controls. This is consistent with previous studies showing that psychogenic stress induces Fos expression in hippocampal neurons (Cullinan et al., 1995) and particularly within vSub (Otake et al., 2002).

By combining Fos immunohistochemistry with FG retrograde tracing, we showed that restraint rats had more Fos+ NAc-projecting vSub neurons compared with nonstressed controls. Thus, acute restraint stress activates NAc-targeting vSub neurons. The distribution of retrogradely labelled vSub neurons was consistent with previous studies showing that the caudomedial portion of NAc receives the densest input from the vSub (reviewed in Strange et al., 2014). The morphology of FG+ neurons was consistent with large pyramidal cells that constitute the population of projection neurons in vSub. In a representative rat exposed to restrain stress, fewer than 5% of NAc-projecting vSub neurons were activated to express Fos, consistent with sparsity in hippocampal networks.

In this study, we employed a compound psychogenic stressor. Thus, the vSub activation observed in the experimental group in our study may be partly driven by the novelty stress experienced in the test room or transport tub and partly driven by the adversity of restraint. However, we suspect that restraint stress is the primary effector because the animals were not deliberately allotted time to encode cues from the test room. Furthermore, Hale and colleagues (2008) examined the subiculum and CA1 c-fos activation in animals exposed to an acute open field exposure (OF group), animals transported to the test room but only handled briefly (HA group), and home cage controls (CO group). They found no difference in c-fos activation between the HA and CO groups, but a large effect resulting from the open field (Hale et al., 2008). Thus, had the control group in our study been transported to the test room and briefly handled rather than taken directly from the home cage, we expect that results would have been similar.

Importantly, the stress-induced Fos expression in vSub reported here reflects a circuit not only recently activated but perhaps also undergoing slow Fos-mediated modifications. The 1- to 2-hour latency of peak Fos expression following experience suggests that Fos may drive consolidation processes (Katche et al., 2010) or metaplasticity (Guzowski, 2002). This facilitates learning (Fleischmann et al., 2003; Katsche et al., 2010) that will presumably prepare the organism for the next threatening encounter.

Based on its anatomical connectivity and its role in fear learning, the vSub appears to be chiefly involved in the facilitation of associative learning between the arousing context and the appropriate behavioral output. Even in response to simple restraint stress, adaptive learning takes place, primarily in the form of habituation. The struggling behavior, ultrasonic vocalizations, tachycardia, and corticosterone stress response habituate across repeated restraint sessions (Grissom et al., 2008; Stamp and Herbet, 2001; Herman, 2013). Furthermore, restrained animals will elicit a vocalization specific to this stressor when exposed to the associated context (Gimaley et al., 2016). Hence, it is conceivable that Fos expression of the vSub-to-NAc pathway following restraint stress reflects plasticity that would facilitate passive-defensive responses such as motionlessness and vocalization while hampering ineffective responses such as struggling.
The vSub and NAc are also known to cooperate in the disinhibition of the ventral tegmental area dopaminergic neurons via the ventral pallidum. The resultant increase in dopaminergic tone may further facilitate behavioral learning. This disinhibition transitions the dopamine neurons from a hyperpolarized, quiescent state to an excitable state, capable of bursting in response to NMDAR-dependent ascending input. This polysynaptic circuit was initially identified as an aberrant circuit in a rodent model of psychosis (Grace, 2016), suggesting that this circuit is a common substrate for stress and psychosis. Indeed, the results reported here indicate that in a healthy animal, vSub stimulates NAc in response to a simple stressor and does so monosynaptically, implying that the same pathway may be activated both in psychosis and stress. In the healthy animal exposed to an acute stressor, activation of this pathway and the ensuing increased dopaminergic tone may engender an appropriate, temporary state of vigilance, whereas as long-lasting hypervigilance may occur in the pathological case. Indeed, an increase in dopaminergic excitability is not just observed in psychosis but also following acute stressors. Both acute and repeated restraint stress can increase the number of tonically active dopamine ventral tegmental area neurons in a vSub-dependent manner for at least 24 hours after the stressor (Valenti et al., 2011). Extracellular dopamine levels of NAc and mPFC have also been shown to increase following acute restraint (Imperato et al., 1991).

Stressful experience or anxious traits have long been thought to exacerbate schizophrenia symptoms, but the pursuit of empirical support of the vulnerability-stress model from patient data has been fraught with methodological difficulty (Norman and Malla, 1993). Our findings highlight the use of animal models as an alternative strategy to probing the interaction of these phenomena. Specifically, we posit the vSub-NAc projection as a critical point of convergence for both hyperdopaminergia and stress-responsivity. Much work lies ahead in characterizing this likely complex interaction.

Supplementary Material

Supplementary data are available at International Journal of Neuropsychopharmacology online.

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Statement of Interest

None.

References

Bienkowski MS, Rinaman L (2011) Immune challenge activates neural inputs to the ventrolateral bed nucleus of the stria terminals. Physiol Behav 104:257–265.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64:477–505.

Fanselow MS, Dong HW (2010) Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65:7–19.

Fleischmann A, Hvalby O, Jensen V, Streeková T, Zacher C, Layer LE, Kvello A, Reschke M, Spanagel R, Sprengel R, Wagner EF, Gass P (2003) Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. J Neurosci 23:9116–9122.

Floresco SB (2015) The nucleus accumbens: an interface between cognition, emotion, and action. Annu Rev Psychol 66:25–52.

Grace AA (2016) Dysregulation of the dopamine system in the pathophysiology of schizophrenia and depression. Nat Rev Neurosci 17:524–532.

Grimsley JM, Sheth S, Vallabh N, Grimsley CA, Bhattal J, Latsko M, Jasnow A, Wenstrup JJ (2016) Contextual modulation of vocal behavior in mouse: newly identified 12 kHz “mid-frequency” vocalization emitted during restraint. Front Behav Neurosci 10.

Grisson N, Kerr W, Bhatnagar S (2008) Struggling behavior during restraint is regulated by stress experience. Behav Brain Res 191:219–226.

Guzowski JF (2002) Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. Hippocampus 12:86–104.

Hale MW, Hay-Schmidt A, Mikkelsen JD, Poulsen B, Bouwknecht JA, Evans AK, Stamper CE, Shekhar A, Lowry CA (2008) Exposure to an open-field arena increases c-Fos expression in a subpopulation of neurons in the dorsal raphe nucleus, including neurons projecting to the basolateral amygdaloid complex. Neuroscience 157:733–748.

Hasselmo ME (2008) The scale of experience. Science 321:46.

Herman JP (2013) Neural control of chronic stress adaptation. Front Behav Neurosci 7:1–12.

Imperato A, Puglisi-Allegra S, Casolini P, Angelucci L (1991) Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis. Brain Res 538:111–117.

Katche C, Bekinschtein T, Slipczuk L, Goldin A, Izquierdo IA, Cammarota M, Medina JH (2010) Delayed wave of c-Fos expression in the dorsal hippocampus involved specifically in persistence of long-term memory storage. PNAS 107:349–354.

Kjelstrup KB, Solstad T, Brun VH, Hafting T, Leutgeb S, Witter MP, Moser EI, Moser MB (2008) Finite scale of spatial representation in the hippocampus. Science 321:140–143.

Kovacs KJ (1998) c-Fos as a transcription factor: a stressful (re) view from a functional map. Neurochem Int 33:287–297.

McLean JW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083.

Norman RM, Malla AK (1993) Stressful life events and schizophrenia. II: conceptual and methodological issues. Br J Psychiatry 162:166–174.

O’Donnell P, Grace AA (1995) Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. J Neurosci 15:3622–3639.

Otaké K, Kin K, Nakamura Y (2002) Fos expression in afferents to the rat midline thalamus following immobilization stress. Neurosci Res 43:269–82.

Reynolds SM, Berridge KC (2003) Glutamate motivational ensembles in nucleus accumbens: rostrocaudal shell gradients of fear and feeding. Eur J Neurosci 17:2187–2200.
Rinaman L, Stricker EM, Hoffman GE, Verbalis JG (1997) Central c-Fos expression in neonatal and adult rats after subcutaneous injection of hypertonic saline. Neuroscience 79:1165–75.
Stamp J, Herbert J (2001) Corticosterone modulates autonomic responses and adaptation of central immediate-early gene expression to repeated restraint stress. Neuroscience 107:465–479.
Strange BA, Witter MP, Lein ES, Moser EI (2014) Functional organization of the hippocampal longitudinal axis. Nat Rev Neurosci 15:655–669.
Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10:397–409.
Valenti O, Lodge DJ, Grace AA (2011) Aversive stimuli alter ventral tegmental area dopamine neuron activity via a common action in the ventral hippocampus. J Neurosci 31:4280–4289.
Watson RE, Wiegand ST, Clough RW, Hoffman GE (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155–159.
Weinberg MS, Johnson DC, Bhatt AP, Spencer RL (2010) Medial prefrontal cortex activity can disrupt the expression of stress response habituation. Neuroscience 168:744–756.