Title: Psychogenic stress activates c-fos in nucleus accumbens-projecting neurons of the hippocampal ventral subiculum

Category: Brief Report

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ABSTRACT

BACKGROUND: The ventral subiculum (vSub) 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 (vHPC) are involved in context-dependent processing and can guide the learning of appropriate action selections in response to threatening contexts. Artificial activation of the vSub can excite medium spiny neurons of the nucleus accumbens (NAc) 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 vSub engages NAc monosynaptically.

METHODS: To address this, the retrograde tracer Fluorogold (FG) was used to label neurons projecting to the caudomedial NAc. One to two weeks later, the same rats were exposed to psychogenic stress (i.e., acute restraint in a novel test room) or served as non-handled controls, followed by dual immunocytochemical localization of retrogradely transported tracer and nuclear Fos.

RESULTS: Compared to controls, rats exposed to psychogenic stress displayed larger numbers of FG-positive vSub neurons that were double-labeled for Fos.

CONCLUSION: This study establishes that the direct pathway from ventral subiculum (vSub) to the caudomedial nucleus accumbens (NAc) is activated by stressful experience.

SIGNIFICANCE 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.

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 (a) more succinctly capture contextual information and (b) be more relevant for behaviors involving large distances, such as the large foraging range of rodents and escape from a predator.

Consistent with its posited role in action selection, the downstream targets of vHPC outflow include areas directly involved in orchestrating behaviors, including the ventromedial prefrontal cortex (vmPFC), medial aspects of the nucleus accumbens (NAc), basolateral amygdala, and hypothalamic nuclei that modulate social behaviors and hormonal responses (for anatomical review, **Strange et al, 2014).** One of the clearest illustrations of vHPC involvement in *coping* to psychogenic stressors is its feedback inhibition over the HPA axis (via inhibitory relays regulating the hypothalamic paraventricular nucleus; **Herman, 2013**). The vHPC also contributes to the habituation of the corticosterone response via a vmPFC-vHPC pathway **(Weinberg et al, 2010; Herman, 2013).**

However, the vHPC also prominently projects to NAc. Since the vHPC afferents are particularly potent in their ability to shift bistable NAc medium spiny neurons to a more depolarized membrane potential (O'Donnell and Grace, 1995), activation of the vHPC-to-NAc pathway is expected to have a large effect on overall NAc activity. It is unclear whether the vHPC-to-NAc pathway would be activated in response to an aversive stressor, given that it is a region long implicated in reward processing from studies of intracranial self-stimulation and studies of dopaminergic neural correlates to reward (reviewed in Floresco, 2015). On the other hand, identical microinjections of agonists along the NAc rostrocaudal extent can elicit both appetitive and defensive behaviors (**Reynolds and Berridge 2003**), and a broader interpretation of NAc function has been put forward (**Floresco 2015**).

Here, we examined whether psychogenic stress, namely acute restraint in a novel test room, would activate the vHPC-to-NAc projection. In contrast to physical stressors such as pain, respiratory distress, blood loss, or infection, psychogenic stressors are triggered by a polysensory anticipation of pain, rather than noxious or painful cues (Ulrich-Lai and Herman, 2009). We employed a

psychogenic stressor for this study because forebrain structures, such as the hippocampal formation, are critical for the animal's response to psychogenic stressors but not physical stressors (Ulrich-Lai and Herman, 2009). To achieve this, the retrograde tracer Fluorogold (FG) was used to label vSub neurons projecting to medial NAc. We report that restraint stress led to an increase in the number of vSub neurons immunoreactive for both FG and the immediate early gene marker of neuronal activity Fos, evidence that acute stress activates the vSub-NAc projection. Since this projection has a particularly potent excitatory effect on medium spiny neurons (O'Donnell and Grace, 1995), these results imply that vHPC activates the NAc during psychogenic stress. These results also indicate that a relatively mild acute, stressor is sufficient to activate the vSub-NAc pathway.

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 (300–350g; Harlan Laboratories) were housed individually in stainless steel cages in a controlled environment (20–22 °C, 12 hour light-dark cycle; lights off at 1900 h) 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, backfilled with a 1–2% solution of FluoroGold (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 iontophoretically ejected for 5 min (7s pulses of positive current, 5 μA). Ejections were performed bilaterally in all animals except for one rat in each experimental condition, in order to assess the uptake of tracer into the contralateral hemisphere. The micropipette tip was left in place for 5 min 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 7–14 days post-surgery, 6 FG-injected rats were exposed to 30 min restraint stress (Restraint rats) in a clear plastic cylindrical tube (Kent Scientific Corporation, Torrington, CT), 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 min. Rats were then returned to their home cage for an additional 60 min, in order to ensure maximal stress-induced neural Fos expression, which generally peaks 60 min after treatment-induced neural stimulation (Kov**acs, 1998)**. Rats were then deeply anesthetized and perfused with fixative (see *Perfusion and histology*). Control rats were not handled for at least 24 hrs before perfusion. All manipulations were performed during the light phase of the rats' diurnal cycle.

Perfusion and histology. Rats were transcardially perfused as previously described (Bienkowski and Rinaman, 2011). Fixed brains were removed from the skull, post-fixed overnight at 4°C, and then cryoprotected in 20% sucrose solution for 24–72 h. 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 (1:50,000; 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 (DAB) 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 Vectastatin ABC Elite reagents followed by a non-intensified DAB-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 two-way ANOVA was used with experimental treatment (Restraint vs. Control) and hemisphere (left vs. right) as independent variables. Post-hoc 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 iotophoretic 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 iontophoretic deposits were concentrated in the shell versus 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 to 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, $p<0.01$; 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 to non-stressed 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 caudo-medial 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.

Importantly, the stress-induced Fos expression in vSub reported here reflects a circuit not only recently activated by perhaps also undergoing slow Fos-mediated modifications. The 1-2 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.

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 group, 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.

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 (**Gimsley 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 (VTA) 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**). 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 VTA 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.

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Statement of Interest: None

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FIGURE CAPTIONS

Figure 1 Overview of methods. (A & B) Example of double immunolabeled sections from Control (A; 5x objective) and Restraint (B; 5x objective) cases. Blue-black nuclear labeling is visible in the Restraint case, but virtually absent in the Control case. (C) Higher magnification of double-labeled neurons in a tissue section from a Restraint case (10x objective). D. Example of two double-labeled neurons (arrowheads) in a section from a Restraint case viewed under high magnification (40x objective). E. Placement of iontophoretically-delivered FG

deposits in the NAc. Circles represent the extent of FG deposits in Control animals (green), and Restraint animals (red).

APir – amygdalopiriform transition area; hif – hippocampal fissure.

Figure 2 Quantified observations. (A) Rats exposed to acute restraint stress displayed significantly more double labeled neurons in the vSub compared to unhandled control rats. (* indicates significant difference between the mean number of double labeled neurons per section in each group, $p<0.01$, t-test) (B) Distribution of double-labeled neurons in the rostro-caudal axis of the region of interest. FG / Fos double-labeled neurons were most densely concentrated in the middle portion of the vSub, approximately -6.0 mm from bregma.

Supplemental Figure 1 Example of the distribution of retrogradely labeled neurons in the left (1) and right (2) ventral hippocampal formation corresponding to cases where FG was iontophoresed in the caudal (B, +1.0 from bregma) and rostral (C, +2.0 from bregma) portions of the NAc. Arrowheads in B indicate the dense concentration of FG immunolabeling in the distal portion of the vSub. Arrowheads in C indicate a more sparse distribution of FG immunolabeling in the proximal vSub and the CA1.

Figure 1.

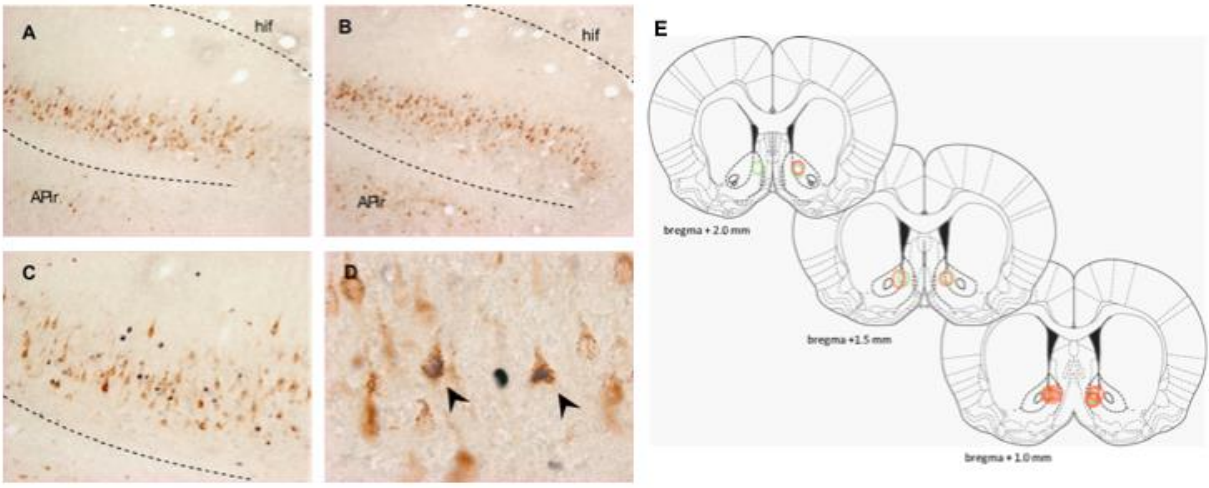

Figure 2.