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Knockout of the norepinephrine transporter and pharmacologically diverse antidepressants prevent behavioral and brain neurotrophin alterations in two chronic stress models of depression

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Abstract

Diverse factors such as changes in neurotrophins and brain plasticity have been proposed to be involved in the actions of antidepressant drugs (ADs). However, in mouse models of depression based on chronic stress, it is still unclear whether simultaneous changes in behavior and neurotrophin expression occur and whether these changes can be corrected or prevented comparably by chronic administration of ADs or genetic manipulations that produce antidepressant-like effects such as the knockout (KO) of the norepinephrine transporter (NET) gene.

Here we show that chronic restraint or social defeat stress induce comparable effects on behavior and changes in the expression of neurotrophins in depression-related brain regions. Chronic stress caused down-regulation of BDNF, NGF and NT-3 in hippocampus and cerebral cortex and up-regulation of these targets in striatal regions. In wild-type mice, these effects could be prevented by concomitant chronic administration of five pharmacologically diverse ADs. In contrast, NETKO mice were resistant to stress-induced depressive-like changes in behavior and brain neurotrophin expression. Thus, the resistance of the NETKO mice to the stress-induced depression-associated behaviors and biochemical changes highlight the importance of noradrenergic pathways in the maintenance of mood. In addition, these mice represent a useful model to study depression-resistant behaviors, and they might help to provide deeper insights into the identification of downstream targets involved in the mechanisms of antidepressants.

Keywords

Mouse models of depression; norepinephrine transporter knockout; neurotrophins; behavior

The precise neurobiological processes involved in depression are still unclear and it is largely unknown why it takes up to 6 weeks for antidepressant drugs (ADs) to exert their clinical antidepressant effects. Most ADs cause an acute increase in synaptic monoamine

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concentration in the central nervous system through inhibition of either the norepinephrine transporter (NET; e.g., the selective norepinephrine reuptake inhibitor [SNRI] reboxetine) or the serotonin transporter (e.g. the selective serotonin reuptake inhibitor [SSRI] citalopram) or of both transporters (e.g., the tricyclic AD imipramine) (Bönisch and Bruss, 2006; Manji *et al.* 2003). These primary pharmacological effects, however, cannot explain the delay in the onset of their therapeutic actions, and even less the clinical efficacy of some “atypical” ADs, which inhibit neither the transporters nor the metabolizing enzymes of monoamines such as tianeptine (Kasper and McEwen, 2008) or trimipramine (Berger and Gastpar, 1996).

Recently, several ADs including tianeptine have been demonstrated to increase cell proliferation and neurogenesis in distinct brain regions, e.g. the hippocampus (Czeh *et al.* 2001; Malberg *et al.* 2000). Furthermore, chronic AD administration has been shown to block down-regulation of the neurotrophin brain-derived neurotrophic factor (BDNF) mRNA in the rat hippocampus in response to chronic stress (Nibuya *et al.* 1995). These observations led to the hypothesis that chronic stress-induced depression may involve neurodegeneration and implicated that ADs increase neuronal regeneration, plasticity and neurotrophin expression (Berton and Nestler, 2006; Cunningham and Watson, 2008). While most of these studies have focussed on the role of BDNF (see Krishnan and Nestler, 2008), little is known about other neurotrophins such as nerve growth factor (NGF) and neurotrophin-3 (NT-3). NGF and NT-3 are both essential neurotrophins in the brain. NGF is widely distributed in the central nervous system (CNS); it plays an important role in the development and maintenance of neurons where it exerts trophic effects (Tucker *et al.* 2001). NT-3 is crucial for the differentiation and proliferation of neurons in the CNS (Zhou und Rush 1996). Both neurotrophins are thought to be potentially involved in the development of depression and in mediating antidepressant action (Schulte-Herbrüggen *et al.* 2006; Otsuki *et al.* 2008).

Conditional or inducible knockout of the gene encoding BDNF from forebrain regions has been shown to block AD effects (Monteggia *et al.* 2004; Monteggia, 2007), a result which strongly supports the antidepressant action of BDNF. Among several mouse models of depression (Cryan and Holmes, 2005; Kalueff *et al.* 2007), chronic stress models such as chronic restraint stress (Kim and Han, 2006) or chronic social defeat stress (Kudryavtseva and Avgustinovich, 1998) seem to be the most promising. While 10-day social defeat mainly produces anxiety, chronic social stress for 20 days leads to depression (Avgustinovich *et al.* 2003). In fact, chronic stress is a key factor in the development of depression (Bale, 2006; de Kloet *et al.* 2005). Whether both kinds of chronic stress cause comparable changes in the expression of BDNF and other neurotrophins is largely unknown.

Recently, NET knockout (NETKO) mice have been developed (Wang *et al.* 1999; Xu *et al.* 2000). In the tail suspension test, NETKO mice resembled that of wild-type controls treated with ADs either acutely (Xu *et al.* 2000) or for several days (Dziedzicka-Wasylewska *et al.* 2006). In a study by Perona *et al.* (2009) NETKO mice showed a reduction in immobility time in the FST and TST compared to wild-type mice but an unaltered consumption of sucrose. The results for FST and TST are in accordance with previous results of Xu *et al.* (2000) and Dziedzicka-Wasylewska *et al.* (2006). In a recent study, we could demonstrate NETKO-induced brain region-specific changes in the expression of some neurotrophins (Haenisch *et al.* 2008). In brief, we detected NETKO-induced changes for NT-3 in olfactory bulb, brainstem and whole brain at the mRNA and for olfactory bulb at the protein level and for NGF mRNA and protein in olfactory bulb, cerebellum and brainstem. BDNF levels, however, remained largely unaltered.

Here, we report that in wild-type mice two forms of chronic stress, restraint and social defeat, induce depression-like behavioral changes and brain region-specific changes in the

expression of BDNF, NGF and NT-3, that can be prevented by pharmacologically diverse ADs whereas NETKO mice are resistant to the effects of chronic stress.

Materials and methods

Animals

Heterozygous NET^{+/-} mice (genetic background C57BL/6J) were transferred from Duke University, Medical Center, Durham, NC, USA and mated to produce homozygous NET^{+/+} (wild-type) and NET^{-/-} (NETKO) mice as previously described (Xu *et al.* 2000). For all experiments we used age-matched adult (3–5 months) male littermates with the genetic background C57BL/6J. Prior to stress procedures mice were housed in groups of 3–5 animals per cage.

The genotypes were confirmed by PCR as described previously (Haenisch *et al.* 2008). The animals were housed at a constant room temperature (24 ± 1°C) under an automatic 12-h light/dark cycle (light on at 07:00) with ad libitum access to food and water. Animals were handled according to the guidelines of the European Union (86/609/EEG) and protocols in this study were approved by a governmental Animal Care Committee.

Antidepressant treatment

Mice received different ADs (reboxetine, trimipramine, citalopram, bupropion, imipramine; 20mg/kg each) or equivalent volumes of saline by daily i.p. injections for 21 days. The amount of the drug was adopted from the literature (Billes and Cowley, 2007; Dziedzicka-Wasylewska *et al.* 2006; Öztürk *et al.* 2006). Antidepressants were given immediately before exposure to stress.

Tissue collection

Brains from decapitated wild-type and NETKO mice were rapidly removed and brain regions (whole cerebral cortex, brainstem, hippocampus [including dentate gyrus and CA1-CA3 regions], striatum and hypothalamus) were dissected on ice. Brain regions were collected after the last stress session and subsequent behavioral test. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

qPCR

qPCR was essentially carried out as described previously (Haenisch *et al.* 2008). In brief, RNA from brain regions was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Total RNA (2µg per sample) was reverse transcribed (RevertAid cDNA Synthesis Kit, Fermentas, St. Leon-Rot, Germany; using random hexamer primers). For qPCR 35µl of amplification mixture (QuantitectSYBRGreen Kit, Qiagen) was used containing 20 ng reverse transcribed RNA and 300nM primers. Primer sequences used were: 5'-TCCATCATGAAGTGTGACGT-3' and 5'-GAGCAATGATCTTGATCTTCAT-3' sense and antisense primers for β-actin, 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'-sense and antisense primers for GAPDH, 5'-TGACACTGGTAAAACAATGCA-3' and 5'-GGTCCTTTTCACCAGCAAGCT-3' sense and antisense primers for HPRT1, 5'-CCTTACTATGGTTATTTCACTTCGGTT-3' and 5'-TCAGCCAGTGATGTCGTCGTC-3' sense and antisense primers for BDNF, 5'-TGATCGGCGTACAGGCAGA-3' and 5'-GAGGGCTGTGTCAAGGGAAT-3' sense and antisense primers for NGF, 5'-CCGGTGGTAGCCAATAGAACC-3' and 5'-GCTGAGGACTTGTCGGTCAC-3' sense and antisense primers for NT-3. Reactions (triplicates, 10 µl) were run on an Mx 3000P real-time cyler (Stratagene, Amsterdam, Netherlands) using the previously described cycling conditions (Haenisch *et al.* 2008): 15 min polymerase activation at 95°C and 45 cycles at 95°C for 30 s, at 58°C for 30 s and at

72°C for 30 s. Each assay included negative controls and a standard curve for each gene. The identity of the PCR products was confirmed by melt point analysis after each real-time reaction and then by agarose gel electrophoresis and by dideoxy chain termination sequencing. The relative mRNA expression was calculated from the ratio treatment group (tg)/control (con) according to Pfaffl (2001) and as described previously (Haenisch *et al.* 2008) (see Eqs. 1–3). The relative quantity was determined using the mean calculated efficiency (E) (Eq. 1) of all experiments. This parameter was measured with standard curves for each gene including the three house-keeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β -actin and HPRT 1 (hypoxanthine phosphoribosyl-transferase 1). The result for each target gene was normalized separately by means of the results of the three house-keeping genes and displayed as $\log_2$ -values (Eq. 3) and corresponding fold-values.

$$E=10^{-1/\text{slope}} \quad \text{Eq. 1}$$

$$R=E^{C_{\text{tcon}}-C_{\text{tgt}}} \quad \text{Eq. 2}$$

$$\log_2(\text{Ratio})=\log_2(R_{\text{target}}/R_{\text{housekeeper}}) \quad \text{Eq. 3}$$

ELISAs

ELISA assays were carried out as described previously (Haenisch *et al.* 2008). Protein levels of NT-3, NGF and BDNF were measured using two-site ELISAs (Promega, Mannheim, Germany). All antibodies used were purchased from Promega (Mannheim, Germany). Tissue samples were weighed and homogenized by ultrasonication in lysis buffer. Samples were centrifuged at 6000g for 15 min at 4°C and supernatants diluted with “Block and Sample Buffer” (B&S B) provided in the kit. For quantification of NT-3, Nunc Maxisorp 96-well plates were coated with anti-human NT-3 polyclonal antibody (pAb) and incubated overnight at 4°C. After blocking the plate with B&S B for 1 h, diluted samples and standards were added and shaken for 6 h at room temperature (RT). The antigen was then incubated with anti-NT-3 monoclonal Ab (mAb) overnight at 4°C. Thereafter, anti-mouse IgG horseradish peroxidase (HRP)-conjugate was added and incubated for 2.5 h at RT. The following incubation with tetramethylbenzidine (TMB) was stopped after 10 min using 1M HCl. Absorbance was measured at 450 nm using a microplate reader.

Quantification of NGF started with coating the plate with anti-NGF pAb. After an overnight incubation at 4°C the plate was blocked with B&S B for 1 h. Then diluted samples and standards were added and shaken for 6 h at RT. The anti-NGF mAb was added and incubated overnight at 4°C. After the incubation of anti-rat IgG HRP-conjugate for 2.5 h at RT and with orbital shaking, the substrate TMB was added. The reaction was stopped with 1M HCl and absorbance recorded at 450 nm.

BDNF was quantified as follows: anti-BDNF mAb was used for coating the plate overnight at 4°C. After blocking the plate with B&S B for 1 h, diluted samples and standards were added and shaken for 2 h at RT. The antigen was then incubated with an anti-human BDNF pAb for 2 h at RT with orbital shaking. Then anti-IgY HRP-conjugate was added and incubated for 1 h at RT with orbital shaking. The substrate TMB was added and the reaction stopped with 1M HCl. The reaction product was measured at 450 nm.

The assay sensitivity ranged from 4.6 to 300 pg/ml for NT-3, from 7.8 to 250 pg/ml for NGF, and from 15.6 to 500 pg/ml for BDNF. The cross-reactivity with the other tested neurotrophins was <3% in each case. The recoveries for added NT-3, NGF and BDNF were 90.8 ± 1.9 , 88.1 ± 1.2 and 92.7 ± 2.2 %, respectively (n= 4 each).

Mouse models of depression and behavioral evaluation

Restraint stress—Wild-type and NETKO mice were subjected to restraint stress by placing them in well-ventilated polypropylene-tubes (internal diameter 2.7 cm, length 11.5 cm). The test duration was 4 h daily for 21 days. During immobilization stress mice did not have access to food and water.

Social defeat stress—The social defeat stress model of depression is based on the resident-intruder paradigm (Berton *et al.* 2006; Kudryavtseva and Avgustinovich, 1998). Wild-type and NETKO mice (test-mice) were exposed to an adult (2–3 months) male CD1 aggressor mouse for 10 min each day over a total of 21 days. After contact, the test-mouse was separated from the CD1-mouse using a cage that was divided into two parts by a perforated Plexiglas divider. Thus, the test-mouse and the CD1-mouse were maintained in visual and sensory contact for the next 24 h without physical contact. Every day the test-mouse was exposed to a new resident cage. Unstressed control mice were housed in equivalent cages with mice of the same strain. CD1 aggressor mice were selected for their level of aggressiveness by counting the latency to attack a naive C57BL/6J mouse. CD1 mice were selected as aggressor mice when they attacked within 2 min in 4 independent experiments. CD1 aggressor mice were housed alone one week prior to the social defeat stress test to make sure they accepted their cage as home cage.

Forced swim test—Mice were individually placed into a transparent glass cylinder (20 cm diameter) filled with warm water (25–26°C). The waterline was 20 cm above the cylinder bottom and the cylinder top 15 cm above the waterline. Immobility was defined as making only those movements necessary to keep the head above water (Porsolt *et al.* 1977). Immobility was measured during the last 4 min of a 6 min test session. Two experienced observers who were not aware of the genotype, stress procedure or drug administration performed the test 24 h after the last stress procedure. To address the problem that the FST might be interpreted as being open to the confounding effects of hyperactivity and other behavioral changes we further examined another model, the sucrose preference test. The sucrose preference test covers another aspect of depressive behavior, anhedonia, and has been used to appraise the effects of different models of depression (El Yacoubi *et al.* 2003; Papp *et al.* 1991).

Sucrose preference test—Mice were given free choice between 1% sucrose-solution and tap water for 24 h. One week before starting, test-mice had been acclimatized to two-bottle choice conditions. To prevent possible effects of side preference in drinking behaviour, the position of the bottles was switched after 12 h. No previous food or water deprivation was applied before the test. The consumption of tap water and sucrose-solution was estimated simultaneously in control and experimental groups by weighing the bottles. The preference for sucrose was calculated as percentage of consumed sucrose-solution to the total amount of liquid drunk.

Social interaction test—We used a video tracking system to record behavioral consequences (social interaction/avoidance) towards an unfamiliar CD1 aggressor mouse. Test-mice were placed into a test-cage (42 × 42 × 30 cm) with an additional cage (12 × 10 × 6 cm) including a CD1 aggressor mouse at one end. The test-cage was divided into different zones including the interaction zone (10 cm surrounding the CD1 mouse cage) and the

corners (12 × 12 cm opposite of the CD1 mouse cage). The test-mouse was put into the cage on the side opposite to the CD1 aggressor mouse. One test-session lasted 5 min. Automatic video-tracking system Videomot2 (TSE-Systems, Germany) was used to evaluate the time spent in different zones of the test-cage.

Data analysis

Results were analysed by means of Prism 5.0 (GraphPad, San Diego, USA). Data are presented as mean ± SEM. Statistical analysis was performed by using the two-tailed Student's t-test or one-way ANOVA (analysis of variance) followed by Dunnett's post hoc test.

Materials

Bupropion hydrochloride, citalopram hydrobromide, imipramine hydrochloride and reboxetine mesylate hydrate were obtained from Sigma-Aldrich (Germany). Trimipramine mesylate was a gift from Aventis (Germany).

Results

Chronic stress for 21 days caused a significant loss of body weight in wild-type mice, namely by 4.7 ± 3.1 % (n=7) under restraint and by 7.6 ± 3.1 % (n=7) under social defeat stress, whereas the body weight of unstressed wild-type mice increased by 5.9 ± 3.0 % (n=14). In NETKO mice, however, restraint and social defeat stress did not significantly affect body weight compared to unstressed NETKO controls. This analysis was done using the paired Student's t-test.

Effects of NETKO and different classes of antidepressants on behavioral consequences after exposure to different stressors

Forced swim test (FST)—Immobility time was significantly lower in unstressed saline-treated NETKO mice (91.6 ± 8.7 s) compared to unstressed saline-treated wild-type controls (144.1 ± 7.7 s; n=9–10; t-test: $p < 0.001$; Fig 1). In reboxetine-treated stressed wild-type mice immobility time in the FST was significantly lower after 21 days of restraint or social defeat stress (85.3 ± 14.1 s and 120.8 ± 10.3 s, respectively) compared to saline-treated restraint or social defeat stressed wild-type mice (202.0 ± 2.7 s and 208.7 ± 3.0 s, respectively; Fig 1a and b). Unstressed saline-treated wild-type mice displayed a reduced immobility time in comparison to saline-treated restraint or social defeat stressed mice, respectively (Fig. 1a and b). In NETKO mice, however, immobility time was not significantly influenced by reboxetine treatment compared to saline treatment after 21 days of restraint or social defeat stress. Moreover, the tricyclic AD imipramine - like reboxetine - did not cause an additional effect in immobility time in restraint or social defeat stressed NETKO mice (see Supplementary Table S1). Saline-treated unstressed NETKO mice showed no significant difference in immobility time compared to restraint or social defeat stressed NETKO mice (Fig. 1a and b). Responses of social defeat stressed wild-type mice in the FST following administration of ADs are presented in Table 1. Chronic administration of the tricyclic AD imipramine, the NET inhibiting reboxetine, the NET and dopamine transporter inhibiting AD bupropion, the SSRI citalopram or the atypical AD trimipramine, i.e., all examined ADs, irrespective of their primary mode of action, caused a significant decrease in immobility time in the FST compared to stressed saline-treated wild-type mice.

Sucrose preference test—The preference for sucrose was significantly higher in unstressed saline-treated NETKO control mice (75.00 ± 2.3 %) compared to unstressed saline-treated wild-type controls (64.00 ± 1.2 %; n = 10; t-test: $p < 0.01$; Fig 2). Restraint or social defeat stressed wild-type mice receiving reboxetine treatment showed a significant

increase in sucrose preference (62.1 ± 1.3 % and 63.1 ± 2.1 %, respectively) compared to saline-treated restraint or social defeat stressed wild-type mice (43.0 ± 2.4 % and 37.5 ± 0.8 %, respectively Fig 2a and b). The relatively low sucrose consumption in restraint and social defeat stressed wild-type mice compared to unstressed saline-treated wild-type mice indicates anhedonia (Fig 2a and b). In contrast, the amount of sucrose-solution consumed by unstressed saline-treated NETKO control mice (75.00 ± 2.3 %) did not significantly differ from that of saline-treated NETKO mice exposed to either restraint (72.5 ± 2.3 %) or social defeat stress (68.8 ± 3.9 %). Chronic administration of ADs other than reboxetine (trimipramine, bupropion, citalopram, and imipramine) in restraint or social defeat stressed wild-type mice caused a significant increase in sucrose preference in comparison to saline-treatment (Table 1). No effect concerning sucrose preference was detected in restraint- or social defeat-stressed NETKO mice receiving reboxetine (71.3 ± 3.6 %; Fig 2a and b) if compared with saline-treated stressed NETKO mice. Like reboxetine, imipramine failed to influence sucrose intake in NETKO mice exposed to restraint or social defeat stress in comparison to saline-treated NETKO controls (see Supplementary Table S1). The total amount of liquid drunk (tap water and sucrose solution) did not differ between saline- and AD-treated groups (see Supplementary Table S2).

Social interaction test—After 21 days of social defeat stress reboxetine-treated wild-type mice spent significantly more time in the interaction zone than saline-treated stressed wild-type mice (87.6 ± 15.9 s and 16.00 ± 5.1 s, respectively; $p < 0.001$; Fig 3a). In contrast, the time spent in the corners was markedly decreased in reboxetine-treated wild-type mice exposed to social defeat stress in comparison to saline-treated stressed controls (69.3 ± 16.8 s and 215.1 ± 29.5 s, respectively; $p < 0.001$; Fig 3b). Saline-treated unstressed wild-type controls showed a significant increase in time in interaction zone and a significant decrease in time in corner compared to saline-treated social defeat stressed wild-type mice (Fig 3a and b). In NETKO mice, however, social defeat stress caused no behavioral effect in this test (Fig 3a and b). Next to reboxetine chronic treatment with other ADs of different classes (trimipramine, bupropion, citalopram, imipramine; see Supplementary Table S3) led to a significant increase in time in interaction zone and to a significant reduction in time in corner in social defeat stressed wild-type mice compared to saline-treated social defeat stressed wild-type mice. Total locomotion in saline-treated unstressed wild-type controls and saline-treated stressed wild-type mice was not significantly different (620.90 ± 79.58 cm [$n=5$] and 469.90 ± 43.73 cm [$n=6$], respectively), and also no significant difference was observed between wild-type controls (see above) and NETKO controls (866.30 ± 145.00 cm [$n=5$]).

Effects of NETKO and pharmacologically diverse antidepressants on brain neurotrophin expression after exposure to different stressors

Fig 4 shows that one week chronic restraint stress caused only a slight reduction of BDNF mRNA expression in the hippocampus of wild-type mice while social defeat stress induced a 1.4-fold decrease. The down-regulation of BDNF mRNA continued in week 2 and was markedly more pronounced after 3 weeks of restraint and social defeat stress (3.3-fold and 3.8-fold, respectively).

Chronic (21 days) restraint or social defeat stress caused a significant down-regulation (2.5 to 4-fold) of BDNF mRNA in hippocampus and cerebral cortex of saline treated wild-type mice (Fig 5a). In these mice, chronic stress also reduced BDNF protein expression: in hippocampus and hypothalamus by 40 to 50% and in cerebral cortex by even 60 to 80 % (Fig 5b). In the hypothalamus restraint and social defeat stress induced a significant 1.5- and 2-fold decrease in BDNF mRNA expression, respectively, and a significant down-regulation by 40–45% in BDNF protein levels (see Supplementary Fig. S1). For NGF significantly

reduced mRNA expression after both stressors was detected in hippocampus and cerebral cortex (3 to 4-fold) while only a slight down-regulation in brainstem could be detected for social defeat stressed wild-type mice (1.5-fold; Fig 6a). Chronic stress-induced down-regulation was also observed at the protein level: NGF was significantly down-regulated (up to 50%) in the hippocampus and cerebral cortex after both stressors and by about 35 % in the brainstem after social defeat stress (Fig 6b). In hippocampus and cerebral cortex regulation of NT-3 protein corresponded well with that of NT-3 mRNA (Fig 7a and 7b). Both stressors induced a decrease in NT-3 protein by 60 to 70% in the hippocampus as well as in the cerebral cortex (Fig 7b). However, in brainstem NT-3 mRNA was not significantly affected by restraint or social defeat stress while NT-3 protein was significantly up-regulated by 45% after restraint stress and tended to be up-regulated after social defeat stress (Fig 7b).

Restraint and social defeat stress induced an increase (2–3-fold) in the mRNA expression of BDNF, NGF and NT-3 in the striatum (Fig 5a, 6a, 7a). This regulation was confirmed (at least for social defeat stress) at the protein level since BDNF and NT-3 were up-regulated by about 50% and NGF by 100 % (Fig 5b, 6b, 7b). A significant up-regulation in striatum after restraint stress was also detected for BDNF and NGF, but not for NT-3 (Fig 5b, 6b, 7b).

The observed hippocampal and cortical mRNA regulations in chronically stressed wild-type mice could be prevented by chronic administration of each of the five examined ADs (for reboxetine see Fig 5a, 6a, 7a; for effects of other tested ADs on BDNF expression in the hippocampus of stressed wild-type mice, see Table 2). This protective effect was also observed at the protein level (as shown for reboxetine in Fig 5b, 6b, 7b). Interestingly, in NETKO mice BDNF mRNA and protein expression in the hippocampus and cerebral cortex were not significantly affected by restraint or social defeat stress (Fig 8a and b). In fact, these mice displayed similar expression profiles for the examined targets as AD-treated stressed wild-type mice. Treatment of NETKO mice with reboxetine did not induce further effects in BDNF expression (Fig 8a and b). Similar results were obtained for NGF and NT-3 (see Supplementary Table S4). We would like to mention that treatment of unstressed wild-type and NETKO mice with reboxetine (20 mg/kg for 21 days) had no effect on mRNA expression of BDNF, NGF and NT-3 compared to saline treated controls (see Supplementary Table S5).

Discussion

Although several studies have been published examining the effects of ADs on regulation of BDNF, many of them have been limited in scope, mostly testing a single drug, a single target, one brain region or the application to only naive animals without an induced depression-like behavior. The present study compares the effects of five pharmacologically diverse ADs on the regulation of three neurotrophins (BDNF, NGF and NT-3) in several mouse brain regions after exposure to chronic restraint or social defeat stress. We also examined how NETKO mice respond to different kinds of stressors without and with additional AD treatment.

To study depression-like behavior we used the FST and the sucrose-preference test. We observed no major behavioral differences between the two kinds of stressors concerning the chronic stress-induced increase in immobility time in the FST or the decrease in sucrose-preference. Unstressed NETKO mice displayed a significant decrease in immobility time in the FST compared to unstressed wild-type mice. These findings are in line with previous results (Dziedzicka-Wasylewska *et al.* 2006; Xu *et al.* 2000) confirming that naive NETKO mice behave like AD-treated wild-type mice in the FST. This also holds true for the behavior of NETKO mice in the sucrose-preference test since unstressed NETKO mice

showed significantly increased sucrose-intake in comparison with unstressed wild-type mice. We wondered if the NETKO phenotype would also hold in stress-based animal models of depression. While in wild-type mice immobility time was dramatically increased by restraint and social defeat stress, NETKO mice were not affected in swimming behavior by both stressors. Additionally, and in contrast to wild-type mice, both stress forms did not worsen sucrose preference in NETKO mice. The observed unaltered social interaction with an unfamiliar separated aggressor in social defeat stress is a further hint for an insusceptibility of NETKO mice to depression-like changes. In wild-type mice, social avoidance could be prevented by daily treatment with the AD reboxetine. Thus, NET deficiency seems to be associated with depression-resistant behavior. This is in line with results reported by Keller *et al.* (2006) who showed a lowering of baseline blood pressure and heart rate in NETKO mice in response to repeated exposure to fearful environments.

The role of noradrenergic and serotonergic neurotransmission in depression or antidepressant effects is not quite clear. However, it is known that there exist functional interactions between the noradrenergic and serotonergic system in the brain which could make antidepressant responses interdependent on both mechanisms (for review see e.g. Ressler and Nemeroff 2000). Serotonergic neurons in the dorsal raphe nucleus (DRN) are under tonic excitatory control by α_1 -adrenoceptors activated by NE released from noradrenergic innervation originating from the locus coeruleus as well as nearby noradrenergic nuclei in the brainstem (Baraban and Aghajanian 1980, 1981). Blockade of α_1 -adrenoceptors decreases serotonergic cell firing and the release of 5-HT, whereas blockade of α_2 -adrenoceptors enhances both 5-HT release and serotonergic cell firing probably by enhancing noradrenergic transmission in the DRN (Baraban and Aghajanian 1980; Bortolozzi and Artigas 2003; Pudovkina *et al.* 2003). NE release in the DRN has been shown to regulate behavioral effects of the SSRI fluoxetine in the TST (O'Leary *et al.* 2007). On the other hand, it is known that systemic administration of 5-HT_{1A} receptor agonists enhances noradrenergic transmission, whereas systemic administration of 5-HT_{2A} receptor agonists dampens noradrenergic transmission (Chen and Reith 1995; Gobert and Millan 1999; Hajos-Korcsok *et al.* 1999; Suzuki *et al.* 1995; Szabo and Blier 2001a,b). In addition, stress-induced plasticity of NE and 5-HT axons as well as morphological interactions between these monoamine axons during axonal regeneration have been observed (Liu and Nakamura 2006). The functional interactions indicate that effects of SSRIs or SNRIs might not be mediated exclusively by either neurotransmitter. Thus, these mutual interactions could explain why SSRIs as well as SNRIs lowered immobility time in the FST and increased sucrose preference in stressed wild-type mice compared to saline treatment observed in the present study. However, these interactions cannot explain the observed effects of trimipramine. Thus, it remains to be shown whether this antidepressant eventually blocks the protein kinase-A-regulated TREK-1 potassium channels which has been demonstrated to be inhibited by diverse antipsychotics (Thümmeler *et al.* 2007) and whose gene inactivation results in a depression-resistant phenotype (Heurteaux *et al.* 2006). Since in NETKO mice noradrenergic neurotransmission is increased (see Introduction), it may be justified to assume that a chronically increased NE firing in the DRN in NETKO mice may also lead to a chronic elevation of brain 5-HT release; this would explain the resistance of NETKO mice to stress-induced behavioral changes as well as neurochemical changes discussed below.

Neurotrophin-induced changes in hippocampal neurogenesis seem to be a key factor in the pathophysiology of depression and in mediating AD action (Sahay and Hen, 2007). To further clarify the neurochemical background underlying susceptibility to stress in wild-type mice as well as NETKO-associated resilience, we studied mRNA and protein levels of the neurotrophins BDNF, NGF and NT-3 after restraint and social defeat stress.

After one week, immobilization stress failed to significantly reduce BDNF mRNA levels whereas social defeat stress caused a profound down-regulation in BDNF mRNA. After three weeks, both stressors induced a similar and pronounced decrease in BDNF expression. These results indicate that comparisons of consequences of different chronic stress forms should only be performed after a long (three weeks) period. Our results are in line with those of Avgustinovich *et al.* (2003) who found that only a long period (20 days) of social defeat stress causes a depression-like behavior.

We could demonstrate that chronic restraint or social defeat stress causes brain region-specific changes in the expression of the neurotrophins BDNF, NGF and NT-3 in wild-type mice.

In the hippocampus, mRNA and protein expression of BDNF, NGF and NT-3 were decreased. Our findings concerning BDNF and NGF mRNA agree with those of Alfonso *et al.* (2006) who had shown that chronic immobilization stress of mice causes a down-regulation of hippocampal BDNF and NGF mRNA. Chronic stress-induced down-regulation as well as AD-induced up-regulation of hippocampal BDNF (Surget *et al.* 2008) have largely contributed to the neurotrophic hypothesis of depression (Castren *et al.* 2007; Duman and Monteggia, 2006; Krishnan and Nestler, 2008; Wang *et al.* 2008). To our knowledge, we are the first who could demonstrate that two different kinds of chronic stress decrease hippocampal expression of all three neurotrophins (BDNF, NGF and NT-3) not only at the mRNA but also at the protein level. This indicates that all three neurotrophins are involved in the chronic stress-induced depression-like behavior. This is supported by the recent observation in another animal model of depression that maternal separation of rat pups induces reduced expression of NGF and NT-3 in the dorsal hippocampus (Marais *et al.* 2008).

In the striatum, chronic restraint and/or social defeat stress induced up-regulation of all three examined neurotrophins at the mRNA and/or protein level, and this increase was attenuated by treatment with the SNRI reboxetine. The BDNF increase in a dopaminergic brain region is in accordance with prolonged social stress-induced increase of BDNF in the nucleus accumbens (also called ventral striatum) reported by Berton *et al.* (2006). In addition, direct infusion of BDNF into ventral tegmental area/nucleus accumbens circuit was shown to increase depression-related behavior (Eisch *et al.* 2003) whereas a selective knockout of the BDNF gene from this circuit had antidepressant-like effects (Berton *et al.* 2006). Thus, the mesolimbic dopamine reward circuit is also involved in depression (Nestler and Carlezon, 2006). Whether an increased BDNF expression in the nigro-striatal system may contribute to depression-like behavior is unknown.

The cerebral cortex (especially its frontal region) together with the hippocampus are of special interest in depression since these regions are particularly associated in cognitive abnormalities often seen in depressed patients (Berton and Nestler, 2006). In the present study, both forms of chronic stress caused a significant decrease in mRNA and protein expression of all three examined neurotrophins in the cerebral cortex. This result underlines the importance of all three neurotrophins and the involvement of this brain region in chronic stress-induced depression. Our findings are in line with the observation that repeated antidepressant treatment increases BDNF expression not only in the hippocampus but also in the cerebral cortex (Balu *et al.* 2008; Rogoz *et al.* 2005).

In the brainstem, no chronic stress-induced regulation of BDNF, NGF and NT-3 mRNA were observed, however, at the protein level NGF was decreased after social and NT-3 increased after chronic restraint stress. Presently, we have no explanation for these changes, and for NGF nothing is known about stress-induced brainstem-specific changes. On first

sight, our results seem to be conflicting but they agree with those of Hock *et al.* (2000) who found increased NT-3 levels in the cerebrospinal fluid of patients suffering from major depression, and also with those of Smith *et al.* (1995) who showed significant decrease in NT-3 mRNA in the locus coeruleus of rats after chronic treatment with tricyclic ADs. The chronic stress-induced brain region-specific changes in neurotrophin expression confirm the hypothesis that a distributed network of several brain structures is involved in depression-related neuronal states (Stone *et al.* 2007).

The above mentioned regulations of BDNF, NGF and NT-3 in hippocampus, cerebral cortex and striatum could be prevented by chronic treatment with reboxetine. In addition, the stress-induced downregulation of BDNF in the hippocampus and cerebral cortex were prevented by all examined ADs, including trimipramine. Interestingly, a similar preventive effect has been described for tianeptine, another AD with unknown primary mode of action (Alfonso *et al.* 2006). Obviously all ADs have a common final pathway addressing the reconstitution of normal levels of neurotrophins and their signaling for maintenance of neurogenesis. Our results are in line with results of several studies about AD-induced increases in brain expression of neurotrophins (see above), but we showed these effects for diverse ADs and not only in the hippocampus but also in other brain regions. However, further investigations are required to disclose the role of brain regions other than the hippocampus in mediating AD effects. Recent studies indicate that ADs rapidly activate TrkB signaling and induce a long-lasting increase in BDNF production (Castren *et al.* 2007; Rantamäki *et al.* 2007). Since our study shows that all examined ADs prevented stress-induced expression changes of all three neurotrophins it seems possible that the p75 neurotrophin receptor which is a common receptor for all these neurotrophins might be of more importance. This assumption is fostered by the very recent observation that this receptor regulates hippocampal neurogenesis and related behaviors (Catts *et al.* 2008).

NETKO mice displayed a different behavior compared to wild-type mice since they showed no stress-induced changes in brain expression of the examined neurotrophins. Furthermore, NETKO mice did not develop depression-like behaviors after chronic stress, and in behavioral tests for depression they previously have been shown to behave like AD-treated wild-type mice after acute and prolonged application of ADs (Dziedzicka-Wasylewska *et al.* 2006; Xu *et al.* 2000). The robust behavior and “stress resistance” of these NETKO mice which have enhanced levels of norepinephrine in the synaptic cleft of central noradrenergic neurones underlines the importance of noradrenergic pathways and signaling in mood and behavior. In fact, Blier and co-workers have demonstrated that noradrenergic mechanisms are of great importance within the monoaminergic crosstalk and the actions of diverse antidepressants, including SSRIs (Blier, 2001; Brunello *et al.* 2003; Guiard *et al.* 2008).

In conclusion, we show that two mouse models of depression induce comparable effects on behavior and changes in brain neurotrophin expression in wild-type mice. The chronic stress-induced effects in brain neurotrophin expression could be prevented by concomitant administration of pharmacologically diverse ADs. Additionally, all tested antidepressants significantly decreased immobility time in the FST and increased sucrose preference in stressed wild-type mice compared to saline treatment. Furthermore, we could demonstrate that NETKO mice are not susceptible to stress-induced depressive-like changes in behavior and brain neurotrophin expression. Thus, NETKO mice represent a useful model to study depression-resistant behavior, a phenomenon of resilience also found in humans; in addition, NET-deficiency can provide deeper insights in the elucidation of downstream mechanisms of antidepressants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADs	antidepressant drugs
BDNF	brain-derived neurotrophic factor
FST	forced swim test
KO	knockout
NE	norepinephrine
NET	norepinephrine transporter
NGF	nerve growth factor
NT	neurotrophin
SNRI	selective norepinephrine reuptake inhibitors
SSRI	selective serotonin reuptake inhibitors

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Figure 1.

Behavior of (a) restraint- and (b) social defeat- stressed wild-type and NETKO mice in the FST following chronic administration of saline or reboxetine (20mg/kg i.p.). Shown are means ($\pm$ SEM) of $n = 6-10$ mice per group. Statistically significant differences (within the wild-type or the NETKO group) were calculated using one-way ANOVA followed by Dunnett's post hoc test (***) $p < 0.001$ vs stressed saline-treated mice); Student's t-test was used for a comparison of unstressed saline-treated wild-type with saline-treated NETKO mice (### $p < 0.001$).

Figure 2. Preference for sucrose in (a) restraint- and (b) social defeat-stressed wild-type and NETKO mice following chronic administration of saline or reboxetine (20mg/kg i.p.). Shown are means ($\pm$ SEM) of $n = 6-8$ mice per group. Statistically significant differences (within the wild-type or the NETKO group) were calculated using one-way ANOVA followed by Dunnett's post hoc test (*** $p < 0.001$ vs stressed saline-treated mice) Student's t -test was used for a comparison of unstressed saline-treated wild-type with saline-treated NETKO mice (## $p < 0.01$).

Figure 3.

Response of social defeat-stressed wild-type and NETKO mice in the social interaction test following chronic (21 days) treatment with saline or reboxetine (20mg/kg i.p.). Shown are a) time spent in interaction zone and b) time spent in the corners of the test-cage. Values are displayed as means ($\pm$ SEM) of 5–10 mice per group. Statistically significant differences (within the wild-type group) were calculated using one-way ANOVA followed by Dunnett's post hoc test (** $p < 0.01$; *** $p < 0.001$ vs stressed saline-treated mice). Student's t-test was used for a comparison of social defeat-stressed NETKO mice and saline-treated unstressed NETKO mice.

Figure 4.

Time course of relative BDNF mRNA expression in the hippocampus over 3 weeks of restraint and social defeat stress. Shown are means ($\pm$ SEM) of 3 mice per group. Statistical significance was calculated using one-way ANOVA followed by Dunnett's post hoc test (## $p < 0.01$; ### $p < 0.001$ vs 0 (before stress) and t-test to compare the different stressors at each time point (* $p < 0.05$).

Figure 5. Relative mRNA expression (a) and protein levels (b) of BDNF in different brain regions of wild-type mice following restraint and social defeat stress and chronic administration of reboxetine. Results are presented as fold- and log₂-values (for mRNA expression) and as % of saline-treated wild-type controls (for protein levels). Shown are means ($\pm$ SEM) of 4–7 mice per group. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs saline-treated unstressed wild-type).

Figure 6.

Relative mRNA expression (a) and protein levels (b) of NGF in different brain regions of wild-type mice following restraint and social defeat stress and chronic administration of reboxetine. Results are presented as fold- and log₂-values (for mRNA expression) and as % of saline-treated wild-type controls (for protein levels). Shown are means ($\pm$ SEM) of 4–7 mice per group. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs saline-treated unstressed wild-type)

Figure 7. Relative mRNA expression (a) and protein levels (b) of NT-3 in different brain regions of wild-type mice following restraint and social defeat stress and chronic administration of reboxetine. Results are presented as fold- and log₂-values (for mRNA expression) and as % of saline-treated wild-type controls (for protein levels). Shown are means ($\pm$ SEM) of 4–7 mice per group. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs saline-treated unstressed wild-type).

Figure 8.

Relative mRNA expression (a) and protein levels (b) of BDNF in the hippocampus and cerebral cortex of NETKO mice following restraint and social defeat stress and chronic administration of reboxetine. Shown are means ($\pm$ SEM) of 4–7 mice per group. No significant differences between groups were detected using one-way ANOVA followed by Dunnett's post hoc test.

Table 1

Responses of wild-type mice in the forced swim test and the sucrose preference test following restraint or social defeat stress and chronic administration of ADs. Immobility is expressed as means ($\pm$ SEM) of 6–10 mice per group. Sucrose intake is displayed as percent of total liquid drunk within 24h. Shown are means ($\pm$ SEM) of 6–8 mice per group.

Stressor	Treatment	Immobility time (s)	Preference for sucrose (%)
restraint stress	saline	202.0 $\pm$ 2.7	43.0 $\pm$ 2.4
	reboxetine	85.3 $\pm$ 14.1 s ***	62.1 $\pm$ 1.2 ***
	trimipramine	nq	59.9 $\pm$ 2.5 ***
	bupropion	nq	61.7 $\pm$ 1.9 ***
	citalopram	nq	60.9 $\pm$ 1.6 ***
	imipramine	nq	60.9 $\pm$ 1.7 ***
no	saline	144.1 $\pm$ 7.7 ***	62.4 $\pm$ 1.6 ***
social defeat stress	saline	208.7 $\pm$ 3.0	37.5 $\pm$ 0.7
	reboxetine	120.8 $\pm$ 10.3 ***	63.1 $\pm$ 2.1 ***
	trimipramine	126.0 $\pm$ 11.2 ***	62.0 $\pm$ 3.2 ***
	bupropion	120.8 $\pm$ 12.5 ***	60.4 $\pm$ 3.2 ***
	citalopram	121.3 $\pm$ 9.8 ***	61.7 $\pm$ 4.2 ***
	imipramine	117.2 $\pm$ 9.4 ***	64.4 $\pm$ 4.1 ***
no	saline	148.9 $\pm$ 7.2 ***	64.0 $\pm$ 1.1 ***

Statistical analysis was calculated using one-way ANOVA following Dunnett's post hoc test for comparison of treatment groups (restraint or social defeat stress and unstressed) against saline-treated stressed mice

***
p < 0.001; nq: not quantified.

Table 2

mRNA expression of BDNF in the hippocampus of wild-type mice following restraint or social defeat stress and chronic administration of ADs. Shown are means ($\pm$ SEM) of 5–7 mice per group.

Stressor	Treatment	mRNA expression ($\log_2$ treatment group/control)
restraint stress	saline	-1.93 ± 0.21
	reboxetine	-0.33 ± 0.14 ***
	trimipramine	-0.46 ± 0.10 ***
	bupropion	-0.45 ± 0.14 ***
	citalopram	-0.38 ± 0.18 ***
	imipramine	-0.49 ± 0.20 ***
no	saline	-0.18 ± 0.12 ***
social defeat stress	saline	-2.00 ± 0.10
	reboxetine	-0.48 ± 0.18 ***
	trimipramine	-0.25 ± 0.11 ***
	bupropion	-0.29 ± 0.09 ***
	citalopram	-0.41 ± 0.14 ***
	imipramine	-0.40 ± 0.18 ***
no	saline	-0.09 ± 0.12 ***

Statistical analysis was performed using one-way ANOVA following Dunnett's post hoc test for comparison of treatment groups (restraint or social defeat stress and unstressed) against saline-treated stressed mice

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p < 0.001.