Early Intervention With Intranasal NPY Prevents Single Prolonged Stress-Triggered Impairments in Hypothalamus and Ventral Hippocampus in Male Rats

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Intranasal administration of neuropeptide Y (NPY) is a promising treatment strategy to reduce traumatic stress-induced neuropsychiatric symptoms of posttraumatic stress disorder (PTSD). We evaluated the potential of intranasal NPY to prevent dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, a core neuroendocrine feature of PTSD. Rats were exposed to single prolonged stress (SPS), a PTSD animal model, and infused intranasally with vehicle or NPY immediately after SPS stressors. After 7 days undisturbed, hypothalamus and hippocampus, 2 structures regulating the HPA axis activity, were examined for changes in glucocorticoid receptor (GR) and CRH expression. Plasma ACTH and corticosterone, and hypothalamic CRH mRNA, were significantly higher in the vehicle but not NPY-treated group, compared with unstressed controls. Although total GR levels were not altered in hypothalamus, a significant decrease of GR phosphorylated on Ser232 and increased FK506-binding protein 5 mRNA were observed with the vehicle but not in animals infused with intranasal NPY. In contrast, in the ventral hippocampus, only vehicle-treated animals demonstrated elevated GR protein expression and increased GR phosphorylation on Ser232, specifically in the nuclear fraction. Additionally, SPS-induced increase of CRH mRNA in the ventral hippocampus was accompanied by apparent decrease of CRH peptide particularly in the CA3 subfield, both prevented by NPY. The results show that early intervention with intranasal NPY can prevent traumatic stress-triggered dysregulation of the HPA axis likely by restoring HPA axis proper negative feedback inhibition via GR. Thus, intranasal NPY has a potential as a noninvasive therapy to prevent negative effects of traumatic stress. (Endocrinology 155: 3920–3933, 2014)

Posttraumatic stress disorder (PTSD) is a disabling, prevalent, and difficult-to-treat psychiatric disorder, which develops in a subset of individuals after exposure to traumatic stress. Increased fear, anxiety, hyperarousal, and avoidance are central to PTSD symptomatology (1). One of the core neuroendocrine abnormalities involves dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (2–4). PTSD patients also display elevated CRH in the cerebrospinal fluid and plasma (5–7). During stress, CRH is released from the hypothalamus and stimulates ACTH synthesis and release from the anterior pituitary, which evokes glucocorticoid secretion from the adrenal cortex into circulation. Glucocorticoids mediate many of their physiological responses to stress via the glucocorticoid receptor (GR). Within the hypothalamus, GR plays a crucial role in direct glucocorticoid feedback by repressing CRH biosynthesis and release and thus enabling appropriate termination of the stress response.

The hippocampus, and especially its ventral part, also participates in the feedback control over the HPA axis (8–12). The hippocampus has abundant GR expression, which can, via stimulation by glucocorticoids, facilitate...
regulation of the HPA axis. Noteworthy, increased GR expression was found in hippocampi of animals subjected to the single prolonged stress (SPS) and predator scent stress, animal models of PTSD (13–17), and smaller hippocampal volume has been observed in PTSD patients (18, 19), emphasizing the role of this structure in PTSD pathology. Abnormalities in GR function within brain regions crucial for proper regulation and termination of the HPA axis activity might be responsible at least for some of the PTSD-related features (15, 20). However, the activation and function of GR are complex processes involving various mechanisms (21). The cochaperone FK506-binding protein 5 (FKBP5) is one of modulators of GR function positively correlated with PTSD (22). By preventing binding, folding, and trafficking of the GR to the nucleus, FKBP5 contributes to glucocorticoid resistance (23–25). Because FKBP5 expression is induced via GR activation by glucocorticoids, it contributes to an ultrashort feedback loop that regulates GR sensitivity and bioavailability. Hence, recalibration and restoration of GR responsiveness and glucocorticoid signaling could represent an additional target of pharmacological interventions.

Neuropeptide Y (NPY) is a promising candidate to treat PTSD, because it is positively associated with resilience or improved recovery from the traumatic stress (26–29), and its intranasal application represents an innovative approach for rapid noninvasive delivery to the brain avoiding potential side effects elicited by peripheral administration. We have recently demonstrated that intranasal NPY given to rats before SPS ameliorated development of the PTSD-associated behavioral symptoms of anxiety, depression, and hyperarousal, prevented HPA axis hyperactivity, and inhibited the GR overexpression in the hippocampus (16). Because traumatic events are often unexpected, pretreatment is not always feasible. Early intervention with nasally infused NPY immediately after SPS stressors also prevented development of several PTSD-associated behavioral symptoms in a similar but not identical fashion as intranasal NPY administered before SPS (16).

Here, we examined some of the molecular mechanisms underlying stress-induced dysregulation of the HPA axis as well as the possible effectiveness of intranasal NPY to reduce these impairments. We determined the effect of early intervention with NPY administered intranasally immediately after the traumatic stress on SPS-triggered changes in GR and CRH expression within the hypothalamus and hippocampus, 2 key brain regions involved in PTSD pathophysiology. The results show that NPY infusion reduced overactivation of the HPA axis, likely by restoring GR activity and hence repressing CRH gene transcription in the hypothalamus. The treatment also prevented overexpression, phosphorylation, and nuclear translocation of GR in the ventral hippocampus and restored the SPS-induced impairments of the CRH expression within this region.

Materials and Methods

Animals

All experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at New York Medical College. Male Sprague-Dawley rats (Charles River), 6 weeks old, weighing 150–160 g at arrival, were housed in a barrier area on a 12-hour light, 12-hour dark cycle at 23 ± 2°C with ad libitum access to food and water. After 14 days of acclimation (4 per cage), animals (8 wk old) were randomly assigned to experimental or control groups.

Intranasal administration

To evaluate intranasal NPY delivery and penetration to different brain regions, we infused 20 μL (10 μL per nare) of 250 pmol of fluorescein amide (FAM)-labeled porcine NPY (Phoenix Pharmaceuticals, Inc) mixed with 100-μg NPY (NioBiosci) in distilled water under light isofluorane anesthesia. Extreme care was taken to avoid contact with intranasal mucosa. After administration, the head of the animal was held in tilted back position for approximately 15 seconds to prevent loss of the solution from the nares. After 30 minutes, rats were decapitated and brains immediately removed and fixed in ice-cold 4% paraformaldehyde for 4 days, then cryoprotected in 15% sucrose for 24 hours followed by 30% sucrose solution for 48 hours, embedded in M-1 Embedding Matrix (Thermo Fisher Scientific), and stored at −80°C until sectioning. Brains were cut coronally at 40 μm in Leica CM1850 cryostat. Every seventh section was removed for cresyl violet staining for cytoarchitectonic verification, and remaining sections were kept at −20°C in cryoprotectant solution (0.01M PBS [pH 7.4], 30% glycerol, and 30% 2-methoxyethanol) until mounted onto 1% gelatinized slides. Care was taken to minimize exposure of the sections to light. Sections were visualized under identical exposure settings on Nikon 90i Eclipse microscope equipped with 495- to 519-nm excitation/ emission filter cube.

Single prolonged stress

SPS was performed as previously described (16, 30). Briefly, rats were immobilized for 2 hours by taping the limbs and restricting the head movement, then immediately subjected to forced swim for 20 minutes, dried, and after a 15-minute recovery exposed to ether vapor until loss of consciousness. Although still under the influence of ether, 20 μL of NPY (150 μg) or vehicle (water, 10 μL per nare) was infused intranasally as described above. The SPS procedure was performed between 9 AM to 2 PM. After SPS, animals were housed 2 per cage and left undisturbed for 7 days to allow PTSD symptoms to develop. Control rats were not stressed.

Seven days after SPS stressors, rats were decapitated between 9 AM to 12 PM in a separate room. Each animal was brought individually into the procedure room, and time between the
transfer of the rat from its cage and decapitation was 35–40 seconds in average, and thus, it could not affect HPA axis activation (31). Blood was collected into EDTA containing tubes with 0.6-TIU aprotinin (Sigma). For the biochemical experiments, mediobasal hypothalamus containing paraventricular nucleus (PVN) and hippocampus, and in some experiments the dorsal and ventral hippocampus, were isolated and frozen at −80°C. For the immunocytochemistry, brains were stored and processed as described above.

### Plasma ACTH and corticosterone

Plasma ACTH and corticosterone levels were determined using ACTH ELISA kit (MyBioSource) and double antibody 125I RIA kit (MP Biomedical Diagnostics) according to the manufacturer’s protocol as previously described (16). The intra- and interassay coefficients of variation for ACTH and corticosterone assays were 9% and 11%, 19.2%, and 13.2%, respectively.

### Real-time PCR

Changes in mRNA levels were measured by RT-quantitative PCR as previously described (16). Briefly, total RNA was isolated with RNeasy Mini kit (QIAGEN), and concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription of 1000 ng of RNA was performed with RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) using oligo dT primer. The cDNA (2 μL) was mixed with 12.5 μL of Fast-Start Universal SYBR Green Master Rox (Roche Diagnostics) according to the manufacturer’s protocol as previously described (16). The intra- and interassay coefficients of variation for ACTH and corticosterone assays were 9% and 11%, 19.2%, and 13.2%, respectively.

### Western blot analysis

Total protein from selected brain regions was isolated using Qproteome Mammalian Protein Prep kit (QIAGEN) containing phosphatase inhibitors (Cell Signaling Technology, Inc) according to manufacturer’s instructions. Protein concentration was determined by DC Protein Assay (Biorad) with Bio-Tek plate reader (Bio-Rad). Western blotting was carried out as described previously (16). Briefly, 10 μg of total protein were separated on 4%–15% Tris-HCl gradient precast gels (Criterion; Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). After blocking in Tris-buffered saline (TBS) containing 5% dry milk or 5% BSA (for phosphorylated protein) and 0.1% Tween 20 (TBS with Tween 20 [TBST]), membranes were incubated with primary anti-GR or antiphospho-GR at Ser232 (pGRSer232) overnight at 4°C. Primary antibody specifications are described in Table 1. After incubation with secondary antirabbit antibody (sc-2004, 1:3000; Santa Cruz Biotechnology, Inc) for 1 hour, membranes were developed with SuperSignal West Pico Chemiluminescent kit (Thermo) and exposed to x-ray films. The blots were reprobed for β-actin (Table 1). Films were scanned by GS-800 Calibrated Densitometer (Bio-Rad) within the linear range, and optical density of individual bands was evaluated by PCNAS 2.08e software (Raytest).

### Isolation of cytosolic and nuclear protein fractions from ventral hippocampus

Isolation of cytosolic and nuclear fractions was performed as described by others (32, 33) with small modifications. All procedures were performed at 4°C. Ventral hippocampus from individual animals was homogenized in 400 μL of B1 buffer (0.32M sucrose, 0.1mM EGTA, and 1mM HEPES) supplemented with 0.5mM dithiothreitol and protease/phosphatase inhibitors (Cell Signaling) with Dounce Tissue Grinder (Wheaton; Fisher Scientific). After centrifugation (2000g, 10 min), the supernatant was removed, centrifuged (10 000g, 15 min), and retained as cytosolic fraction. The pellet from the first centrifuga-

### Table 1. Specification of Antibodies

<table>
<thead>
<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, or Name of Source</th>
<th>Species Raised in Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>N/A</td>
<td>GR (M-20)</td>
<td>sc-1004; Santa Cruz Biotechnology, Inc</td>
<td>Rabbit polyclonal</td>
<td>1:800</td>
<td>32</td>
</tr>
<tr>
<td>pGRSer232</td>
<td>N/A</td>
<td>Phospho-glucocorticoid receptor (Ser211)</td>
<td>4161; Cell Signaling</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>37</td>
</tr>
<tr>
<td>β-Actin</td>
<td>N/A</td>
<td>β-Actin (C4) HRP</td>
<td>sc-47778HRP; Santa Cruz Biotechnology, Inc</td>
<td>Mouse monoclonal</td>
<td>1:50 000</td>
<td>118</td>
</tr>
<tr>
<td>Histone H3</td>
<td>N/A</td>
<td>Histone H3 (D1H2)XP</td>
<td>4499; Cell Signaling</td>
<td>Rabbit monoclonal</td>
<td>1:5000</td>
<td>119</td>
</tr>
<tr>
<td>GAPDH</td>
<td>N/A</td>
<td>GAPDH (14C10)</td>
<td>2118; Cell Signaling</td>
<td>Rabbit monoclonal</td>
<td>1:5000</td>
<td>120</td>
</tr>
<tr>
<td>CRH</td>
<td>N/A</td>
<td>Anti-CRF</td>
<td>T-5007; Peninsula Laboratories</td>
<td>Guinea pig polyclonal</td>
<td>1:500</td>
<td>121</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not available.
Immunohistochemistry

Brains were removed, fixed, cryoprotected, sectioned, and stored at −20°C same as described above. Free-floating coronal sections containing the PVN, and the ventral hippocampus (bregma −1.80 mm to bregma −1.88 mm and bregma −4.80 mm to bregma −5.20 mm, respectively) (34), were rinsed twice with 0.1M TBS (pH 7.4) and once with TBST (0.3% Triton X-100 in 0.1M TBS), blocked for 1 hour at room temperature (1% BSA, 10% goat serum [Vector Labs] in TBST), and incubated with guinea pig anti-CRH (Table 1) at 4°C for 18 hours in blocking solution. Sections were then rinsed 3 times with 0.1M TBST and incubated with secondary antibody (Alexa Fluor 594, 1:400 [Invitrogen], in 1% BSA, 1% goat serum in 0.1M TBST) and incubated with Alexa Fluor 594 (37). Levels of total GR protein were similar in all animals

Stained sections were analyzed using Zeiss Imager A1 bright field microscope (Zeiss) equipped with AxioCam MrM camera (Zeiss) with filter cube for Alexa Fluor 594 excitation/emission spectrum (590–617 nm) and coupled to Zeiss AxioVision software suite. All images were taken using identical exposure and light source and saved as ZVI files. Only sections representing the same coronal plain (determined by structural markers; 2–3 sections per animal) were chosen for quantification. ZVI files were imported to Fuji suite for analysis (image processing package based on Image); National Institutes of Health. The PVN region was delineated using the freehand selection tool, saved as an ROI, and then applied uniformly to all other sections. Similarly, 2 regions lateral to the PVN or the ventral hippocampus were delineated, saved as ROIs, and then applied to all other sections. Fluorescence intensity inside ROIs was measured and compared across groups.

Results

Rapid delivery of intranasal NPY to the brain

First, we assessed delivery of nasally administered NPY into the brain using FAM-labeled NPY. Thirty minutes after intranasal infusion, its fluorescence was clearly visible in many brain areas. Representative images of brain regions used for further analysis are shown in Figure 1. Intensive fluorescence was detected in the PVN (Figure 1A) and in the CA1, CA3, and dentate gyrus subfields of the ventral hippocampus (Figure 1B).

Intranasal NPY altered the SPS-triggered changes of the HPA axis

Plasma ACTH (F = 5.5, P < .01) and corticosterone (F = 7.2, P < .01) levels were significantly different among experimental groups (Figure 2). Unstressed controls and NPY-treated group had similar levels of both ACTH and corticosterone. These hormone levels were significantly higher in rats treated with vehicle after SPS (SPS/V).

Next, we measured CRH mRNA levels in the medio-basal hypothalamus containing the PVN. Significant differences were found in CRH mRNA levels among the experimental groups (F = 4.4, P < .05) (Figure 3A). CRH mRNA levels in SPS/V animals were 4-fold above levels in unstressed controls. Intranasal NPY infusion completely prevented SPS-induced rise of CRH mRNA. The abundance of the CRH transcript in SPS/NPY rats was comparable with the control group. Immunoreactivity of the CRH peptide in the PVN was analyzed by immunofluorescence (Figure 3B). The fluorescence intensity also differed between the groups (F = 7.1, P < .01) and was significantly lower than controls in both groups subjected to SPS, with or without NPY infusion.

Because GR is the major player in glucocorticoid-dependent feedback mechanism regulating CRH gene expression in the hypothalamus (35, 36), we examined expression of total GR and its transcriptionally active form phosphorylated on Ser232 (pGRSer232), corresponding to phosphorylated GR at Ser211 in humans (Figure 4A) (37). Levels of total GR protein were similar in all animals

Statistical analysis

Data were analyzed using JMP 9 (SAS Institute) and Prism 4 (GraphPad) software. Changes between the experimental groups were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test or by Student’s t test to compare 2 means. Results are expressed as mean ± SEM. Values of P < .05 were considered significant.
However, pGRSer232 levels significantly differed among groups (F = 6.2, P < .01). Significantly decreased pGRSer232 was found in the vehicle- but not in NPY-treated rats comparing with controls. The ratio between pGRSer232 and total GR among the experimental group (controls: SPS/V: SPS/NPY) was 1:0.5:1, respectively. GR mRNA levels were also analyzed (Figure 4B), and there were significant differences among the groups (F = 6.1, P < .05). GR mRNA was higher in the vehicle compared with the control and NPY-infused rats. SPS/NPY group did not differ from the control.

Changes in expression of FKBP5, involved in the regulation of GR signaling and linked to PTSD manifestation, were also examined. One-way ANOVA revealed significant effect of the treatment (F = 6.6, P < .01) (Figure 4C). FKBP5 mRNA level was higher in the SPS/V compared with the control and SPS/NPY groups, whereas in the SPS/NPY animals, it was similar to unstressed controls.

**SPS-elicited changes of GR and CRH in hippocampus**

GR protein levels were examined in the hippocampus, because it has been associated with PTSD pathology. There was significant impact of experimental manipulations on total GR protein in the whole hippocampus (F = 5.8, P < .05). Elevated level of GR protein was observed in SPS/V animals compared with the control and SPS/NPY group as detected by Western blotting (Figure 5). The functional roles of the dorsal and the ventral hippocampus are different with the ventral part more related to psychogenic stressors and emotions (38). Therefore, we examined GR protein levels in dorsal and ventral hippocampus separately. Expression of GR protein was region specific with significant alterations in the ventral (F = 5.0, P < .05) but not in the dorsal (F = 1.1, P > .05) hippocampus (Figure 5). In the ventral hippocampus of SPS/V rats, the GR levels were significantly higher than in the SPS/NPY-treated or control animals.

We further analyzed changes induced by SPS and subsequent NPY infusion on total GR protein and pGRSer232 in the cytosolic and nuclear fractions of the
ventral hippocampus (Figure 6). The purity of fractionation was confirmed by Western blotting with GAPDH detected primarily in the cytosolic, and H3 in nuclear fraction with low cross-contamination (Figure 6A). The levels of nuclear GR were dependent on the experimental procedures (F = 4.27, P < .05). Increased nuclear GR was found in the SPS/V group but not in SPS/NPY animals compared with the controls (Figure 6B). A similar pattern of changes was found for pGRSer232 (F = 3.5, P < .05). The levels of nuclear pGRSer232 were significantly increased above the controls in the SPS/V but not in SPS/NPY animals (Figure 6C). There was no difference between rats infused with intranasal NPY and the controls. We did not observe any changes in GR or pGRSer232 in the cytosolic fraction of the ventral hippocampus (data not shown). In addition, there were no variations in FKBP5 mRNA levels between the experimental groups for the ventral hippocampus (Figure 6D).

To examine whether SPS and intranasal NPY administration affect CRH gene expression, which can be increased by stress exposure (39, 40), we determined levels of CRH mRNA and peptide in the ventral hippocampus. One-way ANOVA revealed significant influence of the treatment on CRH mRNA levels (F = 6.9, P < .01) (Figure 7A), which were significantly higher in SPS/V group compared with the control rats. The CRH mRNA levels in the

Figure 4. Effect of NPY on SPS-induced changes in GR expression, pGRSer232 protein levels, GR mRNA, and FKBP5 mRNA levels in the mediobasal hypothalamus. Summary data and representative Western blotting for total GR and pGRSer232 (A); GR mRNA (B) and FKBP5 mRNA (C) levels. Data are presented as mean ± SEM. *, P < .05 vs control; #, P < .05; ##, P < .01 SPS/V vs SPS/NPY; n = 12–14 per group. V, Vehicle; C, control.

Figure 5. Effect of NPY on SPS-induced changes on GR protein in the hippocampus. Representative Western blottings and summary data are shown. Data are presented as mean ± SEM. *, P < .05 vs control; #, P < .05 SPS/V vs SPS/NPY; n = 8–14 per group. V, Vehicle; C, control.
SPS/NPY animals did not differ from the controls or SPS/V group. CRH protein levels were analyzed in different subfields of the ventral hippocampus by immunofluorescence. One-way ANOVA revealed a significant impact of the treatment on CRH peptide in CA3 subfield (F/11005)/H11349.05) (Figure 7B) with significant differences between SPS/V and control groups. In CA1 subregion, the CRH fluorescence intensity was significantly lower in SPS/V than in SPS/NPY animals (P<.05) (Figure 7B).

Discussion

This study revealed, for the first time, that early intervention with intranasal NPY prevented development of several abnormalities in the HPA axis induced by traumatic stress and 2 brain regions where accumulation of FAM-labeled NPY was detected. A week after exposure to SPS stressors, rats treated with NPY, in contrast to vehicle, displayed physiologically normal levels of plasma ACTH and corticosterone, as well as CRH mRNA, pGRSer232, and FKBP5 mRNA levels in the mediobasal hypothalamus. In the ventral hippocampus, NPY infusion normalized CRH expression and nuclear levels of total GR and pGRSer232.

SPS-induced changes in the HPA axis and mediobasal hypothalamus

Dysregulation of the HPA axis is one of the core features in PTSD, and a variety of abnormalities in this system...
are repeatedly reported in patients and animal models (41, 42). Here, using the SPS model of PTSD, we found increased plasma ACTH and corticosterone 7 days after SPS stressors in vehicle-treated rats. These results are similar to our previously published data, where the vehicle was infused 30 minutes before SPS and under the light isoflurane anesthesia (16). Thus, the conditions of vehicle infusion did not affect the SPS-induced long-term elevation of plasma ACTH and corticosterone.

Although hypocortisolism has been reported in PTSD individuals (3), several groups observed elevated cortisol in blood, saliva, urine, and hair of these patients (43–47) as well as increased or unchanged plasma corticosterone in PTSD animal models (15–17). These findings may reflect an initial elevation of glucocorticoids in the aftermath of the trauma followed by gradual normalization and finally low glucocorticoid secretion (48, 49). Alternatively, the development of hypo- vs hypercortisolemia might depend on the previous history of traumatic event. Accordingly, individuals with past trauma history had lower cortisol levels, likely due to down-regulation of the HPA axis activity, whereas those with trauma-free history showed higher cortisol levels, shortly after the last trauma. In both groups, cortisol levels were associated with PTSD symptoms measured 1 month later (50–53). Because SPS involves a single exposure to traumatic stress, our data correspond with the latter situation. However, the dynamics of changes in ACTH and corticosterone after SPS remains to be determined. Levels of CRH mRNA in mediobasal hypothalamus were also markedly increased, indicating hyperactivation of the HPA axis also at the levels of the PVN, leading to elevated plasma ACTH and corticosterone, respectively. Surprisingly, in contrast to the increased CRH mRNA, CRH peptide of both SPS-subjected groups was lower than in controls. There are several possible explanations for this discrepancy. It might reflect changes in mRNA translatability as well as CRH mRNA and protein stability. In this regard, stability of CRH transcript in the PVN is influenced by glucocorticoid signaling (54). However, because ACTH was elevated in SPS/V rats, it is attractive to speculate that lower peptide levels might rather reflect higher transport of CRH from cell bodies and release into the portal system. In line with this hypothesis, increased plasma CRH was found in PTSD patients (7).

With stress, high glucocorticoid levels mediate their effects on the brain via GR. However, ligand-dependent regulation of GR functions is complex and involves multiple mechanisms at posttranscriptional and posttranslational levels, which can further affect transcriptional ac-

Figure 7. Effect of NPY on SPS-induced changes of CRH gene expression in ventral hippocampus. A, CRH mRNA levels. B, CRH immunoreactive protein in CA1 and CA3 subregions. Representative images and summary data of fluorescence intensity are shown. Data are presented as mean ± SEM. *, P < .05; **, P < .01 vs control; #, P < .05 SPS/V vs SPS/NPY group; n = 8 per group. Scale bar, 50 μm.
tivity of GR (55). Although we found increased GR mRNA in the hypothalamus of vehicle-treated animals, the total GR protein level was unchanged despite elevated plasma corticosterone. This may suggest insufficient translation process as a result of potential posttranscriptional degradation mechanisms, such as by microRNAs (56–58). However, the level of pGRSer232, an indicator of GR transcriptional activity (37), was reduced and could result from SPS-elicited reduction in cyclin-dependent kinase 5 activity, which is important for GR phosphorylation on Ser232 specifically in the brain (59). Reduced pGRSer232 might indicate less GR binding to the CRH promoter and diminished suppression of the CRH gene transcription (60). One of the other mechanisms contributing to GR sensitivity to glucocorticoids, is a large chaperone protein complex, including FKBP5. When FKBP5 is bound to the GR via heat shock protein 90, the receptor has lower affinity for its ligand and is retained in the cytoplasm instead of translocation to the nucleus. Furthermore, alleles associated with enhanced FKBP5 induction with cortisol together with impaired negative feedback have been associated with higher risk to develop PTSD (61, 62). Thus, we suggest that long-term reduction of pGRSer232 and elevation of FKBP5 mRNA in the hypothalamus lead to glucocorticoid resistance and impaired negative feedback mechanism. In this regard, disruption of the GR gene in CRH-producing neurons of murine PVN resulted in CRH overexpression and elevated plasma ACTH and corticosterone (63), which is consistent with our findings here.

**SPS-induced changes in hippocampus**

GR is abundantly expressed in the hippocampus, and its role has long been recognized in this structure (11, 12, 64). Hippocampal abnormalities associated with changes in GR signaling have been proposed to play an important role in the pathogenesis of PTSD (15, 20). Elevated levels of GR after SPS have been repeatedly demonstrated in total hippocampal tissue by numerous studies, including ours (15, 16, 20, 65). These changes were accompanied by deficits in long-term potentiation (LTP) in CA1 hippocampal subfield and behavioral alterations, which were reversed by GR antagonist (15).

Here, we further analyzed consequences of SPS on GR signaling in the ventral hippocampus, because its neuronal connections and functions are distinct. Although the dorsal part plays a key role in learning and long-term memory formation, the ventral hippocampus exerts control over the HPA axis, formation of emotional memories, and stress behavior (66). Moreover, GR activation affects LTP in an opposite manner in the ventral compared with the dorsal hippocampus (67). In contrast to the hypothalamus, total GR and pGRSer232 were increased in the nuclear fraction of the ventral hippocampus. FKBP5 mRNA levels were unchanged, similar to results shown by others with different stressors (68). Because at least 100 genes can be regulated by GR in hippocampus (69), its increased transcriptional activity, especially in its ventral part, could have tremendous impact on this and other structures involved in stress-associated neuronal circuits. During stress, the ventral hippocampus participates in inhibition of CRH release from the PVN projections and thus attenuates the HPA axis activation (10, 70–73). Overexpression of GR in hippocampus has been associated with enhanced inhibitory tone to the HPA axis (reviewed in Ref. 74). However, prolonged stimulation of GR by glucocorticoids has been shown to mediate stress-elicited hippocampal atrophy (64) and reduction of synaptic plasticity observed in PTSD patients and animals exposed to SPS (15, 75–77). Furthermore, stress and glucocorticoids via GR also promote structural and cellular reorganization, retraction of apical dendrites, and reduction of the spine density in pyramidal cells and loss of synapses, especially in CA3 subregion (78–82), although more pronounced changes have been observed primarily in the dorsal hippocampus (82). Therefore, we suggest that increased GR expression and transcriptional activity in the ventral region and prolonged elevation of plasma corticosterone may induce morphological changes accompanied by alterations in neurotransmission, decreased synaptic plasticity, and reduced output (83) and, thus, may contribute to disinhibition of the HPA axis (72, 73, 84), elevated CRH transcription, and eventually increased levels of plasma hormones found in this study.

In addition to elevated GR, CRH mRNA was also increased within the ventral hippocampus. A few studies also reported elevated hippocampal CRH mRNA after the stress exposure (39, 40). Although the hippocampus is an important target for CRH with substantial CRH receptor expression (85), this peptide is also produced locally (86). Most CRH peptide is synthesized and stored in γ-aminobutyric acid interneurons of CA1 and CA3 pyramidal cell layers (87, 88). For the first time, this study shows that GR and possibly the GR-dependent regulation of CRH gene expression differ in hypothalamus vs the ventral hippocampus after SPS. Although CRH mRNA was increased in both regions, they varied in the FKBP5 mRNA levels, total and phosphorylated GR. There are many cases of tissue-specific regulation of genes by steroid hormones. Accordingly, stress and corticosterone down-regulate CRH in the PVN but up-regulate it in the central amygdala and bed nucleus of stria terminalis. Diversity of GR signaling comes from neuron-specific actions of various negative and positive glucocorticoid-responsive elements,
differences in binding depending on chromatin accessibility and exposure of the glucocorticoid-responsive element, multiple receptor isoforms generated by alternative splicing, posttranslational modifications, and different recruitment of GR coactivators or coinhibitors (55). These diverse responses may be also driven by tissue-specific distribution and function of steroid receptors coactivator (SRC) isoforms. Although SRC-1a isoform is highly expressed in both the PVN and the hippocampus, SRC-1e is significantly higher in the hippocampus compared with the PVN (90).

Similar to the hypothalamus, immunofluorescence showed attenuated CRH peptide in CA3 subregion and a tendency for a decrease in CA1 subfield of the ventral hippocampus in vehicle indicating exaggerated CRH neurotransmission or increased turnover. Long-term exposure of hippocampal neurons to CRH promotes loss of dendritic arborization and destabilization of dendritic spines (91, 92). In stressed rodents, longer exposure to CRH led to reduction of pyramidal cell number and apical dendritic spine integrity in CA3 area resulting in selective disruption of LTP and cognitive deficits (93), similarly as observed with prolonged GR stimulation. Strikingly, hippocampal volume loss in PTSD patients is most pronounced particularly in the CA3 subregion (19).

Preventive effects of NPY on SPS-induced neuroendocrine impairments

For the first time, we also demonstrated that administration of NPY given immediately after traumatic stress prevented development of most of the molecular consequences of SPS on the HPA axis and the hippocampus. NPY was delivered by intranasal infusion allowing peptides to rapidly and directly enter the brain via extra- and intracellular neuronal olfactory and trigeminal pathways bypassing the blood-brain barrier (94–96). In addition to our previous data showing elevation of NPY in the cerebrospinal fluid but not plasma 30 minutes after intranasal infusion (16), here, we demonstrated that FAM-labeled NPY penetrated into many brain regions, including the PVN and the ventral hippocampus. This is consistent with other studies using intranasal delivery of different peptides to the brain, peaking 30 minutes after infusion and still detected up to 6 hours (97–100). Although we cannot rule out partial degradation of NPY peptide, it could directly exert its effects on both structures to protect against development of neuroendocrine abnormalities resulting from SPS. NPY likely acts on NPY receptors in these regions within several hours after the administration but is no longer present 1 week later when the changes were analyzed.

There are considerable data supporting a role of NPY in resilience or recovery from harmful effects of stress (29, 101, 102). Although intranasal NPY delivery to the brain can counteract the negative consequences of stress, the overall effects may involve a number of structures and various NPY receptor subtypes. Y1, Y2, and Y5 receptors exhibit dense and overlapping gene expression in hypothalamus and hippocampus (103). Our study revealed that intranasal NPY greatly attenuated the SPS-elicited rise of CRH, FKBP5 mRNAs and a decrease of pGRSer232 in the mediobasal hypothalamus, although it did not prevent reduction of CRH immunofluorescence in the PVN. Although a number of studies indicate that under certain conditions NPY increases CRH gene expression and release (104–107), its inhibitory effects has also been demonstrated (108). Accordingly, NPY administered into the cerebral ventricle reduced CRH mRNA in the PVN (109, 110) and proopiomelanocortin mRNA in the pituitary (110). The CRH-producing cells of the PVN have the highest density of NPY-containing nerve terminals (111). It has been proposed that although different stressors initiate rapid CRH release that facilitates the stress response, NPY released during the later phase mediates an adequate termination of the stress reaction (16, 112, 113). The results obtained here somewhat support this hypothesis, because NPY was administered during the recovery stage after the termination of SPS stressors, when plasma ACTH and corticosterone levels are still very high (16). Under normal homeostatic conditions, stress up-regulates NPY transmission, which helps to normalize the HPA axis functions and consequently reduces fear and anxiety (101).

NPY might also have direct effect on the NPY receptors in the hippocampus. Intranasal NPY prevented the SPS-triggered induction of nuclear levels of total GR and pGRSer232 and changes in CRH expression particularly in CA3 region. Injection of NPY or Y1 agonist into hippocampus 1 hour after predator-scent stress model of PTSD also attenuated increased GR expression and reduced behavioral symptoms (26). The prolonged HPA axis activation after SPS may exhaust NPY sources (26) and reduce neurotransmission that can be replenished by direct exogenous NPY delivery into the hippocampus and the PVN shortly after exposure to traumatic stress. In addition, NPY can compete with CRH in extrahypothalamic regions to reduce its anxiogenic effects (114–116). Functional antagonism between NPY and CRH has been observed in the amygdala (103), where they regulate γ-aminobutyric acid transmission in an opposite manner via their respective receptors (117). A similar mechanism might also exist in the hippocampus.

Taken together, we demonstrated that early intervention with intranasal NPY triggered long-term effects on the HPA axis, especially on CRH and GR expression and function in the hypothalamus and the ventral hippocam-
pus. These changes might mediate previously observed benefits in reducing the development of PTSD-associated anxiety, depression, and hyperarousal after traumatic stress (16). Thus, intranasal NPY represents potential novel treatment strategy to reduce the harmful effects of traumatic stress after a proper examination of its effects per se in the future experiments.

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