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Deafferentation of the hypothalamic paraventricular nucleus (PVN) exaggerates the sympathoadrenal system activity in stressed rats

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Objective. The hypothalamic paraventricular nucleus is a key structure in the regulation of the autonomic and neuroendocrine systems response to acute and chronic stress challenges. In this study, we examined the effect of a mechanical posterolateral deafferentation of the PVN on the activity of sympathoadrenal system (SAS) and hypothalamo-pituitary-adrenal (HPA) axis by measuring plasma concentrations of epinephrine (EPI), norepinephrine (NE), and corticosterone (CORT) in rats exposed to acute immobilization (IMO) stress.

Methods. The surgical posterolateral deafferentation of the PVN (PVN-deaf) was performed by Halasz knife, in brain of the adult male Sprague Dawley rats, according to coordinates of a stereotaxic atlas. Sham-operated (SHAM) animals underwent a craniotomy only. The animals were allowed to recover 14 days. Thereafter, the tail artery was cannulated and the animals exposed to acute IMO for 2 h. The blood samples were collected via cannula at the time points of 0, 5, 30, 60, and 120 min of the IMO. Concentrations of plasma EPI, NE, and CORT were determined by radioimmunoassay.

Results. The IMO-induced elevation of plasma EPI concentrations in the PVN-deaf rats reached statistical significance at 60 min of the IMO, when compared to SHAM rats. Similarly, the stress-induced elevation of the NE plasma levels in the PVN-deaf rats was significantly exaggerated at all time intervals of IMO in comparison with SHAM rats, whereas plasma CORT levels were significantly reduced.

Conclusions. In contrast to the traditional view of excitatory role of the PVN in response to stress, our data indicate that some projections from the PVN to caudally localized hypothalamic structures, the brainstem or the spinal cord, exert inhibitory effect on the SAS system activity during acute IMO stress. The data indicate that stress-induced activation of the HPA axis is partially dependent on inputs from the brainstem to the PVN.

Key words: catecholamines, corticosterone, immobilization, paraventricular hypothalamic nucleus, sympathoadrenal system

View on the role of brain structures in the regulation of neuroendocrine stress response is still incomplete. Published data indicate that the PVN represents a crucial structure in regulating the activity of the SAS and the HPA axis during stressful situations (Chrousos 1998; Benarroch 2005; Ulrich-Lai and Herman 2009). Furthermore, the PVN appears to play a pivotal role in the integration of signals related to physical, psychosocial, immune, and metabolic stressors (Smith and Vale 2006).

The PVN regulates the neuroendocrine stress response via different subpopulations of neurons, including the corticotrophin-releasing hormone (CRH)-synthesizing neurons directed to the median eminence

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and the neurons projecting to the brainstem, the sympathetic premotor neurons, and the spinal sympathetic preganglionic neurons (Sawchenko and Swanson 1982; Sawchenko et al. 2000; Herman et al. 2003; Ferguson et al. 2008). Moreover, the PVN regulates the stress response also via projections to the nucleus of the solitary tract (NTS), which regulates the activity of the sympathetic and the parasympathetic branches of the autonomic nervous system (Kvetnansky et al. 2009; Geerling et al. 2010). Conversely, the NTS neurons are activated, via afferent pathways of the vagus nerve and the spinal afferent neurons, by many stressors such as hypoxia, cytokines, and IMO and relay visceral information to PVN (Smith and Vale 2006). Interactions between the PVN and NTS are responsible for the precise orchestration of the stress response (Herman et al. 2002).

The role of the PVN in the regulation of stress responses has been well described (Pacak et al. 1992; Herman et al. 2002; Charmandari et al. 2005; Herman et al. 2008). However, the consequence of the transection of pathways interconnecting the PVN with other brain structures on the activity of the SAS and HPA axis in response to stressful situations has been characterized only partially (Kvetnansky et al. 1988). Therefore, the aim of our work was to investigate the effect of a mechanical disconnection of the PVN neurons from other brain structures on SAS and HPA axis activities during the exposure of rats to acute IMO stress. The activity of the SAS and the HPA axis was characterized by determination of the plasma concentrations of EPI, NE, and CORT.

Materials and Methods

Animals. Adult male Sprague Dawley rats (Charles River, Germany) with an initial body weight of 225 - 250 g, were used. The animals were housed in standard housing in groups of four per cage in controlled conditions (temperature 22 ± 1°C and on a 12:12-hour light-dark cycle, lights on at 6:00 a.m.) with food and water *ad libitum*. Rats were allowed to acclimate for at least one week before starting the experiments. All the experiments were approved in accordance with the institutional guidelines of the Animal Health and Welfare Division of the State Veterinary and Food Administration of the Slovak Republic under the approved protocols and were carried out in accordance to the European Communities Council Directive (86/609/EEC).

Surgical procedures. The animals (n=28) were anaesthetized with an intramuscular injection of a mixture containing ketamine (Narkamon 5% - 1.2 ml/kg b.w.) and xylazine (Rometar 2% - 0.4 ml/kg b.w.) and assigned to one of the two groups for the posterolateral PVN-deaf (n=16) or sham procedure (SHAM; n=12). The animals were placed into a stereotaxic apparatus (David-Kopf Instruments) and their heads were fixed in a 3° nose-down position. Their scalp was exposed and rectangular hole (2.0 x 4.0 mm) was drilled into the skull. The posterolateral PVN-deaf was performed by Halasz knife (Halasz and Pupp 1965) according to coordinates in a stereotaxic atlas (Paxinos and Watson 1997). The ventral tip of the knife was placed perpendicularly to the midline plane of brain during penetration into the brain tissue. After reaching the appropriate point in the brain, the knife was twisted to the left and right by 90°, and then removed (Mravec et al. 2007). The selected stereotaxic coordinates for the posterolateral PVN-deaf were: antero-posterior (from the level of the bregma) -2.9 mm, vertical (from the top of the skull) -10.0 mm, medio-lateral (from the midline) 0.0 mm. The cut interrupted the anatomical connections between the PVN and caudal hypothalamic, brainstem, and spinal cord structures (Fig. 1). SHAM rats underwent the same craniotomy as PVN-deaf rats including the head fixing, drilling, and penetration of Halasz knife into the brain tissue, but without the twisting of the knife. After the transection of the brain pathways, animals were allowed to recover 14 days (Fig. 2A).

Two weeks after surgery (the recovery period), the tail artery of rats was cannulated to allow the collection of blood samples. During the cannulation procedure the rats were anaesthetized with a mixture of ketamine-xylazine for the placement of a permanent cannula into the tail artery, as described previously (Chiueh and Kopin 1978). A polyethylene tube (silicon tubing, PE 50; Becton-Dickinson, Parsippany, NJ) was filled with heparinized saline (300 IU/ml), passed subcutaneously through the back to the base of the neck, protected with a flexible stainless steel spring, and bandaged by an adhesive tape to the neck. The free end of the cannula was passed through the top of the cage, allowing a repeated blood sampling from the freely moving rats. After cannulating, the rats were housed individually. The IMO and blood sampling started on the next day after the overnight recovery.

Immobilization and blood sampling. First blood samples (baseline) were collected from unstressed animals via cannula while housed in their home cages. After the collection of the baseline blood samples, rats were exposed for 2 h to IMO. The IMO started at 8.00

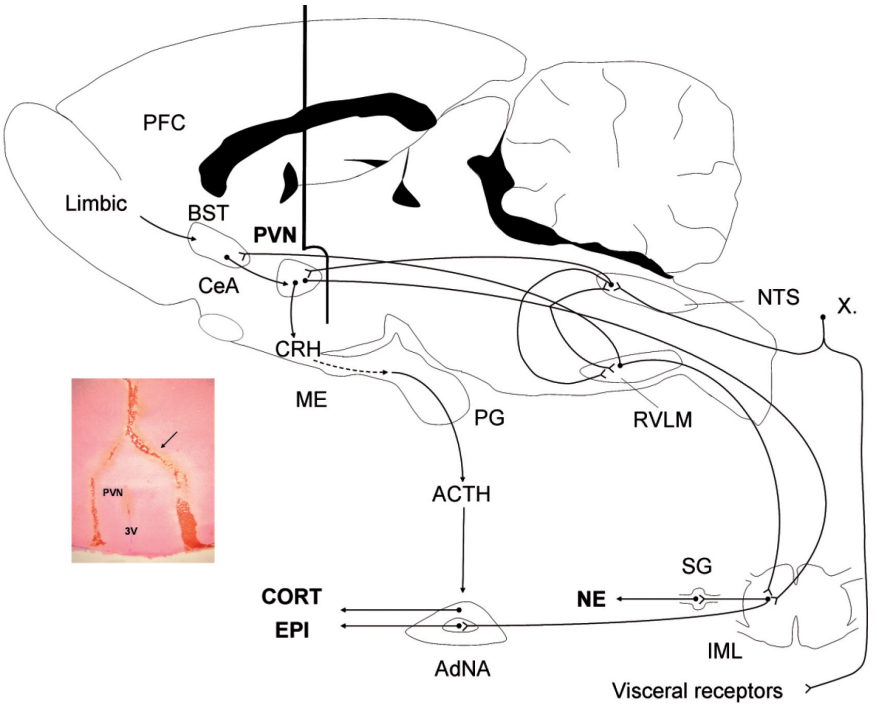

Fig. 1. Schematic illustration of a posterolateral deafferentation of the PVN by using the Halasz knife. Only the major brain structures involved in the SAS and the HPA axis regulation during stressful situations and pathways that have been interrupted are shown. The catecholaminergic inputs of the NTS neurons and the A1/C1 neurons to the PVN represent major excitatory drive in the response to stressors that relay visceral signals through the afferent pathways of the vagus nerve and the spinal afferent neurons. Activated PVN neurons projecting to the sympathetic preganglionic neurons and the RVLM have stimulatory effect on the SAS activity, while inputs to the NTS exert the inhibitory influence. Moreover, the limbic system including the hippocampal formation, the amygdala, and the prefrontal cortex projects to the effector neurons of the PVN via the intermediary neurons of the BST. A stained image shows a degree of cut. Abbreviations: ACTH, adrenocorticotropic hormone; AG - adrenal gland; BST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CORT, corticosterone; CRH, corticotropin-releasing hormone; EPI, epinephrine; IML, intermediolateral nucleus; ME, median eminence; NE, norepinephrine; NTS, nucleus of the solitary tract; PFC, prefrontal cortex; PG, pituitary gland; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SG, sympathetic ganglion; 3V, third ventricle. H&E x 20. Adapted from (Bourque 2008).

a.m. as described previously (Kvetnansky and Mikulaj 1970). Briefly, the animals were fixed in a prone position on a wooden board by taping its limbs to a stainless platform with its head placed through a steel loop attached to a board. The blood samples were collected via cannula at the time points of 0, 5, 30, 60, and 120 min of the IMO (Fig. 2B). As a replacement for the taken blood (0.6 ml), the rats received an equal volume of heparinized saline (50 IU/ml). The vials with blood were immediately placed on ice and centrifuged at 3000× g for 15 min at 4°C. The plasma was separated and then stored at -70°C until used for analysis. After the last blood collection, the animals were decapitated. The brains were removed, frozen on dry-ice, and stored at -70°C. Then, the brains were sliced on cryostat and stained with hematoxylin and eosin. The preciseness of the PVN-deaf was evaluated under the light microscopy. Only the rats with the correct PVN-deaf were included into the study (n=12; Fig. 1).

Plasma biochemical analysis. The plasma EPI and NE concentrations were determined by radioimmunoassay (Peuler and Johnson 1977). Plasma catecholamines aliquots were converted into their labeled O-methylated derivatives by using S-[3H]-adenosylmethionine (Amersham, Little Chalfont, UK) and a lyophilized catechol-O-methyltransferase isolated from rat liver. Then the O-methylated derivatives of the amines were extracted with unlabeled carrier compounds, separated by thin-layer chromatography, eluted, and reacted with periodate. The detection limit was 5 pg of EPI or NE per tube. The plasma CORT concentration was determined by a commercially available radioimmunoassay kit according to the manufacturer's instructions (CORT rat/mouse RIA kit, DRG Diagnos-

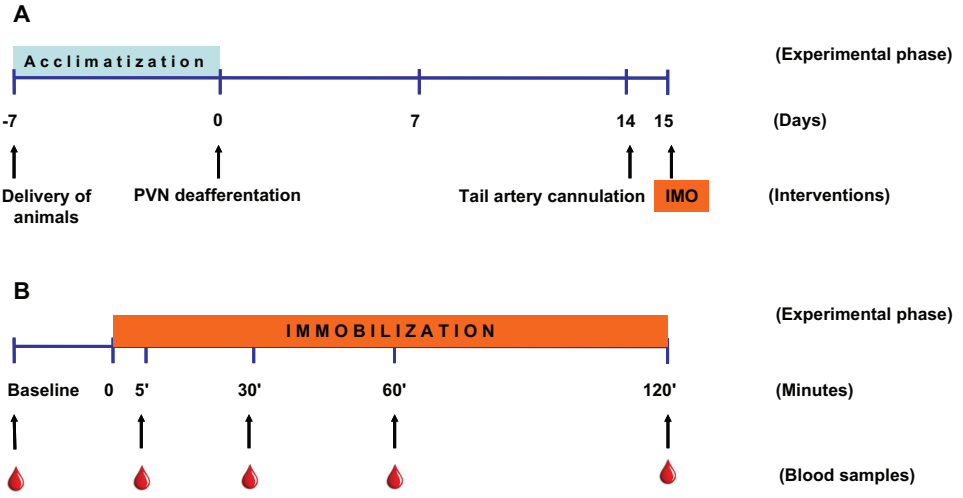

Fig. 2. Design of the experiment in which the effect of the posterolateral deafferentation of the paraventricular nucleus of the hypothalamus (PVN) on plasma catecholamine and corticosterone levels was investigated in rats exposed to a single acute immobilization (IMO) stress 14 days after mechanical transection (A). Time frame during which rats were exposed to immobilization and time of the blood samples withdrawal (B).

tic, Germany). The minimum detectable concentration of CORT in this kit was 7.7 ng/ml.

Data analysis. Statistical analysis was performed using a GraphPad Prism 5 program (GraphPad Software, San Diego CA, USA). Statistical differences between the groups were determined by one- or two-way analyses of variance (ANOVA) followed by *post hoc* pair wise comparisons using a Bonferroni's correction. Differences of $p<0.05$ were considered as statistically significant. An area under the curve (AUC) was calculated based on the concentration × time as a measure of the magnitude of the response. The AUC represents an integrated value of the total amount of hormone released during the 120 min of IMO. Data are presented as mean ± SEM and represent the mean for 12 PVN-deaf and 10 SHAM rats.

Results

The effect of PVN-deaf on baseline plasma catecholamine and CORT levels. The posterolateral PVN-deaf significantly increased both the baseline (unstressed) plasma EPI (PVN-deaf: 83 ± 16 pg/ml *vs.* SHAM: 32 ± 13 pg/ml; $p<0.05$) (Fig. 3A) and NE levels (PVN-deaf: 522 ± 73 pg/ml *vs.* SHAM: 277 ± 32 pg/ml; $p<0.01$) (Fig. 3B). The baseline (unstressed) plasma levels of CORT in the PVN-deaf rats showed a slight but not significant elevation when compared to the sham-operated animals ($p=0.0897$; Fig. 3C).

The effect of PVN-deaf on plasma catecholamine and CORT levels in the IMO rats. The EPI, NE, and CORT responses to a single IMO in SHAM and PVN-deaf animals are shown in Fig. 4. Both groups showed a stress-induced increase in the plasma catecholamines and CORT levels with a marked peak value at the fifth minute of the IMO. Significant effects of the PVN-deaf ($F_{(1,100)} = 21.55$, $p<0.0001$), time interval of IMO ($F_{(4,100)} = 17.29$, $p<0.0001$), and significant interaction between these two factors ($F_{(4,100)} = 1.69$, $p=0.1578$) were observed in the EPI plasma levels. The *post hoc* comparisons showed that the stress-induced elevation of EPI plasma levels in the PVN-deaf rats was significantly exaggerated at 60th min of acute IMO (PVN-deaf: 2315 ± 440 pg/ml *vs.* SHAM: 987 ± 144 pg/ml; $p<0.01$) (Fig. 4A). Similarly, there were significant effects of the PVN-deaf ($F_{(1,100)} = 74.36$, $p<0.0001$), time interval of IMO ($F_{(4,100)} = 27.49$, $p<0.0001$), and significant interaction between these two factors ($F_{(4,100)} = 2.78$, $p=0.0309$) in the NE plasma levels. The *post hoc* comparisons revealed that the stress-induced elevation of the NE plasma levels in the PVN-deaf rats was significantly exaggerated at all time intervals of IMO ($p<0.01$, $p<0.001$; Fig. 4B) compared to SHAM animals. In contrast, a significant reduction of CORT plasma levels was observed in the PVN-deaf rats at 5 min (PVN-deaf: 258 ± 27 ng/ml *vs.* SHAM: 488 ± 70 ng/ml; $p<0.01$), 60 min (PVN-deaf: 369 ± 47 ng/ml *vs.* SHAM: 574 ± 68 ng/ml; $p<0.01$), and 120 min (PVN-deaf: 322 ± 26 ng/ml *vs.* SHAM: 528 ± 47 ng/ml; $p<0.01$) interval of IMO compared to sham-operated rats (Fig. 4C). There was a significant effect of PVN deafferentation ($F_{(1,100)} = 26.47$, $p<0.0001$) and

time interval of IMO ($F_{(4,100)}$ = 18.00, p<0.0001), as well as a significant interaction between these two factors ($F_{(4,100)}$ = 3.03, p=0.0210) on CORT plasma levels.

The total amount of hormones released during the two hour episode of the acute IMO was calculated as an area under the curve. There was no significant effect of the PVN-deaf on the total plasma EPI (p=0.0996; Fig. 4A) release, while the total amount of NE released was significantly greater in the PVN-deaf rats compared to the SHAM animals (PVN-deaf: 183.5 ± 24.1 ng/ml.min *vs.* SHAM: 95.9 ± 15.4 ng/ml.min; p<0.01) (Fig. 4B). In contrast, the total amount of CORT released during the IMO was significantly lower in the PVN-deaf rats compared to the SHAM rats (PVN-deaf: 61.4 ± 4.9 μg/ml.min *vs.* SHAM: 40.8 ± 4.2 μg/ml.min; p<0.01) (Fig. 4C).

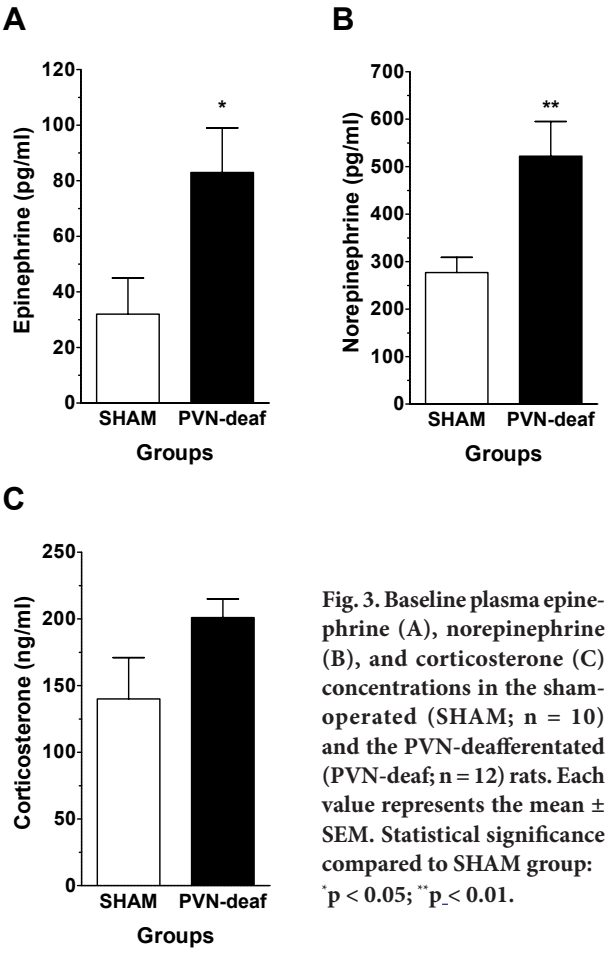

Fig. 3. Baseline plasma epinephrine (A), norepinephrine (B), and corticosterone (C) concentrations in the sham-operated (SHAM; n = 10) and the PVN-deafferentated (PVN-deaf; n = 12) rats. Each value represents the mean ± SEM. Statistical significance compared to SHAM group: $^{*}p < 0.05$; $^{**}p < 0.01$.

Discussion

IMO stress markedly stimulated the activity of the SAS and the HPA axis, resulting in a significant elevation of the plasma EPI, NE, and CORT levels in rats (Kvetnansky et al. 1978; Kvetnansky et al. 1979). Previously published data have clearly shown that the PVN is a crucial structure participating in regulation of stress responses (Benarroch 2005; Ulrich-Lai and Herman 2009). Moreover, we have already shown that anterolateral deafferentation of the medial basal hypothalamus significantly affects the neuroendocrine stress response as well as the transmission of stress related signals to the PVN in rats (Kvetnansky et al. 1988). In the present study, we investigated the effect of a mechanical posterolateral PVN-deaf on the plasma catecholamine and CORT levels in IMO rats. Here, we showed that a non-specific posterolateral PVN-deaf significantly exaggerated stress-induced catecholamine release from the SAS. In contrast, the stress-induced response of the HPA axis was reduced in the PVN-deaf animals. Moreover, in PVN-deaf animals significantly increased baseline plasma EPI and NE levels were found.

The posterolateral PVN-deaf leads to an interruption of several afferent and efferent interconnections between the PVN and the caudally localized structures such as the hypothalamus, brainstem, and spinal cord (Palkovits 1999). Transection of afferent pathways that relay signals from the NTS and the A1 noradrenergic cell group to the parvo- and magnocellular neurons of the PVN is important from the neuroendocrine stress response regulation point of view (Sawchenko and Swanson 1982; Pacak and Palkovits 2001). Firstly, the NTS and the A1 noradrenergic neurons are activated in response to the internal stressors such as hypovolemia, hypotension, or IMO stress, especially by signals transmitted via afferent pathways of the vagus nerve and the spinal afferent neurons (Sawchenko et al. 2000; Pacak and Palkovits 2001){Geerling, 2009 #328}. Stressful stimuli activate the parvocellular CRH neurons of the PVN as a part of the HPA axis consequently increasing the release of glucocorticoids (Keller-Wood and Dallman 1984; Lightman et al. 2002). The activation of the sympathetic premotor neurons of the PVN leads to stimulation of the SAS directly through the PVN projection to the sympathetic preganglionic neurons and indirectly through the PVN projection to the rostral ventrolateral medulla (RVLM) (Kvetnansky et al. 2009; Kc and Dick 2010). Activated PVN neurons project to the sympathetic preganglionic neurons and the RVLM neurons exerting stimulatory effect on the SAS activity. Moreover, the PVN neurons regulate the SAS activity via innervation of the NTS neurons. As such, the PVN neurons projecting to the NTS may exert inhibitory influence on the SAS activity (Badoer 2001; Badoer et al. 2002; Geerling et al. 2010).

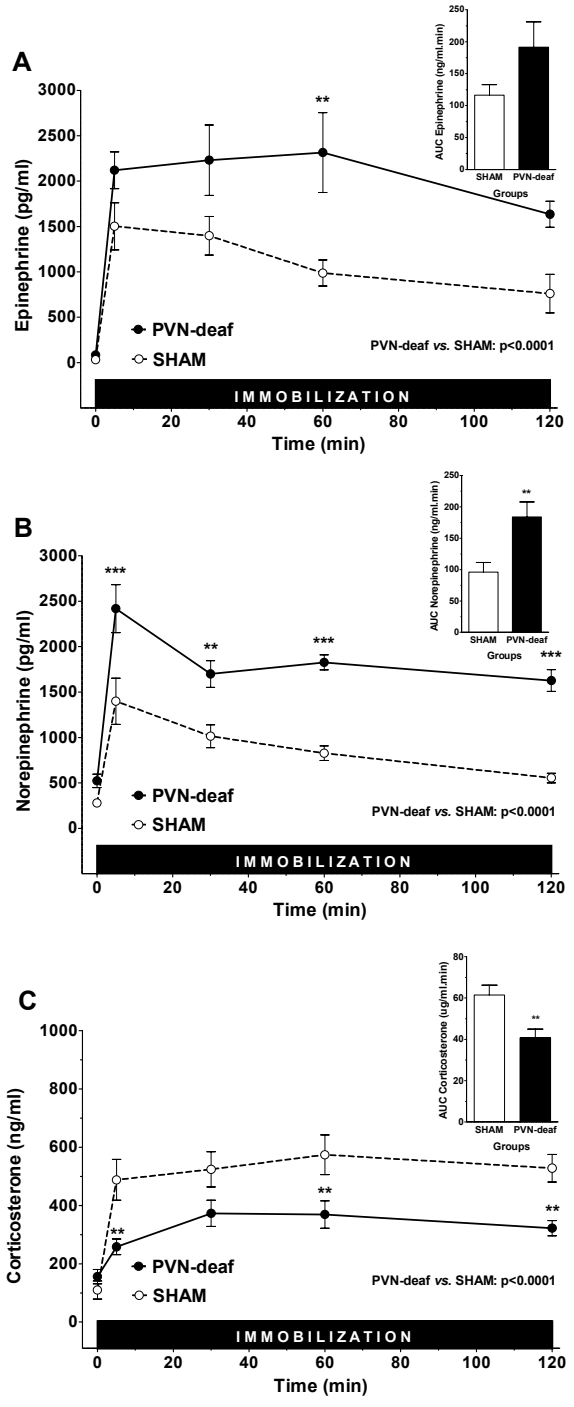

Fig. 4. Plasma epinephrine (A), norepinephrine (B), and corticosterone (C) concentrations in the PVN-deafferentated (n = 12), and sham-operated (n = 10) rats before (0 min), and at 5, 30, 60, and 120 min after the beginning of the 120 min long immobilization stress. Areas under the curve (AUC) were measured from 0 to 120 min of immobilization for the plasma catecholamines and corticosterone concentrations. Each value represents the mean ± SEM. Statistical significance compared to SHAM group: $^{**}p < 0.01$; $^{***}p < 0.001$.

Published data have shown the stimulatory effect of the PVN on the SAS activity in response to stress (Kvetnansky et al. 1995; Pacak et al. 1995). Our data indicate a partial inhibitory effect of the PVN neurons on the SAS activity in IMO rats. There are two mechanisms that could explain this inhibition. First, the posterolateral PVN-deaf may lead to the interruption of those afferent pathways that exert stimulatory effect on sympathetic premotor neurons of the PVN as well as pathways originating in the PVN and terminating in the sympathetic preganglionic neurons. Second, the PVN-deaf may lead to a transection of pathways innervating the NTS neurons that exert an inhibitory influence on the activity of the SAS (Geerling et al. 2010). It is possible that the posterolateral PVN-deaf may lead to the attenuation of the stimulatory as well as inhibitory effect of the PVN neurons on SAS activity. We hypothesize that the inhibitory pathways are dominant over the stimulatory pathways. As such, their interruption leads to a disinhibition of the SAS activity in the IMO rats. However, we cannot exclude the possibility that the observed stimulatory effect of the PVN-deaf on the SAS activity may result from an interruption of other inputs that are not related directly to the activation of the PVN neurons. Another possible explanation of our results might be the fact that the PVN-deaf could additionally reduce multiple inputs from brainstem to forebrain structures that exert an inhibitory effect on the stress-induced activation of the SAS (e.g. bed nucleus of the stria terminalis) (Herman et al. 2003; Choi et al. 2007). Decreased activation of those structures by the deafferentation may lead to a disinhibition of the SAS activity as well.

It has been previously shown that stress-induced activity of the HPA axis, resulting in the increased secretion of glucocorticoids from adrenal cortex, is mediated via the PVN (Kenney et al. 2003; Ferguson et al. 2008). Stressors that activate brainstem structures have stimulatory effect on the HPA axis activity via catecholaminergic inputs from the NTS (e.g. neurons in the A2/C2 region of the NTS) and A1/C1 neurons to the PVN (Cunningham and Sawchenko 1988; Cunningham et al. 1990; Pacak and Palkovits 2001). These catecholaminergic inputs represent a major excitatory drive that increases the release of CRH and ACTH as well as the CRH gene expression (Plotsky 1987; Plotsky et al. 1989; Smith and Vale 2006). The stress-induced activation of the HPA axis is stressors-specific. It has been shown that the stressors act on the HPA axis activity either directly, via the catecholaminergic inputs from the NTS to the PVN (e.g. physical stressors activate CRH in the PVN) or indirectly, through a multisynaptic pathway including the limbic

structures (e.g. psychological stressors stimulate the amygdaloid CRH system) (Pacak et al. 1992; Feldman et al. 1995; Makino et al. 2002; Forray and Gysling 2004). An inhibitory effect of the posterolateral PVN- deaf on the CORT release in rats exposed to IMO stress, found in this study, is in accordance with the published data indicating the attenuated HPA axis response by a lesion of the afferent catecholaminergic inputs to the PVN (Herman and Cullinan 1997; Cecchi et al. 2002; Ulrich-Lai and Herman 2009). Therefore, we suggest that the inhibitory effect of the PVN-deaf observed in our study is a consequence of an interruption of the afferent inputs from the NTS to the PVN parvocellular neurons synthesizing CRH in response to acute IMO stress.

Basal PVN activity is modulated by integration of excitatory and inhibitory inputs from local hypothalamic areas, forebrain, and limbic structures (Han et al. 2002; Kc and Dick 2010; Gunn et al. 2011). The study of Khasar et al. (2003) has showed that the vagal afferent input to the PVN from the NTS inhibits the activity of the adrenal medulla, i.e. the release of EPI. Furthermore, the PVN sympathoexcitatory neurons that increase the sympathetic outflow are tonically inhibited by GABA-ergic mechanisms in the PVN (Li et al. 2006). In accordance with these findings we also observed that the posterolateral PVN-deaf induced a significant increase of the catecholamine plasma concentrations in unstressed rats. The basal plasma CORT levels were slightly increased after the posterolateral PVN-deaf. Therefore, it is possible that the basal SAS and HPA axis activity is under the inhibitory influence of signals transmitted to the PVN from brainstem structures.

Limitations of the study

The major limitation of this study is the method of PVN-deaf. Transection of pathways by Halasz knife severed several pathways interconnecting brainstem with forebrain structures. Even if pathways directly interconnecting the PVN with brainstem structures were transected, other pathways were affected as well. Therefore transection of these pathways may potentially affect neuroendocrine stress response in deafferentated animals. Moreover, mechanical transection can induce non-specific effects including inflammation, vascular, and tissue damages, which may affect the stress response, too.

Conclusion

In conclusion, our data indicate that the PVN not only exerts a stimulatory effect on the SAS activity, but this hypothalamic nucleus may also participate in the inhibitory regulation of the SAS during restful conditions including exposure of organism to acute stress. Disruption of the PVN may be accompanied by an alteration in the homeostatic regulation of the activity of the HPA axis and the SAS under various pathological situations including heart failure and affective disorders. However, to better understand the inhibitory role of the PVN neurons in the SAS stress response, further studies are required.

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