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Stress-activated afferent inputs into the anterior parvicellular part of the paraventricular nucleus of the hypothalamus: Insights into Urocortin 3 neuron activation

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Abstract

Urocortin 3 (Ucn 3) is a member of the corticotropin-releasing factor family, which plays a major role in coordinating stress responses. Ucn 3 neurons in the anterior parvicellular part of the paraventricular nucleus of the hypothalamus (PVHap) provide prominent input into the ventromedial nucleus of the hypothalamus (VMH), a well known satiety center, where Ucn 3 acts to suppress feeding and modulate blood glucose levels. In the present study, we first determined that Ucn 3 expression in the PVHap was stimulated by acute restraint stress. We then performed retrograde tracing with fluorogold (FG) combined with immunohistochemistry for *Fos* as a marker for neuronal activation after restraint stress to determine the stress-activated afferent inputs into the PVHap. Substantial numbers of FG/*Fos* double labeled cells were found in the bed nucleus of the stria terminalis, lateral septal nucleus, the medial amygdala, and a number of nuclei in the hypothalamus including the VMH, the arcuate nucleus, the posterior nucleus, and the ventral premammillary nucleus. In the brainstem, FG/*Fos* positive cells were found in the periaqueductal gray, the nucleus of the solitary tract, and the ventrolateral medulla. In conclusion, the present study showed that acute stress rapidly stimulates Ucn 3 expression in the PVHap and identified specific stress-sensitive brain areas that project to the PVHap. These areas are potentially important in mediating the stress-induced activation of Ucn 3 neurons in the PVHap.

Keywords

stress; CRF; Urocortin 3; CRF₂; paraventricular nucleus of the hypothalamus

1. Introduction

Urocortin 3 (Ucn 3) is a peptide of the corticotropin-releasing factor (CRF) family initially identified in the brains of humans and rodents (Hsu and Hsueh, 2001; Lewis et al., 2001). It

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has high affinity for the type 2 CRF receptor (CRF₂) and low affinity for the type 1 CRF receptor (Hsu and Hsueh, 2001; Lewis et al., 2001). Ucn 3-expressing neurons are concentrated in the medial amygdala (MEA) and the hypothalamus (Lewis et al., 2001; Li et al., 2002). In the murine hypothalamus, the major Ucn 3 cell population is found near the rostral perifornical area (Li et al., 2002), with Ucn-3 positive somata clustered around the fornix. This cell group then extends rostrally into the anterior parvicellular part of the paraventricular nucleus of the hypothalamus (PVHap) and laterally into the posterior part of the bed nucleus of the stria terminalis (BSTp). In addition to the rostral perifornical area, a small population of Ucn 3-positive cells is located in the median preoptic nucleus (Li et al., 2002). Ucn 3-positive nerve fibers and terminals are found predominantly in the forebrain, including the VMH, the lateral septal nucleus (LS), the MEA, and the bed nucleus of the stria terminalis (BST) (Li et al., 2002). These areas also express high levels of CRF₂ (Chalmers et al., 1995; Van Pett et al., 2000), supporting the notion that Ucn 3 is a CRF₂-selective ligand.

Functional studies have determined that Ucn 3 in the brain is involved in regulating energy homeostasis; central administration of Ucn 3 suppresses feeding, elevates blood glucose levels, and induces hyperthermia (Jamieson et al., 2006; Ohata and Shibasaki, 2004; Telegdy et al., 2006). Site-specific injection studies revealed that Ucn 3 injection into the VMH results in suppression of feeding and elevation of blood glucose (Chen et al., 2010; Fekete et al., 2006), suggesting that the VMH is a critical brain area for mediating the effect of central Ucn 3 on feeding and energy modulation. Our previous anatomical study showed that the main source of Ucn 3 input into the VMH originates in the PVHap and the adjacent BSTp. The MEA provides moderate Ucn 3 afferent projections (Chen et al., 2011). Ucn 3 cells in the median preoptic nucleus supply negligible input into the nucleus (Chen et al., 2011). Interestingly, Ucn 3 neurons in the rostral perifornical hypothalamus just caudal and lateral to the PVHap area do not project to the VMH; these cells instead send projections to the LS (Chen et al., 2011). This Ucn 3 population in the rostral perifornical area thus appears to be anatomically heterogeneous.

The expression of Ucn 3 in the brain is sensitive to stress and metabolic challenges. In the MEA, acute restraint stress increases Ucn 3 mRNA levels, while hemorrhages and food deprivation decrease Ucn 3 expression (Jamieson et al., 2006). Restraint stress increases Ucn 3 expression in a time dependent manner in the rostral perifornical area as well (Jamieson et al., 2006; Venihaki et al., 2004). As mentioned above, the Ucn 3 cell population in the rostral perifornical area can be sub-divided into two groups (PVHap/BSTp and rostral perifornical hypothalamus) based on their anatomical projections; it remains to be determined whether stress differentially regulates Ucn 3 expression in these two different sub-populations.

In the present study, we first confirmed the earlier report (Jamieson et al., 2006) that restraint stress stimulates Ucn 3 expression in the MEA and further demonstrated that acute restraint stress greatly increases Ucn 3 mRNA levels in the PVHap/BSTp area, but not in the rostral perifornical hypothalamus. This finding, combined with the PVHap providing prominent Ucn 3 input into the VMH, raised an important question of what brain areas provide stress-activated input into the PVHap. Thus, using retrograde tracing combined with

Fos, an immediate early gene product as a marker for neuronal activation, we then examined stress-sensitive afferent inputs into the PVHap. Our results show that the PVHap is innervated by a broad range of areas that are activated by stress, revealing an intricate circuitry of input into the PVHap that potentially conveys stress signals to activate Ucn 3 neurons.

2. Results

2.1 Effect of restraint stress on Ucn 3 mRNA levels in the PVHap, rostral perifornical hypothalamus, and MEA

Male rats were restraint stressed for 30 minutes or 4 hours to determine the effect of stress on Ucn 3 expression in the perifornical hypothalamic area. As shown in Fig. 1, the Ucn 3 mRNA hybridization signal was significantly elevated in the PVHap area and in the adjacent BSTp after 30 minutes of restraint stress compared to non-stressed controls ($p < 0.05$); a two-way ANOVA showed significant effects of stress [$F_{(1, 24)} = 33.6$, $p < 0.001$] and interaction between stress and time [$F_{(1, 24)} = 62.4$, $p < 0.001$] on Ucn 3 mRNA levels in the PVHap. Conversely, Ucn 3 mRNA signals near the fornix showed a non-significant trend of elevation [$F_{(1, 24)} = 0.33$, $p < 0.57$] in acute stressed animals compared to that of non-stressed controls (Fig. 1). Consistent with an earlier report (Jamieson et al., 2006), a significant effect of stress [$F_{(1, 24)} = 4.2$, $p < 0.05$] and interaction between stress and time [$F_{(1, 24)} = 10.9$, $p < 0.05$] were found on Ucn 3 mRNA levels in the MEA. Ucn 3 mRNA hybridization signal was significantly elevated in the MEA of rats subject to 30 min restraint stress ($p < 0.05$) (Fig. 1). Ucn 3 mRNA in rats stressed for 4 hrs showed a trend toward lower levels as compared to non-stressed animals in the areas examined; however, the differences did not reach statistically significant levels (Fig. 1).

2.2 Identification of stress-activated afferent inputs into PVHap

2.2.1 Injection of a retrograde tracer, fluorogold, to identify neurons that project to PVHap—To determine neuronal projections to the PVHap that are activated by acute stress, rats were first injected with a retrograde tracer, fluorogold (FG), into the PVHap, where Ucn 3 cells are concentrated (Chen et al., 2011; Lewis et al., 2001) (Fig. 2), before being subjected to acute restraint stress. Out of twenty tracer-injected animals, five cases with injection sites centered in the PVHap were included for analysis. Due to the proximity of the target area to other parts of the paraventricular nucleus of the hypothalamus (PVH), there was minor spillover in some of the cases into the periventricular nucleus and the medial parts of the PVH (Fig. 2).

2.2.2 PVHap input as identified through FG-positive cells—Neurons that project to the PVHap were identified by brown precipitate in the cell body, marking them as FG-positive. Rostrally, FG-positive cells were found in parts of the prefrontal cortex including the prelimbic and infralimbic cortex, particularly in the deep layers close to corpus callosum (Fig. 3, Fig. 4, Table 1). Moderate numbers of tracer-labeled neurons were found in the BST (Fig. 3, Fig. 4, Table 1), particularly the medial part. Compared to other brain areas (Table 1), the LS had a high concentration of FG-labeled cells; abundant FG-positive cells were found in the ventral part of the nucleus, with reduced density toward the rostral division

(Fig. 4, Fig. 5). Scattered FG cells were also found in the caudal parts of the LS (Fig. 4). The hypothalamus had a large number of FG-labeled cells (Table 1), including in the VMH (Fig. 3, Fig 4), the posterior hypothalamic nucleus (PH, Fig. 4), and the ventral premammillary nucleus (Fig. 4). Areas of the hypothalamus with moderate numbers of FG-labeled cells included the anterior hypothalamic area (Fig. 4), the arcuate nucleus (ARH, Fig. 4), and dorsomedial hypothalamus (Fig. 4). In the amygdala, FG-positive cells were found in the MEA (Table 1), particularly concentrated in the anterodorsal and posterior parts (Fig. 3, Fig 4).

In the brainstem, discrete clusters of FG-positive cells were observed throughout the rostral-caudal axis, with decreasing concentrations toward the caudal levels (Table 1). Rostrally, abundant FG-labeled cells were found throughout the periaqueductal gray (PAG, Fig. 4, Fig. 6). Lateral to the PAG, the peripeduncular nucleus had a moderate number of FG cells (Fig 4). At the caudal levels of the PAG, FG-labeled neurons were concentrated in the laterodorsal tegmental nucleus (Fig. 4), while a few FG-positive cells were noted in the pedunculo pontine area (Fig. 4). On the ventral surface of the brainstem, there were moderate levels of tracer labeled cells in the ventral tegmental area (Fig. 3, Fig. 4) and interpeduncular nucleus (Fig. 3, Fig. 4). There were a few FG-labeled cells found in the nucleus brachium inferior colliculus (Fig. 3). At the caudal levels of the brainstem, there were scattered FG-positive cells in the pontine central gray (Fig. 3, Fig. 4), the nucleus of the solitary tract (NTS, Fig. 4, Fig. 6), and the lateral reticular nucleus in the rostral ventrolateral medulla (RVLM, Fig. 4, Fig. 6).

2.2.3 Stress-activated neurons as identified by Fos after restraint stress—To determine stress-activated afferent inputs into the PVHap, tracer-injected rats were subjected to acute restraint stress and *Fos* was used to identify cells activated by stress. In general, restraint stress precipitated a stereotyped pattern of neuronal *Fos* expression, similar to those characterized previously (Cullinan et al., 1995). An additional group of non-stressed rats were processed in parallel as a control. The non-stressed rats showed minimal *Fos* activation throughout the brain (data not shown).

Fos-positive cells were seen throughout the brain of stressed rats (Table 1). The brain areas with the densest numbers of *Fos* cells were the pontine gray on the ventral surface of the pons, and the piriform cortex in the ventrolateral region of the brain (Table 1). There were also extensive *Fos*-positive cells in hypothalamic areas such as the PVH, the ARH, dorsomedial nucleus, the PH, and the premammillary nuclei. Prominent *Fos* expression was also observed in the prefrontal cortex, dorsal and ventral parts of the LS, the nucleus accumbens, and in the posterodorsal part of the MEA. Moderate numbers of *Fos* positive cells were found in a variety of areas including the anteromedial part of BST, the VMH, thalamic areas (paraventricular thalamic nucleus), the dentate gyrus, and the lateral habenular nucleus (Fig. 4). In the brainstem, there were a moderate number of *Fos*-positive cells in the PAG, peripeduncular nucleus, interpeduncular nucleus, laterodorsal tegmental nucleus, superior colliculus, and the trapezoid and olive bodies. There was a light smattering of *Fos* cells in the ventral tegmental area, pedunculo pontine area, pontine central gray, NTS, and RVLM (Fig. 4).

2.2.4 Stress-activated input to PVHap as identified by FG and Fos labeling—

Stress-induced *Fos* staining combined with FG retrograde labeling was used to determine stress-activated afferent inputs in the PVHap. FG/*Fos* double-labeled cells were observed in a wide range of structures throughout the rostral to caudal axis of the brain (Table 1). In the forebrain, double labeled cells were concentrated in the ventral part of the LS near the lateral ventricle, extending with a lighter density of double labeled cells into the rostral parts of the nucleus (Fig. 4, Fig. 5). A few scattered double labeled cells were also noted in the caudal part of the LS close to the lateral ventricle (Fig. 4). In the BST, double labeled cells were observed in the posterior division of the principal nucleus (Fig. 4, Fig. 5). FG/*Fos*-positive cells were observed throughout the hypothalamus with prominent double labeled cells found in the VMH, (Fig. 4), the PH (Fig. 4), and the ventral premammillary nucleus (Fig. 4), with a moderate number in the ARH (Fig. 4). In the VMH, FG/*Fos* positive cells were observed mainly in the ventrolateral part of the nucleus (Fig. 4, Fig. 5). In the amygdala, abundant FG/*Fos* double labeled cells were found in the anterodorsal part of the MEA, with scattered double labeled cells observed in the posterodorsal and posteroventral parts of the MEA (Fig. 4, Fig. 5). There were a few double labeled cells found in the anterior hypothalamus and dorsomedial nucleus of the hypothalamus (Table 1).

In the brainstem (Table 1), FG/*Fos* double labeled cells were found throughout the rostral-caudal axis of the PAG (Fig. 4, Fig. 6). At the levels of the caudal PAG, there was a cluster of neurons double labeled with *Fos* and FG in the laterodorsal tegmental nucleus (Fig. 4). A moderate number of double labeled cells were observed in the peripeduncular nucleus (Fig. 4) and the pedunclopontine nucleus (Fig. 4). In the NTS there was a small cluster of FG and *Fos* double labeled neurons close to the central canal (Fig. 4, Fig. 6). The RVLM had a small tangle of FG-labeled neurons in the lateral reticular nucleus with a high degree of colocalization with *Fos* (Fig. 4, Fig. 6). Though the absolute number of double labeled cells in the NTS and RVLM was not high (Table 1), the percentage of FG-positive cells that were also *Fos*-positive was remarkable (approximately 50%).

3. Discussion

Ucn 3 plays a critical role in the brain in regulating energy homeostasis (Chen et al., 2010; Fekete et al., 2006; Jamieson et al., 2006; Ohata and Shibasaki, 2004; Pelleymounter et al., 2004; Telegdy et al., 2006) and, like other members of the CRF family, Ucn 3 expression in the brain is regulated by stress (Jamieson et al., 2006; Venihaki et al., 2004). Our previous work determined that the PVHap/BSTp is the main source of Ucn 3 afferent input to the VMH, a brain area well known to modulate energy balance and sympathetic outflow (Chen et al., 2011; Chen et al., 2012; King, 2006). Consistent with an earlier report (Jamieson et al., 2006), the present study confirmed that acute restraint stress stimulated Ucn 3 expression in the MEA. Notably, we found that restraint stress rapidly enhances Ucn 3 expression in the PVHap/BSTp, but not in neurons in the rostral perifornical hypothalamus lateral to the PVHap, suggesting that stress selectively stimulates Ucn 3 neurons in the PVHap/BSTp area. Interestingly, Ucn 3 expression in animals after 4 hours restraint stress showed a non-significant trend toward decreased levels compared to controls. It is possible that additional signals may be recruited under extended stress conditions as a feedback mechanism to negatively regulate Ucn 3 expression in the PVHap. This slightly lowered Ucn 3 expression

in 4hr stressed animals may be due to an overshoot by the feedback mechanism in reducing Ucn 3 expression. Consistent with this notion, it has been shown that CRF expression in the PVH is slightly reduced in rats with repeated cold stress compared to naïve rats (Hatalski et al., 1998).

To investigate the possible neural mechanisms by which acute stress stimulates Ucn 3 expression in the PVHap, the current study combined retrograde tracing and stress-induced *Fos* staining to determine stress-sensitive afferent input into the PVHap. In general, stress-activated afferent input into the PVHap originates from a wide range of brain areas including the rostral and ventral parts of the LS, various parts of the hypothalamus (ARH, VMH, PH, and ventral premammillary nucleus), and the MEA, with a lesser input from the BSTp and brainstem areas (PAG, the laterodorsal tegmental nucleus, the peripeduncular nucleus, the NTS, and the RVLM) (Fig. 7).

3.1 Forebrain stress-activated afferent input into the PVHap

3.1.1 Hypothalamic input

3.1.1.1 Arcuate nucleus of the hypothalamus: Consistent with earlier reports of ARH projections (Atasoy et al., 2012; Li et al., 2000; Magoul et al., 1994), FG-positive cells were observed in the ARH of rats injected with FG in the PVHap. A significant number of these FG-positive cells were double labeled with *Fos*, indicating that the arcuate-PVHap pathway is stress sensitive. The ARH is an established regulator of energy homeostasis, serving as a converging point for peripheral and central signals concerning peripheral energy status (Sasaki and Kitamura, 2010). After integrating various inputs, the ARH then signals multiple downstream targets including the PVH, lateral hypothalamus, and amygdala to modulate energy homeostasis (Bagnol et al., 1999; Balthasar et al., 2005). For example, orexigenic neurons from the ARH inhibit oxytocin neuron activity in the PVH to stimulate feeding (Atasoy et al., 2012). Thus, it is conceivable that stress activates anorexigenic neurons in the ARH, which in turn target and stimulate Ucn 3 cells in the PVHap to inhibit feeding in response to stress. It will be of great interest to identify the phenotypes of neurons in the ARH and their projections into the PVHap to further substantiate this possibility.

3.1.1.2 Ventromedial hypothalamus: Our previous study showed that the PVHap projects to the VMH (Chen et al., 2011) and the present study demonstrated that there are afferent projections from the VMH to PVHap, indicating reciprocal connections between the two areas. VMH projections to the PVHap have been previously confirmed through anterograde tracing (Canteras et al., 1995). While there were FG labeled cells throughout the VMH, the stress-activated neurons appeared to be concentrated in the ventrolateral portion of the nucleus, which has been implicated in regulating behaviors including aggression (Lin et al., 2011) and female sexual behavior (King, 2006). The connection from the ventrolateral VMH to the PVHap could be involved in modulating stress-associated behaviors.

Currently, the role of the ventrolateral VMH in energy balance is less clear. Interestingly, a number of recent studies have implicated the estrogen receptor in the VMH in energy balance. Stimulation of the estrogen receptor in the VMH promotes thermogenesis in brown adipose tissue (Martinez de Morentin et al., 2014) and mice with estrogen receptor knockout

in the VMH exhibit altered energy balance and are obese (Correa et al., 2013). Most of the estrogen receptors in the VMH are found in the ventrolateral part of the nucleus (Cao and Patisaul, 2011; Gonzales et al., 2012; Simerly et al., 1990), suggesting a potential role of the ventrolateral VMH in regulating energy balance. This raises the possibility that the ventrolateral VMH-PVHap pathway may be involved in regulating energy balance in response to stress.

3.1.1.3 Posterior hypothalamic nucleus and ventral premammillary nucleus: The PH and ventral premammillary nucleus contained a moderate number of cells double labeled with FG and *Fos* in animals injected with FG into the PVHap. The projection of both areas to the PVHap has been confirmed by previous studies (Canteras et al., 1992; Silverman et al., 1981). The PH has been associated with the regulation of cardiovascular function through sympathetic nervous system activity and locomotion; stimulation of the nucleus results in hypertension and increased movement (Eferakeya and Buñag, 1974; Martin et al., 1988; Young et al., 2011). The current result suggests that the PH may regulate sympathetic activity and locomotion by communicating with the PVHap in response to stress. The ventral premammillary nucleus has been shown to play a crucial role in regulating various aspects of reproduction including maternal aggression, pheromone sensing in male rats, and reproductive hormone secretions in response to nutrition (Cavalcante et al., 2006; Donato and Elias, 2011; Motta et al., 2013). More studies are needed to determine the precise role of the ventral premammillary nucleus projections to the PVHap in acute stress.

3.1.2 Extra-hypothalamic input

3.1.2.1 Lateral septal nucleus: Previous studies found that the LS provides modest projections to the PVHap, with most of the PVHap-projecting cells located in the ventral part of the LS (Risold and Swanson, 1997). Consistent with this earlier report, the present study found prominent FG-positive cells in the ventral LS with scattered double labeled cells in the caudal part of the LS. Many of the FG-positive cells were activated by acute stress. The LS has been implicated in feeding (Angeles-Castellanos et al., 2007; Koppell and Sodetz, 1972; Mitra et al., 2011), with both suppression and stimulation of feeding reported. Infusing noradrenaline into the LS increases feeding, whereas infusion of Ucn 1 decreases feeding (Scopinho et al., 2008; Wang and Kotz, 2002), suggesting the functional role of the nucleus in feeding is signal dependent. The present study suggests that the lateral septal neurons may mediate stress-associated energy adaptation by recruiting PVHap cells, including Ucn 3 neurons, which subsequently modulate feeding and sympathetic outflow.

3.1.2.2 Bed nucleus of the stria terminalis: Both anterograde and retrograde studies (Dong and Swanson, 2004; Gaykema et al., 2007) have demonstrated that neurons in the BST provide projections to the PVHap, and the current study identified many of the PVHap-projecting cells as *Fos* positive. The BST receives inputs carrying information about olfaction (Dong et al., 2001) and gustation (Li and Cho, 2006), and has been functionally linked to integration of behavioral and autonomic responses to stress (Cecchi et al., 2002; Davis et al., 1997; Khoshbouei et al., 2002). Moreover, a number of studies have indicated that the BST negatively regulates food intake (Ciccocioppo et al., 2003; Kocho-Schellenberg et al., 2014; Roman et al., 2012). Taken together, it is conceivable that the BST is involved

in conveying the olfactory and gustatory aspects of acute stress to the PVHap to modulate physiological functions including feeding.

3.1.2.3 Medial amygdala: Consistent with earlier reports (Canteras et al., 1995; Coolen and Wood, 1998), the present study found neurons in the MEA that project to the PVHap, and further defined the pathway by showing that it is activated by acute stress. Functional and genetic studies show that the MEA can act as a satiety center (Rollins and King, 2000; Xi et al., 2012), suggesting that the MEA-PVHap pathway may contribute to hypophagia induced by acute stress. In addition to feeding and stress regulation, the MEA is a major regulator of defense behaviors and emotional responses to olfactory stimuli (Dielenberg et al., 2001; Keshavarzi et al., 2014; Lehman et al., 1980). Lesions of the MEA eliminate defensive responses to predator odor (Martinez et al., 2011) and sexual response to odors of potential mates in mice (Lehman et al., 1980). The MEA-PVHap pathway may potentially be involved in regulating these behaviors in response to stress.

3.2 Brainstem stress-activated afferent input into the PVHap

3.2.1 Periaqueductal gray—The PAG contained FG labeled cells throughout its rostral to caudal axis, many double labeled with *Fos*, confirming the previously described projection (Pelosi et al., 2009). The PAG is well known for its role in modulating pain (Lau and Vaughan, 2014), but is also involved in a wide range of activities from hunting to fear learning. It has been shown that the PAG is activated by negative and life threatening experiences (Kinchski et al., 2012), and stimulation of the PAG in humans induces panic attacks and intense feelings of terror, panic, and anxiety (Schenberg et al., 2014). As restraint stress is a negative psychological experience, it is possible that the projection to the PVHap is involved in processing the negative nature of acute restraint stress.

3.2.3 Rostral ventrolateral medulla—Previous anterograde tracing studies have shown that the RVLM sends moderate projections to the PVHap (Cunningham and Sawchenko, 1988; Rinaman et al., 1995). A small number of FG-positive cells were observed in this area with a high percentage of *Fos* double labeling, indicating that this pathway is important in conveying stress signals. The medulla-PVH pathway has been implicated in inhibition of food intake by cholecystokinin, a gut secreting peptide (Rinaman et al., 1995). Thus, it is possible that this medullary projection to the PVHap may be involved in stress-induced hypophagia.

The RVLM is also extensively involved in sympathetic nervous system regulation and vascular tone (Allen et al., 2009; Allen, 2011; Pilowsky et al., 2008). A range of physical challenges activate C1 neurons in the RVLM, which then maintains cardiac, respiratory, and glucose homeostasis (Guyenet et al., 2013; Verberne et al., 2014). Though we did not identify input to Ucn 3 cells specifically, it is conceivable that the RVLM projection targets Ucn 3 neurons in the PVHap, which project to the VMH. As Ucn 3 acts in the VMH to increase blood glucose levels (Chen et al., 2010), the RVLM may recruit the PVHap-VMH pathway to increase blood glucose levels during acute stress.

3.2.4 Nucleus of the solitary tract—In the present study, a small number of FG cells were found in the NTS, which is consistent with previous anterograde studies that observed moderate projections from the NTS to the PVHap (Cunningham and Sawchenko, 1988; Rinaman et al., 1995). Importantly, a high percentage of these cells were double labeled with *Fos*. The NTS has an established role as a center that receives, integrates, and relays visceral gastrointestinal, gustatory, cardiovascular, and respiratory information to higher centers (Bradley and Grabauskas, 1998; Rinaman, 2010; Zhang and Mifflin, 2010; Zoccal et al., 2014). The NTS is also stress-responsive; during hypoxic stress, the NTS integrates neural signals from chemoreceptors and then initiates responses to increase blood oxygen (Zoccal et al., 2014). Thus, it is conceivable that the NTS is relaying visceral information to the PVHap during acute restraint stress.

3.3 Conclusion

The present study shows that, during acute restraint stress, the PVHap receives information from a diverse range of areas such as those concerning learning and behavior (LS, BST, amygdala) and autonomic regulation (hypothalamus, brainstem) (Fig. 7). Areas associated with behavioral regulation (peripeduncular nucleus, laterodorsal tegmental nucleus) also contained double labeled FG/*Fos* neurons, though the precise role of these stress-activated projections to the PVHap remains to be determined. Although these stress-activated inputs do not necessarily or specifically activate Ucn 3 neurons or enhance Ucn 3 mRNA production in the PVHap, it is likely that at least some of these inputs target Ucn 3 neurons, as the FG injections covered the entire PVHap area in which Ucn 3 cells reside. As such, the data provide a basic framework to further determine the neural inputs that activate Ucn 3 cells in the PVHap during stress.

4. Experimental Procedure

4.1 Animals

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250–300 g were housed in a temperature-controlled room with lights on between 0600–1800 hr. All experimental protocols and procedures were approved by The University of Virginia Animal Use and Care Committee, University of Virginia protocol number 3537.

4.2 Stress paradigm

To determine the effect of stress on Ucn 3 gene expression, male rats (6–7 animals per group) were subjected to restraint stress for 30 minutes or 4 hours with a rat restrainer before euthanization. Control animals remained in their home cages. Brains were quickly removed after stress, deep frozen in powdered dry ice, and stored at –80°C until use.

4.3 Tracer injection

A separate cohort of rats was anesthetized with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). A glass micropipette with a tip diameter of 25–30 µm was filled with a retrograde tracer, FG (2% w/v in physiological saline), and inserted into the PVHap. FG was then injected by iontophoresis with 5 µA current pulsed at 7 second intervals for 10 minutes. The stereotaxic coordinates for the PVHap were: 1.1 mm caudal

and 0.2 mm lateral to the bregma, and 7.6 mm ventral to the dura. Ten days after tracer injection, animals were subjected to 30 minutes of restraint stress and were perfused with 4% formaldehyde (pH 9.5) in borate buffer 90 minutes after the onset of the stress paradigm. The brains were removed and sectioned coronally at 25 μ m thickness and stored in cryoprotectant at -20°C until use.

4.4 In situ hybridization

Frozen rat brains were sectioned (20 μ m thickness) coronally with a cryostat. *In situ* hybridization for detecting Ucn 3 mRNA was performed as previously reported (Li et al., 2002). Briefly, a Ucn 3 complementary RNA (cRNA) probe was transcribed from a linearized vector containing a full-length rat Ucn 3 sequence and flanking the 30bp untranslated region (total 528 bp) in the presence of the 33 P-labeled UTP (Perkin-Elmer, Oak Brook, IL). Brain sections were treated with a fresh solution containing 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) followed by a rinse in 2X SSC. The sections were then exposed to the cRNA probe overnight at 55°C . After hybridization, the brain sections were washed in increasingly stringent SSC, in RNase, and in 0.1X SSC at 60°C , dehydrated through a graded series of alcohol, and dried. Slides were dipped in NTB emulsion (Kodak, New Haven, CT), exposed for 7 days at 4°C , and developed. Following development, the slides were counterstained with cresyl violet to identify anatomical landmarks. Results were examined on a Nikon light microscope, and images were acquired with a Qimaging digital camera (Biovision) controlled by iVision imaging software.

4.5 Immunohistochemistry

For *Fos* immunohistochemistry, brain sections were washed with potassium phosphate-buffered saline (KPBS) and then were incubated in rabbit anti-*Fos* antibody (Santa Cruz, 1:20,000) in KPBS with 0.4% Triton-X 100 for 48 hrs at 4°C . After incubation, the sections were washed and incubated in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, 1:600) for 1 hr at room temperature. They were then incubated for 1 hr at room temperature in an avidin-biotin complex solution (Vectastain ABC Elite Kit, Vector Laboratories). Nickel enhanced 3,3-diaminobenzidine (DAB) reaction was used as a chromogen to visualize *Fos* immunoreactivity. After the color development, brain sections were washed with KPBS and were incubated in rabbit anti-FG antibody (Millipore, 1:15,000) in KPBS with 0.4% Triton-X 100 for 48 hrs at 4°C . The sections were then incubated in biotinylated donkey anti-rabbit IgG for 1 hr at room temperature, followed by a 1 hr incubation in an avidin-biotin complex solution. The FG antibody-peroxidase complex was revealed with a DAB reaction. Brain sections were then mounted onto gelatin-coated slides, dehydrated, and cover slipped with Permount.

4.6 Antibody characterization

FG antiserum (Millipore, AB153): The FG antiserum recognized fluorogold (hydroxystilbamidine) and aminostilbamidine with immunohistochemical analyses and has been extensively used to label the retrograde tracer (manufacturer's datasheet). The antiserum labeled cells and processes in FG-injected, but not in non-injected, animals (Chen et al., 2011; Li et al., 1999b).

Fos antibody (Santa Cruz, sc-52): The antibody recognized *Fos* in cell lysates of HEK293T cells transfected with mouse *Fos* cDNA with western blot analysis and in phorbol ester-induced HeLa cells and Hep G2 cells with immunofluorescence (manufacturer's datasheet). The antibody is affinity purified rabbit polyclonal IgG raised against the N-terminus residues 3–16 of the human *Fos* (FSGFNADYEASSSR) (manufacturer's datasheet). The antibody labeled nuclei in neurons in rat brains after stimulation (Hou et al., 2008; Li et al., 1999b).

4.7 Data analysis

For *in situ* hybridization, one-in-four series of the coronal brain sections that were processed for the experiments were first anatomically matched across animals using the rat brain atlas of Swanson (1998) to match brain areas. Three parallel coronal sections that evenly contained the rostrocaudal levels of the PVHap were used for quantifying hybridization signal by ImageJ. For the rostral perifornical hypothalamus and MEA, five and seven sections were used, respectively, for quantification. The hybridization signals were quantified as previously described (Li et al., 1999a) using ImageJ image analysis system. Briefly, the system identified silver grains as the brightness of the image under darkfield illumination in a marked area that covered the area of interest in each tissue section. Hybridization signal for each area of interest was obtained by subtracting a background reading taken from nearby brain regions (the striatum for the PVHap and rPFH, and the cortex for the MEA) where no discernible Ucn 3 hybridizing signal was found (Li et al., 2002). Results for each brain area are presented as mean $\pm$ SEM. The combined effects of stress and time on Ucn 3 mRNA levels were analyzed using a two-way ANOVA followed by post hoc analysis for between group comparisons, with a significance level set at 0.05.

Results from the FG/*Fos* double label tracing study were examined and captured by a Nikon 80i microscope coupled with a QImaging CCD camera (BioVision, Exton, PA) controlled by iVision software. The images were adjusted to balance brightness and contrast in Photoshop before importing into Canvas (version 8.0) for assembly into plates. An FG immunoreactive neuron was considered to be double labeled if the nucleus of the FG cell had a black nickel-DAB reaction product indicative of *Fos* immunoreactivity. Brain atlas plates by Swanson (1998) were used as templates to plot the distribution of *Fos*, FG, and double labeled cells in the rat brains. The number of *Fos*, FG, and double labeled cells in the areas of interest in each animal were counted, and the mean number of cells per section for each of the areas was then determined for each animal. Data are presented as mean $\pm$ SEM in Table 1.

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Glossary

Ucn 3	Urocortin 3
CRF	corticotropin-releasing factor
CRF₂	type 2 CRF receptor
ACAd	Dorsal part of the anterior cingulate area
AHN	Anterior hypothalamic nucleus
AHNc	Central part of the anterior hypothalamic nucleus
AHNp	Posterior part of the anterior hypothalamic nucleus
AQ	Cerebral aqueduct
ARH	Arcuate nucleus of the hypothalamus
BA	Bed nucleus of the accessory olfactory tract
bic	Brachium of the inferior colliculus
BN	Basal nucleus of the dorsal horn
BST	bed nucleus of the stria terminalis
BSTp	Posterior division of the bed nucleus of the stria terminalis
BSTad	Anterodorsal area of the anterior division of the bed nucleus of the stria terminalis
BSTpr	Posterior part of the principal nucleus of the bed nucleus of the stria terminalis
COAa	Anterior part of the cortical nucleus of the amygdala
COApl	Lateral zone of the posterior part of the cortical nucleus of the amygdala
DMHa	Anterior part of the dorsomedial nucleus of the hypothalamus
fa	Anterior forceps of the corpus callosum
fx	Columns of the fornix
ILA	Infralimbic area
IPN	Interpeduncular nucleus
LDT	Laterodorsal tegmental nucleus
LHA	Lateral hypothalamic area
LRN	Lateral reticular nucleus
LS	lateral septal nucleus
LSc	Caudal part of the lateral septal nucleus
LSr	Rostral part of the lateral septal nucleus
LSv	Ventral part of the lateral septal nucleus
mcp	Middle cerebellar peduncle

MDRNv	Ventral part of the medullary reticular nucleus
MEA	Medial amygdala
MEAad	Anterodorsal part of the medial amygdala
MEApd	Posterodorsal part of the medial amygdala
MEApv	Posteroventral part of the medial amygdala
ml	Medial lemniscus
mlf	Medial longitudinal fascicle
MPN	Medial preoptic nucleus
NB	Nucleus brachium inferior colliculus
NTS	Nucleus of the solitary tract
opt	Optic tract
PAG	Periaqueductal grey
PCG	Pontine central grey
PH	Posterior hypothalamic nucleus
PL	Prelimbic area
PMv	Ventral premammillary nucleus
PP	Peripeduncular nucleus
PPN	Pedunculopontine nucleus
PVH	Paraventricular nucleus of the hypothalamus
PVHap	Anterior parvicellular part of the paraventricular nucleus of the hypothalamus
rPFH	Rostral perifornical hypothalamus
RVLM	Rostral ventrolateral medulla
SC	Superior colliculus
sm	Stria medullaris
V3	Third ventricle
VL	Lateral ventricle
VMH	Ventromedial nucleus of the hypothalamus
VMHvl	Ventrolateral part of the ventromedial nucleus of the hypothalamus
VTa	Ventral tegmental area
vtd	Ventral tegmental decussation

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Highlights

- Ucn 3 neurons in the PVHap are known to be the main Ucn 3 afferent input to the VMH
- Acute restraint stress increases Ucn 3 mRNA levels in neurons in the PVHap
- A retrograde tracer and restraint stress revealed stress-activated input to the PVHap
- The extended amygdala and hypothalamus convey stress signals to the PVHap
- Stress activates neurons in the PAG and RVLM that project to the PVHap

Fig. 1.

Ucn 3 mRNA expression after restraint stress. Representative darkfield photomicrographs showing silver grains by in situ hybridization for Ucn 3 mRNA in the paraventricular nucleus of the hypothalamus, anterior parvicellular part (PVHap) and posterior part of the bed nucleus of the stria terminalis (BSTp) (A–D), rostral perifornical hypothalamus (F–I) and medial amygdala (MEA) (K–N) with 30 min (B,G,L) or 4 hrs (D,I,N) restraint stress. Non-stress control for 30 min (A,F,K) and 4 hrs (C,H,M) in each area were also shown. E,J,O: Summary of Ucn 3 mRNA levels in the PVHap (E), rostral perifornical hypothalamus (J) and MEA (O). ** $P < 0.01$, * $P < 0.05$ vs. non stressed control. Scale bars = 50 μ m

Fig. 2. Representative darkfield photomicrograph showing injection sites (A) and schematic drawing showing a reconstruction of injection sites (B) in the PVHap. Circles indicate extent of dye placement for each case.

Fig. 3.

Representative brightfield photomicrographs showing fluorogold (FG, light brown precipitate in cytosol) staining in the prefrontal cortex (A), bed nucleus of the stria terminalis (BST) (B), the ventromedial hypothalamus (VMH) (C), the MEA (D), the ventral tegmental area (E), the nucleus brachium inferior colliculus (F), the interpeduncular nucleus (G), and the pontine central gray (H) of animals injected in the PVHap. Scale bars = 100 μ m.

Fig. 4.

Representative illustrations showing neurons stained with *Fos* (black), FG (light brown), and double labeled (blue) in the prefrontal cortex (A), lateral septal nucleus (LS) (B–C), principal nucleus of the posterior division of the BST, PVHap, and medial preoptic nucleus (D), PVHap, anterior hypothalamic nucleus (E), paraventricular nucleus of the hypothalamus, anterior hypothalamic nucleus, arcuate nucleus of the hypothalamus (ARH), and MEA (F), posterodorsal part of the MEA, anterior part of the dorsomedial nucleus of the hypothalamus, ARH, and ventrolateral part of the VMH (G), PH and ventral premammillary nucleus (H), periaqueductal gray (PAG), peripeduncular nucleus, and ventral tegmental area (I), PAG and interpeduncular nucleus (J), PAG, laterodorsal tegmental nucleus, and pedunculopontine nucleus (K), pontine central gray (L), and nucleus of the solitary tract (NTS), lateral reticular nucleus in the rostral ventrolateral medulla (RVLM) (M) of animals injected in the PVHap.

Fig. 5.

Representative brightfield photomicrographs showing immunoreactive FG (light brown precipitate in cytosol) and *Fos* (dark nuclear precipitate) staining in the forebrain areas of the LS (A), BST (C), the VMH (E), and the MEA (G) of animals injected in the PVHap. B, D, F, H: High power magnification of boxed area in A (B), C (D), E (F) and G (H). Representative FG/*Fos* double labeled cells were indicated by blue arrowheads. Scale bars = 50 μ m (A, C, D, E, G), and 20 μ m (B, D, F, H).

Fig. 6.

Representative brightfield photomicrographs showing immunoreactive FG (light brown precipitate in cytosol) and *Fos* (dark nuclear precipitate) staining in the brainstem areas of the PAG (A), the NTS (C), and the lateral reticular nucleus of the RVLM (E) of animals injected in the PVHap. B, D, F, H: High power magnification of boxed area in A (B), C (D), and E (F). Representative FG/*Fos* double labeled cells were indicated by blue arrowheads. Scale bars = 50 μm (A, C, D, E, G), and 20 μm (B, D, F, H).

Fig. 7. Summary of the stress-activated input to the PVHap. Circles represent areas with FG and *Fos* double labeling. Pink arrows represent PVHap neural circuit inputs.

Quantification of fluorogold (FG), Fos, and FG/Fos double labeled cells in the brains of animals that received acute stress

Table 1

Area	FG	SEM	Fos	SEM	FG/Fos	SEM
Prefrontal cortex						
Prelimbic	92	9.4	102	30.8	2	0.5
Infralimbic	98	16.5	86	16.3	1	0.6
Piriform cortex	0	0.0	159	12.8	0	0.0
Nucleus accumbens	1	0.3	69	8.6	0	0.0
Lateral septum	114	26.4	144	16.4	18	1.8
Bed nucleus of the stria terminalis	56	5.8	43	10.8	13	0.8
Medial preoptic nucleus	27	2.4	12	3.4	1	0.2
Thalamus						
Paraventricular thalamic nucleus	9	1.8	60	9.7	1	0.2
Anterior hypothalamic area	65	10.1	25	2.2	3	0.4
Hypothalamus						
Paraventricular nucleus of the hypothalamus	* note		141	13.3	* note	
Periventricular hypothalamic nucleus	20	6.4	34	4.1	0	0.0
Arcuate nucleus	32	4.0	37	5.7	7	0.6
Ventromedial hypothalamus	128	27.2	53	5.3	19	2.5
Dorsomedial nucleus	48	8.4	79	10.7	4	1.2
Posterior hypothalamic nucleus	99	11.8	63	4.5	16	3.1
Ventral preamillary nucleus	64	5.7	69	12.3	16	3.4
Amygdala						
Medial amygdala	78	9.3	105	9.3	14	1.0
Brainstem						
Periaqueductal gray	105	12.1	137	12.9	14	1.8
Peripeduncular nucleus	63	11.4	100	25.8	10	2.9
Superior colliculus	17	10.0	81	17.1	1	0.8
Pontine gray	2	0.3	440	65.7	0	0.0
Laterodorsal tegmental nucleus	39	7.2	53	12.5	10	2.7
Pedunculopontine area	18	2.1	27	3.7	7	0.6
Trapezoid/olivary bodies	6	2.7	73	5.0	1	0.6
Ventral tegmental area	27	7.4	29	10.1	2	0.7
Interpeduncular nucleus	18	1.1	44	5.3	1	0.4
Nucleus brachium inferior colliculus	16	1.4	57	7.0	2	0.4
Pontine central gray	19	4.2	37	8.7	1	0.5

Area	FG	SEM	Fos	SEM	FG/Fos	SEM
Nucleus of the solitary tract	10	1.7	23	3.1	5	0.4
Rostral ventrolateral medulla	13	2.3	14	3.1	5	0.6

* note: FG cells were not determined due to excessive accumulation of FG near the injection site.