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The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses

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Abstract

The lateral septum (LS) has been shown to play a key role in emotional processes and stress responses. However, the exact role of the LS upon stress modulation is not clear, since previous lesion studies mostly used electrolytic lesions, thereby destroying the whole septal area including medial components and/or fibres of passage. The aim of the present study was therefore, to investigate the effects of selective excitotoxic ablation of the LS on neuroendocrine and behavioral stress responses in rats. Bilateral ibotenic-acid lesions of the LS increased hypothalamo-pituitary-adrenocortical (HPA) axis responses to forced swim stress indicated by enhanced plasma ACTH and corticosterone responses and higher stress-induced c-Fos-like immunoreactivity in the paraventricular hypothalamic nucleus. Moreover, LS-lesioned animals displayed a more passive coping-style in the forced-swim-test indicated by increased floating and reduced struggling/swimming-behavior compared to sham-lesioned controls. Interestingly, intraseptal corticosteroid-receptor blockade modulated behavioral stress-coping but failed to change HPA-axis stress responses. Further experiments aimed at elucidating underlying neurochemical mechanisms revealed that intraseptal administration of the selective 5-HT_{1A} receptor antagonist WAY-100635 increased and prolonged stress-induced ACTH and corticosterone levels mimicking lesion effects, while the agonist 8-OH-DPAT suppressed HPA-axis activity facilitating the inhibitory role of the LS. Additionally, 8-OH-DPAT injected animals showed increased active and decreased passive coping strategies during forced swimming suggesting antidepressant efficacy. Taken together, our data suggest that the LS promotes active stress coping behavior and is involved in a HPA-inhibitory mechanism that is at least in part mediated by septal 5-HT_{1A} receptors and does not involve a glucocorticoid mediated feedback mechanism.

Key words: forced swimming, HPA axis, ACTH, corticosterone, glucocorticoids, serotonin

Introduction

Considerable evidence suggests that a significant element in the etiology of numerous psychiatric disorders including depression and posttraumatic stress disorder is a dysregulated stress hormone function (De Kloet et al., 2005; Krishnan and Nestler, 2008). The release of stress hormones such as glucocorticoids is regulated mainly by the hypothalamo-pituitary-adrenocortical (HPA) axis and it is well documented that glucocorticoids play an important role in the adaptation of the organism to stress, including the coordination of appropriate behavioral response to stressors (McEwen, 2007). The importance of maintaining glucocorticoid secretion within tolerable limits requires efficient mechanisms for inhibiting neuroendocrine stress responses. To avoid excessive stress reactions glucocorticoid levels are regulated by a negative feedback mechanism, which mainly acts at the level of hypothalamus and pituitary (Keller-Wood and Dallman, 1984; De Kloet et al., 1998). In parallel with steroid feedback there is also evidence for the existence of further glucocorticoid-independent inhibitory pathways that might emanate from neuronal sources including GABAergic neurons from multiple brain areas including neurons scattered in the immediate surroundings of the hypothalamic paraventricular nucleus (PVN) (Herman et al., 2003, 2004, 2005; Cullinan et al., 2008). Additional studies have now provided evidence that the lateral septum (LS) is involved in HPA axis activity modulation (Herman et al., 1996, 2005). However, the exact role of the LS in HPA axis regulation is presently obscure, because of inconsistent findings from prior studies. For example, basal plasma corticosterone levels following septal lesions are elevated in some studies (Usher et al., 1974; Dobrakovova et al., 1982) but not in others (Seggie and Brown, 1971; Seggie et al., 1974; Uhlir et al., 1974; Dobrakovova et al., 1982; Seggie, 1987). Moreover, the impact of LS-neurons on the expression of

anxiety-related behaviors are similar controversial as both reduced (Pesold and Treit, 1992; Menard and Treit, 1996) as well as increased anxiety levels (Albert and Chew, 1980; Yadin et al., 1993) were found after septal lesions. These discrepant findings might be related to the fact that most of the above mentioned lesion studies used large electrolytic lesions, thereby damaging both lateral and medial divisions of the septal area as well as extensive portions of the fornix system and thus fibres passing through this region. So far, only one study investigated the effects of selective LS-lesions on the HPA-axis activity by using the excitotoxic lesion agent ibotenic acid, that spares fibres of passage (Herman et al., 1996). However, since this study was performed under basal conditions only, where no modulatory function of the LS was found, the specific role of LS neurons under stress conditions is still unknown.

The aim of the present study was to investigate the effects of axon-sparing excitotoxic lesions of LS neurons on neuroendocrine and behavioral stress responses. Moreover, we examined whether the LS is involved in the glucocorticoid-mediated negative feedback mechanism, as a high density of glucocorticoid receptors was found in the LS of rodents and primates (Morimoto et al., 1996; Patel et al., 2000). Finally, we aimed to study the underlying neurochemical mechanisms of how LS neurons modulate neuroendocrine and behavioral stress responses.

Materials and Methods

Animals

All experiments were carried out on adult male Sprague-Dawley rats (250 – 320g). Before use, the animals were housed in groups of four to six under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 07:00, $21 \pm 1^\circ\text{C}$, 60% humidity, pelleted food and water *ad libitum*) for at least 1 week after delivery from the supplier. All experiments were approved by the local Ethical Committee on Animal Care and Use of the Austrian governmental body.

Surgery

All surgeries were conducted under sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.) anaesthesia. Moreover, before incision the local anaesthetic xylocaine (AstraZeneca, Vienna, Austria) were injected on the head surface of animals. Postoperatively, rats received a one-time injection of buprenorphine (0.03 mg/kg i.m.).

Excitotoxic lesions: Excitotoxic lesions were produced bilaterally by microinjections of ibotenic acid (Sigma-Aldrich, Steinheim, Germany) into the LS with a Hamilton microsyringe stereotaxically inserted according to a brain atlas (Paxinos and Watson, 1998). The implantation coordinates were: 0.6 mm rostral to bregma, 0.8 mm lateral to the midline. To ensure ablation that implies most of the LS, lesions were placed 7.0 and 5.5 mm ventral to skull surface. Each injecting point was infused with 0.5 μl of ibotenic acid (5 $\mu\text{g}/\mu\text{l}$) during a time period of 5 min. After injections the injection cannula was left in position for an additional 5 min to facilitate drug diffusion and minimize dragging of the injected liquid along the injection tract. Sham-lesioned animals received the same surgical treatment as a burr hole was drilled in the skull

and the dura was removed in the location dorsal to LS lesion placements, except that no ibotenic acid was applied.

Implantation of microinjection cannulae: For microinjection experiments, 25 gauge stainless steel cannulae (15 mm length) used as guide cannulae were bilaterally implanted with their tip 2 mm above the LS (implantation coordinates: 0.6 mm rostral to bregma, 1.6 mm lateral to the midline, 3.7 mm below the surface of the skull with an angle of 10° to avoid sagittal sinus damage) and fixed to the skull with two jeweller's screws and dental cement.

Implantation of a microdialysis probe: A homemade, U-shaped microdialysis probe was stereotaxically implanted two days prior to experiments as previously described (Ebner et al., 2004) with its tip reaching the right LS (implantation coordinates: 0.6 mm rostral to bregma, 1.6 mm lateral to the midline, 7.0 mm below the surface of the skull with an angle of 10° to avoid sagittal sinus damage). The probe consisting of an 18 kDa dialysis membrane (Hemophan, Membrana, Wuppertal, Germany) with an active surface length of 2 mm was fixed to the skull with two jeweller's screws and dental cement. The two endings of the probe were attached to 5-cm-long pieces of polyethylene tubing for connection with the infusion pump.

Implantation of a jugular venous catheter: A silastic-tipped vinyl catheter was inserted into the left jugular vein, routed under the skin and exteriorized at the neck of the animal as described previously (Ebner et al., 2005). The wounds were closed using metal clips. The catheter was filled with sterile saline containing gentamycin (30.000 IU/rat, Centravet, Bad Bentheim, Germany) and flushed with the same solution 2 days after surgery. On the day of the experiment the catheters were connected to 1-ml plastic syringes via approximately 40 cm long pieces of PE-50 tubing 2h before starting the experiment. Blood sampling through a pre-implanted jugular vein catheter

allows repeated blood sampling from conscious, freely moving rats without restraining animals.

Experimental protocol

After surgery, rats were housed individually in transparent plexiglas cages until testing. Operated animals were allowed to recover from surgery for 2-3 days during they were handled for 3 min twice daily to familiarize them with the experimental procedure and to minimize nonspecific stress responses during the experiments. At least 16 hours before experiments, animals were placed in the experimental room and allowed to habituate. All experiments were performed between 07:00 and 16:00. Exposure to the stressor was always completed between 11:00 and 14:00 to minimize circadian rhythm-related variations in stress responses.

Effects of LS-lesions on stress responses

The experiment started with the collection of a first blood sample (0.3 ml) under basal conditions, 30 min prior to stressor. After sampling of the basal blood sample, lesioned and sham-lesioned animals were exposed to forced swimming according to the procedure described previously (Ebner et al., 2008). Briefly, the rats were forced to swim for 5 min in a square plastic tank (40 cm in diameter) filled to a depth of 30 cm with tap water ($20 \pm 1^\circ\text{C}$). During the forced swimming session the behavior was recorded by a video system and scored by a trained observer blind to the animals' treatment quantifying absolute time measurements. The behavior of the animals was assigned to one of the three following behavioral categories: (1) struggling, defined as movements during which the forelimbs broke the water's surface; (2) swimming, defined as movement of the animal induced by movements of the fore and hind limbs

without breaking the water surface; and (3) floating defined as the behavior during which the animal used limb movement just to keep its equilibrium without any movement of the trunk (Ebner et al., 2008). After the 5-min swimming session, animals were gently dried using a towel and returned to their home cage. To determine the time course of plasma ACTH and corticosterone release in response to forced swim stress, additional blood samples were collected 10, 30, and 60 min after onset of the stressor. Sampled blood volumes were immediately replaced with an equal volume of heparinized saline.

Effects of intraseptal administration of selective corticosteroid receptor antagonists on stress responses

In a separate group of animals, the stylets of guide cannulae were replaced by microinjection cannulae (31 gauge), which were 2 mm longer than the guide cannulae, thus reaching the LS. Injection cannulae were connected to a syringe mounted on a microinfusion pump (TSE-Systems, Bad Homburg, Germany) and filled either with artificial cerebrospinal fluid (aCSF; pH 7.2; 140 mM NaCl, 3.0 mM KCl, 1.25 mM CaCl₂, 1.0 mM MgCl₂, 1.2 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, and 3.0 mM glucose) or aCSF containing the corticosteroid receptor antagonists mifepristone or RU-28318 (30 and 150 µM; Tocris Bioscience, Bristol, UK). The dose range of RU-28318 and mifepristone used in our experiments was based on previous studies demonstrating a modulatory effect on HPA axis activity as well as emotional behavior in rats after intracerebroventricular, intrahippocampal or intraseptal injections (Ratka et al., 1989; Korte et al., 1995; Van Haarst et al, 1997; Calvo and Volosin, 2001; Gesing et al., 2001; Calfa et al, 2006). After a habituation period of 60 min, experiment started with the collection of two blood samples (0.3 ml) under basal

conditions, 30 and 15 min before stress exposure. Bilateral infusions of the corticosteroid receptor antagonists into the LS started 12 min before onset of the stressor. The injection time of 7.5 min with a defined flow rate of 0.2 $\mu\text{l}/\text{min}$ achieved a total volume of 1.5 μl (=20 and 100 ng) for each injection side followed by 2.5 min where the injectors were kept in place to allow absorption of the injection bolus into the tissue. After injections another blood sample was taken and one minute later animals were exposed to the forced swimming procedure for 5 min as described above. During the swim session behavioral output was scored and analyzed as described above. Additional blood samples were collected 10, 30, and 60 min after onset of the stressor and again equal volume of heparinized saline was reinfused.

Effects of intraseptal administration of 5-HT_{1A} receptor ligands on stress responses

In a further series of experiments, the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (10 $\mu\text{g}/\text{side}$; Sigma-Aldrich, Steinheim, Germany) was bilaterally microinjected into the LS as described above. In order to intraseptally administer the selective 5-HT_{1A} receptor antagonist WAY-100635 (Sigma-Aldrich) over an extended period of time with no further perturbation to the surrounding tissue (Bourne, 2003) we decided to infuse the compound via retrodialysis into the LS as described previously (Ebner et al., 2008). Briefly, at the day of experiment the microdialysis probe was connected to a syringe mounted onto a superfusion pump and dialysed with aCSF. After an equilibration period of 120 min, experiment started with the collection of two blood samples (0.3 ml) under basal conditions, 120 and 60 min before stress exposure, followed by infusion of WAY-100635 (10 μM) in aCSF or aCSF alone (controls) with a flow-rate of 3.3 $\mu\text{l}/\text{min}$. Thirty minutes after the start of retrodialysis another blood sample was collected. The drug reached the LS after approximately 5–6 min and

forced swimming was applied 30 min later as described above. During the swim session behavioral output was scored and analyzed as described above and afterwards three additional blood samples were collected 10, 30 and 60 min (sample 4-6) after onset of the stressor.

ACTH and corticosterone radioimmunoassay

Plasma ACTH and corticosterone concentrations were measured by radioimmunoassay using commercially available kits (MP Biomedicals, Orangeburg, NY, USA) with an intra and inter-assay variability of less than 10% and a lower limit of detection of 6 pg/ml for ACTH and 8 ng/ml for corticosterone.

Immunocytochemistry

Two hours after stress exposure, lesioned and sham-lesioned animals were deeply anaesthetized with an overdose of sodium pentobarbital (200 mg/kg) and transcardially perfused with 100 ml of 0.9% saline followed by 100 ml of 4% paraformaldehyde in 0.1 mol/l phosphate buffered solution (PBS, pH 7.4). The 2 h time point represents the interval at which maximal c-Fos protein expression has been reported in acute stress paradigms (Viau and Sawchenko, 2002). Brains were then removed, postfixed for 2 h, and cryoprotected at 4°C overnight in 10% sucrose (in 0.1 M phosphate buffered saline, pH 7.4). Coronal sections (40 µm) were cut through the PVN using a Cryostat (Leica CM 1850, Leica-Microsystems, Nussloch, Germany) and collected in PBS. The sections were processed for c-Fos-like immunoreactivity as described previously (Smith and Day, 1993; Buller et al., 1999). Briefly, brain sections were incubated for 48 h in rabbit anti-Fos (1:50.000, Santa Cruz, CA, USA), followed by a 2h incubation in a biotinylated donkey anti-rabbit

(1:300, Jackson ImmunoResearch, West Grove, PA, USA). Sections were then incubated for a further 2 h in a solution of avidin–biotin–horseradish peroxidase complex (ABC Vector Elite Kit, Burlingame, CA, USA) before being exposed to a nickel 3,3-diaminobenzidine solution to allow visualization of the horseradish peroxidase activity. Cells containing a nuclear brown-black reaction product were considered as c-Fos positive cells. The reaction was terminated once an optimal contrast between specific cellular and nonspecific background labelling was reached. To minimize variations in immunolabelling, sections from each experimental group were processed simultaneously. Sections were then mounted on chrome-alum subbed slides, dehydrated in alcohol, cleared in xylene and cover-slipped.

Histology

At the end of the experiments animals were killed by an overdose of pentobarbital and their brains were removed. For histological verification of either the extension of LS lesions - on the basis of gliosis and neuronal cell loss (Fig. 1) - or placement of microinjection cannulae and microdialysis probes (Fig. 2), brains were sectioned using a Cryostat and 20-40 µm coronal sections were stained with cresyl violet. Lesion verification and judgment of successful implantation of cannulae and probes into the LS was made before analyzing neuroendocrine and behavioral experiments.

Statistics

Experimental subjects were included in the statistical analysis only if the lesions, microinjection or microdialysis cannulae were confirmed to be localized within the LS. Statistical analysis was performed using a computer software package (GB-Stat 6.0, Dynamic Microsystems, Silver Springs, USA). Plasma concentrations of ACTH and

corticosterone were analyzed using a two-way ANOVA (treatment/lesion x time) with repeated measures on the last factor followed by appropriate post-hoc analysis. Behavioral data and counts of c-Fos-positive nuclei within the PVN after stress exposure were analyzed using Student's two-tailed t-test comparing lesioned or drug treated animals with respective controls. Anatomical boundaries defining the subareas of interest within the PVN were evaluated with reference to morphological and cytoarchitectonic organization of the PVN (Swanson and Sawchenko, 1980). Data are presented as means \pm SEM. Statistical significance was accepted if $p < 0.05$.

Results

Lesion placements

Lesions were evaluated with reference to morphological and cytoarchitectonic organization of the LS (Alonso and Frotscher, 1989; Risold, 2004). Three subregions were of primary interest: the dorsal, the intermediate and the ventral part (Fig. 1A, left panel). Lesion placement and extent was verified by light microscopic examination of cresyl violet-stained sections showing substantial cell degeneration and cell loss resulting from ibotenic acid injections in lesioned compared to sham-lesioned animals (Fig. 1A, right panel). Analysis of LS-lesions indicated significant damage in all subregions as well as over the entire rostro-caudal extension of the LS (Fig. 1B).

Lesion effects on neuroendocrine stress response

Exposure to forced swimming caused an increase in plasma ACTH and corticosterone levels in both LS-lesioned animals and sham-lesioned controls (Fig. 3A). Statistical analysis of ACTH levels by two-way ANOVA revealed a significant effect of the main factors (lesion: $F_{1,22}=8.56$, $p=0.0078$; time: $F_{3,66}=82.87$, $p<0.0001$) as well as a significant interaction between main factors ($F_{3,66}=3.24$, $p=0.0275$). Post hoc analysis indicated significant differences of ACTH levels between lesioned animals and controls immediately after stress exposure with a higher ACTH response to forced swimming at 10 ($p<0.01$) and 30 min ($p<0.05$) after onset of the stressor in lesioned animals compared to sham-lesioned controls. In contrast, basal levels of ACTH did not vary as a function of lesion status (Fig. 3A, -30 min).

Plasma corticosterone levels showed similar main effects (lesion: $F_{1,22}=4.97$, $p=0.03$; time: $F_{3,66}=69.89$, $p<0.0001$) and interaction of main factors ($F_{3,66}=2.94$, $p=0.03$). Lesion status differentially affected the magnitude and longevity of the response, with

lesioned animals displaying higher and prolonged plasma corticosterone levels after forced swim stress than sham-lesioned controls. Basal levels of corticosterone were not different between groups.

Lesion effects on stress-induced PVN activation

Next we examined whether ablation of the LS is associated with an increased neuronal activity in the PVN, the apex of HPA axis regulation. Therefore, we compared the stress-induced expression of the immediate early gene product c-Fos in the PVN of LS-lesioned animals and controls. As shown in Fig. 3B, sham-lesioned animals subjected to forced swim stress displayed moderate numbers of c-Fos-positive cells in the PVN. Moreover, stress-induced c-Fos expression in the PVN especially in the medial parvocellular part of the PVN (mpPVN) was considerably enhanced (by 140%) in LS-lesioned animals compared to sham-lesioned controls ($p < 0.001$).

Lesion effects on behavioral stress response

To determine the role of LS neurons on stress-related behavioral regulation we monitored stress coping behavior during forced swimming in lesioned animals and controls. In this experiment we found that LS-lesions significantly affected the animals' behavior during forced swimming. As illustrated in Fig. 4, LS-lesioned animals compared to sham-lesioned controls showed increased floating behavior ($p < 0.001$) and a decreased time spent with active coping such as struggling and swimming ($p < 0.001$).

Effects of intraseptal administration of selective corticosteroid receptor antagonists on stress responses

In a further series of experiments, we aimed to clarify whether HPA inhibitory effects of the LS are mediated by septal glucocorticoid and/or mineralocorticoid receptors. Bilateral infusion of the selective glucocorticoid receptor antagonist mifepristone into the LS at different concentrations (20 and 100 ng) had no effect on HPA axis activity since neither basal nor stress-induced ACTH (Fig. 5A) or corticosterone levels (Fig. 5B) differed between mifepristone and aCSF injected control animals. Moreover, intraseptal administration of the selective mineralocorticoid receptor antagonist RU-28318 had no effect on HPA axis activity. There was no difference either in basal ACTH (basal controls: 14.6 ± 1.6 pg/ml) or corticosterone levels (basal controls: 60.3 ± 15.2 ng/ml) between vehicle injected controls and RU-28318 treated rats. Also, the stress-induced ACTH and corticosterone levels did not differ between controls (peak levels ACTH: 337.4 ± 86.3 pg/ml; CORT: 759.5 ± 64.2 ng/ml) and antagonist treated animals (20 ng RU-28318: ACTH: 320.3 ± 68.1 pg/ml; CORT: 726.1 ± 23.1 ng/ml; 100 ng RU-28318: ACTH: 304.9 ± 60.8 pg/ml; CORT: 767.7 ± 31.7 ng/ml). However, septal glucocorticoid receptor blockade affected the animals' stress coping behavior during forced swimming. As illustrated in Fig. 5C, mifepristone injected animals showed increased struggling and swimming behavior ($p < 0.01$) and decreased time spent floating ($p < 0.01$) compared to aCSF injected controls. In contrast, mineralocorticoid receptor antagonism did not influence stress coping behavior as floating (20ng: 145.8 ± 10.1 ; 100 ng: 144.5 ± 17.4) or struggling/swimming behavior of RU-28318 treated rats (20ng: 153.2 ± 10.2 ; 100 ng: 154.1 ± 18.0) did not differ to that of vehicle treated controls (floating: 137.6 ± 8.6 ; struggling/swimming: 161.8 ± 8.5).

Effects of intraseptal administration of 5-HT_{1A} receptor ligands on stress responses

As shown in Fig 6, our data indicate significant differences in the plasma ACTH and corticosterone response to forced swimming between 5-HT_{1A} receptor agonist treated animals and controls. Statistical analysis of ACTH levels by two-way ANOVA showed a significant effect of the factor time ($F_{5,85}=89.57$, $p<0.0001$) and a significant interaction between main factors ($F_{5,85}=3.80$, $p=0.0037$). Post hoc analysis revealed that the administration of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT locally into the LS resulted in a significant attenuation of swim stress-induced ACTH levels. The attenuation of the plasma ACTH response to forced swimming after intraseptal 8-OH-DPAT administration was evident only at the peak level at 10 min after stressor onset, while at 30 min where ACTH levels were still significantly elevated this difference was not observed. As shown in Fig. 6B, all experimental groups exhibited increased plasma corticosterone levels in response to the 5 min forced swim stress exposure with peak levels at 30 min after stressor onset. Similarly to ACTH, 8-OH-DPAT significantly reduced the stress-induced rise of plasma corticosterone levels. Statistical analysis by two-way ANOVA revealed significant effects of the main factors (treatment: $F_{1,17}=6.88$, $p=0.0178$; time: ($F_{5,85}=71.42$, $p<0.0001$) and a significant interaction between main factors ($F_{5,85}=3.05$, $p=0.0137$). Notably, attenuated stress-induced corticosterone levels after intraseptal 8-OH-DPAT administration were observed only in the post-stress period with peak corticosterone levels at 30 and 60 min after stressor onset. In contrast, basal ACTH and corticosterone levels were not different between groups. Furthermore, intraseptal administration of 8-OH-DPAT significantly affected the animals' behavior during forced swimming, indicated by increased active coping such as struggling and

swimming behavior ($p < 0.01$) and decreased time spent floating ($p < 0.01$) in agonist treated animals compared to controls (Fig. 6C).

Fig. 7 illustrates the effects of intraseptal 5-HT_{1A} receptor blockade on basal and stress-induced ACTH and corticosterone levels. Infusion of the selective 5-HT_{1A} receptor antagonist WAY-100635 into the LS changed ACTH responses to forced swimming. Statistical analysis by two-way ANOVA showed a significant effect of the main factors (treatment: $F_{1,10} = 11.46$, $p = 0.00$; time: $F_{5,50} = 75.02$, $p < 0.0001$) as well as a significant interaction between main factors ($F_{5,50} = 7.38$, $p < 0.0001$). Post hoc analysis revealed that intraseptal administration of WAY-100635 resulted in higher and prolonged swim stress-induced ACTH levels. Notably, this effect was long-lasting and observable even at 60 min after stress exposure because at this time point antagonist treated animals but not controls showed elevated ACTH levels compared to basal. Similarly to ACTH, intraseptal WAY-100635 administration resulted in higher stress-induced corticosterone levels (notably at 30 and 60 min after stressor onset) than in controls with significant main factors (treatment: $F_{1,10} = 38.61$, $p < 0.0001$; time: $F_{5,50} = 276.86$, $p < 0.0001$) as well as a significant interaction between main factors ($F_{5,50} = 4.41$, $p = 0.002$). In contrast, basal ACTH and corticosterone levels were not different between groups. Moreover, intraseptally administered WAY-100635 did not affect the animals' stress coping behavior in the forced swim test as floating (113.8 ± 15.9) or struggling/swimming behavior (153.2 ± 10.2) of WAY-100635 treated rats did not differ to that of vehicle treated controls (floating: 95.4 ± 9.8 ; struggling/swimming: 161.8 ± 8.5).

Discussion

The results of the present study demonstrate an important role of the LS in the modulation of behavioral and neuroendocrine stress responses. Ablation of the LS was associated with disinhibited HPA axis stress responses indicated by enhanced plasma ACTH and corticosterone levels and an exaggerated c-Fos expression in the mpPVN. In contrast, lesions did not affect basal plasma hormone levels. The enhanced neuroendocrine stress response in LS-lesioned animals was accompanied by alterations in the stress-coping behavior during forced swimming, indicated by increased floating and reduced struggling/swimming behavior. Additionally, we found that this HPA inhibitory mechanism of the LS is not mediated via septal corticosteroid receptors as intraseptal glucocorticoid and mineralocorticoid receptor blockade failed to change HPA axis activity. However, we identified septal 5-HT_{1A} receptors to play an important role in this mechanism as intraseptal 5-HT_{1A} receptor activation suppressed while blockade of these receptors increased and prolonged stress-induced ACTH responses. Moreover, septal 5-HT_{1A} receptors also seem to be implicated in the modulation of behavioral stress coping as intraseptal administration of a 5-HT_{1A} receptor agonist increased active and decreased passive stress-coping strategies.

Role of LS neurons on neuroendocrine stress response

In the present work we investigated the effects of selective lesions of the LS on plasma ACTH and corticosterone levels under basal and stress conditions. Notably, we could not observe any differences in basal ACTH or corticosterone levels between LS-lesioned animals and controls. Thus, our data are in contrast to previous findings showing higher corticosterone levels after ablation of the septum suggesting

a tonic inhibitory effect of septal neurons on HPA axis activity (Uhlir et al., 1974; Bouille and Bayle, 1975; Dobrakovova et al., 1982). Methodological differences might be a possible explanation of this discrepancy. Since these early lesion studies used large electrolytic lesions, thereby destroying the whole septum and extensive portion of the fornix system (see introduction) such kind of destruction can likely lead to a damage of putative inhibitory input to PVN neurons descending through the septal region. Thus, the results of these early studies are difficult to interpret with regard to the specific role of distinct subregions of the septum in HPA axis regulation. In line with our results are data from J.P. Hermans group showing no changes in basal corticosterone levels in rats with selective lesioning of the LS by using the neurotoxic agent ibotenic acid (Herman et al., 1996).

However, a novel finding in the present study was that ACTH and corticosterone responses to swim stress increased after selective lesioning of LS neurons. In support of this, we observed increased neuronal activation (indicated by an enhancement of c-Fos-positive cells) in the mpPVN, the key region of HPA axis regulation that contains the main neuropeptidergic adrenocorticotropin secretagogues such as CRF and arginine vasopressin (Antoni, 1986; Whitnall, 1993). Thus, our data suggest an inhibitory role of LS neurons on stress-induced HPA axis activity and extend previous findings demonstrating higher corticosterone levels after electrolytic lesioning of the whole septum in rats (Brown et al., 1974; Usher et al., 1974; Dobrakovova et al., 1982; Seggie, 1987). In contrast to previous studies where only corticosterone levels were measured our study provides information on HPA axis activity at different levels from hypothalamus, pituitary to adrenal level. Moreover, blood sampling through a jugular catheter as was done in our study offers the advantage of collecting sequential samples to monitor temporal changes in

plasma stress hormones during and after stress exposure. Thus, in contrast to previous studies where blood samples were collected by decapitation our study provides some intra-individual time course information.

Next, we examined whether this HPA inhibitory mechanism of the LS is mediated by corticosteroid receptors which are highly expressed in the LS (see below). Previous studies have shown that stress-induced HPA axis activity is regulated by a glucocorticoid-driven feedback inhibition that is mediated at least in part by limbic forebrain structures (Herman et al., 2005). Since both glucocorticoid and mineralocorticoid receptors are expressed in the LS (Arriza et al., 1988; Ahima and Harlan, 1990; Ahima et al., 1991; Cintra et al., 1994) this area might be a potential target for mediating glucocorticoid effects. However, neither injecting the selective glucocorticoid receptor antagonist mifepristone nor the selective mineralocorticoid receptor antagonist RU-28318 into the LS changed ACTH responses to forced swimming suggesting that this LS HPA-inhibitory mechanism is independent of a glucocorticoid mediated negative feedback mechanism. Thus, our data provide further evidence that the HPA axis can also be regulated by glucocorticoid-independent inhibition from neuronal sources. This is in line with findings from adrenalectomized animals that still can inhibit ACTH stress responses although lacking glucocorticoid feedback signals (Jacobson et al., 1988).

Role of LS neurons on behavioral stress response

In addition to HPA axis regulation we found that the LS modulates stress coping behavior in the forced swim test, a widely used test to assess antidepressant properties of drugs. As LS-lesioned animals showed a more passive coping style

which is often interpreted as behavioral despair (Porsolt et al., 2001) our finding identifies the LS as a key brain area especially important in mediating active stress-coping strategies. Thus, our data strengthen the suggestion that the septum and especially the LS belongs to a “behavioral integration system” responsible for promoting the expression of relevant active behavioral responses in aversive and stressful situations (De Oca and Fanselow, 2004; Sheehan et al., 2004). Interestingly, previous studies have shown that LS-lesions in rats produced an anxiogenic-like phenotype (Yadin et al., 1993). Accordingly, this effect was defined as “septal rage syndrome” - representing a generalized disinhibition of fear (Brady and Nauta, 1953; Albert and Chew, 1980; Sheehan et al., 2004). Furthermore, septal damage has been found to be correlated with an increase in the expression of defensive behaviors, which is suggestive of an increase in fear (Sparks and LeDoux, 1995). However, different LS lesion studies suggest some anxiolytic-like effects in the elevated plus-maze and shock-probe burying test after LS ablation (Pesold and Treit, 1992; Menard and Treit, 1996). Thus, the exact role of the LS in modulation of anxiety and emotion-related behaviors is still not fully understood. Methodological differences such as extent of lesions and ways of application might be responsible for the disparity between findings. It might be speculated that even subregions of the LS mediate different and distinct behavioral functions and therefore it depends on the exact position and extent of lesions which effects are finally produced.

Interestingly, previous studies point to a critical role of the LS in mediating behavioral symptoms of depression. For instance, in the learned helplessness paradigm in rats, a widely used animal model of depression, it has been shown that helpless animals show lower stress-induced neuronal activation within the LS than controls (Steciuk et

al., 1999). However, stress resistant rats which received the same inescapable shock but did not become helpless showed no reduction in septal activity indicating that a normal functioning of the LS is required for effective coping responses to inescapable stress. Conversely, substantial evidence suggests that antidepressants ameliorate the behavioral manifestations of depression by restoring the neuronal activity in the LS (Contreras et al., 1989, 1990, 2001; Lino-de-Oliveira et al., 2001; Muigg et al., 2007). Therefore, we injected mifepristone, a selective glucocorticoid receptor antagonist with anxiolytic and antidepressant properties (De Kloet et al., 1988; Korte et al., 1995; DeBattista and Belanoff, 2006; Schatzberg and Lindley, 2008; Wulsin et al., 2010) locally into the LS and measured behavioral stress coping behavior in the forced swim test. Notably, we found that local administration of mifepristone into the LS increased active coping and reduced floating behavior indicating an antidepressant-like effect. This behavioral effect seems to be mediated by glucocorticoid receptors since intraseptal administration of a mineralocorticoid receptor antagonist had no effect. Thus, our data extend previous findings demonstrating that intraseptal injection of mifepristone normalizes the anxiogenic response in the elevated plus-maze test induced by either previous social defeat or systemic corticosterone administration (Calfa et al., 2006). However, mifepristone is not an ideal glucocorticoid-receptor antagonist, because it has also some affinity to progesterone receptors. Since progesterone receptors are also expressed in the lateral septum (MacLusky and McEwen, 1980; Sar and Parikh, 1986) and intraseptally administered progesterone has been shown to influence forced swim behavior (Estrada-Camarena et al., 2002), it is possible that these receptors are involved in the mediation of the behavioral effects of mifepristone. Further studies

with more specific ligands for the glucocorticoid receptor are required to exclude this possibility.

Involvement of septal 5-HT_{1A} receptors in stress responses

After identifying a stress-inhibitory role of the LS we wanted to gain insight into the neurochemistry involved this effect. Previous studies suggest that the septal 5-HT system plays an important role in mediating behavioral and physiological responses to emotionally significant events. Both a dense serotonergic innervation of the LS from the raphe nuclei (Köhler et al., 1982) and various 5-HT receptors primarily of the 5-HT_{1A} subtype are highly expressed in the LS (Kia et al., 1996; Lanfumey and Hamon, 2000). Therefore, we bidirectionally modulated septal 5-HT_{1A} receptors by administration of selective 5-HT_{1A} receptor agonist and antagonists into the LS and examined the role of this receptor on neuroendocrine and behavioral stress responses. We found a suppression of the stress-induced ACTH levels after 5-HT_{1A} receptor agonist administration and an increased and prolonged ACTH response after 5-HT_{1A} receptor blockade. Thus, our data suggest an inhibitory role of endogenous 5-HT within the LS on stress-induced HPA axis activation via intraseptal 5-HT_{1A} receptors. Notably, blockade of septal 5-HT_{1A} receptors by WAY-100635 mimics lesion effects while activation of these receptors facilitates the inhibitory role of the LS. The exact mechanism, however, mediating this inhibitory effect to the PVN remains unclear. It was shown previously that LS 5-HT_{1A} receptors were located primarily on calbindin-positive neurons indicative for septal GABAergic interneurons (Aznar et al., 2003; Lüttgen et al., 2005). Moreover, electrophysiological experiments have established that septal 5-HT_{1A} receptor activation causes neuronal hyperpolarisation (Joels et al., 1987; Van den Hooff and Galvan, 1992). Thus, it is

conceivable that a reduced 5-HT function in the LS either during psychological stress exposure (Kirby et al., 1995; Ebner et al., 2008) or after 5-HT_{1A} receptor blockade is associated with an enhanced intraseptal inhibitory function (presumably via GABAergic interneurons). Since a major part of LS output neurons are GABAergic (Gallagher et al., 1995; Risold and Swanson, 1997) and given that several LS projections to the PVN are GABAergic this GABA-to-GABA pathway would result in disinhibition of HPA axis stress responses. However, it is also conceivable that increased HPA axis stress responses are mediated not directly through GABAergic efferents to the PVN but indirectly through glutamatergic projections to inhibitory PVN projecting regions, including the peri-PVN region (Herman et al., 2004; Cullinan et al., 2008). Both scenarios would lead to enhanced stress hormone levels during forced swimming resulting from a decreased inhibitory action of the LS on the PVN. Further studies using tract tracing methods combined with immunohistochemical techniques (e.g. c-Fos, GABA) should clarify the exact pathways and mechanisms underlying this septal HPA axis regulatory mechanism.

Interestingly, the suppression of stress-induced ACTH levels after intraseptal 5-HT_{1A} receptor activation was accompanied by behavioral alterations during forced swimming, including reduction of immobility and an increase in active coping (e.g. struggling, swimming). Thus, our data confirm earlier studies also demonstrating antidepressant-like effects after intraseptal 8-OH-DPAT administration in the classical forced swim test (Schreiber and De Vry, 1993) and learned helplessness model (Martin et al., 1990). In the present study, we used the modified version of the forced swim test (Cryan et al., 2005) to assess the rodent's reactivity to the novel stress environment without any potential confounds of learning/memory processes

associated with the pretest session performed in the traditional test. These modifications make the test more sensitive for serotonergic antidepressant drugs such as SSRIs (Detke et al., 1995; Cryan et al., 2005). Indeed, preliminary data from our lab shows a reduction of floating behavior and an enhancement of struggling behavior in our modified forced swim test after intraseptal administration of citalopram (Ebner et al, unpublished data). Interestingly, we could not observe any behavioral changes during the forced swim session in animals that were intraseptally infused with the selective 5-HT_{1A} receptor antagonist WAY-100635. This might be surprising in respect to the observed effects of WAY-100635 in modulating the neuroendocrine stress response. However, although the reason for the behavioral ineffectiveness is currently unknown, it could be related to the low endogenous 5-HT tonus in the LS during forced swimming (Kirby et al., 1995; Ebner et al., 2008). Therefore, it is conceivable that under those conditions the endogenous 5-HT level is not sufficient for significant behavioral effects after 5-HT_{1A} receptor blockade. Indeed, the lack of a behavioral effect in the forced swim test after 5-HT_{1A} receptor blockade was also reported after systemic administration (De Vry, 1995; De Vry et al., 2004; Cryan et al., 2005).

Taken together, our results show that the LS plays an important role in promoting a HPA-axis inhibitory mechanism and active coping strategies during stress exposure. Furthermore, we found that this HPA inhibitory mechanism of the LS acts independent from glucocorticoid mediated negative feedback mechanisms. However, our data indicate a critical role of septal 5-HT_{1A} receptors in mediating stress-preventing actions of the LS as activation of septal 5-HT_{1A} receptors inhibits neuroendocrine and facilitates behavioral stress responses. Thus, our study supports

a model in which enhanced activity of LS neurons is required for effective stress coping responses which provides improved protection from the deleterious effects of exaggerated psychological stress exposure.

Disclosure/Conflict of Interest

The author(s) declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Figure legends

Fig. 1. Placement and extent of bilateral LS-lesions. **A)** Schematic drawing of a coronal section illustrating the dorsal, intermediate and ventral part of the LS (left panel) and photomicrographs showing cresyl-stained coronal sections of a lesioned and sham-lesioned control animal (right panel). LS-lesions reduced the number of cells in the LS. Neuronal cell degeneration and cell loss was found in all subregions of the LS. **B)** Schematic coronal sections of the rat brain adapted from the atlas of (Paxinos and Watson, 1998) illustrating rostro-caudal extension of lesions in the LS. The largest (bright grey) and smallest (dark grey) lesions are plotted at different levels through the LS. ac, anterior commissure; cc, corpus callosum; LSd, lateral septum dorsal; LSi, lateral septum intermediate; LSv, lateral septum ventral.

Fig. 2. Schematic drawing and representative microphotographs of cresyl-stained coronal sections of the rat brain showing the localization of bilaterally implanted microinjection cannulae (left) and the active membrane tip of a microdialysis probe (right) within the LS. ac, anterior commissure; cc, corpus callosum; LV, lateral ventricle; MS, medial septum.

Fig. 3. Effects of LS-lesions on HPA axis responses to forced swimming. **A)** Plasma ACTH (left) and corticosterone (CORT, right panel) responses to forced swimming (FS; 5-min, 20°C, grey shaded bars). Swim stress caused a significant increase in plasma ACTH and corticosterone concentrations in both LS-lesioned and sham-lesioned animals. Compared with sham-lesioned animals, LS-lesioned animals displayed higher plasma ACTH and corticosterone levels during and after forced swimming but not under basal conditions. **B)** Effects of LS-lesions on swim stress-

induced c-Fos expression in the mpPVN. Depicted are representative bright-field photomicrographs (left panel) and c-Fos quantification presented as bar graphs (right panel) illustrating higher c-Fos expression in the mpPVN in lesioned animals compared to sham-lesioned controls. 3V, third ventricle; mPVN, magnocellular PVN; vPVN, ventral PVN; mpPVN, medial parvocellular PVN. Data expressed as means +SEM. * $p < 0.05$, ** $p < 0.01$ vs. basal value; # $p < 0.05$, ## $p < 0.01$ vs. respective value in the sham-lesioned controls. ++ $p < 0.001$ vs. sham-lesioned controls (Newman-Keuls post hoc test or Student's t-test).

Fig. 4. Effects of LS-lesions on stress coping parameters during a 5-min forced swimming session as indicated by the time the animals spent struggling, swimming and floating. Lesioned animals displayed less active coping indicated by reduced time struggling and swimming, and enhanced immobility (floating behavior) than sham lesioned controls. Data are expressed as means + SEM. ** $p < 0.001$ vs sham-lesioned controls (Student's t-test).

Fig. 5. Effects of intraseptal administration of the glucocorticoid receptor antagonist mifepristone (20 or 100 ng/side; black arrow) on neuroendocrine and behavioral stress response. Administration of mifepristone had no effect on either basal or stress-induced increase of plasma ACTH (**A**) or corticosterone (CORT, **B**) levels because there was no significant difference between mifepristone treated animals and controls. **C**) Mifepristone injected animals displayed an increased active coping style indicated by an enhancement of struggling and swimming and a reduction of floating behavior during the forced swim exposure. Data are expressed as means +

SEM. ** $p < 0.01$ vs basal values; ++ $p < 0.001$ vs vehicle-treated controls (Newman-Keuls post hoc test or Student's t-test).

Fig. 6. Effects of intraseptal administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT (10 µg/side; black arrow) on neuroendocrine and behavioral stress response. Plasma ACTH (**A**) and corticosterone (CORT, **B**) responses to forced swimming were significantly attenuated by the 8-OH-DPAT treatment. However, basal levels did not differ between groups. **C**) Rats injected with 8-OH-DPAT displayed increased active coping indicated by an enhanced time the animals spent struggling and swimming, and reduced floating behavior during the forced swim exposure. Data are expressed as means + SEM. ** $p < 0.01$ vs basal; ## $p < 0.01$ vs respective value in the vehicle-treated controls; ++ $p < 0.001$ vs vehicle-treated controls (Newman-Keuls post hoc test or Student's t-test).

Fig. 7. Effects of intraseptal infusion of the 5-HT_{1A} receptor antagonist WAY-100635 (10µM) via retrodialysis (black bar) on basal and swim stress-induced ACTH (**A**) or corticosterone levels (CORT, **B**). While basal ACTH and corticosterone levels did not differ between groups, the forced swim stress-induced ACTH and corticosterone levels were significantly higher in WAY-100635 treated animals compared to controls. Data are expressed as means + SEM. * $p < 0.05$, ** $p < 0.01$ vs basal values; # $p < 0.05$, ## $p < 0.01$ vs vehicle-treated controls (Newman-Keuls post hoc test).













