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Differential responses to anxiogenic drugs in a mouse model of panic disorder as revealed by Fos immunocytochemistry in specific areas of the fear circuitry

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Summary. Sensitivity to pharmacological challenges has been reported in patients with panic disorder. We have previously validated transgenic mice overexpressing the neurotrophin-3 (NT-3) receptor, TrkC (TgNTRK3), as an engineered murine model of panic disorder. We could determine that TgNTRK3 mice presented increased cellularity in brain regions, such as the locus ceruleus, that are important neural substrates for the expression of anxiety in severe anxiety states. Here, we investigated the sensitivity to induce anxiety and panic-related symptoms by sodium lactate and the effects of various drugs (the α2-adrenoceptor antagonist, yohimbine and the adenosine antagonist, caffeine), in TgNTRK3 mice. We found enhanced panicogenic sensitivity to sodium lactate and an increased intensity and a differential pattern of Fos expression after the administration of yohimbine or caffeine in TgNTRK3. Our findings validate the relevance of the NT-3/TrkC system to pathological anxiety and raise the possibility that a specific set of fear-related pathways involved in the processing of anxiety-related information may be differentially activated in panic disorder.

Keywords: Panic disorder – Fos immunohistochemistry – Amygdala – Locus ceruleus – TrkC – NT-3 – Yohimbine – Caffeine

Introduction

Panic disorder is a severe anxiety disorder characterized by recurrent panic attacks that affects 5% of the population, and is associated with substantial morbidity and reduced quality of life (Longley et al., 2006; Kessler et al., 1994; Hirschfeld, 1996; Katon et al., 2002). Severe anxiety states accompanied by fear, hypervigilance, and autonomic activation can be elicited in human volunteers by administration of certain pharmacologic agents. In panic disorder patients, there is an increased sensitivity to pharmacological challenges, possibly due to a dysregulation of the fear system. They are particularly susceptible to panic-like responses after chemical stimuli such as sodium lactate (Liebowitz et al., 1984), CO_2 inhalation (Gorman et al., 1994), or pharmacological challenges, including the benzodiazepine inverse agonist, FG-7142; m-chlorophenyl piperazine (mCPP), a nonselective 5-HT2C receptor agonist; caffeine, an adenosine receptor antagonist; and yohimbine, an α2-adrenoceptor (AR) antagonist (Dorow et al., 1983; Bourin et al., 1998; Coplan et al., 1992, 1999; Murphy et al., 1998; Price et al., 1995). The brain areas activated by each of these anxiogenic drugs have been previously identified using Fos expression (Singewald and Sharp, 2000; Singewald et al., 2003). These areas included forebrain and hindbrain fear-related regions (for reviews, see Bernard and Bandler, 1998; Charney et al., 1987; Gorman et al., 2000). The regions activated included the periaqueductal gray and locus ceruleus (LC), and forebrain areas such as the amygdala, hypothalamus, and prefrontal cortex (Charney et al., 1987; Gorman et al., 2000; McNaughton and Gray, 2000; Rosen and Schulkin, 1998). Our previous work (Dierssen

et al., 2006) demonstrated anxiety-related behavior and panic reaction in transgenic mice overexpressing the full-length neurotrophin-3 receptor, TrkC (TgNTRK3) in the central nervous system, along with significant increases in noradrenergic neurons in LC and other catecholaminergic nuclei, thus suggesting a possible noradrenergic dysregulation. Moreover, the anxiety-like behavior was reversed by benzodiazepam administration in transgenic mice.

Neurotrophic factors were initially implicated in neuronal plasticity and development, but their role in the etiology and drug treatment of a number of psychiatric disorders including depression, eating disorders or obsessive-compulsive disorder (Russo-Neustadt, 2003; Poo, 2001) has now been widely recognized. Very recently, brain derived neurotrophic factor (BDNF) dysfunction has been suggested to play a role also in anxiety disorders (Castren, 2004, 2005). Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behavior in mice (Yee et al., 2006; Zhu et al., 2006), and mice expressing the BDNF variant $BDNF^{Met/Met}$ exhibited increased anxiety-related behavior in stressful situations, that was not normalized by fluoxetine (Chen et al., 2006). In this line, transgenic overexpression of the neurotrophin BDNF has a facilitatory effect on anxiety-like behavior, concomitant with increased spinogenesis in the basolateral amygdala (Govindarajan et al., 2006). However, the role of the NT-3/TrkC system in anxiety is less clear. NT-3 is known to be trophic for LC neurons, and some of the effects of stress and antidepressants on LC function and plasticity are mediated through NT-3 (Smith et al., 1995).

In the present study, we have selected sodium lactate to evaluate the sensitivity to induction of anxiety- and panic-related symptoms by this agent in TgNTRK3 mice using the mouse defense test battery (MDTB), that evaluates the panicogenic/panicolytic properties of drugs (Griebel et al., 1995a, b, 1996, 1999; Blanchard et al., 2001), to specifically address the panic reactions. We also determined the possible differential activation of the fear circuit using anxiogenic drugs (yohimbine and caffeine) induced Fos immunohistochemistry on specific areas of the hindbrain and forebrain, recognized as important to anxiety and fear mechanisms.

Materials and methods

Two lines of transgenic TgNTRK3 mice (Dierssen et al., 2006) were used in order to exclude positional effects. Adult male TgNTRK3 and wild-type littermates (5–7 months of age) from different litters were used. Same sex littermates were group-housed (4–6 animals per cage) in standard macrolon cages ($40 \times 25 \times 20$ cm) under a 12-h light/dark schedule (lights on 0600 to 1800) in controlled environmental conditions of humidity (60%) and temperature (22 ± 2 °C) with food and water supplied *ad libitum*. All animal procedures met the guidelines of the European Communities directive 86/609/EEC and the Society for Neurosciences regulating animal research, and were approved by the Local Ethical Committee.

Mouse defense test battery

The MDTB was conducted following the procedure of Griebel et al. (1999), with slight modifications as previously described (Dierssen et al., 2006). Fifteen TgNTRK3 and twelve wild-type littermates were randomly distributed into saline and sodium lactate group. Sodium lactate (2.5 mEq/kg) was administered intraperitoneally in a volume of 0.1 ml/10 g of mouse weight. Experiments were performed under ambient red fluorescent light (10 Lux). Briefly, six different subtests were used: (I) pre-test familiarization period: line crossings, wall rears, wall climbs and jump escapes were recorded for 3 min. (II) Predator avoidance test. Immediately after the familiarization period a hand-held male rat (Long Evans, Charles River, Elbeuf, France) killed by CO_2, was introduced at the opposite end of the apparatus and brought up to the subject at a speed of approximately 0.5 m/s. The number of avoidances, mean avoidance distance (distance between the rat and the mouse at the point of flight in cm), and maximum flight speed (an average of three measures of uninterrupted straight flight, over 1 m linear segment of the runway) were recorded. This procedure was repeated five times with an inter-trial interval of 15 s. (III) Chase/flight test. The hand-held rat was brought up to the mouse at speed of approximately 2.0 m/s avoiding contact. Chase was terminated when the subject had traveled a distance of 18 m. Number of stops (pause in locomotion) orientations (subject stops and then orients the head toward the rat) and reversals (subjects stops, turns, and then runs in the opposite direction) were calculated. (IV) Straight alley test. Thirty seconds after the chase/flight test, the runway was converted to a straight alley (a segment of 0.6 m) in which the subject was constrained. Three confrontations at a stimulus-subject distance of 0.4 m, 15 s each, were made. Immobility time, closest distance between the mouse and the rat, and the number of approaches/withdrawals (subject must move more than 0.2 m forward the closed wall and then return to it) were measured. (V) Forced contact test. The experimenter brought the rat in contact with the subject along 1 min. For each contact, bites, vocalizations, attacks and jump escapes were recorded. (VI) Post-test period. The doors and predator were removed and the mouse was allowed to explore freely the runway along a 3-min session. Line crossings, and contextual defense (wall rears, wall climbs and jump escapes) were recorded.

Fos immunoreactivity

Ten TgNTRK3 and twelve wild-type littermates, randomly distributed in yohimbine or caffeine-treated and vehicle-injected groups were used. They were acclimated to handling one week before the experiment. Yohimbine (Sigma, St. Louis, MO) and caffeine (Sigma-Aldrich, Germany) were dissolved in saline solution (0.9%) and administered intraperitoneally at 5 and 50 mg/kg, respectively. The dose selected has already been shown to induce anxiogenic-like effects in mice (Southwick et al., 1993). The behavioral effects caused by the drugs (freezing, increased grooming, restlessness, burrowing behavior, etc.) were not quantified in this study during the 45-min survival time to prevent any possible confounding effects in addition to the sole drug effects. One hour after injection of drug or vehicle, animals were deeply anaesthetized with isoflurane. They were then perfused with paraformaldehid 4% and brains were removed, postfixed for 4 h, and cryoprotected overnight in 30% sucrose solution. 50-μm coronal frozen sections were obtained in a cryostat. Three serial groups of free-floating sections were obtained and stored in 0.1 M phosphate buffer containing 5% sucrose and 0.02% sodium azide at 4 °C. For

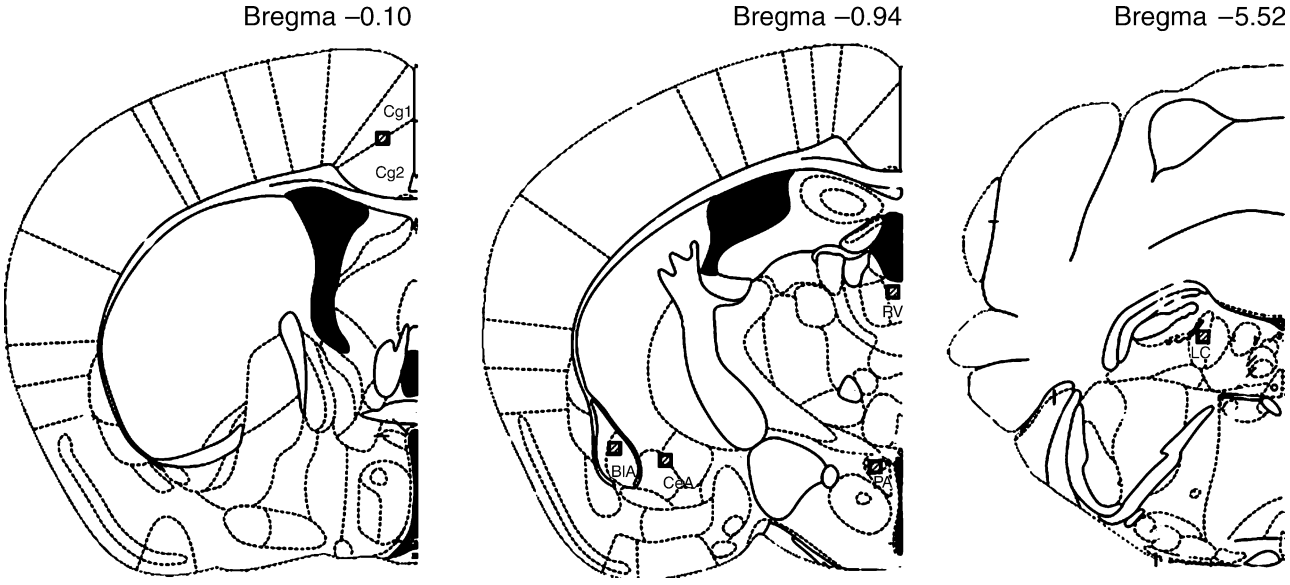


Fig. 1. Schematic diagrams, took from the atlas of Paxinos and Watson (2001) showing the areas in which Fos expression was quantified. The squares indicate the placement of optical dissectors for counting Fos positive cells. Bregma −0.10: Cg1, cingulated cortex1; Cg2, cingulate cortex2; Bregma −0.94: BlA, basolateral amygdaloid nucleus; CeA, central amygdaloid nucleus; PV, paraventricular thalamic nucleus; PA, paraventricular hypothalamic nucleus; Bregma −5.52: LC, locus ceruleus

Fos immunohistochemical detection, sections were incubated for 2 h in 3% normal goat serum and 0.3% Triton X-100 in 0.1 M phosphate buffer (NGS-T-PB). Then, sections were incubated overnight in a rabbit polyclonal antibody anti-Fos (Ab-5; Calbiochem, La Jolla, CA) 1:100.000 diluted in NGS-T-PB. Sections were washed in 0.1 M PB and incubated for 1 h in a goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA) 1:500 in NGS-T-PB. Sections were washed again in 0.1 M phosphate buffer and incubated for 2 h in avidin-biotin peroxidase complex (ABC Elite; Vectastain, Vector Laboratories). After washes in Tris buffer (0.15 M, pH 7.6), sections were incubated for 2 min in a solution containing 0.05% of 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO) and 0.2% ammonium nickel sulfate in 0.15 M Tris buffer. Increasing doses of hydrogen peroxide were added every 5 min. Finally, the reaction was stopped by washes in 0.1 M phosphate buffer.

For quantitative analysis of Fos positive cell density, coronal sections through the cingulate cortex (Bregma 1.34 to −0.82 mm), hypothalamus (Bregma −0.58 to −1.22 mm) and thalamus (Bregma −0.22 to −2.30 mm), basolateral amygdala (BLA) (Bregma −0.58 to −3.16 mm), central amygdala (CeA) (Bregma −0.58 to −2.06 mm), and LC (Bregma −5.34 to −5.80 mm) were analyzed (Singewald and Sharp, 2000), in the specific locations shown in Fig. 1. Estimation of the volume (Vref) of these regions was performed with the Cavalieri method, and the optical dissector method was used to estimate neuronal density (Nv). 10 dissector probes of 1739.926 μm^2 (Sdis) with a thickness (Hdis) of 10 μm [V (dis) = Sdis × Hdis = 17399.260 μm^3; guard zone = 3 μm to the surface of section] were analyzed per section, using a 40× objective to count neuronal nuclei in sampling probes. Estimation of total number of neurons was obtained according to the formula: N (neu) = Nv × V (ref), and the coefficient of error, CE = SEM/mean was calculated to evaluate the precision of the estimates. Sampling was optimized to produce a coefficient of error (CE) under the observed biological variability (West and Gundersen, 1990). CE, the estimated intra-animal coefficient of error, was calculated according to Gundersen and Jensen (1987). To estimate the volumetric shrinkage factor (SV), the thickness before and after processing was analyzed using the computer-driven z-axis of the microscope. This analysis revealed an average thickness shrinkage factor of about 0.86 that was similar in wild type and TgNTRK3 mice.

α2A-AR and α2B-AR expression

Three TgNTRK3 and three control littermates were sacrificed and brains were rapidly removed and brainstem area was then dissected on ice. Samples were homogenized in lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1 mM $MgCl_2$, PBS 0.2% Triton and protease inhibitor (Roche, Mannheim, Germany). After clearance of the lysates by centrifugation (14,000 × *g*, 20 min at 4 °C), protein quantification was performed following the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) protocol. For each sample, 80 μg (for α2A-AR and α2B-AR) of protein was electrophoresed on a denaturing 10% polyacrylamide gel and transferred to a Hybond-P membrane (Amersham Bioscience, Germany). A polyclonal anti-α2A-AR and anti-α2B-AR antibody (1:100; Santa Cruz Biotechnology, California) were used with a secondary peroxidase-conjugated anti-mouse and anti-goat IgG respectively (1:2000; DAKO, UK). Protein detection was done following the ECL system protocol (Pierce, Rockford, IL). The quantification of the obtained bands was done by densitometric analysis (Quantity One Image software). β-actin (1:2000; Sigma, St. Louis, MO) controls were used and relative levels of protein were analyzed.

Data analysis

Parametric data are reported as mean ± standard error of mean (SEM) and nonparametric data are reported as median ± semi-inter-quartile range (S.I.R.). When no significant differences were detected between transgenic mice of the two lines used, the obtained results were combined. Statistical analysis was performed by two-way ANOVA (genotype and treatment or genotype and gender as between group factors), followed by one-way ANOVA when significant ($P < 0.05$) interaction between factors was found. For the analysis of the western blot, Student's *t*-test analysis was employed. Posthoc comparisons were performed with independent *t*-tests for between group comparisons when appropriate. Nonparametric tests were used for data that did not show homoscedasticity or did not fit the parametric assumptions. For the paired analysis of pre- vs. post-exposure data in the mouse defense test battery, the Wilcoxon matched pair test was used. The statistical analysis was performed using the SPSS 12.0 software.

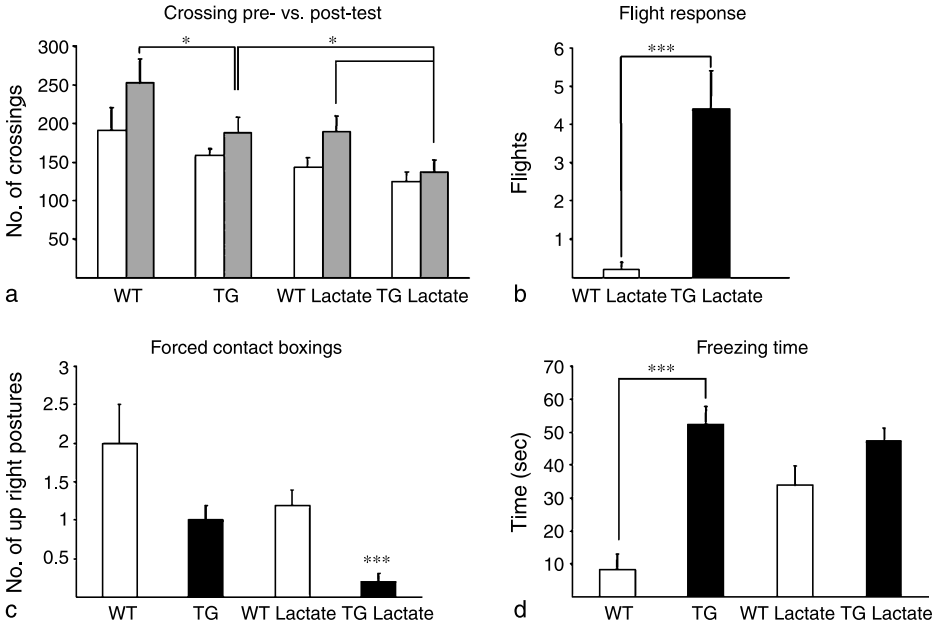


Fig. 2. Mouse defense test battery in TgNTRK3 mice. **a** Differences in pre-test (white bars) vs. post-test (grey bars) between genotypes and treatment in zone crossings; **b** predator avoidance test. Flight response in wild type (white bars) and TgNTRK3 littermates (black bars) after administration of sodium lactate; **c** forced contact test. Reduced up-right behavior (boxing) in TgNTRK3 after administration of sodium lactate; **d** increase in immobilization time in TgNTRK3 mice (black bars) with respect to wild types (white bars) after confrontation with the rat before and after administration of sodium lactate. Data are expressed as mean ± SEM. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

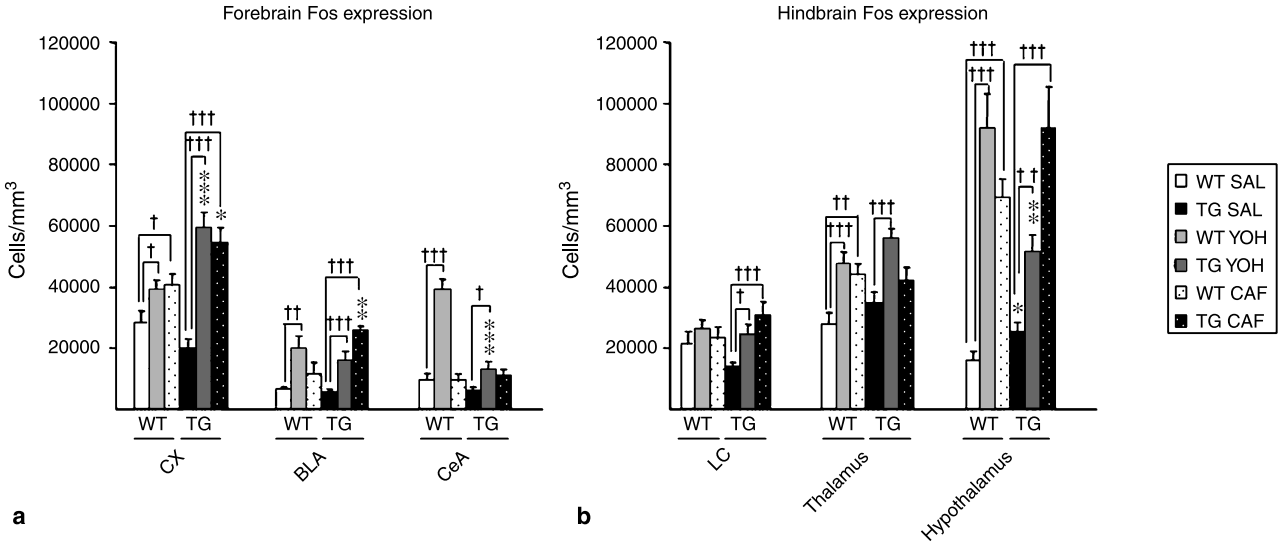
Results

Increased sensitivity to sodium lactate in TgNTRK3 mice

In the pre-test familiarization period of the mouse defense test battery (MDTB), the number of zone crossings were not affected by genotype or treatment ($F(1,26)=0.126$; $P=0.726$); Two-way ANOVA), thus indicating a similar reaction to the apparatus. After confrontation and removal of the rat (post-test), significant differences between groups and treatment were observed in the runway activities that evaluate contextual defense behaviors. Transgenic mice traveled significantly less distance ($F(1,26)=4.479$; $P<0.05$), in comparison to control mice, these differences between genotypes being increased under lactate treatment ($F(1,26)=8.476$; $P<0.05$; Fig. 2A). This paradoxical reduction in activity was due to a very marked increase in duration of immobilization in transgenic vs. wild type mice ($F(1,26)=14.297$; $P<0.001$; Fig. 2D). Consistently, comparison of pre- vs. post-exposure data for each group (two-tailed Wilcoxon matched pair test) revealed a significant increase in zone crossings in the post-test with respect to the pre-test in control mice, with ($P<0.01$) and without ($P<0.05$) treatment. In the predator avoidance test, overexpression of TrkC gave rise to a significant increase in flight response in transgenic mice with ($F(1,26)=7.706$; $P=0.01$) and also without treatment ($F(1,26)=56.738$; $P<0.001$). Moreover, the number of flight responses, a parameter sensitive to panicogenic agents, was dramatically increased in TgNTRK3 mice with lactate ($F(1,26)=20.914$; $P<0.001$; Fig. 2B). No differences were observed in the number of orientations, reversals and number of stops in the chase/flight test or in the number of approaches in the straight alley test in TgNTRK3 mice. Finally, in the forced contact test one-way ANOVA revealed an increase in number of upright postures but not in vocalizations and bites in wild-type treated mice ($F(1,26)=18.056$; $P<0.001$; Fig. 2C).

Yohimbine and caffeine induced a differential Fos expression pattern in TgNTRK3 mice

We investigated the consequences of the administration of yohimbine and caffeine on Fos expression in TgNTRK3 mice and wild-type littermates. Mice habituated to the test environment and injected with saline vehicle displayed little or no Fos immunoreactivity in most of the brain areas investigated (see below). Immunohistochemical analysis of Fos expression, analyzed 45 min after administration of yohimbine (5 mg/kg) or caffeine (50 mg/kg), showed a strong up-regulation of Fos immunoreactive cells in numerous brain areas implicated in anxiety- and



	WT	TG	WT	TG	WT	TG
SALINE	↑	↓	↑	↑	↑	↑
YOHIMBINE	↑↑	↑↑↑	↑↑	↑↑	↑↑↑	↑↑
CAFFEINE	↑↑	↑↑↑	↑	↑↑↑	↑	↑
	CORTEX		BLA		CeA	

	WT	TG	WT	TG	WT	TG
SALINE	↑	↓	↑	↑	↑	↑↑
YOHIMBINE	↑	↑	↑↑↑	↑↑↑	↑↑↑	↑↑/↑↑↑
CAFFEINE	↑	↑↑	↑↑	↑	↑↑↑	↑↑↑
	LC		THALAMUS		HYPOTHALAMUS	

Fig. 3. Fos immunoreactive cells in various brain regions of TgNTRK3 and control mice after vehicle, yohimbine (5 mg/kg) or caffeine (50 mg/kg) administration in TgNTRK3 and wild-type littermates. **a** Fos positive nuclei density in cingulate cortex (CX), basolateral (BLA) and central (CeA) nuclei of the amygdala in TgNTRK3 vs. wild type mice after saline, yohimbine or caffeine treatment. **b** Fos positive nuclei density in locus ceruleus (LC), thalamus and hypothalamus in TgNTRK3 vs. wild type mice after saline, yohimbine or caffeine administration. Tables below show an outline of the increase or decrease of Fos expression after each treatment. Data are expressed as density of Fos immunopositive nuclei (mean ± SEM). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ and $^{\dagger}P<0.05$ for genotype differences; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ for treatment differences

fear-related circuits such as thalamus, hypothalamus, cingulate cortex, BLA and CeA nuclei of the amygdala, and LC in wild-type and transgenic mice (Fig. 3). Two-way ANOVA on the cell count data revealed a significant interaction between genotype and treatment after yohimbine administration in the cingulate cortex ($F(1,103) = 13.614$; $P<0.001$), hypothalamus ($F(1,69) = 6.352$; $P<0.05$), and CeA ($F(1,36) = 20.987$; $P<0.001$) but not in BLA, thalamus, or LC. A significant genotype per treatment interaction was also observed after caffeine injection in cingulate cortex ($F(1,108) = 7.723$; $P<0.01$), BLA ($F(1,33) = 11.914$; $P<0.01$), and LC ($F(1,29) = 5.009$; $P<0.05$) but not in the hypothalamus, the thalamus, or the CeA.

Cingulate cortex

Very low levels of Fos expression were observed in the cingulate cortex after saline treatment being significantly lower in TgNTRK3 mice (Fig. 3). One-way ANOVA revealed a marked increase in Fos positive cells in TgNTRK3 vs. wild type mice in cingulate cortex after yohimbine administration ($F(1,50) = 12.034$; $P<0.001$) and ($F(1,55) = 5.214$; $P<0.05$) being the increments significantly greater in TgNTRK3 mice (yohimbine 39.05% in wild type vs. 196.60% in TgNTRK3; $t = 3.69$, $P<0.001$; caffeine 43.71% in wild type vs. 172.35% in TgNTRK3; $t = 2.77$, $P<0.01$). Compared to saline treated animals, the increase in Fos positive cells was significant in yohimbine in wild type mice ($F(1,52) = 4.774$; $P<0.05$) but was more marked in TgNTRK3 mice ($F(1,49) = 45.663$; $P<0.001$). Similar differences were observed after caffeine administration, where a significant effect was also attained both in wild type ($F(1,59) = 4.221$; $P<0.05$) and more marked in TgNTRK3 mice ($F(1,47) = 37.856$; $P<0.001$).

Hypothalamus

In the hypothalamus one-way ANOVA revealed a significantly higher number of Fos positive cells in TgNTRK3 vs. wild type mice after saline administration

($F(1,22) = 4.992$; $P < 0.05$). Yohimbine treatment significantly increased Fos positive cells in both genotypes (wild type: $F(1,35) = 16.041$; $P < 0.001$; TgNTRK3: $F(1,32) = 13.728$; $P < 0.01$) compared to saline treated animals, which was significantly less important in transgenic mice ($F(1,45) = 8.755$; $P < 0.01$). Consequently, the percentage of increase in Fos expression after yohimbine administration was significantly lower in TgNTRK3 mice (476.75% in wild type vs. 101.21% in TgNTRK3; $t = -2.52$, $P < 0.05$). Caffeine administration also induced an increase in Fos positive cells (wild type: $F(1,24) = 39.381$; $P < 0.001$; TgNTRK3: $F(1,32) = 17.662$; $P < 0.001$), with no differences between genotypes.

Thalamus

In the thalamus no genotype effect was detected after either treatment. One-way ANOVA revealed a significant increase in Fos positive cells after yohimbine administration in wild type mice ($F(1,52) = 12.014$; $P < 0.001$), and in transgenic mice ($F(1,46) = 16.651$; $P < 0.001$) as compared to saline. After caffeine administration, a significant effect was also attained in thalamus in wild type ($F(1,44) = 9.885$; $P < 0.01$) but not in transgenic mice.

Amygdala

No genotype effect was detected after yohimbine treatment in BLA. However, caffeine administration produced an increase in Fos positive cells in TgNTRK3 mice ($F(1,13) = 9.241$; $P < 0.01$) that was significantly higher than in wild type (79.39% in wild type vs. 362.91% in TgNTRK3; $t = 3.45$, $P < 0.01$). In the CeA no genotype effect was detected after treatment with saline or caffeine, but yohimbine induced a significantly lower increase in Fos positive cells in transgenic mice as compared to wild type ($F(1,18) = 55.366$; $P < 0.001$). The percentages of increase were 298.46% in wild type vs. 107.66% in transgenic mice ($t = -4.58$, $P < 0.001$). When compared to saline administration an increase in Fos positive cells after yohimbine administration was detected in wild type mice in BLA ($F(1,17) = 10.386$; $P < 0.01$) and CeA ($F(1,18) = 55.366$; $P < 0.001$). This increased Fos expression was also attained in transgenic mice in the BLA ($F(1,16) = 11.191$; $P < 0.01$) and CeA ($F(1,16) = 6.127$; $P < 0.05$). After caffeine administration, no significant effects were attained in wild type mice neither in BLA nor in CeA. Contrarily, Fos positive cells were significantly increased in the BLA ($F(1,14) = 135.964$; $P < 0.001$), but not in CeA of transgenic mice.

Locus ceruleus

In the LC, whereas no significant increase in Fos positive cells was detected in wild type mice after yohimbine treatment, one way ANOVA revealed a significant increase in Fos expression after yohimbine treatment in transgenic mice ($F(1,19) = 8.034$; $P < 0.05$), but the genotype dependent differences in the percentage of increase, did not reach statistic significance (24.82% in wild type vs. 72.70% in TgNTRK3). Caffeine administration did not increase the number of Fos positive cells in wild type, but a significant increase was observed in TgNTRK3 mice ($F(1,15) = 25.237$; $P < 0.001$) with respect to saline treated animals. The percentage of increase of Fos expression was significantly higher in TgNTRK3 mice (10.21 vs. 119.04%; $t = 2.23$, $P < 0.05$).

α2A-AR and α2B-AR expression is increased in TgNTRK3 mice

Western blot analysis of the brainstem region revealed a significant increase in α2A-AR ($F(1,5) = 7.439$; $P = 0.05$)

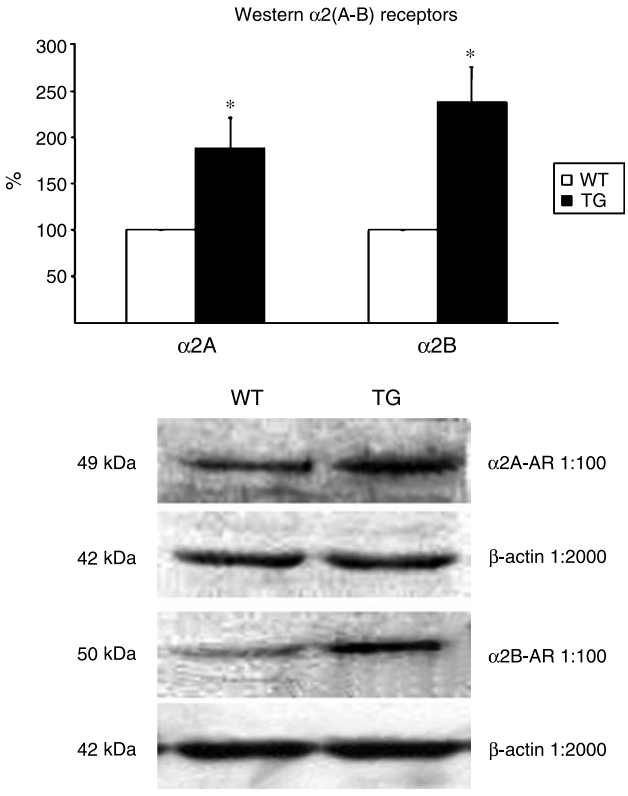


Fig. 4. Western blot analysis of α2A-AR and α2B-AR in TgNTRK3 (filled bars) and wild-type littermates (open bars). β-actin was used as loading control. Relative NTRK3 immunoreactivity was determined densitometrically and quantification is represented as percentage of increase with respect to basal values; $^{*}P < 0.05$, Student's *t*-test

and α2B-AR ($F(1,5) = 13.622$; $P < 0.05$) levels in transgenic NTRK3 mice vs. control mice (Fig. 4).

Discussion

Panic patients show increased sensitivity to panicogenic/anxiogenic drugs probably due to dysregulation of the fear system. We have evaluated the behavioral and neural pattern of response to drugs that evoke anxiety or panic in mice overexpressing TrkC. In our experiments, TgNTRK3 mice showed increased lactate sensitivity in specific panic-like reactions (flight response) in the MDTB. Moreover, exposure to the panicogenic agents, yohimbine, or caffeine increased Fos levels in the brain in a number of limbic and autonomic regulatory centers that have been implicated in the pathophysiology of panic disorder and lactate sensitivity (Gorman et al., 2000; Shekhar et al., 2003). The differential pattern of Fos activation in the brain of transgenic mice may be seen as a fingerprint of panic-like phenotype.

In the present study, the baseline levels of defensive behaviors of control animals in the MDTB were qualitatively similar to those reported previously (Griebel et al., 1995a, b; 1996; Dierssen et al., 2006). Prior to confrontation with the rat, spontaneous locomotor activity in the apparatus was not affected by genotype or treatment. After removal of the rat (post-test), the contextual defense behaviors were expressed in transgenic mice as increased immobilization that was more marked under lactate treatment and was also observed in lactate-treated control mice. This paradoxical reduction in activity was due to a very important increase in duration of immobilization in transgenic mice an effect that was induced by lactate treatment in control mice. Moreover, lactate treatment significantly increased the immobility in transgenic mice. Since inhibition of motor responses during fear/arousal (freezing) appears to be mediated via the periaqueductal gray and medial hypothalamus (Bandler and Shipley, 1994), this pathway might be altered in TgNTRK3 mice.

In the predator avoidance test, TgNTRK3 mice showed significantly higher flight responses (number of avoidances and flight speed) that were dramatically increased after sodium lactate. This escape behavior and is considered a measure of panic reaction in rodents, highly sensitive to panicogenic and panicolytic agents was also observed in wild type mice after sodium lactate treatment (Blanchard et al., 1997, 2001; Dierssen et al., 2006). Defensive threat and attack reactions, such as vocalizations, bites, upright postures, and jump attacks, that have been found to be sensitive to anxiolytic drugs (Blanchard et al., 1997), were not modified by treatment in TgNTRK3 but wild type mice showed an increase in number of upright postures after sodium lactate treatment. Finally, in our previous study we observed reduced risk assessment behaviors in the chase-flight test and a lower number of approaches in the straight alley test in TgNTRK3 mice, that could not be reproduced in the present experiments. However, it is known that both cognitive and affective oriented behaviors are less sensitive to panicogenic agents (Blanchard et al., 1997, 2001).

In the second series of experiments, we used Fos immunohistochemistry to study the response to anxiogenic drugs (yohimbine and caffeine) that are clinically effective to increase panic reaction. These two pharmacological agents increase the availability of noradrenergic in the synaptic cleft (Reith et al., 1987), and several lines of evidence suggest that the specific pattern of Fos expression evoked is associated with the state of anxiety that they produce (Singewald et al., 2003). In our study, strong up-regulation of Fos immunoreactive cells was detected in brain areas anatomically connected and implicated in anxiety- and panic-related circuitries, such as the thalamus, hypothalamus, amygdala and cingulate cortex in yohimbine and caffeine treated vs. saline-injected wild type and transgenic mice The pattern of activation was similar to that previously described (Singewald et al., 1999, 2003), although we observed discrepancies in some brain regions in wild type mice. Moreover, overexpression of TrkC influenced both the intensity and distribution of Fos expression. In particular, the increase of Fos immunoreactivity was higher in the cingulate cortex and LC of TgNTRK3 mice, whereas the transgenic amygdala showed a less important response than wild types.

In the cingulate cortex both caffeine and yohimbine increased Fos expression, this activation being significantly higher in transgenic mice (yohimbine: 39.05% in wild type vs. 196.6% in TgNTRK3 and caffeine: 43.7% in wild type vs. 172.3% in transgenic mice). However, lower basal activity of cingulate cortex was detected in saline treated transgenic mice. The cingulate cortex influences the induction of anxiety and fear-related behavior and may play a role in modulating autonomic reactions during stress and anxiety (Espejo, 1997; Morgan and LeDoux, 1995). Previous studies showed increased Fos expression after administration of yohimbine, caffeine and other anxiogenic drugs in prefrontal areas of the cortex (Singewald et al., 2003) that are also activated in animals exposed to the elevated plus maze (Duncan et al., 1996) or conditioned fear paradigms (Beck and Fibiger, 1995; Campeau et al., 1997). Interestingly, lack of appropriate

governance by frontal cortical regions has been hypothesized to be central in the pathophysiology of panic disorder. Lesions of the medial prefrontal cortex enhance anxiety-like behavior in rodents (Jinks and McGregor, 1997) and potentiate the anxiogenic effect of FG-7142 in rats (Jaskiw and Weinberger, 1990). Reduced basal activity of the prefrontal cortex predicts panic vulnerability to respiratory challenge across subjects, suggesting that input from this cortical region may be important in suppressing fear responding (Kent et al., 2005). Thus, the lower activity of the cingulate cortex in saline treated TgNTRK3 and the higher levels Fos expression induced by anxiogenic drugs could reflect a malfunctioning of the cortical areas controlling the fear system leading to reduced cortical control.

In our experiments yohimbine but not caffeine elicited a significant response in the amygdaloid complex of wild type mice. This result does not reproduce previous findings showing that both caffeine and yohimbine increased Fos expression in the amygdala (Singewald et al., 2003). Methodological differences may account for these discrepancies, since we sacrificed the animals 45-min after injection, whereas in previous studies at least two hours were allowed, thus suggesting that the activation pattern may reflect different stages in the panicogenic response. Interestingly, in transgenic mice both yohimbine and caffeine elicited a significantly lower response than in wild types in CeA but a significantly higher response to caffeine but not to yohimbine in the BLA. Activation of the amygdala, a structure proposed to be a central point of dissemination of fear information, results in behavioral and physiological responses associated with anxiety and panic-like behavior, whereas lesions reduce such effects (Davis, 1992; Killcross et al., 1997; Sajdyk and Shekhar, 2000). However, the specific role of the CeA is controversial and some inconsistencies appear when evaluating in emotional responses evoked by psychogenic stressors such as immobilization (Dayas and Day, 2002; Ma and Morilak, 2004), with previous reports describing little or no induction of Fos expression in CeA by acute restraint stress (Arnold et al., 1992; Chen and Herbert, 1995). Moreover, it has been reported that the dynamic pattern of regional cerebral blood after a panicogenic challenge includes activations and deactivations in the amygdala and other forebrain regions (Boshuisen et al., 2002). During anticipatory anxiety, PD patients show hypoactivity in several brain areas including the amygdala, compared to control subjects. Kent et al. (2005) were unable to demonstrate a relationship between panic anxiety and central amygdala activation in patients.

In the hypothalamus, which coordinates neuroendocrine, autonomic, and behavioral responses for a variety of homeostatic mechanisms (Bernardis and Bellinger, 1998; DiMicco et al., 2002; Chou et al., 2003), each of the anxiogenic drugs examined in our study exerted a particularly pronounced effect on Fos expression in this brain region. As observed in the amygdala, TgNTRK3 mice showed a different activation pattern upon administration of the drugs, with significantly less activation upon yohimbine administration. Systemic administration of yohimbine induces release of noradrenaline in target regions such as hypothalamus and hippocampus (Tjurmina et al., 1999), and elicits behaviors consistent with anxiety and fear (Handley and Mithani, 1984; Johnston and File, 1989). Stimulation of the lateral hypothalamus seems to be important for cardiovascular expressions of fear and anxiety (Charney et al., 1987; Fendt and Fanselow, 1999). Moreover, activation of the hypothalamus by blockade of GABA-A neurotransmission in rats elicits panic-like responses such as increases in heart rate, respiratory rate, mean arterial blood pressure, and experimental anxiety (DiMicco et al., 1986; DiMicco and Abshire, 1987; Shekhar, 1993). A significantly higher number of Fos positive cells after saline administration was detected in TgNTRK3 vs. wild type mice, that may reflect a reduced control over this region.

Finally, in the LC no significant increase in Fos positive cells was detected in wild type mice after either treatment but instead, an increase in Fos in the LC was induced by all the anxiogenic drugs tested in TgNTRK3 mice. LC is a site of integration between the limbic forebrain and somatosensory, cardiovascular and visceral information and extensive preclinical and clinical evidence indicates a key role for the LC in panic and anxiety mechanisms (for reviews, see Linthorst, 2005) probably facilitating panic reaction (Coplan and Lydiard, 1998). Enhanced Fos expression in the LC has been observed in animals subjected to various aversive paradigms, including the elevated plus maze (Silveira et al., 1993) and conditioned fear (Beck and Fibiger, 1995; Campeau et al., 1997) and there is evidence that stimulation of the LC elicits fear and panic-like behavior in rats (Butler et al., 1990; Priolo et al., 1991; Funk et al., 2006) and monkeys (Redmon et al., 1976). Electrophysiological evidence suggests that sprouting of LC axons may also occur after chronic stress (Sakaguchi and Nakamura, 1990). Neurotrophic factors are logical candidates for the modulation of LC plasticity as they not only affect neuronal development and survival but also influence phenotypic expression of transmitters and neuropeptides and neurite

outgrowth. In this regard, our previous work demonstrated that overexpression of TrkC in transgenic mice led to increased numbers of TH and of TH/TrkC positive-cells in LC. Although in the present experiments we did not investigate the neurochemical identity of the neurons expressing Fos in the LC, it is very likely that most if not all of the neurons are noradrenergic. This assumption is supported by previous studies reporting an increased Fos immunolabeling in noradrenergic neurons in the ventrolateral medulla, nucleus of the solitary tract, and LC after yohimbine identified by retrograde neural tracing.

As explained above, one interesting result in our study is the differential pattern of response to yohimbine and caffeine that was observed in the amygdala and hypothalamus, yohimbine eliciting a lower response in TgNTRK3. The mechanisms of action of these two drugs may account for these differences. Yohimbine is a competitive α2-AR antagonist (Tsujino et al., 1992) that may also interact with α1-AR, serotonin and dopamine receptors and inhibit monoamine oxidase (Dukes, 1988; Bhattacharya et al., 1991). On the other hand, caffeine is a methylxanthine with adenosine receptor blockade properties (Hadfield and Milio, 1989). Presynaptic α2-AR in mammals present two predominant somatodendritic α2-autoreceptors, α2A/D-autoreceptors and a minor population of either α2B- or α2C-autoreceptors (for review see Starke, 2001). Western blot analysis of the brainstem of TgNTRK3 mice revealed a significant increase in the α2A-AR and α2B-AR subtypes. α2-AR exist both pre- and postsynaptically, at which sites they have very different functions. In TgNTRK3, the increased TH-positive cellularity in catecholaminergic nuclei could account for these increased α2A and α2B-AR population. However, studies with antagonists in the LC have shown that somadendritic α2-AR mediate hyperpolarization and a decrease of firing of noradrenergic neurons (Nörenberg et al., 1997; Mateo and Meana, 1999) and electrophysiological studies in LC of TgNTRK3 mice showed increased firing rates in this nucleus (J. Pineda, unpublished). It is difficult to predict the final effects of the chronic elevation of α2-AR population. A number of studies have shown that adaptive changes of α2-AR or α1-AR in the presence of chronically administered agents may either interfere with or enhance noradrenergic neurotransmission. It could thus be argued that, in our model, the increased α2-AR population reflects a compensatory mechanisms trying to restores the chronically increased noradrenergic transmission.

In fact, consequences on other neurotransmitter systems could also appear. The α2-heteroreceptors also occur on cerebral serotoninergic terminal axons where they inhibit the release of serotonin (Trendelenburg et al., 1994). In our model extracellular serotonin levels showed no differences in basal conditions but reduced serotonin release upon lactate administration in TgNTRK3 mice (R. Trullás and A. Zapata, unpublished observations), that may reflect the increase in release-inhibiting α2-adrenoceptors. In our experiments, TgNTRK3 mice showed increased freezing response in the MDTB that may be dependent on an aberrant serotonergic/noradrenergic system response (Marchesi et al., 2006; Linthorst, 2005).

Neuroanatomical studies have established that the LC has connections with limbic forebrain regions such as the amygdala, lateral hypothalamus and prefrontal cortex. It has been described that neurons activated by yohimbine included medullary and pontine neurons that project to the central nucleus of the amygdala and to the lateral bed nucleus of the stria terminalis (Myers et al., 2005). Thus, the reduced activation attained by yohimbine in the amygdala and hypothalamus may reflect the chronic alteration of α2-adrenoceptors in TgNTRK3 mice. In fact, response to yohimbine was higher in the cingulate cortex, but lower in the hypothalamus and the amygdala, thus suggesting that the elevation of extracellular noradrenergic levels induced by acute administration of the α2-receptor antagonist yohimbine has specific effects on amygdala and hypothalamus in conditions where increased α2-adrenoceptors exist.

An aberrant cross-talk between LC and forebrain regions may thus be of crucial importance in the altered responses to yohimbine observed in our study. The increased numbers of TrkC neurons within the peri-LC dendritic field in TgNTRK3 mice (Dierssen et al., 2006), raises the possibility that the integration of afferent inputs that regulate activity in the LC-noradrenergic system (Jones, 1991) is altered in TgNTRK3 mice and potentially in panic disorder. Afferents to the amygdala that modulate its activity during anxiogenic situations may influence the expression of anxiety-like behavior.

In summary, we report that overexpression of TrkC increases the sensitivity to prototype anxiogenic/panicogenic drugs with very diverse pharmacological properties. Since anxiogenesis is a common property of the drugs tested, the differential drug-evoked increase in Fos expression is the result of the acute state of anxiety induced by the drugs and reflects functional differences of anxiety-associated circuits in transgenic mice. The ascending noradrenergic neurotransmitter system, that is dysregulated in TgNTRK3 mice, is activated during panic redaction and provides a dense innervation to the amygdala, hy-

pothalamus and prefrontal cortex (Moore and Bloom, 1979; Groenewegen and Uylings, 2000). Therefore, our data support the theory of an alteration in the integrated forebrain and hindbrain neuronal system that is important for anxiety states evoked by drugs and environmental manipulations.

We conclude that the perturbation in brain noradrenergic homeostasis of TgNTRK3 mice is drastically enhanced when the mice are challenged with anxiogenic drugs. The supersensitivity of these mice to the behavioral and neurochemical effects of sodium lactate and the differential pattern of Fos obtained upon administration of panicogenic drugs indicates a crucial involvement of the NT-3/TrkC system in the modulation of the fear system with consequences in panic disorder.

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