INTRODUCTION

Interleukin-1β (IL-1β) is one of the major proinflammatory cytokines involved in the regulation of hypothalamic-pituitary-adrenal (HPA) axis and stress responses in the brain (1-5). Cytokines can access a cytokine network in the brain and influence neurotransmission within stress regulatory brain circuits and induce hormonal changes similar to those observed following stress exposure (6). Cytokines may also markedly affect neurotransmission within regulatory brain circuits for emotion,
mainly in the prefrontal cortex (PFC) (7). Pro-inflammatory cytokines are able to induce the release of a corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamic paraventricular nucleus (PVN) and augment neurochemical stimulation of the pituitary gland (8). Stressors such as footshock and immobilization (IMO) have been shown to induce hypothalamic interleukin-1 (IL-1) production, while other stressors such as restraint, maternal separation or social isolation have no effect on hypothalamic IL-1 levels (9). Stress-induced IL-1β levels in the circulation directly stimulates pituitary cells to secrete adrenocorticotropic hormone (ACTH). Exposure to some predominantly emotional and systemic stressors has been found to induce long-term sensitization of the HPA responsiveness to further superimposed stressors (10). It is possible that in an intact animal the major or initial effect of IL-1 is mediated through the hypothalamus. Chronic psychosocial stress decreased IL-1β mRNA levels in the hippocampus, the IL-1 receptor in pituitary and increased the plasma corticosterone level (11). During stress combined networks from brainstem nuclei to specific limbic system structures exercise their regulatory function on HPA axis activity and glucocorticoid regulation. The key target of these various direct and indirect pathways is the PVN of the hypothalamus (12, 13). The specific role of the hippocampus, PFC, and brainstem nuclei in regulating the HPA axis under stress condition depends on the type of stressor (14). Different types of stressors, such as reactive versus anticipatory stressors induce stimulation of the HPA axis through activation changes in distinct brain regions involved in HPA regulation (2, 9). Stressful stimuli, whether physical, metabolic or psychological activate all levels of the HPA axis as indicated by the increased release of brain CRH, anterior pituitary ACTH and consequent increase of plasma corticosterone or cortisol concentration.

Accumulated evidence indicates that nitric oxide (NO) is widely distributed in the central nervous system (CNS) and subserves the functions via its synthetic and signal transduction mechanisms. The special proteins NO receptors are coupled to cyclic guanosine monophosphate (cGMP) formation (15, 16) and cytokines modulate of NO production. A large number of reviews on the role of NO in CNS regulatory function in neuroendocrinology of stress system have been published in the last years (15, 17-20). NO is synthesized by two constitutive enzyme isoforms: neuronal nitric oxide synthase (nNOS) and endothelial NO synthase (eNOS) by conversion of L-arginine to citrulline. The third type inducible iNOS, although generally undetectable under basal conditions, can be constitutively expressed in different brain regions of rats mainly by astrocytes in normal, undisturbed mammalian brains and in microglia during immunological challenge (21). In neurons nNOS is located mainly at the post-synaptic terminal. NO functions in the mammalian CNS like a conventional neurotransmitter, because of its ability to freely cross a cell membrane. NO can act in an autocrine and paracrine manner also on distant targets (22). Brain nNOS exists in particulate and soluble forms and the differential subcellular localization of nNOS may contribute to its diverse functions. nNOS is involved in a variety of synaptic events, modulating physiological functions and in a number of human diseases (23). NO participates in signal transduction pathways in physiological processes such as corticosterone release from the adrenal gland (24-26). NO is widely involved in interaction between neuroendocrine and neuroimmune systems in physiological and pathological processes. nNOS is implicated in modulating learning, memory and neurogenesis and is involved in a number of human diseases, including depression. nNOS is highly expressed by cells of the hypothalamic PVN, the convergence point for the sympathetic-adrenal system, the HPA axis and the hypothalmo-hypophyseal regulatory systems (27). These systems are key regulators of the neuroendocrine stress responses. Constitutive iNOS activity would interfere with neuronal processes that favor stress adaptation (28).

We have recently found that repeated restraint for 3 days markedly altered the homotypic stress induced IL-1β level and iNOS level in brain structures and influenced HPA axis activity (29, 30). These changes were brain structure-specific and different in magnitude. Despite numerous studies on the involvement of central IL-1β and NO systems in stress reactions, their role and functional adaptation during prolonged stress have not yet been elucidated. The aim of the present study was to evaluate the expression of nNOS, iNOS and IL-1β in brain structures involved in stress responses and to determine adaptional changes of IL-1β and NO-synthases induced by chronic restraint for the homotypic stress. We also investigated the involvement of plasma IL-1β in the functional adaptation of pituitary-adrenocortical axis during prolonged stress.

MATERIALS AND METHODS

Animals

All the experiments were performed on unanesthetized (n = 480) male Wistar rats (6 weeks old, 190 – 220 g) housed in groups of 5 per cage (52 × 32 × 20 cm) under standard conditions with an artificial 12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.), constant temperature 22 ± 2°C, food and tap water available ad libitum. The animals were given 1 week of habituation period before the experimental session. All the procedures were approved by Local Bioethics Commission for Animal Experiments at the Institute of Pharmacology, Polish Academy of Sciences in Cracow and met the requirements of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC).

Experimental procedures

To avoid circadian variability, acute stress protocols were carried out at light cycle between 10 – 12 a.m. All the decapitations were performed rapidly by a skillful, always the same person, within few seconds after taking the animal from its cage. This procedure did not affect the basal plasma ACTH and corticosterone levels. After decapitation, the brains were removed from the skulls and three whole structures prefrontal cortex, hippocampus and hypothalamus were excised on an ice-cold glass plate, immediately frozen on dry ice and stored at –70°C until assayed.

The animals were divided into four groups. Animals from the control group were not subjected to any restraint (the first group). Rats from the second group were restrained for 10 min only once, on the day of the experiment, and decapitated 0, 1, 2 and 3 hours afterwards. Rats from the third group were subjected to 10 min restraint stress sessions twice a day for 3, 7 and 14 consecutive days. The break between stress sessions was at least 8 hours. The use of a single time point in the present investigation might not allow for the determination of peak variations in some of the NO synthases and IL-1β. Therefore, the changes in these components levels were measured during 3 hours in the post-stress period. Restraint stress was carried out using metal tubes with ample holes for ventilation. After stress the animals were returned to the same cages. Some of the rats which were subjected to single restraint stress for 10 min, were injected i.p. with a single dose of IL-1 receptor antagonist (IL-1Ra) (100 µg/kg, Sigma-Aldrich) 15 min before the stress session and decapitated 0, 1, 2 and 3 hours afterwards (the fourth group). The dose of 100 µg/kg of IL-1Ra was selected as effective antagonist of IL-1β-induced effect in our earlier study (31).
**RESULTS**

Repeated restraint-induced nNOS, iNOS and IL-1β responses to homotypic stress in brain structures

In the prefrontal cortex, a single restraint for 10 min initially induced a modest increase of nNOS and iNOS protein levels at the time of the cessation of restraint. Then a progressive, significant decrease of the iNOS level appeared 1–2 hours later, and a subsequent, non-complete return occurred 3 hours later (Anova F(4, 46) = 6.707; P < 0.0002). The changes of the nNOS level were parallel to those of iNOS but not significant (Anova F(4, 46) = 1.993; P = 0.1083).

In PFC, a single restraint immediately after its termination moderately increased the IL-1β protein level, which significantly increased 2 and 3 hours later (Anova F(4, 44) = 12.61; P < 0.0001). The level of IL-1β in PFC changed parallel to the alterations of nNOS and iNOS, but appeared at significantly higher than control level, whereas the nNOS and iNOS levels decreased below the control values in non-stressed rats. (Fig. 1A). In PFC, repeated restraint for 3 days markedly enhanced the homotypic stress-induced nNOS level 1 hour after stress termination, when compared to the level in the non-stressed group (Anova F(4, 46)=5.287; P < 0.0002). Concomitantly, the iNOS level induced by subsequent homotypic stress increased considerably, immediately after restraint cessation (Anova F(4, 42) = 11.92; P < 0.0001) and slowly decreased 1–3 hours later (Fig. 1B). In PFC, repeated restraint for 3 days significantly enhanced the IL-1β level, induced by homotypic stress, during the first 2 hours to the maximum value (Anova F(4, 46) = 6.456; P < 0.0001) and later it declined to the same level as immediately after stress termination (Fig. 1B).

In PFC restraint for 7 days blunted the increase of the homotypic stress induced nNOS level after prior restraint for 3 days (Anova F(4, 42) = 1.866; P = 0.3744) and markedly impaired the increase of iNOS level, thought it remained at significantly higher level when compared with control level in previously non stressed rats (Anova F(4, 42) = 4.350; P < 0.0011). Restraint for 7 days also blunted the significant increase of the IL-1β level in rats restrained for 3 days but did not appreciably alter the IL-1β level induced by homotypic stress in rats stressed for 3 days (Anova F(4, 46) = 1.154; P = 0.3361) (Fig. 1C). Repeated restraint for 14 days further diminished but did not abolish the homotypic stress-induced increase of iNOS after 7 days of restraint (Anova F(4, 42) = 5.386; P < 0.0002). Homotypic stress after repeated restraint for 14 days, significantly lowered the nNOS level when compared to the level after 3 and 7 days of prior restraint, as well as after single restraint (Anova F(4, 48) = 3.945; P = 0.0023). After restraint for 14 days the IL-1β level in PFC did not significantly differ from its control level in previously non-restrained rats (Anova F(4, 46) = 1.345; P = 0.2590) (Fig. 1D).

In the hippocampus a single restraint induced transient and significant elevation of the nNOS level 2 hours after stress termination (Anova F(4, 44) = 3.467; P < 0.0103) and a significant decrease of the iNOS level 1 hour later (Anova F(4, 46) = 2.860; P < 0.0275). Single restraint gradually increased IL-1β level which reached the highest value 3 hours after stress cessation (Anova F(4, 46) = 3.741; P < 0.0084) (Fig. 2A). In the hippocampus repeated restraint for 3 days markedly enhanced the homotypic stress-induced increase of the nNOS level 1 hour later (Anova F(4, 46) = 3.678; P < 0.0038) and evoked significant increase of the iNOS level in the whole post-stress period, when compared to the level in control non-stressed rats (Anova F(4, 42) = 8.762; P < 0.0001) (Fig. 2B). Repeated restraint for 3 days also significantly enhanced the homotypic stress-induced increase of the IL-1β level (Anova F(4, 44) = 3.704; P < 0.0045) (Fig. 2B). After longer restraint, for 7 days, homotypic stress induced a moderate decrease of nNOS level immediately after stress termination (Anova F(4, 46) = 2.925;
P < 0.0187) and a significant increase of iNOS throughout the whole post stress period (Anova F(4, 46) = 3.760; P < 0.0039). Repeated stress for 7 days transiently increased the restraint stress-induced IL-1β level 1 hour after stress termination (Anova F(4, 42) = 0.8667; P = 0.5073) (Fig. 2C). In the hippocampus 14 days of restraint significantly decreased the nNOS level induced by homotypic stress (Anova F(4, 46) = 4.667; P < 0.0008) and substantially impaired the increase of iNOS level during 2 hours after stress termination (Anova F(4, 44) = 6.260; P < 0.0001). Prolonged restraint also markedly increased the homotypic stress-induced IL-1β level during the post stress period (Anova F(4, 42) = 4.693; P < 0.0014) (Fig. 2D).

**Fig. 1.** Neuronal (nNOS) and inducible (iNOS) nitric oxide synthase and interleukin 1β (IL-1β) content in prefrontal cortex in rats exposed to single restraint stress (1 × RS) for 10 min. Rats were restrained in metal tubes (for 10 min) and decapitated at the termination of restraint and 1, 2 and 3 hours later (Fig. 1A). Effect of prior restraint for 10 min two times per day for 3 (Fig. 1B), 7 (Fig. 1C) and 14 (Fig. 1D) days on 10 min RS-induced nNOS, iNOS and IL-1β content in prefrontal cortex. Data was assessed by one-way ANOVA followed by Tukey’s multiple range test. Each point represents the mean ± S.E.M. of 10 – 12 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. non-stressed control group. Right panels show the representative immunoblots showing the expression of nNOS, iNOS and IL-1β in prefrontal cortex.
In the hypothalamus a single restraint markedly enhanced nNOS level after stress cessation (Anova F(4, 55) = 4.795; P < 0.0022) and it significantly decreased iNOS level 3 hours after restraint (Anova F(4, 46) = 3.9; P < 0.0057). Single restraint stress failed to markedly affect IL-1β level in this structure (Anova F(4, 53) = 0.2750; P = 0.9257), moderate changes were parallel to these in iNOS level (Fig. 3A). In hypothalamus, repeated stress for 3 days slightly increased nNOS level induced by homotypic stress, compared to corresponding control levels in non-stressed rats (Anova F(4, 46) = 2.041; P = 0.0787) (Fig. 3B). The response of iNOS to the following homotypic stress was considerably enhanced and most pronounced immediately after

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**Fig. 2.** Neuronal (nNOS) and inducible (iNOS) nitric oxide synthase and interleukin 1β (IL-1β) content in hippocampus in rats exposed to single restraint stress (1 × RS) for 10 min. Rats were restrained for 10 min and decapitated at the termination of restraint and 1, 2 and 3 hours later (Fig. 2A). Effect of prior restraint for 10 min two times per day for 3 (Fig. 2B), 7 (Fig. 2C) and 14 (Fig. 2D) days on 10 min RS-induced nNOS, iNOS and IL-1β content in hippocampus. Data was assessed by one-way ANOVA followed by Tukey’s multiple range test. Each point represents the mean ± S.E.M. of 10 – 12 rats per group. ++P < 0.05, +++P < 0.01, +++P < 0.001 vs. non-stressed control group. Right panels show the representative immunobots showing the expression of nNOS, iNOS and IL-1β in hippocampus.
stress cessation (Anova F(4, 44) = 9.189; P < 0.0001), then gradually declined but persisted at significantly higher than control level during the whole post-stress period. Repeated restraint for 3 days also significantly increased the homotypic stress-induced IL-1β level after 1 hour at the maximum (F(4, 46) = 6.130; P < 0.0001) and then slightly declined (Fig. 3B). A longer period of repeated restraint, for 7 days, significantly diminished nNOS level (Anova F(4, 42) = 7.076; P < 0.0001), evoked by subsequent homotypic stress (Fig. 3C). The parallel increase of iNOS level (Anova F(4, 44) = 3.790; P < 0.0046) was

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**Fig. 3.** Neuronal (nNOS) and inducible (iNOS) nitric oxide synthase and interleukin 1β (IL-1β) content in hypothalamus in rats exposed to single restraint stress (1 × RS) for 10 min. Rats were restrained in metal tubes (for 10 min) and decapitated at the termination of restraint and 1, 2 and 3 hours later (Fig. 2A). Effect of prior restraint for 10 min two times per day for 3 (Fig. 2B), 7 (Fig. 2C) and 14 (Fig. 2D) days on 10 min RS-induced nNOS, iNOS and IL-1β content in hypothalamus. Data was assessed by one-way ANOVA followed by Tukey’s multiple range test. Each point represents the mean ± S.E.M. of 10 – 12 rats per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. non-stressed control group. Right panels show the representative immunoblots showing the expression of nNOS, iNOS and IL-1β in hypothalamus.
Fig. 4. Comparison of the influence of restraint stress for 10 min two times per day for 3 days and 1 × RS, restraint for 10 min two times per day for 7 days and 1 × RS, restraint for 10 min two times per day for 14 days and 1 × RS on nNOS (A), iNOS (B) and IL-1β (C) content in prefrontal cortex. Rats were restrained in metal tubes (for 10 min) and decapitated at the termination of restraint and 1, 2, and 3 hours later. Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test Each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, **P < 0.01, *** P < 0.001 vs. group restrained for 10 min at 0, 1, 2 and 3 hour respectively.

Fig. 5. Comparison of the influence of restraint stress for 10 min two times per day for 3 days and 1 × RS, restraint for 10 min two times per day for 7 days and 1 × RS, restraint for 10 min two times per day for 14 days and 1 × RS on nNOS (A), iNOS (B) and IL-1β (C) content in hippocampus. Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test Each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, ***P < 0.001 vs. group restrained for 10 min at 0, 1, 2 and 3 hour respectively.
considerably less marked compared to the level in rats stressed for 3 days. Prior restraint for 7 days did not substantially alter the homotypic stress-induced IL-1β level (Anova F(4, 46) = 0.2750; P = 0.9257) compared to the control level in non-stressed rats. (Fig. 3C). After prolonged restraint for 14 days, the hypothalamic nNOS (Anova F(4, 44) = 1.849; P = 0.1402) and iNOS (Anova F(4, 48) = 2.041; P = 0.0787) level responses to homotypic stress did not significantly differ from their control values in non-repeatedly stressed rats (Fig. 3D). However, prolonged restraint for 14 days strongly enhanced the homotypic stress-induced increase of IL-1β level in the hypothalamus during 3 hours post stress-period (Anova F(4, 44) = 7.547; P < 0.0001) (Fig. 3D).

Prior restraint period-dependent homotypic stress-induced nNOS, iNOS and IL-1β levels in brain structures

In the prefrontal cortex, prior restraint for 3 days induced a marked, transient increase of nNOS level 1 hour after homotypic stress termination (Anova F(3, 56) = 3.774; P < 0.0156). Repeated restraint for 7 days transiently diminished the homotypic stress-induced nNOS level (Anova F(3, 56) = 5.898; P < 0.0014) and after 14 days of restraint nNOS level significantly decreased (Anova F(3, 56) = 10.62; P < 0.0001) when compared to the levels in control non-stressed rats (Fig. 4A). Repeated restraint for 3 days considerably enhanced the homotypic stress-induced iNOS protein level (Anova F(3, 56) = 6.005; P < 0.0013), during 3 hours of a post-stress period. Prior restraint for 7 days evoked a less pronounced, though significant, increase of the homotypic stress-induced iNOS response (Anova F(3, 56) = 4.288; P < 0.0086) and after 14 days of repeated restraint, iNOS level further decreased although it remained at a significantly higher level than after single restraint (Anova F(3, 56) = 16.99; P < 0.0001) (Fig. 4B). Prior repeated restraint for 3 days also significantly enhanced the homotypic stress-induced IL-1β level in the prefrontal cortex (Anova F(3, 56) = 16.59; P < 0.0001) to the highest level 1 and 2 hours after stress termination. A longer restraint period, for 7 days, did not alter the homotypic stress-induced IL-1β response (Anova F(3, 56) = 2.340; P = 0.083) and prior restraint for 14 days significantly diminished this response (Anova F(3, 56) = 9.239; P < 0.0001), 3 hours after stress termination when compared to the response in control non-stressed rats (Fig. 4C).

In the hippocampus, repeated restraint for 3 days moderately increased the homotypic stress-induced nNOS level immediately after cessation of stress (Anova F(3, 56) = 4.563; P = 0.0063), which gradually vanished in the post-stress period. A longer repeated restraint, for 7 days, moderately lowered the homotypic stress-induced nNOS level (Anova F(3, 56) = 3.534; P = 0.0204), compared to the level in previously non-stressed rats. After 14 days of prior restraint the homotypic stress evoked a marked decrease of nNOS level during 2 hours after cessation of stress and later it significantly increased nNOS response (Anova F(3, 56) = 10.49; P < 0.0001) over the control level in prior non-stressed rats. Previous restraint for 3 days also induced a marked, more pronounced increase of the homotypic stress-induced IL-1β level (Anova F(3, 56) = 3.424; P < 0.0027) in the post-stress period and a less, moderately increased response appeared after 7 days (Anova F(3, 56) = 4.711; P < 0.0053) and 14 days of prior restraint (Anova F(3, 56) = 1.050; P = 0.3779), (Fig. 5C).

Fig. 6. Comparison of the influence of restraint stress for 10 min two times per day for 3 days and 1 × RS, restraint for 10 min two times per day for 7 days and 1 × RS, restraint for 10 min two times per day for 14 days and 1 × RS on nNOS (A), iNOS (B) and IL-1β (C) content in hypothalamus. Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Each point represents the mean ± S.E.M. of 10–12 rats per group *P < 0.05, **P < 0.01, ***P < 0.001 vs. group restrained for 10 min at 0, 1, 2 and 3 hours respectively.
In the hypothalamus, restraint for 3 days did not markedly alter the homotypic stress-induced increase of nNOS protein level (Anova F(3, 56) = 0.6308; P = 0.5982), during the post-stress period. Previous stress for 7 days markedly decreased nNOS level (Anova F(3, 56) = 3.596; P < 0.0190) and after restraint for 14 days, nNOS level induced by homotypic stress altered from an initial fall 1 hour after stress termination to a significant increase (Anova F(3, 56) = 8.676; P < 0.0001) 1 hour later (Fig. 6A). Repeated restraint for 3 days strongly intensified the homotypic stress-induced increase of iNOS level (Anova F(3, 56) = 6.157; P < 0.0011), which slightly declined but remained at a significantly higher level throughout 3 hours post-stress period. Prior restraint for 7 days induced a significant increase of iNOS levels (Anova F(3, 56) = 3.205; P < 0.0300), over the control level. Repeated restraint for 14 days markedly increased the homotypic stress-induced iNOS level when compared to the control level in non-stressed rats (Anova F(3, 56) = 7.850; P = 0.05073), (Fig. 6B). The homotypic stress-induced increase in the hypothalamic IL-1β level was significantly enhanced (Anova F(3, 56) = 4.854; P < 0.0045), by previous restraint for 3 days. Restraint for 7 days did not substantially alter IL-1β level (Anova F(3, 56) = 2.374; P = 0.0798), compared to that in non-stressed rats, and prolonged stress for 14 days evoked the most pronounced enhancement of the homotypic stress-induced increase in IL-1β levels (Anova F(3, 56) = 6.977; P < 0.005), above the control levels in non-stressed rats (Fig. 6C).

**Effect of IL-1Ra on homotypic stress-induced plasma IL-1β, ACTH and corticosterone levels after repeated restraint**

A single restraint for 10 min significantly increased plasma IL-1β level immediately after stress termination (Anova F(4, 44) = 55.22; P < 0.0001) and to a maximum level 1 hour later. IL-1Ra (100 µg/kg i.p.) given 15 min before restraint abolished this increase in the whole 3 hours post-stress period (Anova F(3, 88) = 10.25; P < 0.0001) (Fig. 7A). Repeated restraint for 3 days significantly increased the homotypic stress-induced plasma IL-1β level 1 hour later than a single restraint (Anova F(4, 57) = 5.592; P

![Graph A](image1.png)

**Graph A** shows the influence of IL-1Ra on homotypic stress-induced plasma IL-1β levels after repeated restraint.

![Graph B](image2.png)

**Graph B** illustrates the effect of IL-1Ra on homotypic stress-induced plasma ACTH levels.

![Graph C](image3.png)

**Graph C** depicts the impact of IL-1Ra on homotypic stress-induced plasma corticosterone levels.

**Graph D** represents the combined effect of IL-1Ra on homotypic stress-induced plasma levels of IL-1β, ACTH, and corticosterone.

Note: Each point represents the mean ± S.E.M. of 10–12 rats per group. *P < 0.05, **P < 0.01 vs. group restrained for 10 min at 0, 1, 2 and 3 hour respectively.

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*Fig. 7.* Influence of IL-1 receptor antagonist (IL-1Ra), given i.p. 15 min before single restraint stress for 10 min, on IL-1β plasma levels (Fig. 7A). Influence of IL-1 receptor antagonist (IL-1Ra), given i.p. 15 min before restraint stress for 10 min, on IL-1β plasma levels induced by restraint stress for 3 days (Fig. 7B), 7 days (Fig. 7C), and 14 days (Fig. 7D). On the day of the experiment, rats were exposed to single restraint for 10 min and decapitated at the termination of restraint and 1, 2 and 3 hours later. Data were analyzed by one-way ANOVA followed by Tukey’s multiple range test (each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, **P < 0.01, ***P < 0.001 vs. non-stressed control group) and two-way ANOVA followed by Bonferroni’s multiple comparison test (each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, ** P < 0.01 vs. group restrained for 10 min at 0, 1, 2 and 3 hour respectively).
< 0.0005). IL-1Ra, given i.p. 15 min before homotypic stress blunted this increase to the control level in rats exposed to repeated restraint for 3 days (Anova F(3, 56) = 5.437; P < 0.00241) (Fig. 7B). Previous restraint for 7 days significantly enhanced homotypic stress-induced plasma IL-1β level 0 – 3 h after restraint termination (Anova F(4, 54) = 5.861; P < 0.0003) and IL-1Ra abolished this increase in the whole post stress period (Anova F(3, 56) = 5.019; P < 0.0038) (Fig. 7C). Similarly, chronic restraint for 14 days significantly elevated the homotypic stress-induced plasma IL-1β level (Anova F(4, 60) = 3.006; P < 0.0181) and IL-1Ra administered before restraint totally reduced this increase 1 and 2 hours after stress termination (Anova F(3, 56) = 5.467; P < 0.0023) (Fig. 7D).

Single restraint rapidly and strongly increased the plasma ACTH level which decreased in a fast manner to resting level during 2 hours after stress termination (Anova F(4, 55) = 46.05; P < 0.0001). IL-1Ra given i.p. significantly inhibited the restraint-induced increase of plasma ACTH level immediately and 1 h after stress termination (Anova F(3, 84) = 14.73; P < 0.0001) (Fig. 8A). In rats previously restrained for 3 days IL-1Ra administered 15 min before homotypic stress moderately weakened the increase of plasma ACTH response immediately after termination of stress (Anova F(3, 56) = 2.434; P = 0.0743), (Fig. 8B), modestly diminished this response after 7 days of prior restraint (Anova F(3, 56) = 0.1766; P = 0.9118), (Fig. 8C), and did not alter this response after repeated restraint for 14 days (Anova F(3, 56) = 0.1766; P = 0.9987), (Fig. 8D).

Single restraint immediately after termination rapidly and strongly increased plasma corticosterone level which returned in a similar manner to control level 2 hours later (Anova F(4, 55) = 56.46; P < 0.0001). IL-1Ra administered i.p. before stress significantly diminished the restraint-induced the increase and the time of returning of plasma corticosterone to control level (Anova F(3, 88) = 5.76; P < 0.0001) (Fig. 9A). Plasma corticosterone level significantly increased by homotypic stress in repeatedly restrained rats for 3 days was not altered by pretreatment with IL-1Ra (Anova

![Figure 8](image)

**Fig. 8.** Influence of IL-1 receptor antagonist (IL-1Ra), given i.p. 15 min before single restraint stress for 10 min, on ACTH plasma levels (Fig. 8A) Influence of IL-1 receptor antagonist (IL-1Ra), given i.p. 15 min before restraint stress for 10 min, on ACTH plasma levels induced by restraint stress for 3 days (Fig. 8B), 7 days (Fig. 8C) and 14 days (Fig. 8D). On the day of the experiment, rats were exposed to single restraint for 10 min and decapitated at the termination of restraint and 1, 2 and 3 hours later. Data were analyzed by one-way ANOVA followed by Tukey’s multiple range test (each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, **P < 0.01, ***P < 0.001 vs. non-stressed control group) and two-way ANOVA followed by Bonferroni’s multiple comparison test (each point represents the mean ± S.E.M. of 10 – 12 rats per group *P < 0.05, ** P < 0.01 vs. group restrained for 10 min at 0, 1, 2 and 3 hour respectively).
After repeated restraint for 7 days this antagonist markedly enhanced the homotypic stress-induced rise of plasma corticosterone at the cessation of restraint (Anova $F(3, 56) = 3.797; P < 0.0150$) (Fig. 9C). In rats previously restrained for 14 days IL-1Ra moderately enhanced the fall of plasma corticosterone level 1 and 2 hour after homotypic stress termination (Anova $F(3, 56) = 1.833; P = 0.1517$), (Fig. 9D).

DISCUSSION

Effect of restraint stress on nNOS, iNOS and IL-1β levels in brain structures

In the present study, a single restraint which is a milder experimental type of stressors, affected nitric oxide synthases and IL-1 systems in brain structures regulating stress responses.

In the prefrontal cortex restraint for 10 min induced parallel alterations of nNOS and iNOS protein levels. Immediately after restraint cessation both isoenzyme levels transiently increased and 1 – 2 hours later the iNOS level significantly decreased and returned to the resting level 3 hours after stress termination. A significant fall of iNOS level may suggest either excessive use of a limited amount of constitutive iNOS or its insufficient generation during increased demand in the initial phase of acute stress reaction in PFC. Although it is generally accepted that iNOS is undetectable under basal conditions, some evidence indicates that iNOS can be constitutively expressed in different brain regions of rats (21). Constitutive levels of nNOS and iNOS in PFC may not be sufficient to compensate for an increased demand in the initial phase of acute stress reaction. Neuronal NOS is constitutively expressed in neurons, microglial cells and astroglial cells (32). The generation of NO by the nNOS isoform appears very rapidly since it does not require mRNA synthesis, and can act as a synaptic neurotransmitter. During stress the generation of NO by iNOS is...
long-lasting but in far higher local concentration than by nNOS or eNOS. In our experiment after single restraint both nNOS and iNOS protein levels in PFC initial declined during 2 hours and then approached their control levels which suggests the induction of NOS gene expression by acute restraint stress (33). Single restraint stress caused acute changes in NO producing neurons in the amygdala complex and delayed modifications in the hippocampal formation (34). Our data show that in PFC acute restraint stress induced a moderate, transient decrease of the IL-1β level 1 hour after restraint cessation which significantly increased 2 and 3 hours later probably via a genomic mechanism. IL-1 is regarded as an essential mediator of adaptive stress responses. This cytokine and stressors are able to impair neuronal plasticity and neurotransmission contributing to depression illness (1, 35, 36). In our experiment the changes of the IL-1β levels in PFC were parallel in time to the alterations of nNOS and iNOS, but appeared at a significantly higher level. Cytokines increased by exposure to stress can induce iNOS expression (37) and NO synthesis, which is involved in the modulation of stress induced neuroendocrine action. In the present experiment a single restraint increased the IL-1β level in PFC, whereas the nNOS and iNOS levels were markedly decreased below the control level in non-stressed rats. Our results show that neuro-modulatory changes during acute stress rapidly disrupt PFC network connection of IL-1 and NO systems and markedly impair function of the most sensitive structure to detrimental effects of stress (7).

In the hippocampus a single restraint induced transient elevation of the nNOS level and a significant decrease of the iNOS level 2 and 3 hours, after restraint termination. This may suggest a marked involvement of nNOS and iNOS in an acute stress-induced reaction before further compensation by a genomic mechanism. A single restraint significantly increased the IL-1β level 3 hours after stress termination in the hippocampus, similar to the increase in PFC but not in the hypothalamus. A single restraint after 3 hours induced reciprocal changes in hippocampus iNOS level (a significant decrease) and IL-1β levels (a significant increase), but repeated restraint for 3 days evoked a strong increase of both iNOS and IL-1β levels in that structure suggesting the involvement of NO generated by iNOS and IL-1β in adaptational compensatory response during repeated stress.

In the hypothalamus a single restraint markedly enhanced the nNOS protein level in the whole post stress period, perhaps due to a larger presence of nNOS in the neuronal processes of different neurons involved in the central regulation of stress reactions in that structure. These results suggest that single stress activates the nNOS protein system in brain structures containing a neuronal net, like the hippocampus and hypothalamus. By contrast single stress significantly decreased the iNOS protein level 3 hours after stress termination below the control level in non-stressed rats. Acute restraint stress also significantly declined the IL-1β level parallel to similar decrease of the iNOS level. NO may regulate brain IL-1 gene expression in the hypothalamic PVN which releases CRH (38), the first stimulator of the HPA axis. These changes indicate that in the hypothalamus single restraint stress moderately enhanced nNOS protein level and markedly decreased iNOS and IL-1β levels (and functions).

Repeated stress-induced-adaptational changes of the nNOS, iNOS and IL-1β levels in brain structures induced by homotypic stress

The present data show that in PFC repeated restraint for 3 days consistently induced the strongest increase of the iNOS protein level in response to followed homotypic stress. This strong sensitized increase of iNOS response gradually declined after prior prolonged exposures to restraint for 7 and 14 days, but remained at a markedly higher level when compared to the parallel decreased iNOS response in control prior non-stressed rats. Also the responses of nNOS and IL-1β protein levels to the homotypic stressor in PFC are sensitized to a maximum extent after 3 days of repeated restraint. Our results suggest that the cumulative effects of repeated stress for 3 days may be more vulnerable for animals and humans subjected to a subsequent homotypic stressor.

In the hippocampus, repeated stress for 3 days moderately increased the homotypic stress induced nNOS level. The homotypic stress induced iNOS protein level was significantly and most strongly enhanced after 3 days and to a gradually lesser extent after prolonged exposure to stress for 7 and 14 days. This indicates that also in the hippocampus like in PFC, the highest increase of iNOS level appears after 3 days of prior stress. Repeated restraint for 3 days also significantly enhanced the homotypic stress-induced increase of the IL-1β level but a longer prior restraint did not alter the IL-1β protein level when compared with a single restraint-induced level. Our results indicate that restraint, a prevailing psychological stressor, engages more strongly the hippocampus for adapting the stress-induced NO and IL-1β regulatory functions (39). Our results suggest that in the hypothalamic homotypic stress-induced nNOS, iNOS and IL-1β levels are significantly sensitized by repeated restraint, and that the iNOS and IL-1β levels are particularly enhanced after 3 days of prior stress.

Restraint stress repeated for 3 days selectively enhanced excitatory synaptic inputs to parvocellular neurons of the PVN leading to increased CRH release and HPA axis stimulation (40). In the hypothalamus repeated stress for 3 – 14 days slightly altered the nNOS protein level induced by subsequent homotypic stress as compared with the level after single restraint. Repeated restraint for 7 days significantly diminished this level and after 14 days of restraint a marked increase of the nNOS level in response to successive homotypic stress appeared. On the contrary, prior restraint for 3 days considerably increased the homotypic stress-induced iNOS level during 3 hours after stress cessation. A vanishing increase of iNOS level in response to homotypic stress appeared after 7 and 14 days, after repeated stress. In the hypothalamus repeated restraint for 3 days and more strongly after 14 days enhanced the homotypic stress-induced IL-1β level 1 hour after stress termination. These biphasic adaptational changes of homotypic stress-induced IL-1β in the hypothalamus suggest strong sensitization of the IL-1β response after 3 and 14 days of prior repeated stress treatment. Single administration of IL-1β induced long lasting sensitization of the HPA axis to novelty stress 3 – 22 days later, parallel fast and delayed with biphasic increases of CRH and HPA axis sensitization (41). Prolonged, biphasic increase of IL-1β level in the hypothalamus after 3 and 14 days in our experiment may be dependent in part on HPA system adaptation to chronic emotional stress. Emotional stress in restrained rats can induce a long-lasting sensitization of IL-1β-evoked stimulation of hypothalamic PVN (41, 42). Proinflammatory cytokines in plasma may be involved over a long period in human metabolic liver disease (43). Multiple pathogenic factors can be involved in the regulation of autonomic nervous system. Prolonged emotional stress might disturb neuromodulation in the brain-gut interactions during gastrointestinal disorders (44).

A single restraint, significantly increased the plasma IL-1β level immediately and 1 hour after stress cessation. This increase was abolished by IL-1Ra administered i.p. 15 min before restraint. The data indicate that this initial increase of ACTH level in a significant part depends on the elevated IL-1β level, since IL-
1RA reduced the stress-induced increase in plasma ACTH level after restraint termination. After 3 days of repeated restraint homotypic stress-induced a rapid increase in plasma ACTH level which was moderately diminished by pretreatment with IL-1Ra and after longer repeated restraint, for 7 and 14 days, IL-1Ra did not affect the homotypic stress induced increase of ACTH level. These results suggest that prior repeated stress inhibited completely the IL-1β stimulatory component observed after single restraint stress. Single restraint stress-induced considerable increase of plasma corticosterone level that was significantly diminished by prior i.p. IL-1Ra administration. Repeated stress for 3 and 14 days abolished the inhibitory action of IL-1Ra on homotypic stress-induced corticosterone secretion. However, the enhancement by IL-1Ra the homotypic stress induced corticosterone response after prior repeated stress for 7 days is difficult to explain. Corticosterone release during adaptation to prolonged stress is regulated by a multiplicity of local adrenocortical signaling factors like cytokines, NO, prostaglandins. During stress adaptation the activity of these factors may be suppressed or sensitized in not a parallel time and direction manner. It is known that IL-1β has a marked stimulatory effect in adrenal gland whereas NO is a potent stimulator of corticosterone synthesis and release (42). At present the action of the temporal dependent factors, connected with a local stereoidogenesis in vivo, in regulation of corticosterone secretion during stress adaptation is not explained. The long and commonly accepted view that the functions of the adrenal cortex were mainly, or exclusively, regulated by circulating ACTH was successively completed by pointing out that many neurotransmitters and neuropeptides (45), as well as cytokines and NO also participate in the local paracrine regulation of adrenocortical stereoidogenesis (3, 24, 25). Recent evidence suggests interaction between cytokine pathways and HPA hormones directly at the level of the pituitary or adrenal glands to stimulate the secretion of ACTH and corticosterone (46, 47). IL-1β dose-dependently enhanced plasma corticosterone concentrations. Different stressors induce a rapid and high increase of IL-1β expression within the adrenal glands. NO significantly decreased corticosterone production both in unstimulated and in corticotropin stimulated zona fasciculata adrenal cells. Both nNOS and eNOS were detected in rat adrenal zona fasciculata and several NO donors significantly decreased both basal and ACTH-stimulated corticosterone generation in rats (24). Inflammatory messengers like IL-1β and prostaglandin (PG) E2 influence (in vitro) the release of ACTH and corticosterone by induction production of IL-1β as well as inducible cyclooxygenase (COX-2) in the adrenal immune cell population (47). The adrenal gland adapts to various forms of acute and chronic stress by producing cytokines and upregulation of cyclooxygenase and nitric oxide synthase enzymes that can modulate corticosterone release. Our results indicate that plasma IL-1β is markedly involved in a single restraint stress-induced stimulation of ACTH and corticosterone secretion.

We conclude that chronic stress may both sensitize or decrease specific control circuits to stimulate or inhibit stress-sensitive systems. In PFC repeated restraint for 3 days enhanced most strongly the homotypic stress-induced increase of iNOS and to a lesser extent IL-1β levels. The sensitization of iNOS and IL-1β responses vanished after 14 days. In the hippocampus, the strongest increase of homotypic stress-induced iNOS, nNOS and IL-1β levels appeared after 3 days of prior stress and decreased 7 and 14 days later. In the hypothalamus the iNOS level induced by homotypic stress was strongly enhanced by a prior 3 days of stress and returned to the control level after 7 days. Repeated stress induced a biphasic increase of the IL-1β level in response to homotypic stress after 3 days and 14 days. This suggests that homotypic stress significantly increases the IL-1β response after 3 days, which had vanished after 7 days, and increased more strongly after a further 7 days of stress. Our results present a distinct structure specificity and intensity of cytokine and nitric oxide in brain structures required to preserve or adapt homeostatic and protective cooperation mechanisms.

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