Substance P and the neurokinin 1 receptor

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The effects of antidepressants and an NK_1 antagonist on the proliferation of splenocytes in the tree-shrew chronic psychosocial stress model

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Abstract

Major depressive disorder has been associated with a disturbance of immune function. In the present study we investigate the effect of different types of antidepressants on splenocyte proliferation in a chronic psychosocial stress paradigm in tree shrews. The animals were subjected to a 7-day period of psychosocial stress, before receiving daily oral administration of tianeptine (50 mg/kg/day), L-760735 (10 mg/kg/day), clomipramine (50 mg/kg/day) or fluoxetine (15 mg/kg/day). The psychosocial stress continued throughout the treatment period of 28 days. Daily morning urine was collected to measure cortisol and norepinephrine levels. Chronic psychosocial stress resulted in a significant increase of urinary cortisol and norepinephrine concentrations. None of the treatments with tianeptine, fluoxetine, clomipramine or L-760735 was able to normalize the stress-induced elevation of cortisol or norepinephrine. Stress increased the relative weight of the adrenal glands, but none of the antidepressants in the study was able to reverse this increase. In contrast, stress decreased the relative spleen weights. While tianeptine and clomipramine tended to normalize relative spleen weight, L-760,735 and fluoxetine did not. Five weeks of chronic psychosocial stress increased the responsiveness of splenocytes to stimulation with the mitogen concavalin A. With the exception of L-760,735, four weeks of treatment with the antidepressants normalized the stress-induced increased responsiveness of splenocytes.

3.7 Introduction

Depressed patients display impairments of both immune system function and hypothalamic-pituitary-adrenal (HPA)-axis activity (Steckler et al., 1999). Besides alleviating symptoms of depression, antidepressants are often capable of improving immune system function as well as normalizing HPA-axis function (Himmerich et al., 2006b). Antidepressants also attenuate symptoms of depression associated with cytokine (e.g. inteferon-α and IL-2) treatment (Musselman et al., 2001).

A major pathogenic theory states that chronic stress precipitates symptoms of depression (Holsboer et al., 1984). Stress activates the HPA-axis and the sympathetic adrenal medullary (SAM) system. This leads to an increased release of cortisol from the adrenal cortex and of catecholamines from sympathetic nerve endings and the adrenal medulla. The increased release of cortisol suppresses the immune system (Carrasco & Van de Kar, 2003). It has been shown that patients who experience depression often exhibit sustained activation of the HPA-axis resulting in higher than normal plasma concentrations of cortisol under basal conditions (Maes et al., 1995). It is conceivable that the changes in HPA-axis activity, associated with major depression, will also influence the immune system. This theory is supported by studies in laboratory animals, wherein chronic stress was demonstrated to induce immune system deficits. For instance, the thymus, which is essential in T-cell maturation and plays a decisive role in the immune system, was shown to be extremely sensitive to acute and chronic stressors in rats (Selye, 1936). Another study showed that social defeat in rats results in more persistent and long lasting alterations of thymus function (Engler & Stefanski, 2003). In rats both surgical and physical stress suppress the activation of natural killer (NK) cells (Ben Eliyahu et al., 1999). Suppression of NK cell activation is also seen after chronic application of a α adrenoceptor agonist (Shakhar & Ben Eliyahu, 1998), the sympathetic adrenal medullary system (SAM) is likely to play a role in this suppression too (Padgett & Glaser, 2003). Interestingly, during the initial phase of the stress response activation of NK cells is increased, suggesting dynamic and temporal effects of stress on the immune system (Leonard, 2001).

The intensity and the duration of exposure to a stressor may influence the effect of stress on immune system function. Acute exposure to auditory stressors suppresses the splenocyte reaction to mitogen exposure. However, prolonged exposure to these stressors results in an elevation of mitogenic activity (Monjan & Collector, 1977). Antidepressants with monoaminergic properties tested on isolated lymphocytes in experimental studies showed a dose dependant reduction in lymphocytes (Audus & Gordon, 1982). On the other hand *ex-vivo* data were less convincing, but this may reflect to the heterogeneity of the patient group (Haack et al., 1999).

Tree shrews (*Tupaia belengeri*) are phylogenetically in between insectivores and primates. Their HPA-axis and immune system more closely resemble that of humans, than that of rodents (Flugge et al., 2002). Previously it was demonstrated that the chronic psychosocial stress paradigm in tree-shrews has high construct, face and predictive validity for depression (Kramer et al., 1999). In the present study we have investigated the effect of the antidepressants clomipramine, tianeptine, fluoxetine and the NK_1 receptor antagonist L-760,735 on several stress indices in this model. These include some immunological parameters, like the activation of splenocyte proliferation by concavalin A (conA).

3.8 Materials and Methods

3.8.1 Animals:

Experimentally naive male adult tree shrews (*Tupaia belengeri*) were obtained from the breeding colony of the German Primate Centre in Göttingen. Tree shrews are regarded as an intermediate between insectivores and primates and have been placed in the taxonomic order Scadentia (Martin, 1990). The day active animals were housed singly on a regular day/night cycle (lights on from 08:00 h to 20:00 h) at 26 °C, 55% relative humidity, with free access to food and water (for details see: (Fuchs, 1999). Animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and were approved by the Government of Lower Saxony, Germany. The minimum number of animals required to obtain consistent data was employed.

3.8.2 Drug treatment:

Animals received tianeptine (Stablon®; Servier, Courbevoie, France) dissolved in tap water via drinking bottles, which were light-protected (for more detail see (Czeh *et al.*, 2001)). This route of administration resulted in a mean intake of tianeptine of 50 mg/kg per day. Alternatively the animals received the highly brain-penetrating NK_1 receptor antagonist L-760,735 (10 mg/kg/day, Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Harlow, UK). Since the majority of the available NK_1 antagonists are designed to have a high affinity for the human NK_1 receptor, we conducted a pilot study to establish whether L-760,735 has an adequate affinity for the tree shrew NK_1 receptor. This study was also used to establish the dose that effectively blocks the NK_1 receptor in the brain (for details see for details see (van der Hart *et al.*, 2002)). A third group of animals was treated with) and the tricyclic antidepressant clomipramine (50 mg/kg/day Sigma, St Louis, USA) were dissolved in water and every second day freshly prepared. Finally some animals were administered with 15 mg/kg/day fluoxetine (Ratiopharm, Ulm, Germany), which resulted in a 81-634 ng/mL plasma concentration of norfluoxetine 24 hours after the last administration of the four weeks long treatment period. A similar range has been reported for patients under fluoxetine treatment (Laboratory Corporation of America database). L-760,735, clomipramine and fluoxetine were given in the morning orally. This route of administration was chosen because oral application is the most common route of administration for antidepressants in psychiatric patients and it also circumvents the effects caused by daily injections.

3.8.3 Experimental design

The experimental procedure was identical to the one described by Czeh et al. (2001). The experimental groups (Control; Stress; Stress + Tianeptine; Stress + L-760,735; Stress + Clomipramine; Stress + Fluoxetine) and the experimental design are shown in Fig. 1. The first experimental phase (No Stress) lasted for 7 days, during which all animals remained undisturbed and body weight was recorded daily to control for the general physical health of the animals. During the second phase of the experiment was a 7-days period, during, the animals of the Stress, Stress + L-760,735, and the Stress + Clomipramine group were submitted to daily psychosocial conflict (Stress). The induction of psychosocial conflict was carried out according to our standard procedure (Fuchs *et al.*, 1996). Briefly, one naive male was introduced into the cage of a socially experienced male. This resulted in active competition for control over the territory, and after establishment of a clear dominant/subordinate relationship, a wire mesh barrier separated the two animals. As in earlier studies (Fuchs *et al.*, 1996; van Kampen *et al.*, 2000), all of the naive animals turned out to become subordinate. The barrier was removed every day for approximately 1 hour allowing physical contact between the two males only during this time. By this procedure, the subordinate animal was protected from repeated attacks, but it was constantly exposed to olfactory, visual and acoustic cues from the dominant. Under these conditions, subordinate animals displayed characteristic subordination behavior such as reduced locomotor activity and decreased marking behavior. The third experimental phase consisted of the antidepressant drug treatments lasting for 28 days. During this time the stressed animals remained in the psychosocial conflict situation and were treated daily with the antidepressant drugs or vehicle, respectively. The animals of the Stress + L-760,735 group (10 mg/kg body weight/day) and of Stress + Clomipramine (50 mg/kg body weight/day) group received the compound orally. Previously, we reported that a daily dose of 50 mg/kg clomipramine is effective in reversing stress-induced endocrine and behavioral impairments in male tree shrews (Fuchs *et al.*, 1996). In all cases, the drug solutions were freshly prepared every second day and the solutions were stored light protected and cool. Animals of the Stress group were treated according to the same experimental schedule but received tap water only. The animals of the Control group were run in a separate experiment. They were individually housed and undisturbed in separate quarters elsewhere in the animal facility and received normal tap water. During all experimental phases morning urine was collected daily to assess indices of HPA axis and SAM function.

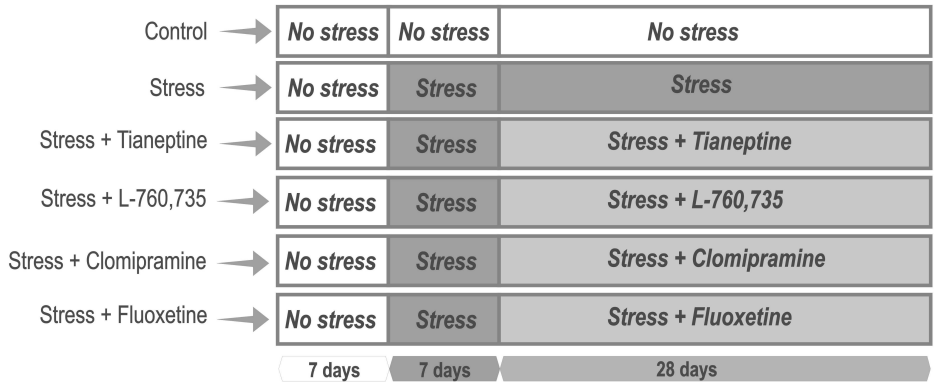

Fig. 1 Experimental setup

3.8.4 Analysis of urinary cortisol and noradrenaline.

Urinary free cortisol was measured by scintillation proximity radioimmunoassy (Udenfried *et al.*, 1985) using anti-rabbit cortisol antiserum (Paesel-Lorei, Frankfurt, Germany). Antirabbit IGG-coated fluormicrosrospheres (scintillation, proximity assay anti-rabbit reagent type I and ^{3}H-cortisol were from Amersham, Braunschweig, Germany. Urinary noradrenaline was quantified by RP-HPLC with coulometric detection after extraction on BioRex 70 cation-exchange columns (BioRad, Munich, Germany). To correct for difference in urine dilutions, the resulting concentrations were related to creatinine concentrations, which were determined with a Beckman Creatinine Analyzer 2.

3.8.5 Organ data

Animals were anaesthetized with an overdose of xylazin/ketamine (50 mg/18 mg per 100 ml). Thereafter under deep sleep, the spleen and the adrenals of the animals were removed and weighed. Organ weights were normalized via bodyweights.

3.8.6 Proliferation data

Spleens were aseptically removed and transferred into phosphate buffered saline. The splenocytes were harvested by pressing the organ through a plastic strainer and collected in a plastic sterile tube. The splenocytes were separated by lymphocyte separation medium (LSM®, ICN Biomedilac, aurora, OH, USA). The yielded monocytes were washed three times with HEPES buffered RPMI 1640 (life technologies, Glasgow, UK) supplemented with 10% FCS, 10 mM MEM with non essential amino acids, 2 mM L-glutamine, 100 U/ml pencillin G, 100 µg/ml

streptomycin and 2 x 10^{-4} M ME and counted. The cells were then resuspended in culture medium at a density 1 x 10^6/ml and distributed to 96-well flat-bottomed plates (100 µl per well, containing 1 x 10^5 cells). Then, the T-cell mitogen concavalin A (conA) was applied in triplicate to the wells. The cells were incubated for 48 hour and subsequently pulsed with 0.5 µCi/well [^{3}H]thymidine for 18 h. Incorporated radioactivity was measured in a matrix 9600 beta-counter (Packerd, Meridan, CT, USA). The results are expressed as the mean stimulation Index (S.I.). In each experiment, a non-treated stress group was tested. To compensate for minor uncontrollable changes, the stress group in each separate experiment was set as 1.

3.8.7 Data analysis

Data was statistically analyzed using Sigmastat 3.5 (Sysstat software, Inc, Chicago, IL, USA). The treatment effect was assessed using Kruskal Wallis test. Subsequently a post hoc test according to Dunn's method was performed to test for significant differences between the groups. Significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

3.9 Results

In Fig. 2 the morning urinary cortisol/ creatinine levels are shown in the last week of the chronic study. Morning urinary cortisol levels were elevated after chronic stress compared to the basal values measures in the control week in the experiment this effect was significant (Czeh *et al.*, 2001; van der Hart *et al.*, 2002). The elevation was pronounced throughout the entire duration of the psychosocial stress. None of the tested compounds were able to reverse this increase, although clomipramine showed a tendency to normalize the elevation. Fluoxetine, on the other hand, further enhanced the increase of cortisol levels. In the last week of the experiment and the chronic stress, the fluoxetine treated animals showed a significantly higher urinary cortisol level ($p<0.01$)

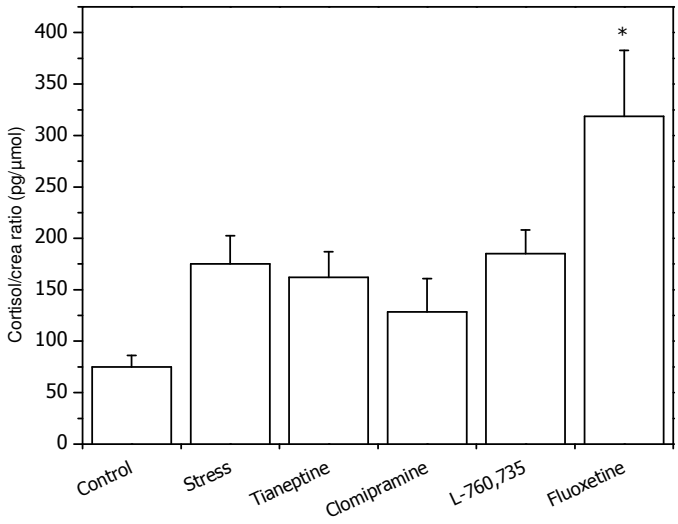

Figure 2 *Mean urinary cortisol/creatinine ratios in pg/µmol in week 6 of the chronic psychosocial stress experiment. * denotes significantly different from control ($p < 0.01$)*

Fig. 3 shows that urinary noradrenaline is elevated in the last week of the experiment, in all groups that underwent chronic psychosocial stress for five weeks. The urinary noradrenaline concentration rises immediately when the psychosocial stress commences. During the five weeks of stress the urinary norepinephrine levels are increased in all the experimental groups that undergoing the chronic psychosocial stress. None of the tested compounds were able to normalize the observed increase. Tianeptine and clomipramine augmented the effect of chronic psychosocial stress. Both tianeptine and clomipramine treated animals had significantly higher norepinephrine concentration in their morning urine compared to the control animals in the fifth week ($p<0.05$).

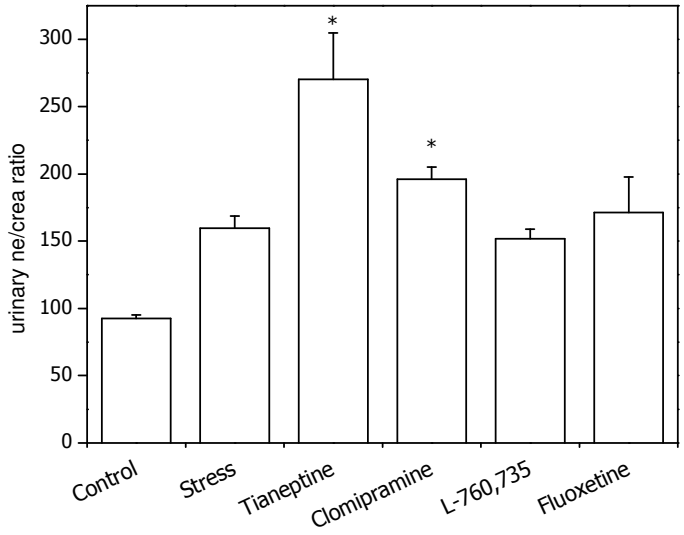

Figure 3 *Mean urinary norepineprhine/creatinine ratio in the last week of the chronic stress experiment. * denotes significantly different from control (p<0.05)*

In Fig. 4 the relative adrenal gland to body weight of the different groups is shown. Chronic stress increased the relative adrenal weight in tree shrews. Four weeks of treatment with any of the compounds did not result in a normalization of the relative weight of the adrenals ($p<0.05$). While tianeptine and L-760 735 showed a tendency to normalization, clomipramine and fluoxetine appeared to increase their relative adrenal weight more than stress alone did. This effect was significant for clomipramine and fluoxetine versus control ($p<0.05$).

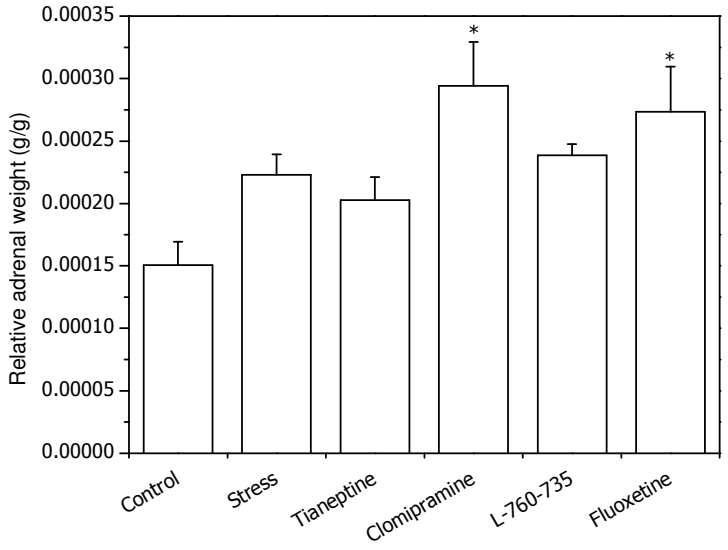

Figure 4 *Relative adrenal weight corrected for bodyweight. * denotes significant different from control (p<0.05)*

Chronic psychosocial stress reduced the relative spleen weight of tree shrews as is shown in Fig. 5. Treatment with either tianeptine or clomipramine showed a tendency towards normalization. Treatment with the NK_1 antagonist, L-760,735 did not show any effect compared to chronic stressed animals alone and fluoxetine further decreased the relative spleen weight, which was significantly smaller than that of animals treated with tianeptine ($p<0.05$)

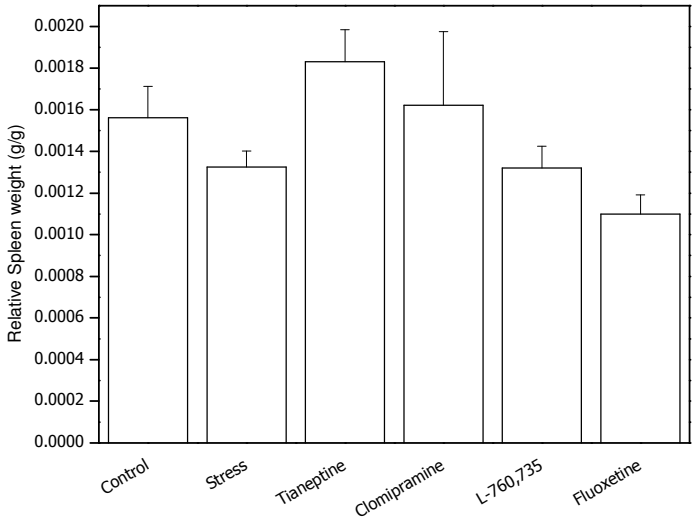

Figure 5 *Relative spleen weight corrected for bodyweight. * denotes significant different from tianeptine (p<0.05)*

Fig. 6 shows that proliferation of spleen cells induced by conA stimulation was significantly increased in the animals after chronic psychosocial stress for 5 weeks ($p < 0.05$). Tianeptine, clomipramine and fluoxetine were able to reverse this increase. However, in the L-760.735 treated group there was still a significantly increased in stimulated splenocyte proliferation rate compared to controls ($p < 0.05$).

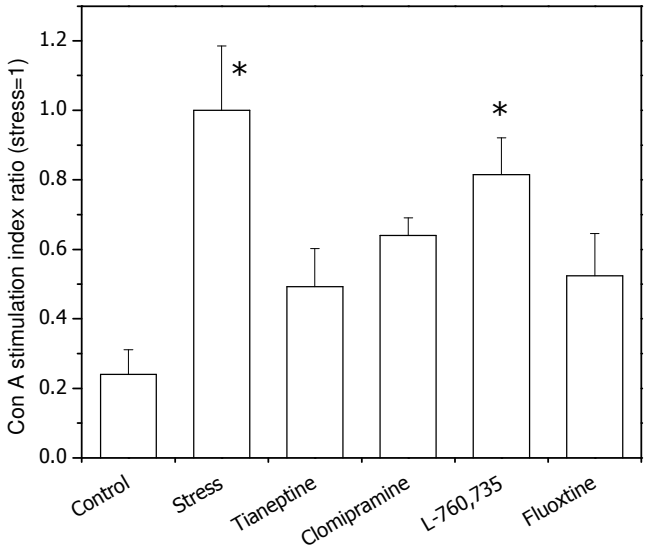

Figure 6 *Splenocyte stimulation index for the mitogen concavalin A normalized for stress. * denotes significant different from control (p < 0.05)*

3.10 Discussion

The present study in tree shrews shows that with chronic psychosocial stress several indices of sympathetic and HPA-axis activity change compared to non-stressed animals. Neither the antidepressants nor the NK_1 antagonist were able to reduce the stress induced changes of SAM and HPA-axis activity. In fact on some occasions the effects were augmented. Chronic psychosocial stress significantly increased the proliferation of splenocytes following conA activation, but relative spleen weight was not significantly altered. Overall the treatments tended to normalize the indices of immune function, but effects were moderate and mostly non significant.

Monocyte proliferation by the non-specific mitogen concavalin A activation might be a suitable index of immune system integrity. Short term stress decreased the proliferation rate of conA stimulated monocytes (Kubera *et al.*, 1995; Shurin *et al.*, 1994). This blunted response has been attributed to increased concentrations of circulating glycocorticoids following activation of the HPA-axis. In the present study we show that five weeks of chronic psychosocial stress increases the proliferation rate of splenocytes after conA stimulation compared to control animals. As cortisol levels still tended to be increased after five weeks of psychosocial stress in our study, this might indicate that the immune cells have become less sensitive to the elevated cortisol levels (Sterzer *et al.*, 2004). The antidepressants tended to normalize the increased proliferation rate of the spleen cells, however this effect could not be observed for the NK_1 antagonist L-760,735. Involvement of the monoaminergic system is in line with a previous study showing that classic antidepressants dose-dependently reduce the number of lymphocytes in vitro (Audus & Gordon, 1982). It is interesting to see that most of the effects in the present study failed to reach significance and that previous *ex-vivo* data were also ambiguous (Haack *et al.*, 1999).

Antidepressants alleviate many chronic psychosocial stress induced changes in tree shrews (van der Hart *et al.*, 2002; van der Hart *et al.*, 2005), but obviously not the increased activation of the HPA-axis and the sympathetic nervous system in the present study. SAM and HPA-axis influence immune function either through direct innervation, or endocrine activation of glycocorticoid and norepinephrine receptors on immune cells and lymphoid organs. The increased SAM and HPA-axis activity are in keeping with previously published studies in tree shrews (Kramer *et al.*, 1999; van der Hart *et al.*, 2002), however the immune system data from this study are novel for this animal model. Our results are consistent with the idea that during stressful events both the sympathetic system and HPA-axis are activated (Koolhaas *et al.*, 1997) and the fact that this may have consequences for immune function (Padgett & Glaser, 2003).

It is known that the central nervous system (CNS) and immune system communicate through various mechanisms, including direct sympathetic

innervation of the primary and secondary lymphoid organs, hormonal activation by the HPA-axis and molecular mechanisms (Glaser & Kiecolt-Glaser, 2005; Ader *et al.*, 1995;Haas & Schauenstein, 2001). Yet, it is far from clear whether a causal relation exists between the stress-induced increase of SAM/HPA-axis activity and an altered immune function. Because stress precipitates symptoms of depression (Holsboer *et al.*, 1984), and antidepressants are capable of ameliorating symptoms of depression, we have investigated the effect of several antidepressants on stress-induced changes of SAM/HPA-axis activity and immune function. The antidepressants used in this study belong to different pharmacological classes, therefore the outcome of the study could have provided information regarding the neuronal systems involved in the communication between CNS and immune system during psychosocial stress.

On a molecular level, there are many candidates that could play a role in this communication.

I. Increased circulating norepinephrine might modulate immune function through ß-adrenergic receptors that are expressed by all lymphocytes (Nance & Sanders, 2007).

II. Glucocorticoids regulate many aspects of immune cell function. They play a role in the adaptive responses of T-helper cells, inhibiting pro-inflammatory cytokines, like TNF-alpha or interleukin-2 (Glaser & Kiecolt-Glaser, 2005).

III. Serotonin could also play a role because selective serotonin reuptake inhibitors are capable of improving immune system function as well as normalizing HPA-axis dysfunction in depression (Himmerich *et al.*, 2006a).

IV. Antidepressants also attenuate symptoms of depression associated with cytokine (e.g. inteferon-α and IL-2) treatment (Musselman *et al.*, 2001).

V. Substance P promotes inflammation in peripheral tissues, and many immune cell types express receptors for substance P, including NK_1 receptors. Afferents of neurokinine neurons innervate immune organs and activation by substance P up-regulates pro-inflammatory cytokines and influences other immunological processes (Rosenkranz, 2007).

Unfortunately, as our results failed to reach significance, it is not possible to identify one clear candidate for the communication between the CNS and the immune system.

The idea of interaction between the CNS and immune system and a mutual influence of these systems is well accepted. Depressed patients often have an impaired immune system (Maes *et al.*, 1995), as witnessed by an increased liability for infections and an impaired wound healing capacity (Kemeny & Schedlowski, 2007). Conversely, patients with an impaired immune system often show symptoms of depression (Konsman *et al.*, 2002). Furthermore, cytokine treatment

can lead to depressive-like symptoms (Valentine *et al.*, 1998). It is conceivable that feedback and other control mechanisms play an important role in the communication between CNS and immune system. Accordingly, concentrating on splenocyte proliferation and spleen weight alone does not merit the complexity of the CNS-immune system interaction, though it might give an impression of its functionality and the capacity of antidepressants to alleviate stress induced symptoms. Other parameters of the immune system have been investigated in relation to stress and antidepressant treatment, but these data are equally inconclusive. For instance, *in vitro* a major impact of antidepressant treatment on cytokine production has been reported but this could not be replicated *in vivo* (Castanon *et al.*, 2002). It is imaginable that the mutual influence of CNS and immune system is so complex that the involved processes is extremely difficult using classical pharmacological tools as used in the present study.

Conclusion

The present data are in line with previous studies indicating that stress may compromise immune function. It is also clear that our data do not support the idea of a clear-cut relation between increased SAM/HPA-axis activity and impaired immune function, which corroborates previously published *in vivo* studies on this item.

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