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Consequences of chronic social stress on behaviour and vasopressin gene expression in the PVN of DBA/2OlaHsd mice—influence of treatment with the CRHR1-antagonist R121919/NBI 30775

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Abstract

Accumulating evidence suggests that corticotropin-releasing hormone (CRH) neurocircuitry modulate the neuroendocrine and behavioural phenotypes in depression and anxiety. Thus, the administration of the selective CRH-receptor 1 (CRHR1)–antagonist R121919/NBI 30775 has proven its ability to act as an anxiolytic in rats. It is still unclear whether vasopressinergic neuronal circuits, which are known to be involved in the regulation of emotionality, are affected by R121919/NBI 30775. Using DBA/2OlaHsd mice, we investigated the effects of chronic social defeat and concomitant treatment with R121919/NBI 30775 on 1) the behavioural profile in the modified hole board test and 2) in-situ hybridization analysis–based expression of arginine vasopressin (AVP) and CRH mRNA in both the hypothalamic paraventricular nucleus and supraoptic nucleus. The results suggest that chronic social defeat leads to increased avoidance behaviour and reduction in directed exploration, general exploration, and locomotion. Chronic treatment with the CRHR1-antagonist was effective in reversing the directed exploration to control level. The dissection of the antagonist-treated group into responders and non-responders using the parameter *time spent on board* revealed further positive effects of R121919/NBI 30775 on avoidance behaviour and locomotion. Behavioural changes were accompanied by alterations in AVP gene expression in the paraventricular nucleus. Taken together, the anxiolytic action of the CRHR1 antagonist was found in a subgroup of animals only, and further studies have to be done to clarify the inter-individual biological differences in response patterns to this compound to optimise its application under clinical conditions.

Key words

chronic social defeat; CRHR1 antagonist; DBA/2OlaHsd mice; hole board test; PVN; vasopressin

Introduction

Corticotropin-releasing hormone (CRH) is a key neuropeptide in the regulation of hormonal, autonomic, and behavioural response to stress (for review: de Kloet, *et al.*, 2005). There is compelling evidence that overactivity of the CRH-related neurocircuitry, resulting in a characteristic dysfunction of the hypothalamic pituitary adrenocortical (HPA) system, plays a causal role in the development of anxiety and other affective disorders (Holsboer, 2000; Nestler, *et al.*, 2002; Pariante and

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Miller, 2001). Thus, pharmacological strategies targeting the synthesis and effects of CRH via its receptors are considered a promising approach to investigate the pathophysiological processes underlying affective disorders and their treatment (Holsboer, 2003). The CRH exerts its biological function by activating CRH receptors type 1 (CRHR1) and 2 (CRHR2). In animals, the CRHR1 appears to be the primary mediator of CRH-induced anxiogenic and depression-like responses, the function of CRHR2 in this context is less clear (Keck, *et al*., 2005; Muller, *et al*., 2003; Smith, *et al*., 1998). One antagonistic compound that targets CRHR1 with high affinity and functional selectivity is NBI 30775, a pyrrolo[1,5-a]pyrimidine derivative bearing a 3-pyridinyl group, also known as R121919 (Chen, *et al*., 2004; Heinrichs, *et al*., 2002). Previous studies in rodents assessing anxiety- and depression-like behaviour showed that R121919 reduces anxiety-like behaviour in several animal models, such as the elevated plus-maze, in a dose-dependent manner under both basal conditions and in response to stress (Keck, *et al*., 2001; Gutman, *et al*., 2003; Heinrichs, *et al*., 2002; Keck, *et al*., 2003a; Post, *et al*., 2005). R121919 was the first CRHR1 antagonist that was applied in a limited phase IIA clinical trial in Major Depressive Disorder (Zobel, *et al*., 2000). This compound was effective in reducing depressive and anxiety symptoms and in improving the sleep electroencephalogram structure in patients with depressive disorders while maintaining normal peripheral HPA system function and overall good clinical tolerability (Held, *et al*., 2004; Kuenzel, *et al*., 2001).

Previous studies investigated mainly the acute effects of R121919 on anxiety- and depression-related behaviour in different animal stress models. Although R121919 shows anxiolytic properties under the condition of acute stress (for review: Chen and Grigoriadis, 2005), little is known about its behavioural effects under chronic stress conditions. However, chronic stress and maladaptive response of the HPA system to chronic stress is thought to be causally linked to human psychiatric disorders, such as depression and anxiety (Arborelius, *et al*., 1999; Holsboer, 2001). Additionally, the majority of currently available antidepressants are only effective when administered chronically, suggesting that the investigation of novel antidepressive and anxiolytic compounds in chronic treatment schedules is indispensable. Therefore, in the present study, we focused on the behavioural effects of R121919 in an animal model of chronic psychosocial stress. Several studies suggest that this animal model is suitable for the investigation of anxiety- and depression-like behaviour and for the effectiveness of antidepressant treatment (Bhatnagar, *et al*., 2006; Keeney, *et al*., 2006). Furthermore, we chose this stress model because it closely mimics the clinical situation where psychosocial stress is regarded a severe risk factor for the development of depressive and anxiety symptoms (Bjorkqvist, 2001).

The brain sites involved in the CRHR1 antagonist–induced anxiolytic actions are not exactly known but one of the most promising sites is the hypothalamic paraventricular nucleus (PVN). In fact, human studies revealed increased CRH mRNA levels and an elevated number of CRH and CRH/AVP–expressing neurons in the PVN in patients with depression (Raadsheer, *et al*., 1994). The AVP is a second major regulator of HPA system and may be involved in the anxiolytic effects of CRHR1 antagonists (Aguilera and Rabadan-Diehl, 2000; Welt, *et al*., 2006). This neuropeptide is synthesised in both the PVN and the hypothalamic supraoptic nuclei (SON) and is a weak secretagogue, but it acts synergistically with CRH in the stimulation of adrenocorticotropic hormone (ACTH) secretion (Antoni, 1993). Similar to CRH, AVP seems to be critically involved in learning, behaviour, and emotional processes in the brain (Ebner, *et al*., 2002; Engelmann, *et al*., 1996; Keck, *et al*., 2003b). Several studies indicate the particular importance of AVP in maintaining HPA system overactivity under the condition of chronic stress with a predominant expression of AVP but not CRH in PVN neurons (Aguilera and Rabadan-Diehl, 2000). Thus, effects of chronic treatment with a putative antidepressant or anxiolytic substance under a stress test paradigm may involve predominantly AVP-related neurocircuits.

The aim of the present study was to investigate if CRHR1 antagonist exerts anxiolytic effects in a chronic treatment schedule under social defeat in DBA/2OlaHsd (DBA/2) mice. Furthermore, we were interested in changes of AVP mRNA expression compared with CRH expression in the PVN and SON in response to chronic R121919 treatment and its behavioural effects. The behavioural changes were assessed using the modified hole board (mHB) (Ohl, *et al*., 2001a). In contrast to other established tests, the mHB paradigm enables to detect alterations in a wide spectrum of behaviours, including anxiety-related behaviour, exploration, and locomotion in one single test. Therefore, multiple testing with possible confounding effects on behavioural data is dispensable. Furthermore, previous studies suggest that while using the mHB, the investigator is able to dissociate anxiolytic effects from sedative effects and general alterations in exploration, which is of particular interest for studies dealing with new pharmacological agents, such as R121919 (Ohl, *et al*., 2003).

Materials and methods

Animals

The animal studies were conducted in accordance with NIH guidelines and the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany. The experiments were performed with naive, male DBA/2 ($n = 55$, age at the beginning of the experiment 8–9 weeks, body weight 25 ± 5 g) mice. The animals were immediately separated after the delivery from the supplier (Harlan Winkelmann Laboratories, Borchen, Germany) and were housed singly under standard laboratory conditions (12:12 h light/dark cycle with lights on at 7.00 a.m., 22 ± 1°C, 60% humidity, pelleted food and water ad libitum) at least 2 weeks before the experiment. Each animal was used for one experiment only.

Treatment with CRHR1 antagonist and saline

The CRHR1 antagonist 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo [2,3-*a*]pyrimidin-7-amine (NBI30775/R121919; kindly provided by Janssen Pharmaceuticals, Beerse, Belgium) was dissolved in 0.9% saline. To avoid confounding stress-related effects of systemic injections or gavage, the drug solution was administered orally (1 mg/kg) with porridge oats daily during a period of 21 days at 6 p.m. We chose the dosage according to the previous results from a study conducted by Post, *et al.* (2005), where acute administration of 1 mg/kg R121919 in DBA/2 mice exerted clear anxiolytic effects on the mHB, whereas dosages of 0.5 mg/kg and 5 mg/kg were ineffective. The vehicle/control group received porridge oats with saline. Only intruder mice were treated with R121919 or saline. The porridge was served on a plastic plate in the home cage, and the intake was directly monitored by the scientist. Three days prior to the experiment, the animals were adapted to the feeding procedure twice daily. The latency to the porridge intake during the experiment was 5–15 sec.

Social defeat procedure

Male C57BL/6JolaHsd mice, 12 weeks old, were selected on attack latency against a non-aggressive intruder (DBA/2, 8 weeks old) during a 1-week training period prior to the experiment. Only mice with a constant attack latency of less than 30s were included in the experiment as residents. The social defeat procedure consisted of placing a naive DBA/2 male in the home cage (42 × 26 × 15 cm) of the male resident mouse (Martinez, *et al.*, 1998). The intruder was attacked and subdued in all confrontations within the first 10–30 sec after transfer into the resident's cage. The physical interaction was terminated after the sign of submission or "attempt to bite", and the mice were than separated by a wire mesh wall for 24 h. By this procedure, the experimental animal was protected from repeated attacks and potential injuries, whereas it was still exposed to visual, acoustic, and olfactory cues from the resident. Social defeat was considered successful if the intruder showed defensive behaviour as indicated by submissive body postures or freezing behaviour for at least the first 30 min after the physical interaction. The procedure was repeated once daily at 10.00 AM for 21 days with the intruder being rotated daily before the defeat into the next unfamiliar resident cage to prevent adaptation between resident and intruder. The resident animals remained in their home cage throughout the experiment. There is limited evidence for a stable anxiogenic effect of chronic psychosocial defeat in mice. Therefore, with DBA/2 mice we used a strain with high innate anxiety-related behaviour under basal condition, which might present a vulnerable phenotype for the induction of behavioural alterations under chronic stress.

Control mice were housed singly (30 × 20 × 14 cm) and were replaced daily into a new cage but were not exposed to either aggressive or non-aggressive animals.

Experimental protocol

DBA/2 mice were randomly divided into three groups: 1) social defeat over 21 days and saline treatment ($n = 20$), 2) social defeat and concomitant treatment with R121919 ($n = 20$), and 3) control group without social defeat or pharmacological treatment ($n = 15$). Daily social defeat at 10.00 AM and pharmacological or vehicle treatment at 18.00 PM were performed for 21 days in the first two groups. To measure stable behavioural changes and not acute effects of social defeat, the animals had a recovery time on day 22. On day 23, animals were behaviourally tested using the mHB test between 8.00 and 11.00 PM. Immediately after testing, the animals were sacrificed, and the brains were prepared for further expression analysis.

Behavioural assessment

The mHB test was used to evaluate the animals' behavioural profile. The behaviour of each mouse was assessed over a period of 5 min. Test sessions were videotaped and were evaluated by a trained observer who was unaware of the treatments.

The mHB represents a test for unconditioned behaviour, which allows for the assessment of a variety of motivational systems, such as anxiety-related behaviour, activity, and exploration (Ohl, 2003). The mHB test used here combines a hole board and an open field. The hole board (60 × 20 cm) was constructed from opaque gray PVC with 23 holes staggered in three lines; the holes were covered by movable lids (gray PVC), which could be easily opened. During testing, the hole board was placed in the middle of a PVC box (100 × 50 × 50 cm) thus representing the central area of an open field. The outer area was divided into 12 quadrants (20 × 16 cm) demarcated by white lines. Complete details of the testing procedure were described previously by Ohl, *et al.* (2001a). Briefly, the test involves measurement of the following parameters: the latency to the first board entry (*latency board)*, number of entries on board (*entries board*), and the percentage of time spent on the board (*ti%bo*) as indicators for anxiety-related behaviour; the number of rearings (*rearings*) as an indicator of general exploration; the total number of holes visited (*holes visited*) and latency to the first hole visit (*latency hole*) as indicators for directed exploration. The parameter *number of line crossings* indicates locomotor activity.

In-situ hybridization

Biochemical analysis was performed on brain tissues from animals sacrificed immediately after the behavioural test on the mHB, at least 36 h after the last administration of the CRHR1 antagonist. The brains were used for the analysis of stress-induced AVP and CRH gene expression. Frozen brains were sectioned at −20 °C in a cryostat microtome (18 μm) in parallel series at the coronal plane spanning the region of the PVN and SON. The sections were thaw-mounted on glass slides and were stored at −80 °C until use. In-situ hybridization was performed as described previously (Schmidt, *et al.*, 2003)

using ^{35}S-UTP cRNA probes transcribed from plasmids containing fragments of cDNAs encoding mouse CRH and AVP. The slides were exposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, New York) and were developed. For CRH and AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co.) and were exposed at 4 °C for 5 days (AVP) or 10 days (CRH). Slides were developed, counterstained with Toluidine Blue staining, and examined with a light microscope with both bright- and dark-field condensers.

Statistics

Univariate analysis of variance was performed to identify possible group effects. The inequality of variances between the groups was tested with Levene's test. In case of inhomogeneity of variances, pairwise Games–Howell post hoc test was carried out. This test was applied for the behavioural data. The Games–Howell procedure maintains the experimentwise alpha near the nominal level like the Bonferroni test but is considered superior, especially in the case of unequal variances (Jaccard, *et al*., 1984). In case of homogeneity of variances, Student Newman Keuls test was performed, as done for in-situ hybridization data. The significance level was set at $\alpha = 0.05$. Data are presented as means ± SEM. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS; Releases 12, SPSS Inc., Chicago, Illinois 60606, USA).

Results

Behavioural effects of chronic social defeat and CRHR1 antagonist

Analysis of variance across the three main groups (control: c, defeated group: def, defeated, and CRHR1 antagonist–treated group: def/R121919) revealed a significant group effect in the behavioural parameters *rearings in the box* [$F(2,58) = 22.74$, $P < 0.001$], *number of entries on the board* [$F(2,58) = 9.12$, $P < 0.001$], *latency board* [$F(2,58) = 4.58$, $P = 0.01$], *latency hole* [$F(2,58) = 4.61$, $P = 0.01$], *number of holes visited* [$F(2,58) = 10.23$, $P < 0.001$], and *number of line crossings* [$F(2,58) = 25.3$, $P < 0.001$]. We failed to find a significant group effect in the parameter *percentage time on board* due to the big standard deviation, especially in the R121919-treated group. Post hoc analysis via Games–Howell test (Figure 1A, *latency board* and *latency hole* not shown) revealed that chronic social defeat procedure induced increased avoidance behaviour towards the unprotected area as seen in the reduced *number of entries on the board* ($P = 0.006$ vs. control) and increased *latency board* ($P = 0.02$ vs. control), reduction in general exploration (*number of rearings*: $P < 0.001$ vs. control), directed exploration (*the total number of holes visited*: $P < 0.001$ vs. control; increased *latency hole*: $P = 0.03$ vs. control) and locomotion (*the number of line crossings*: $P < 0.001$). The treatment of defeated mice with R121919 displayed a modulating effect on defeat-induced changes in directed exploration (*number of holes visited*: $P = 0.12$ vs. control) and *latency board* ($P = 0.11$ vs. control), although there were no significant effects when compared with the defeated group. R121919 treatment has no significant effects on entries on board on general exploration and locomotion. In all seven parameters, there was no statistical difference between the defeated group and the CRHR1 antagonist–treated group.

Under clinical and experimental conditions the effectiveness of the pharmacological treatment and vulnerability to chronic stress are unequal across individuals. Thus, we extended the data analysis and looked for responder and non-responder in the defeated and CRHR1 antagonist–treated groups. The response criterion was defined as the percentage of the time spent on the board (*ti%bo* > 0.5) for R121919 response and non-response to chronic social defeat. The results are displayed in Figure 1B (*latency board* and *latency hole* not shown). Significant group effects were found for all seven behavioural parameters (*rearings*: $P < 0.001$, *ti%bo*: $P = 0.01$, *latency hole*: $P = 0.01$, *number of holes visited*: $P < 0.001$, *latency board*: $P < 0.001$, *entries on board*: $P < 0.001$, and *line crossings*: $P < 0.001$). In the defeated group, 3 (15%) of 20 animals were non-responder to chronic social defeat by the definition. These animals were similar to controls in the directed exploration, locomotion, *latency board*, and *entries on the board*. Interestingly, there was still a significant reduction in general exploration ($P < 0.001$ vs. control) in the defeat non-responders, whereas the locomotion-related parameter *line crossings* was at the level of control animals. In the R121919-treated group, 11 (55%) of 20 animals were treatment responder by definition. The pharmacological treatment in the responder group was effective to attenuate the chronic social defeat effects in the parameters of avoidance behaviour (*latency board*, *entries on board*, and *ti%bo*), directed exploration (*holes visited*), and locomotion (*line crossings*). In the R121919 responder group, we detected a significant effect in the parameter *entries on board* versus R121919 non-responder ($P = 0.029$) and versus defeat responder ($P = 0.035$). General exploration was still significantly reduced in the R121919 treatment responder group (*rearings*: $P = 0.002$ vs. control). In the directed exploration parameter *latency hole*, there was no statistical difference in R121919 responder and R1219119 non-responder versus controls. All remaining parameters were significantly reduced in the R121919 treatment non-responder group versus the control group ($P < 0.001$ vs. control for all six parameters).

AVP and CRH gene expression in PVN and SON

The mean density of expression of AVP and CRH mRNA within the hypothalamic PVN and SON of control, chronically defeated and R121919-treated mice, as determined by in-situ hybridization, is displayed in the Figure 2 for PVN (c: $n = 8$; def: $n = 12$; def/R12: $n = 14$). The numbers are lower in comparison to the behavioural data, because only high quality slices entered the expression analysis. Regarding the three groups, a significant group effect was found for the expression of AVP in the PVN only [$F(2,26) = 4.38$, $P = 0.02$]. Because of the homogeneity of the variances between the groups, we used

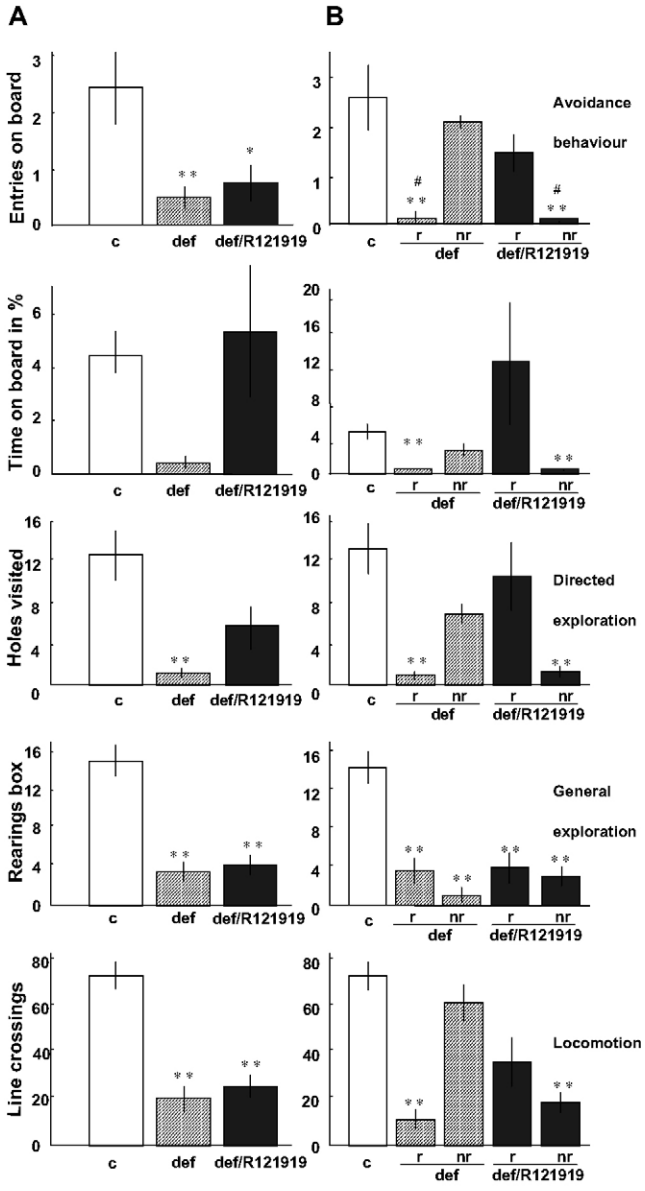

Figure 1 A) Behavioural differences during the exposure to the mHB test on day 23 between the three experimental groups A) control mice (c, white bars, $n = 15$), defeated group (def, striped bars, $n = 20$) and defeated and CRHR1 antagonist treated group (def/R121919, black bars, $n = 20$). Data are expressed as mean value ± SEM. *$P < 0.05$, ** $P < 0.01$ vs. control. B) Behavioural parameters during the exposure to the mHB test on day 23 analysed separately in responder (r) and non-responder (nr) in the defeated group (defr: $n = 17$; defnr: $n = 3$) and defeated and R121919-treated group (def/R121919r: $n = 11$, def/R121919nr: $n = 9$). Data are expressed as mean value ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. control. # $P < 0.05$ vs. responder to R121919.

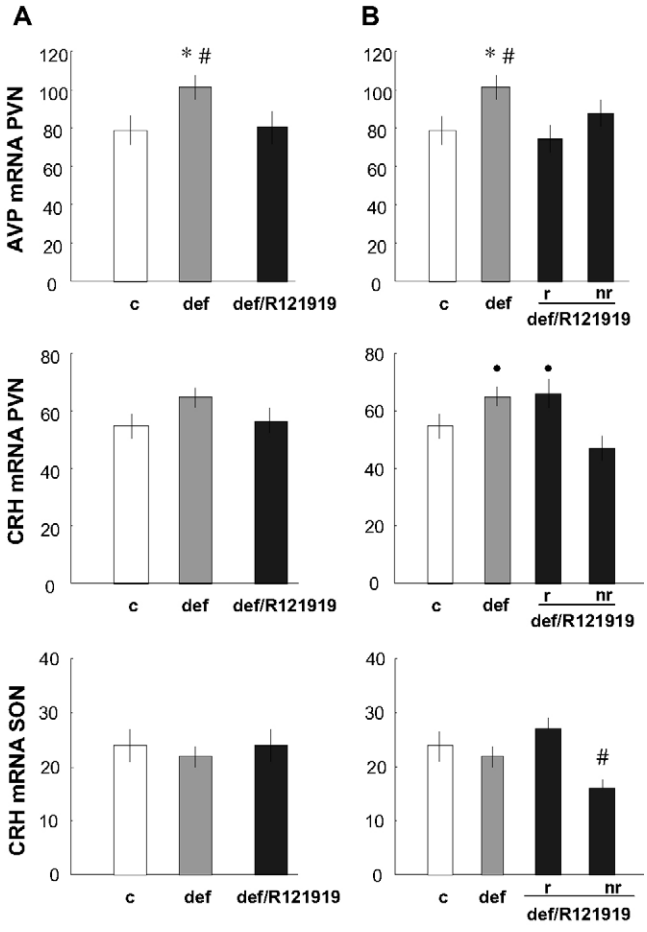

Figure 2 Differences in AVP and CRH mRNA expression in the PVN and CRH mRNA expression in the SON in A) the three experimental groups control (c, white bars, $n = 8$), defeated (def, striped bars, $n = 12$) and CRHR1 antagonist treated mice (def/R121919, black bars, $n = 14$). Data are expressed as mean value ± SEM. * $P < 0.05$ vs. control. # $P < 0.05$ vs. R121919 treatment. B) treatment response groups: control (c: $n = 8$), defeated (def: $n = 12$), CRHR1 antagonist responder (def/R121919r: $n = 8$) and non-responder (def/R121919nr: $n = 6$). Data are expressed as mean value ± SEM. * $P < 0.05$ vs. control. # $P < 0.05$ vs. responder to R121919 treatment. • $P < 0.05$ vs. def/R121919-nonresponder.

Student-Newman-Keuls test for post hoc comparisons. We found a significant increase of AVP mRNA expression in the PVN in the defeated group ($P = 0.02$ vs. control; $P = 0.02$ vs. CRHR1 antagonist treated mice). No statistical differences were found for AVP mRNA expression in the PVN between control and the antagonist treated group. The in-situ microphotographs for the three groups are displayed in the Figure 3.

According to the behavioural data we looked for statistical differences of AVP and CRH expression in responders and non-responders in the group of defeated animals with concomitant CRHR1 antagonist treatment only (c: $n = 8$; def: $n = 12$; def/R12r: $n = 8$; def/R12nr: $n = 6$), the number of defeat non-responder was too low to perform a statistical test ($n = 1$). Significant group effects were found for AVP in the PVN

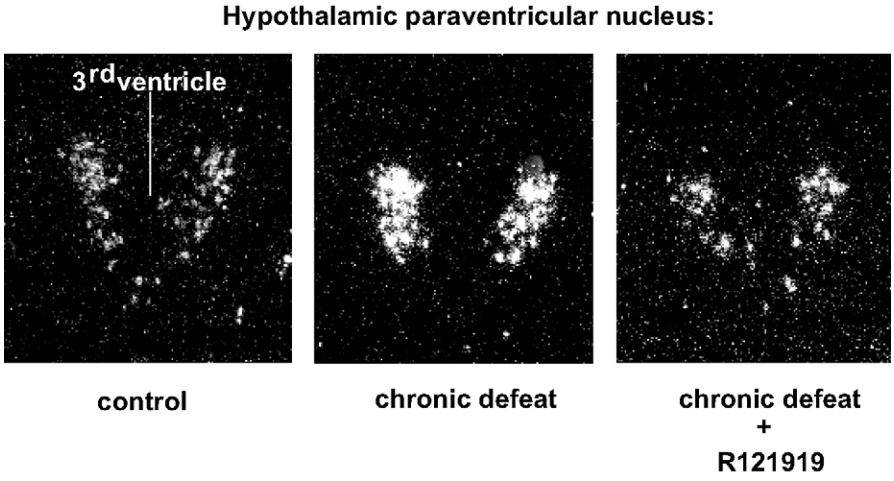

Figure 3 In-situ hybridization microphotographs of AVP mRNA expression in the hypothalamic paraventricular nucleus in the three groups: control, chronic defeat and chronic defeat/R121919-treatment mice.

[$F(3,27) = 5.05$, $P = 0.008$], for CRH expression in the PVN [$F(3,27) = 3.91$, $P = 0.023$] and CRH expression in the SON [$F(3,27) = 4.21$, $P = 0.016$]. Post hoc comparisons revealed a significant increase of AVP mRNA expression in the PVN of defeated mice versus control ($P = 0.01$) and versus responder to the antagonist treatment ($P = 0.01$). No statistical differences were found for AVP mRNA expression in the SON. CRH mRNA expression in the PVN was higher in the defeated mice ($P = 0.03$) and also higher in responders to CRHR1 treatment ($P = 0.04$) when compared to the CRHR1-treatment non-responders. In the SON, CRH mRNA expression was significantly lower in non-responders to the CRHR1 antagonist in comparison to treatment-responder mice ($P = 0.01$).

Discussion

In the present study we focused on the behavioural effects of chronic R121919 treatment under chronic social defeat and the regulation of AVP/CRH expression in both the PVN and SON in DBA/2 mice. Although acute effects of R121919 have been previously shown, its chronic treatment effects under stress condition and concomitant AVP/CRH changes were unclear so far (Chen and Grigoriadis, 2005; Post, *et al*., 2005). The behavioural and molecular effects of potential anxiolytic and antidepressant substances are known to be critically dependent on the choice of a particular mouse strain, the nature of the stress paradigm, and the behavioural tests applied (Blanchard, *et al*., 2001; Ohl, 2003). The results of the present study suggest that chronic social defeat robustly induces anxiogenic behavioural changes as evidenced by the increase of avoidance behaviour and decrease of exploration and locomotion in DBA/2 mice, rendering this stress paradigm a useful model for investigation of anxiety-like behaviour. However, the DBA/2 strain displays high anxiety-related behaviour under basal conditions, and the aptitude of this strain in the chronic social defeat paradigm could be limited by the small amplitude of induced behavioural changes that may be more difficult to measure in the mHB than in the strains with lower anxiety level, such as C57BL/6 (Ohl, *et al*., 2003). However, the stressor applied might be too severe in DBA/2 mice in comparison to the clinically reported stress factors, leading to a relatively high percentage of R121919-treatment non-response (45%). Additional studies with a strain less prone to anxiety would be helpful in specifying the behavioural effects of R121919 under chronic psychosocial stress in mice with different genetic backgrounds and altered anxiety-related behaviour.

In our study the definition of special response criteria was helpful to dissociate the behavioural differences between the treatment groups. We focused on avoidance behaviour because this behaviour was repeatedly shown to be sensitive to compounds with anxiolytic actions in humans, such as benzodiazepines (Chaouloff, *et al*., 1997; Martin, 1998). The response definition brought up a small number of mice (15%), which did not respond to the stress procedure and behaved similarly to the controls concerning the parameters of avoidance behaviour, directed exploration, and locomotion. Taking into the account the different rates of R121919 responder (55%) and defeat non-responder (15%), it is unlikely that defeat non-responders were falsely classified as R121919 responders in the antagonist-treatment group. The differences in vulnerability to uncontrollable chronic stress seen in our experiment has similarity to clinical practice where most, but not all, patients report stress experience as trigger factors for the development of psychiatric symptoms (Caspi, *et al*., 2003; Kendler, *et al*., 2005). Thus, the individual genetic background and gene–environment interactions seem to produce a vulnerable phenotype under experimental and clinical conditions (de Kloet, *et al*., 2005; Holsboer, 2001; Nemeroff and Vale, 2005).

The vulnerability to stress may have additional importance for the efficacy of pharmacological substances targeting the HPA system, such as R121919. The CRHR1 antagonistic compounds may not be effective in all individuals, rendering the analysis of subgroups by defining specific criteria indispensable. Additionally, the investigation of drug-specific effects may have a major effect on the definition of when a drug could be indicated in clinical practice. Regarding the whole treatment group, R121919 was only effective in attenuating the social defeat effects on directed exploration. The definition of responder and non-responder groups using the parameter percentage of *time spent on the board* revealed further positive effects of R121919 on avoidance behaviour and locomotion in the responder group. Interestingly, Post, *et al.* (2005) described positive effects of acute R121919 treatment (1 mg/kg dose) on avoidance and directed exploration behaviour, but not on general exploration, in DBA/2 mice too. Thus, acute and chronic treatments with R121919 display similar behavioural effects in the mHB paradigm when only the responder group in our experiment is considered. This finding suggests a more immediate anxiolytic mode of action of this compound in comparison to other antidepressant substances, which display a longer latency to the onset of action. One limitation of the study is that we applied only one dose of the compound. Further studies are indispensable to find out whether or not we can increase the anxiolytic efficacy of R121919 at higher dosages. It is important to note that the parameters of avoidance behaviour could be influenced by locomotor activity. However, Ohl, *et al.* (2001b) could show by factor analysis in a ratline with high anxiety-related behaviour that decreased general activity and anxiety-related behaviour are not causally related. In the same ratline, the anxiolytic effects of R121919 had no consequences on locomotor activity in comparison to vehicle-treated rats in the open-field test (Keck, *et al.*, 2001). In the present study, the finding that locomotion was reversed to control conditions only in R121919-responder mice suggests a primary effect of this compound on anxiety-related behaviour rather than a general effect on locomotion.

Several preclinical and clinical studies point to an important role of AVP in the regulation of CRH-related circuits, HPA system function, and behaviour (Aguilera and Rabadan-Diehl, 2000). Increased AVP gene expression in the PVN was described not only in different chronic stress animal models but also in depressed suicide patients (Merali, *et al.*, 2006; Nakase, *et al.*, 1998). In our experiment, we found an increase of AVP mRNA expression in the PVN, but not in the SON, in response to chronic psychosocial stress. The elevation of AVP mRNA in the PVN under chronic social defeat condition was also shown by Keeney, *et al.* (2006) in a modified defeat protocol. Treatment with R121919 restored AVP gene expression to control levels in the whole group. This is somewhat surprising as the behavioural effects of R121919 were evident only in one single behavioural parameter, i.e. directed exploration, and the dissection into R121919 responder and non-responder group revealed no differences in AVP gene expression patterns. These results suggest that the behavioural effects of R121919 may not depend on the modulation of intra-PVN AVP gene expression. Thus, the site-specific changes of AVP expression in the PVN in response to chronic stress and to R121919 treatment seem to be secondary and not primarily related to the behavioural changes observed in our study.

Stress-induced regulation of CRH gene expression in the PVN also showed an increase but failed to reach statistical significance. No effects were found in the SON. Interestingly, CRH mRNA expression in the PVN was significantly lower in the R121919 non-responder group in comparison to R121919 responders and defeated animals. The same phenomenon was observed in the SON between R121919 responders and non-responders. The mRNA expression analysis was performed with a lower number of animals, which limits the interpretation of the results. One possible explanation is that the prerequisite for the efficacy of CRHR1 antagonism at the behavioural level is a strong activation of the HPA system and CRH-related circuits to chronic stress. It was repeatedly shown that R121919 has attenuating properties on ACTH and glucocorticoid release under stress conditions (Gutman, *et al.*, 2003; Keck, *et al.*, 2003a) and that the anxiolytic effects of R121919 are correlated with competitive blockade of CRHR1 (Heinrichs, *et al.*, 2002). Therefore, it is conceivable that individuals with a high reactivity of CRH-related circuits and maladaptation processes of the HPA system under chronic stress may display a more favourable therapeutic response to CRHR1 antagonistic compounds than individuals with a low vulnerability to stress at the HPA system level.

Taken together, the chronic social defeat procedure leads to alterations in anxiety-related behaviour. The R121919 displays anxiolytic effects under the experimental condition of chronic psychosocial stress; however, both effects are prominent in a subgroup only. The results suggest that R121919 treatment may be more beneficial in patients with a hyperactivity of central CRH circuits and that the behavioural effects of R121919 may not depend on the modulation of AVP gene expression in the PVN. Further studies are required to confirm the hypothesis that patients with prominent alterations in central CRH circuits, for instance as evidenced in the combined dexamethasone/CRH test in patients suffering form panic disorder (Holsboer, 2000; Erhardt, *et al.*, 2006), may benefit from CRHR1-antagonist treatment and therefore might be the most promising target group for this treatment approach.

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References

Aguilera, G, Rabadan-Diehl, C (2000) Vasopressinergic regulation of the hypothalamic-pituitary- adrenal axis: implications for stress adaptation. Regul Pept *96*: 23–29.

Antoni, FA (1993) Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. Front Neuroendocrinol *14*: 76–122.

Arborelius, L, Owens, MJ, Plotsky, PM, Nemeroff, CB (1999) The role of corticotropin–releasing factor in depression and anxiety disorders. J Endocrinol *160*: 1–12.

Bhatnagar, S, Vining, C, Iyer, V, Kinni, V (2006) Changes in hypothalamic-pituitary-adrenal function, body temperature, body weight and food intake with repeated social stress exposure in rats. J Neuroendocrinol *18*: 13–24.

Bjorkqvist, K (2001) Social defeat as a stressor in humans. Physiol Behav *73*: 435–442.

Blanchard, RJ, McKittrick, CR, Blanchard, DC (2001) Animal models of social stress: Effects on behavior and brain neurochemical systems. Physiol Behav *73*: 261–271.

Caspi, A, Sugden, K, Moffitt, TE, Taylor, A, Craig, IW, Harrington, H, McClay, J, Mill, J, Martin, J, Braithwaite, A, Poulton, R (2003) Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. Science *301*: 386–389.

Chaouloff, F, Durand, M, Mormede, P (1997) Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav Brain Res *85*: 27–35.

Chen, C, Grigoriadis, DE (2005) NBI 30775 (R121919), an orally active antagonist of the corticotropin-releasing factor (CRF) type-1 receptor for the treatment of anxiety and depression. Drug Dev Res *65*: 216–226.

Chen, C, Wilcoxen, KM, Huang, CQ, Xie, YF, McCarthy, JR, Webb, TR, Zhu, YF, Saunders, J, Liu, XJ, Chen, TK, Bozigian, H, Grigoriadis, DE (2004) Design of 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazol o[1,5-a]pyrimidine (NBI 30775/R121919) and structure-activity relationships of a series of potent and orally active corticotropin-releasing factor receptor antagonists. J Med Chem *47*: 4787–4798.

de Kloet, ER, Joels, M, Holsboer, F (2005) Stress and the brain: From adaptation to disease. Nat Rev Neurosci *6*: 463–475.

Ebner, K, Wotjak, CT, Landgraf, R, Engelmann, M (2002) Forced swimming triggers vasopressin release within the amygdala to modulate stress-coping strategies in rats. Eur J Neurosci *15*: 384–388.

Engelmann, M, Wotjak, CT, Neumann, I, Ludwig, M, Landgraf, R (1996) Behavioral consequences of intracerebral vasopressin and oxytocin: Focus on learning and memory. Neurosci Biobehav Rev *20*: 341–358.

Erhardt, A, Ising, M, Unschuld, PG, Kern, N, Lucae, S, Putz, B, Uhr, M, Binder, EB, Holsboer, F, Keck, ME (2006) Regulation of the hypothalamic-pituitary-adrenocortical system in patients with panic disorder. Neuropsychopharmacology *31*: 2515–2522.

Gutman, DA, Owens, MJ, Skelton, KH, Thrivikraman, KV, Nemeroff, CB (2003) The corticotropin-releasing factor(1) receptor antagonist R121919 attenuates the behavioral and endocrine responses to stress. J Pharmacol Exp Ther *304*: 874–880.

Heinrichs, SC, De Souza, EB, Schulteis, G, Lapsansky, JL, Grigoriadis, DE (2002) Brain penetrance, receptor occupancy and antistress in vivo efficacy of a small molecule corticotropin releasing factor type I receptor selective antagonist. Neuropsychopharmacology *27*: 194–202.

Held, K, Kunzel, H, Ising, M, Schmid, DA, Zobel, A, Murck, H, Holsboer, F, Steiger, A (2004) Treatment with the C RH1-receptor-antagonist R121919 improves sleep-EEG in patients with depression. J Psychiatr Res *38*: 129–136.

Holsboer, F (2000) The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology *23*: 477–501.

Holsboer, F (2001) Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. J Affect Disord *62*: 77–91.

Holsboer, F (2003) High-quality antidepressant discovery by understanding stress hormone physiology. Ann N Y Acad Sci *1007*: 394–404.

Jaccard, J, Becker, MA, Wood, G (1984) Pairwise Multiple Comparison Procedures - a Review. Psychol Bull *96*: 589–596.

Keck, ME, Ohl, F, Holsboer, F, Muller, MB (2005) Listening to mutant mice: a spotlight on the role of CRF/CRF receptor systems in affective disorders. Neurosci Biobehav Rev *29*: 867–889.

Keck, ME, Welt, T, Muller, MB, Landgraf, R, Holsboer, F (2003a) The high-affinity non-peptide CRH1 receptor antagonist R121919 attenuates stress-induced alterations in plasma oxytocin, prolactin, and testosterone secretion in rats. Pharmacopsychiatry *36*: 27–31.

Keck, ME, Welt, T, Muller, MB, Uhr, M, Ohl, F, Wigger, A, Toschi, N, Holsboer, F, Landgraf, R (2003b) Reduction of hypothalamic vasopressinergic hyperdrive contributes to clinically relevant behavioral and neuroendocrine effects of chronic paroxetine treatment in a psychopathological rat model. Neuropsychopharmacology *28*: 235–243.

Keck, ME, Welt, T, Wigger, A, Renner, U, Engelmann, M, Holsboer, F, Landgraf, R (2001) The anxiolytic effect of the CRH1 receptor antagonist R121919 depends on innate emotionality in rats. Eur J Neurosci *13*: 373–380.

Keeney, A, Jessop, DS, Harbuz, MS, Marsden, CA, Hogg, S, Blackburn-Munro, RE (2006) Differential effects of acute and chronic social defeat stress on hypothalamic-pituitary-adrenal axis function and hippocampal serotonin release in mice. J Neuroendocrinol *18*: 330–338.

Kendler, KS, Kuhn, JW, Vittum, J, Prescott, CA, Riley, B (2005) The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression – A replication. Arch Gen Psychiatry *62*: 529–535.

Kuenzel, HE, Zobel, AW, Nickel, T, Ackl, N, Uhr, M, Ising, M, Sonntag, A, Holsboer, F (2001) Treatment with the CRH-1-receptor antagonist R121919 shows a high safety in the HPA system and clinical routine. Biol Psychiatry *49*: 51S–51S.

Martin, P (1998) Animal models sensitive to anti-anxiety agents. Acta Psychiatr Scand *98*: 74–80.

Martinez, M, Calvo-Torrent, A, Pico-Alfonso, MA (1998) Social defeat and subordination as models of social stress in laboratory rodents: A review. Aggress Behav *24*: 241–256.

Merali, Z, Kent, P, Du, LS, Hrdina, P, Palkovits, M, Faludi, G, Poulter, MO, Bedard, T, Anisman, H (2006) Corticotropin-releasing hormone, arginine vasopressin, gastrin-releasing peptide, and neuromedin B alterations in stress-relevant brain regions of suicides and control subjects. Biol Psychiatry *59*: 594–602.

Muller, MB, Zimmermann, S, Sillaber, I, Hagemeyer, TP, Deussing, JM, Timpl, P, Kormann, MSD, Droste, SK, Kuhn, R, Reul, J, Holsboer, F, Wurst, W (2003) Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat Neurosci *6*: 1100–1107.

Nakase, S, Kitayama, I, Soya, H, Hamanaka, K, Nomura, J (1998) Increased expression of magnocellular arginine vasopressin

mRNA in paraventricular nucleus of stress-induced depression-model rats. Life Sci *63*: 23–31.

Nemeroff, CB, Vale, WW (2005) The neurobiology of depression: Inroads to treatment and new drug discovery. J Clin Psychiatry *66*: 5–13.

Nestler, EJ, Barrot, M, DiLeone, RJ, Eisch, AJ, Gold, SJ, Monteggia, LM (2002) Neurobiology of depression. Neuron *34*: 13–25.

Ohl, F (2003) Testing for anxiety. Clin Neurosci Res *3*: 233–238.

Ohl, F, Holsboer, F, Landgraf, R (2001a) The modified hole board as a differential screen for behavior in rodents. Behav Res Methods Instrum Comput *33*: 392–397.

Ohl, F, Roedel, A, Binder, E, Holsboer, F (2003) Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. Eur J Neurosci *17*: 128–136.

Ohl, F, Toschi, N, Wigger, A, Henniger, MSH, Landgraf, R (2001b) Dimensions of emotionality in a rat model of innate anxiety. Behav Neurosci *115*: 429–436.

Pariante, CM, Miller, AH (2001) Glucocorticoid receptors in major depression: Relevance to pathophysiology and treatment. Biol Psychiatry *49*: 391–404.

Post, A, Ohl, F, Almeida, OFX, Binder, EB, Rucker, M, Welt, S, Binder, E, Holsboer, F, Sillaber, I (2005) Identification of molecules potentially involved in mediating the in vivo actions of the corticotropin-releasing hormone receptor 1 antagonist, NBI30775 (R121919). Psychopharmacology *180*: 150–158.

Raadsheer, FC, Hoogendijk, WJG, Stam, FC, Tilders, FJH, Swaab, DF (1994) Increased Numbers of Corticotropin-Releasing Hormone Expressing Neurons in the Hypothalamic Paraventricular Nucleus of Depressed-Patients. Neuroendocrinology *60*: 436–444.

Schmidt, M, Oitzl, MS, Muller, MB, Ohl, F, Wurst, W, Holsboer, F, Levine, S, De Kloet, ER (2003) Regulation of the developing hypothalamic-pituitary-adrenal axis in corticotropin releasing hormone receptor 1-deficient mice. Neuroscience *119*: 589–595.

Smith, GW, Aubry, JM, Dellu, F, Contarino, A, Bilezikjian, LM, Gold, LH, Chen, RP, Marchuk, Y, Hauser, C, Bentley, CA, Sawchenko, PE, Koob, GF, Vale, W, Lee, KF (1998) Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron *20*: 1093–1102.

Welt, T, Engelmann, M, Renner, U, Erhardt, A, Muller, MB, Landgraf, R, Holsboer, F, Keck, ME (2006) Temazepam triggers the release of vasopressin into the rat hypothalamic paraventricular nucleus: Novel insight into benzodiazepine action on hypothalamic-pituitary-adrenocortical system activity during stress. Neuropsychopharmacology *31*: 2573–2579.

Zobel, AW, Nickel, T, Kunzel, HE, Ackl, N, Sonntag, A, Ising, M, Holsboer, F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J Psychiatr Res *34*: 171–181.