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Transgenic mice overexpressing the full-length neurotrophin receptor TrkC exhibit increased catecholaminergic neuron density in specific brain areas and increased anxiety-like behavior and panic reaction

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Accumulating evidence has suggested that neurotrophins participate in the pathophysiology of mood disorders. We have developed transgenic mice overexpressing the full-length neurotrophin-3 receptor TrkC (TgNTRK3) in the central nervous system. TgNTRK3 mice show increased anxiety-like behavior and enhancement of panic reaction in the mouse defense test battery, along with an increase in the number and density of catecholaminergic (tyrosine hydroxylase positive) neurons in locus coeruleus and substantia nigra. Furthermore, treatment of TgNTRK3 mice with diazepam significantly attenuated the anxiety-like behaviors in the plus maze. These results provide evidence for the involvement of TrkC in the development of noradrenergic neurons in the central nervous system with consequences on anxiety-like behavior and panic reaction. Thus, changes in TrkC expression levels could contribute to the phenotypic expression of panic disorder through a trophic effect on noradrenergic neurons in the locus coeruleus. Our results demonstrate that the elevated NT3-TrkC tone via overexpression of TrkC in the brain may constitute a molecular mechanism for the expression of anxiety and anxiety.

Keywords: Panic disorder; NT-3; TrkC; Anxiety-like behavior; Mouse defense test battery; Locus coeruleus

Recently, an accumulation of evidence has indicated that neurotrophins may be involved in the pathophysiology of mood disorders (Nestler et al., 2002; Castren, 2004a,b). BDNF and NT-3 have been proposed as susceptibility genes for major depressive disorder and schizophrenia (Lin and Tsai, 2004; Tadokoro et al., 2004; Schumacher et al., 2005) and for other neuropsychiatric disorders (Ribases et al., 2005; Liu et al., 2005). Administration of brain-derived neurotrophic factor (BDNF- Mouse Genome Informatics) or neurotrophin-3 (NT3; NTF3-Mouse Genome Informatics) into the dentate gyrus or CA3 region of hippocampus causes antidepressant-like effects in the forced swim and learned helplessness tests (Shirayama et al., 2002) and activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects (Saarelainen et al., 2003).

BDNF mouse mutants present higher anxiety levels when evaluated using the light/dark test and are hyperactive after exposure to stressors (Rios et al., 2001), although transgenic mice over-expressing the full-length neurotrophin receptor TrkB exhibit reduced anxiety (Koponen et al., 2004). Furthermore, chronic administration of selective serotonin reuptake inhibitors and other antidepressants that suppress panic attacks, increase expression of TrkC and TrkB in the rat cerebral cortex (Nibuya et al., 1995; Castren, 2004a). Recurrent panic attacks define panic disorder, a common and severe anxiety disorder of unknown etiology. Although several chromosomal regions and genes have been suggested to contribute to panic disorder, the phenotypic and genetic complexity of this disorder has precluded the finding of candidate genes (Knowles et al., 1998; Weissman et al., 2000; Crowe et al., 2001; Gratacos et al., 2001; Thorgeirsson et al., 2003). It has been proposed that genetic variants of several candidate genes of neurotransmitter systems, mainly catecholaminergic, may con-

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tribute to the susceptibility to panic disorder (Maron et al., 2005). This is in line with clinical investigations that have demonstrated abnormal noradrenergic (NA) system regulation in panic disorder patients and during panic attacks (Charney et al., 1990; Bremner et al., 1996a,b; Balaban, 2002). Elevated NA activity enhances anxiety-like behavioral responses and inappropriate activation of the locus coreuleus (LC), may participate in the exaggerated stimulus-responsiveness and increased emotionality seen in patients with stress or anxiety disorders (Priolo et al., 1991; Aston-Jones et al., 1996; Goddard and Charney, 1997). On the other hand, antidepressant treatment, that is effective in panic disorder patients, decreases LC firing and tyrosine hydroxylase (TH, rate-limiting enzyme in the synthesis of catecholamines) expression (Smith et al., 1995), and behavioral manipulations that decrease stress, such as postnatal handling modulate the responses of the noradrenergic system (Escorihuela et al., 1995a,b; Baamonde et al., 1999, 2002).

There are indications that LC NA neurons could respond to NT3 during development and is an important regulator of the adult catecholaminergic system (Martin-Iverson and Altar, 1996; Hagg, 1998). NT3 has been found to be particularly efficient at preventing the degeneration of adult LC NA neurons in 6-hydroxydopamine lesion models (Arenas and Persson, 1994; Arenas et al., 1995). In vitro studies in fetal tissue demonstrated that NT3 increases NA neuron survival (Friedman et al., 1993; Reiriz et al., 2002), upregulates the expression of NA markers (Sklair-Tavon and Nestler, 1995), and is involved in the appearance of the NA transporter during embryonic development (Zhang et al., 1997). Interestingly, NT3 promotes the neuritogenesis and hypertrophy of NA neurons in culture (Tsoulfas et al., 1996), suggesting that NT3 might regulate this aspect of NA neuron development. However, some authors have specifically questioned the involvement of NT3 in prenatal LC development since Nt3- or double Gdnf/Nt3-null mutant mice (Ernfors et al., 1994; Fariñas et al., 1994; Holm et al., 2003) do not display clear developmental abnormalities in this region. Neurons of the LC, express high levels of neurotrophin type-2 TrkB (NTRK2-Mouse Genome Informatics) and neurotrophin type-3 receptor TrkC (NTRK3-Mouse Genome Informatics) (Numan et al., 1998; King et al., 1999). However, it remains to be determined whether TrkC ligands regulate the development of LC NA neurons in vivo and if developmental alterations in LC may facilitate an anxiety prone phenotype and predispose to the development of panic disorder.

We have tested the hypothesis that in vivo overexpression of TrkC could produce an increase in survival and/or induction or promotion of TH-positive neurons in the LC with possible consequences on anxiety-like behavior in mice. To this aim we have generated transgenic mice over-expressing the NTRK3 gene and we have studied anxiety-related behavior and panic reaction, as well as structural changes in LC.

Materials and methods

General procedures

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. Same sex littermates were group-housed (4–6 animals per cage) in standard macrolon cages (40 × 25 × 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. Adult TgNTRK3 and wild-type littermates (5–7 months of age) gender-matched F1 from eight different litters were used for the phenotyping studies. Two lines of transgenic mice with insertion of the transgene in different chromosomes were used in order to exclude positional effects. The non-transgenic littermates of TgNTRK3 mice served as controls.

Generation of transgenic mice and genotyping

The NTRK3 cDNA was introduced into the *Eco*RI site of a fragment of the rabbit β-globin gene that includes the last two exons, the last intron and an SV40 enhancer in the pBluescript plasmid. A 1.3 kb fragment of the human PDGFB chain promoter (Resnick et al., 1993) was cloned in the *Xba*I–*Hin*dIII of the plasmid. The β chain promoter drives efficient and specific expression in the brain (Sashara et al., 1991) contains a shear stress response element (Resnick et al., 1993) and has been subjected to deletion analysis in endothelium (Khachigian et al., 1994). The complete expression cassette was designated PDGFB-NTRK3 and was verified by sequencing with specific primers in an automated sequencer (Applied Biosystems 377). The PDGFB/NTRK3 chimeric gene used to obtain transgenic mice is shown in Fig. 1a.

Transgenic mice were generated by standard pronucleus microinjection of the 6.4 kb fragment from the PDGFB-NTRK3 construct on a hybrid B6/SJL-F1J genetic background. The presence of the transgene was tested in DNA from tail biopsies by digestion with *Eco*RI and Southern blot analysis by using the complete NTRK3 cDNA as a probe. Three transgenic lines were obtained and were maintained by backcrossing to a B6/SJL-F1J background in heterozygosity. Genotyping was performed routinely by PCR analysis using the primer pairs: NTRK3 hum/mou-F 5′-cTGTTTGACGAAGTGAGTCCC-3′ and NTRK3 hum/mou-R 5′-TCCAGTGACGAGGGCGTG-3′. Hybrid founders were backcrossed extensively in order to attenuate littermate's genetic differences. All experiments were performed in mice from the F16–F20 generations. In all cases, transgenic mice were directly compared with non-transgenic littermates.

Expression analysis of the transgene

The expression analysis has been repeated along different generations of mice (F1; F01; F18; F20). The number of animals per experiment was at least nine (3 wild type, and 3 transgenic mice per line). For expression analysis of PDGFB-NTRK3 mRNA, total RNA from brain samples of control and transgenic mice was isolated using the RNAeasy Mini Kit (Qiagen) and analyzed by RT-PCR. RT-PCR was carried out by reverse-transcribing total RNAs (1 μg). The cDNA solution was subjected to 30 cycles of PCR amplification by using specific primers from the transgene (NTRK3 hum/mou-F and NTRK3 hum/mou-R). Absence of genomic DNA contamination was determined by the amplification of a 126-bp PCR fragment and the non-amplification of a 235-bp fragment from cDNA samples with primers for gdx (ubiquitin-like protein) transcript: gdx-F 5′-GGCAGCCCCCTGATCTCCAAAGTCCTGG-3′ and gdx-R 5′-AACGTTCGATGTCATCCAGTGTTA-3′.

To determine protein expression levels of TrkC, whole brain tissue extracts from adult (6 month of age) and postnatal (PD14) TgNTRK3 ($n = 8$ per line and experiment) and wild type mice ($n = 8$ per experiment) were prepared in ice-cold RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS).

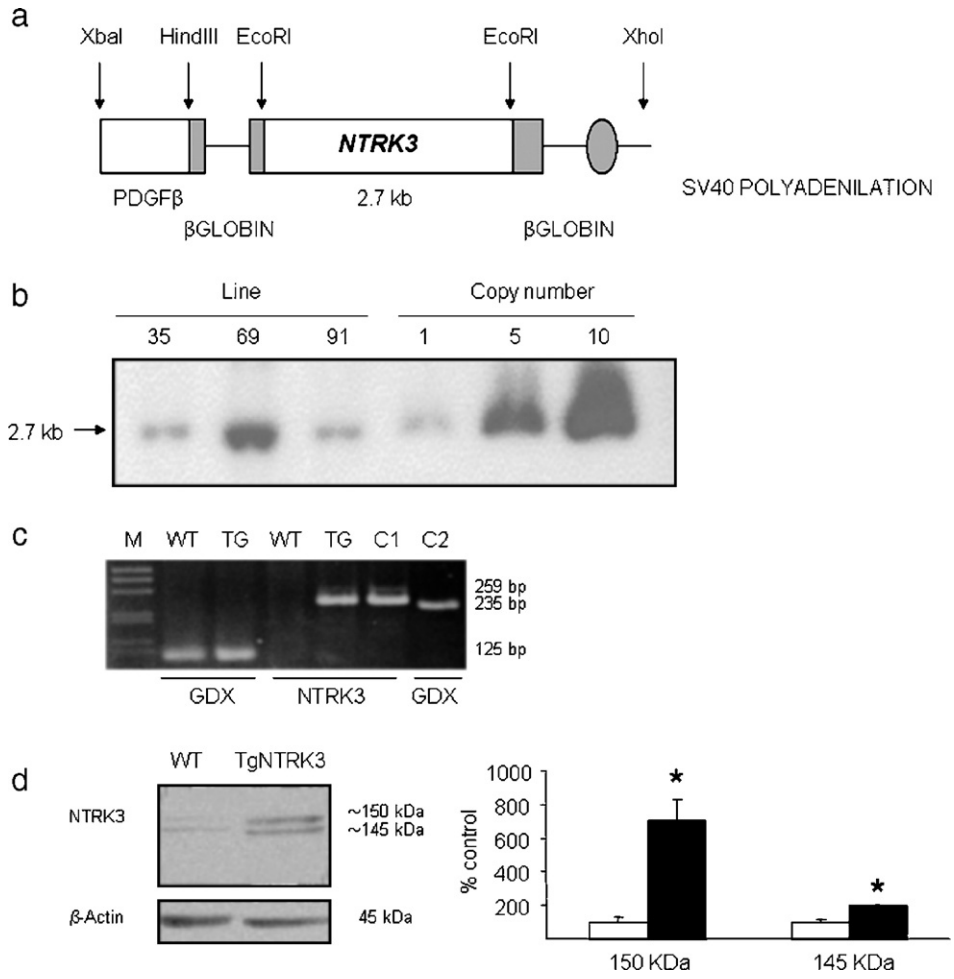

Fig. 1. Generation and expression analysis of TgNTRK3: (a) Schematic representation of the PDGFNTRK3 chimeric gene. (b) Southern blot analysis of three independent transgenic lines (L35, L69 and L91) carrying two (L35 and L91), and five (L69) copies of the transgene. (c) Expression of TrkC transgene by RT-PCR analysis. Lane 1: molecular weight marker; lanes 2 and 3: RT-PCR of murine gdx from total brain RNA to discriminate RNA amplification (125 bp) from DNA contamination (235 bp) in wild type and TgNTRK3 of a B6SJL strain. Lanes 4 and 5: RT-PCR from total brain RNA with NTRK3 hum/mou primers of wild type (no amplification) and TgNTRK3 (259-bp fragment), respectively. Lane 6: control of NTRK3 hum/mou primers using the NTRK3 construct as a template. Lane 7: control of gdx (Ubiquitin-like protein) primers, amplifying mouse genomic DNA. (d) Western blot analysis of TrkC in 3 TgNTRK3 (filled bars) and 3 wild-type littermates (open bars). β-actin was used as loading control. Relative TrkC immunoreactivity was determined densitometrically and quantification is represented as percentage of increase with respect to basal values $*P<0.05$, Student's t test.

The samples were mixed in SDS sample buffer, separated on a 7,5% SDS-polyacrilamide gel and thereafter transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The PVDF sheets were blocked and incubated with anti-TrkC (H-300) antibody (1:200; Santa Cruz, CA, USA) at 4°C overnight. Incubation with anti-rabbit IgG/HRP followed by ECL assay (Pierce, Rockford, IL, USA) allowed detection. β-actin was used as internal standard, using anti-Actin antibody (A-2066, Sigma, St. Louis, MO, USA).

Histological studies

For all histological experiments, mice were deeply anesthetized with isoflurane and then perfused intracardially with 50 ml of 0.1 M PBS, pH=7.4, followed by 150 ml of chilled 4% paraformaldehyde (Sigma, St. Louis, MO). The brains were post-fixed in the same fixative overnight, rinsed and then cryoprotected for 24 h in 30% sucrose-PBS at 4°C.

Expression pattern of TrkC

To determine the TrkC expression pattern in the central nervous system, 4 TgNTRK3 per line and 5 wild type mice were used. Fourteen micrometer coronal frozen sections were incubated for 10 min in sodium citrate (10 mM, pH=6) and then 48 h at 4°C with an anti-TrkC (1:200; Santa Cruz, CA, USA) antibody followed by incubation with a biotinylated secondary anti-rabbit IgG (1:2000; Vector Laboratories, Inc.) and ABC kit (Vector Laboratories, Inc.). Peroxidase activity was visualized with 0.05% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with toluidine. After completion of the staining, sections were dehydrated with increasing concentrations of ethanol and cover slipped with DPX (BDH laboratory supplies, UK).

Quantification of tyrosine hydroxylase positive cells

Stereological estimates of total number of neurons, and anti-TH positive neurons were obtained in 50 μm coronal sections incubated with anti-TH (1:8000; Sigma, St. Louis, MO; 6

TgNTRK3 per line and 7 wild type mice) with the aid of CAST-GRID software package adapted to an OLYMPUS BX51 microscope (Olympus, Denmark), through the substantia nigra pars compacta (SNc) and pars reticulata (SNr) (Bregma −2.46 mm to −4.04 mm) and LC (Bregma −5.34 mm to −5.8 mm) according to the stereotaxic coordinates adopted from the mouse brain atlas (Franklin and Paxinos, 1997). Since the rostral portion of the LC innervates forebrain structures such as the hippocampus, whereas the caudal portion of the LC innervates hindbrain structures such as the cerebellum and spinal cord (Fallon and Loughlin, 1982; Loughlin et al., 1982), the rostral-to-caudal distance of the LC was separately analyzed. Sections were systematically analyzed to include the 30, 50, and 70% levels of the LC, considering the rostral part (or 0%) as the beginning of the trochlear nucleus, and the caudal pole (100%) ended at the rostral level of the trigeminal motor nucleus (Hoogendijk et al., 1999). Estimation of the volume (Vref) of LC and SN 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. The size and shape of TH positive neurons were obtained with the aid of CAST-GRID software using the L03 nucleator IUR that provides a weighted volume estimate of the cell body. 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.

Co-localization of TH and TrkC positive cells

To determine the possible co-localization of TH and TrkC double labeling immunofluorescence experiments were carried out in the LC of transgenic (n=4 mice per line) and wild type (n=4) adult mice. Three adjacent series of 14 μm coronal sections were obtained on a cryostat through the LC [Bregma −5.34 to −5.80, (Franklin and Paxinos, 1997)]. The rostral to caudal distance of the LC was determined as for the stereological studies and sections were systematically taken to include the 30%, 50%, and 70% levels of the LC. Sections were incubated for 10 min in sodium citrate (10 mM, pH=6) and then blocked with goat serum 10% for 1 h at room temperature. Sections were incubated (48 h at 4°C) with anti-TrkC (1:100, Santa Cruz, CA, USA) antibody and 0/N with anti-TH antibody (1:4000; Sigma, St. Louis, MO, USA). Thereafter, slides were incubated with a fluorescent secondary Alexa 546 Anti-Rabbit (1:400; Molecular Probes, Invitrogen Detection Technologies) and an Alexa Fluor 488 goat anti-mouse (1:400; Molecular Probes, Invitrogen Detection Technologies). Following incubation with secondary antibodies during 1 h, the slides were washed with PBS 0,1 M and coverslipped with mowiol. Images of TH/TrkC-IR were taken by a Leica TCS SP2 confocal spectral microscope and then compared to the brain slices from the atlas of Franklin and Paxinos (1997). The number of TH, TrkC and double labeled cells was obtained by systematically examining bilaterally slides at each level of the LC (30%, 50% and 70%) for each subject. All lighting conditions and magnifications were held constant. Measurements were taken specifically over the cell body region of the LC (Fig. 2b).

Quantification of TH expression levels

Eight TgNTRK3 and eight control littermates were sacrificed, 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 for 20 min at 4°C), protein quantification was performed following the BCA protocol. A primary polyclonal anti-TH antibody (1:15,000; Sigma, St. Louis, MO) and a secondary peroxidase-conjugated anti-mouse (1:2000; DAKO, UK) were used. For TH expression studies, 20 μg of protein was electrophoresed on a denaturing 10% polyacrylamide gel and transferred to a Hybond-P membrane (Amersham Bioscience, Germany). Protein detection was done following the ECL system protocol. The quantification of the obtained bands was done by densitometric analysis (Quantity One Image software). β-Actin was used as internal standard, using anti-Actin antibody (A-2066, 1:2000; Sigma, St. Louis, MO, USA).

NT3 Elisa

For the analysis of NT3 levels, five wild type and five transgenic mice per line were deeply anesthetized with isoflurane. Ventral mesencephalon and brainstem were dissected out on ice and rapidly frozen using liquid N_2. Tissue samples were sonicated in lysis buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 10% glycerol and 1% NP-40) plus protease inhibitors (1 mM PMSF, 1 mg/ml leupeptin, 10 mg/ml aprotinin and 0.5 mM sodium orthovanadate) and centrifuged. Supernatants were collected and total protein was measured using the Detergent Compatible Protein Assay (Bio-Rad, Hercules, CA). NT3 contents were determined in duplicate by ELISA using the Emax ImmunoAssay system (Promega, Madison, USA). 200 μg of protein was analyzed for each point diluted in block and sample buffer. Values were calculated as pictograms of NT3 per milligram of protein.

Behavioral characterization of TgNTRK3 Mice

All the behavioral studies were conducted for both lines under basal conditions by the same experimenter in an isolated room and at the same time of the day. Behavioral experimenters were blinded as to the genetic status of the animals. The order of the experimental test was defined to reduce the influence of each test on the others, and was scheduled from less to more aversive. Male TgNTRK3 (n=24 per line) and wild type littermates (n=35) were used for all the behavioral testing. For the Mouse Defense Test Battery, a separate group of animals was used that included male TgNTRK3 (n=16 per line) and wild type littermates (n=25). A separate testing of 35 wild type mice of the reference strain of mouse has been performed in order to ensure the reliability of this task.

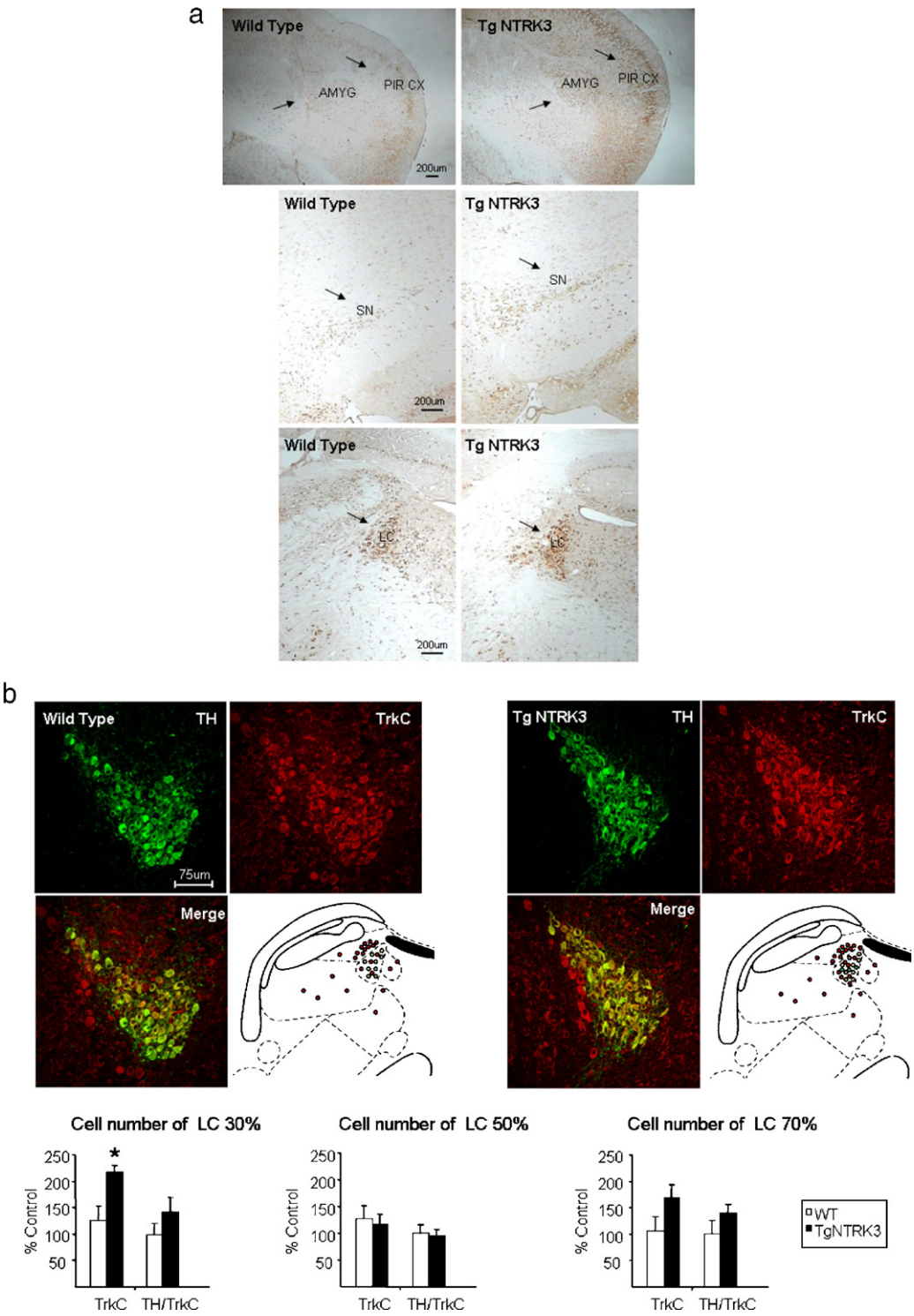

Fig. 2. TrkC immunoreactivity. (a) Photomicrographs of 14 μm coronal sections of wild type (left panel) and TgNTRK3 (right panel) mice illustrate TrkC immunoreactivity in the amygdala (Amyg), piriform cortex (Pir Cx), substantia nigra (SN) and locus coeruleus (LC). Scale bar = 200 μm. (b) Fluorescence photomicrographs of a representative section through the A3 area (SLC) of wild type (left panel) and TgNTRK3 (right panel) mice. Scale bar = 75 μm. The same field photograph was taken under different filters; The left-upper panels illustrate TH immunoreactivity (green), the right-upper panels show TrkC immunoreactivity (red), left-lower panels show the double-labeled LC neurons and right-lower panels a distribution map of TH and TrkC positive neurons. The number of TH/TrkC positive neurons of wild type (open bars) and TgNTRK3 (filled bars) mice in the LC at the 30%, 50%, and 70% rostro-caudal levels are given. Data are expressed as mean percentage of control ± SEM. *$P < 0.05$.

Locomotor activity

Locomotor activity was evaluated by using locomotor activity boxes (9×20×11 cm; Imetronic, France). The boxes contained a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical activity (Otto et al., 2001). TgNTRK3 and wild type littermates were exposed during 3 days to the locomotor activity boxes to be habituated to the test environment (data not shown). On days 4, 5 and 6 the animals were tested. Ambulatory locomotor activity and total (horizontal and vertical) activity were recorded during 15 min in a low illuminated environment (20–25 Lux). Data were recorded in a contiguous PC computer. The statistics for the locomotor activity was assessed only for the final 3 days of testing because the 3 first days correspond to the habituation of the mice to the monitoring boxes.

Open field

The open-field apparatus consisted of a rectangular area (70 cm wide×90 cm long×60 high) made of white Plexiglas in which 63 squares (10 cm×10 cm) were delimited with black lines on the white floor of the apparatus. The experimental protocol was carried out as previously defined (Escorihuela et al., 1995a,b; Altafaj et al., 2001). Briefly, each animal was placed in the center of the field under bright illumination (500 Lux) and during the observation session of 5 min the latency of crossing the first two squares from the central square where the mouse is initially placed; the number of squares crossed in the peripheral and central area of the field; rearings; number of grooming bouts; defecation boli left in the field; and urination events were measured (Maccarrone et al., 2002).

Elevated plus maze

These tests are based on exploration in a novel environment leading to the generation of an approach–avoidance conflict behavior (Otto et al., 2001).

The elevated plus-maze consisted of a black Plexiglas apparatus with four arms (29 cm long×5 cm wide) set in cross from a neutral central square (5 cm×5 cm). Two opposite arms were delimited by vertical walls (closed arms) and the two other arms had unprotected edges (open arms). The maze was elevated 40 cm above the ground and placed in indirect light (100 Lux). At the beginning of the 5-min observation session, each mouse was placed in the central neutral zone, facing one of the open arms. The total numbers of visits to the closed arms and the open arms, and the cumulative time spent in open arms and closed arms were then observed on a monitor through a videocamera system (ViewPoint). An arm visit was recorded when the mouse moved all four paws into the arm.

Thirty-four TgNTRK3 and 36 wild type littermates were randomly distributed in four groups according to the drug and dose injected: diazepam (0.75, 1.5 and 3.0 mg/kg, $n=8$–9 per group and genotype) and saline ($n=8$ per genotype). They were acclimated to handling and injection procedures 1 week before the experiment. Diazepam (Sigma, St. Louis, MO) was dissolved in saline solution (0.9%) and administered intraperitoneally. The behavioral effects observed in the plus-maze (latency to enter the open segment, number of entries and total time spent into the open segment) were measured and reversal caused by the drug was quantified. The anxiolytic effectiveness of a drug is illustrated by a significant statistical augmentation of parameters in open arms (time and/or entries and latency).

Zero-Maze

The zero-maze consisted of a circular path (runway width 5.5 cm, 46 cm diameter) with two open and two closed segments (walls 8 cm high) and was elevated 50 cm above ground. Animals were placed into the closed segments and their movements were recorded for 5 min using a video tracking system (ViewPoint Life Sciences Inc, Otterburn Park, Canada). The latency to enter the open segment, the number of entries and the total time spent into the open segment were measured. This test was also used to measure habituation to an aversive environment. To this aim, mice were exposed to the test for 3 consecutive days.

Light–Dark box

The light and dark box test is based on the innate tendency of mice to seek refuge in a dark box and propensity to escape novel places in which they have been constrained (Belzung and Le Pape, 1994). We used a box consisting of a small (15×20×25 cm) compartment with black walls and black floor dimly illuminated (25 Lux), connected by a 4 cm long tunnel to a large compartment (30×20×25 cm) with white walls and a white floor, intensely lit (500 Lux). Straight lines drawn on the floor of both compartments allowed the measurement of locomotor activity (number of squares crossed). Mice were individually placed in the dark compartment facing the tunnel at the beginning of the 5 min observation session. Number of squares crosses in light and dark zones, and in the tunnel connecting both zones and time spent in each were recorded, as well as the latency to the first visit to the illuminated zone.

Mouse defense test battery

The test was conducted in an oval runway (6.0×0.30×0.40 m) consisting of two 2 m straight segments joined by two 0.4 m curved segments and separated by a median wall (2.0×0.60 m). All parts of the apparatus were made of black wood and the floor was divided in sections of 20 cm to facilitate distance and crossing measurement. The apparatus was elevated 0.7 m from the floor to enable the experimenter to hold the rat easily, while minimizing the mouse's visual contact. All the experiments were video-recorded. Experiments were performed under ambient red fluorescent light (10 Lux). The procedure was carried out as proposed by Griebel et al. (1999). (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 handheld 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), percentage of mice presenting flight response 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 stimulus 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.

Data analysis

Parametric data are reported as mean±standard error of mean (SEM) and nonparametric data are reported as median±semi-interquartile 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 using a 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 and Elisa data, Student's *t* test analysis was employed. Significance levels were set at $P<0.05$. 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.

Results

Generation and general characterization of TgNTRK3 mice

Three transgenic lines, designated as lines 35, 69, and 91, carrying between two and five copies of the transgene (Fig. 1b), were established. Since line 91 did not express the transgene at the protein level, transgenic lines 35 and 69 were used for all the experiments. Physical characteristics such as body weight and the presence of bald patches and appearance of behavioral anomalies in the home cages were registered systematically with no apparent differences detected between wild type and transgenic mice.

PDGFB is distributed in ubiquitous neurons at all stages of development from embryo to adult (Sashara et al., 1991) and the endogenous TrkC transcript is present in the CNS already at late embryonic stages and increases to adult levels during first postnatal week (Numan and Seroogy, 1999). Increased expression of the transgene was shown by RT-PCR at embryonic (E 13.5 data not shown) and adult mice (Fig. 1c), and by Western blot analysis at postnatal stages (PD14) with a significant increase in TrkC levels in transgenic vs. wild type mice ($F=26.88$, $P<0.03$, one way ANOVA, Fig. 1d) that was less pronounced in adult mice. The NT3 content in brainstem was also measured, but in this region the values obtained in TgNTRK3 mice did not differ from wild types (20.22±1.98 pg/mg vs. 20.75±1.23 pg/mg, respectively, $P=0.03$, Student's *t* test). In the SN, NT-3 content was significantly increased in TgNTRK3 mice, being the obtained values 17.62±1.4 pg/mg (mean±SEM) in wild type versus 26.14±2.3 pg/mg in transgenic mice ($P=0.03$, Student's *t* test).

Since it is critical for the interpretation of any behavioral effects, extensive analysis of the pattern of TrkC overexpression across brain areas was carried out. In TgNTRK3 mice, expression of TrkC showed a spatial distribution pattern similar to that described previously for endogenous TrkC (Lamballe et al., 1994, Muragaki et al., 1995) with no ectopic expression (Table 1). Strong expression was detected in the forebrain, in hippocampal pyramidal neurons and dentate granule cells, and in cerebral cortex, specifically in layers II, III, and V, being more intense in TgNTRK3 mice. Moreover, thalamus, amygdala, and cerebellum showed clear, although slightly lower intensity of TrkC. These regions also showed higher intensity level in TgNTRK3. In the mesencephalon and specific nuclei of the medulla–pons, such as LC (Figs. 2a, b), TrkC was expressed in almost all noradrenergic neurons and in adjacent populations as shown by double labeling immunofluorescence (Fig. 2b) showing higher levels of expression and a clear increase in intensity in transgenic mice. Quantification of the TH/TrkC positive neurons showed a tendency for an increase in LC of TgNTRK3 mice, that only reached statistic significance at the more rostral level of the LC (92.4%±14,7%; $F(1,4)=9.66$, $P<0.05$).

Increase in the number of noradrenergic neurons in TgNTRK3

Immunoreactivity for TH presented an overall increase in LC and SNc with an increase in the density of mesostriatal projections (MSP) (Fig. 6a) in TgNTRK3 mice. Western blot analysis to quantify the levels of TH in the medulla–pons region revealed a significant increase in TH levels in transgenic vs. control mice ($t=4.26$, $P<0.01$, Student's *t* test, Fig. 6b). The total volume of the LC was not significantly different between genotypes. As shown in Fig. 6c, the mean density of LC TH-positive neurons was significantly increased in TgNTRK3 (23.1%; $F(1,225)=7.805$, $P<0.01$), and the mean density of the total neuronal population showed non-significant differences ($F(1,225)=0.007$, N.S.). This increased TH-positive cell density was accompanied by a reduction in non-TH cell density in TgNTRK3 ($F(1,225)=4.071$, $P<0.05$). Consequently, the proportion of TH positive neurons was increased in TgNTRK3 (23%; $F(1,225)=7.310$, $P<0.01$, Fig. 6f). When counting the total number of cells through all sections, an increase was detected in the number of TH-positive neurons in transgenic mice (31.8%; $F(1,12)=3.528$, $P=0.085$) but not in the total number of TH-positive plus toluidine blue stained cells. The size and shape of the TH-positive neurons, that might also be expected to change as a result of TrkC over-expression were not modified (wild type, 2.8±0.3 μm^3 vs. TgNTRK3, 3.1±0.1 μm^3 ($F(1,18)=0.54$, N.S.)), thus indicating that cell shape/size were not confounding the cell counts. Because different portions of the LC innervate different structures (Szot et al., 2006), sections were systematically taken to include the 30, 50, and 70% levels of the LC (Fig. 6d). The analysis of the 30% level of the LC (rostral) in TgNTRK3 showed an increased in TH-positive cell density (24.9%; $F(1,129)=3.753$, $P=0.055$). However, such increase was not observed in the 50% level of the LC (medial) in transgenic mice ($F(1,33)=1.379$, N.S.). The caudal level of the LC (70% level) of the TgNTRK3 showed an increase in TH-positive cell density (21%; $F(1,66)=4.904$, $P<0.05$).

To determine if the effect observed in TgNTRK3 could be observed in other catecholaminergic nuclei, we studied the SN, separately analyzing SNc and SNr. In the SNc quantification of the TH-positive neuronal population showed a significant increase

Table 1
Expression pattern of TrkC receptor in TgNTRK3 and wild type mice

Area	Wild type intensity	TG NTRK3 intensity
Forbrain		
Cerebral cortex (general)	**	***
Endopiroform cortex	**	***
Piriform cortex	**·***	***
Entorhinal cortex	**	***
Cortex–amygdala transition zone	**	***
Anterior amygdaloid area	**	**
Lateral septal nucleus	**	**
Triangular septal nucleus	*	*
Bed nucleus anterior commissure	**	**
Thalamus		
Paraventricular thalamic nucleus		
Reticular thalamic nucleus	**	***
Anterodorsal thalamic nucleus	**	***
Laterodorsal thalamus	**	***
Lateral globus pallidus	*	**
Caudate putamen (striatum)	*	*
Accumbens nucleus	*	*
Supraoptic nucleus	***	***
Hypothalamus		
Paraventricular hypothalamus	**	**
Lateral hypothalam area	**	**
Posterior hypothalam area	**	**
Hippocampus		
CA1	**·***	***
CA2	**·***	***
CA3	**·***	***
DG	**	**·***
Medial habenular nucleus	**	**·***
Amygdala		
Lateral amygdaloid nucleus	*·**	**
Basolateral amygadaloid nucleus	**·***	***
Central amygdaloid nucleus	*·**	**
Basomedial amygdaloid nucleus	*·**	**
Medial amygdaloid nucleus	*·**	**
Midbrain		
Substantia nigra		
Substantia nigra reticulata	**	**·***
Substantia nigra compacta	**	**·***
Ventral tegmental area	**	**·***
Red nucleus		
Red nucleus parvocellular	**·***	**·***
Red nucleus magnocellular	***	***
Mamillary nucleus		
Lateral mamillary nucleus	***	***
Supramamillary nucleus	**·***	**·***
Edinger-westhal nucleus	**·***	**·***
Periaqueductal gray	**	**
Paranigral nucleus	**	**
Interstitial nucleus	*·**	*·**
Hindbrain		
Oculomotor nucleus	**·***	**·***
Pontine nucleus	**	**
Ventral tegmental nucleus	*·**	*·**
Reticulotegmental nucleus pons	**·***	**·***
Raphe nucleus		
Dorsal raphe	**	**
Median raphe	**	**
Raphe magnus	**	**

Table 1 (*continued*)

Area	Wild type intensity	TG NTRK3 intensity
Hindbrain		
Locus coeruleus	**	**·***
Mesencephalic trigeminal nucleus	**·***	**·***
Olivary nucleus		
Dorsal periolivary region	**·***	***
Superior paraolivary nucleus	**·***	***
Lateral superior olive	**·***	***
Cerebellum	**·***	***
Molecular layer	*	*
Purkinje cells	**·***	**·***
Granular cells	**·***	**·***
Facial nucleus	**·***	**·***

in the total number of TH-positive neurons, in TgNTRK3 (35.6%; $F(1,13)=6.237$, $P<0.05$), with non-significant effect on non-TH cells nor total cell number. Accordingly, the density of TH-positive neurons was significantly elevated in TgNTRK3 (25.3%; $F(1,169)= 18.562$, $P<0.001$) and the mean density of non-TH and total neuronal population showed a non-significant increase ($F(1,169)= 1.087$, N.S. and $F(1,169)=1.489$, N.S., respectively; Fig. 6e). As a consequence, the proportion of TH-positive neurons was increased in TgNTRK3 (28.5%; $F(1,169)=16.316$, $P<0.001$; Fig. 6f). In contrast, in SNr, that presented no TH immunoreactive cells, the total cell number was not modified in TgNTRK3 (in all $P>0.06$).

TgNTRK3 mice show an anxiety-like phenotype in several behavioral tests

Genetic background is known to influence the phenotype of transgenic mice, and special attention must be paid when interpreting behavioral phenotypes (Crawley, 2000; Crawley et al., 1997). In this study, the B6/SJL hybrid mouse line was used, which helps to avoid problems associated with behavioral abnormalities in parental strains (Dierssen et al., 2002). Any bias due to background genes is unlikely because wild type littermates were used as comparison group for transgenic mice in every test studied. Male TgNTRK3 and control littermates derived from line L35 and L69 were submitted sequentially to several anxiety tests (see Materials and methods). Two-way ANOVA found no significant transgenic line effect for any behavioral measure, and therefore, data were collapsed across line. The analysis of the general neurological and neurosensorial profile, by a modified SHIRPA protocol (Masuya et al., 2005) did not show any differences between genotypes (data not shown).

In the locomotor activity test during the three first sessions the animals were habituated to the test environment (data not shown). On days 4, 5 and 6 the animals were tested. Repeated measures ANOVA with session as main factor revealed significant differences over sessions in both genotypes on the horizontal ($F(2,50)=6,313$ $P<0.01$ in wild type and $F(2,42)=5.599$ $P<0.01$ in TgNTRK3) and total activity ($F(2,50)=6.298$ $P<0.001$ in wild type and $F(2,42)=11.389$ $P<0.01$ in TgNTRK3) indicating a similar habituation pattern in both genotypes. However, no genotype effect was detected in horizontal ($F(1,47)=2,090$ $P=0.155$) and total (horizontal and vertical) activity ($F(1,47)=2.280$ $P=0.136$). These results indicate that the baseline activity and habituation to a novel

environment in a non-aversive environment were similar in both groups.

In the open field test, that measures activity in a more aversive environment, significant differences between genotypes were observed in the number of groomings ($F(1,47)=9.072$ $P<0.01$; Fig. 3a), indicating that some emotionality-related behaviors are increased in TgNTRK3 mice. However, one-way ANOVA calculated for horizontal locomotor activity (number of squares crossed) revealed a non-significant genotype effect neither in the periphery ($F(1,47)=3.548$ $P=0.06$) nor in activity in the center of the apparatus ($F(1,47)=1.656$ $P=0.205$). No differences were found in the other parameters measured.

In the elevated plus maze, a significant reduction in the cumulative time spent in open arms ($F(2,88)=3.162$ $P<0.05$) was observed in TgNTRK3, along with a tendency to a reduction in the percentage of time ($F(2,40)=22.773$ $P<0.052$) and the percentage of entries ($F(2,40)=3.589$ $P=0.037$) in the open arms, indicating a higher anxiety-like behavior in TgNTRK3 mice (data not shown). TgNTRK3 showed an increased sensitivity to diazepam in the elevated plus maze, showing an increase in the percentage of time in open arms in TgNTRK3 mice that reached statistic significance at 3 mg/kg ($F(1,15)=5.822$ $P<0.05$; Fig. 4a) with respect to saline injected transgenic mice. The percentage of time in open arms showed a tendency to a higher increase in TgNTRK3 than in wild type mice ($F(1,16)=1.886$, N.S.; $F(1,15)=2.097$, N.S. and $F(1,15)=4.033$ $P=0.063$ for the doses of 0.75 mg/kg 1.5 mg/kg and 3 mg/kg, respectively). The latency to enter the open arms was reduced by diazepam administration in a dose-dependent manner in TgNTRK3 mice that reached statistical significance at 1.5 mg/kg dose ($F(1,15)=11.149$ $P<0.01$; Fig. 4b) and 3 mg/kg dose ($F(1,15)=7.33$ $P<0.05$) with respect to saline injected mice. Moreover, the ratio of open/total arm entries, that was reduced in saline injected TgNTRK3 mice ($F(1,15)=4.427$ $P=0.05$; Fig. 4c) with respect to wild type animals, showed a significant increase in TgNTRK3 mice at 0.75 mg/kg ($F(1,15)=5.745$ $P<0.05$), 1.5 mg/kg ($F(1,15)=7.354$ $P<0.05$) and 3 mg/kg ($F(1,15)=12.858$ $P<0.01$) with respect to saline injected TgNTRK3.

In the elevated zero-maze, a significant reduction in the time spent and the total number of entries in the unprotected zones was observed in TgNTRK3 ($F(1,47)=7.550$ $P<0.01$ and $F(1,47)=4.083$ $P<0.05$, respectively; Fig. 3b) in the first session. When measuring habituation along three sessions (Fig. 3b), a significant session per genotype interaction was found in TgNTRK3 mice with respect to wild type mice ($F(1,47)=7.758$ $P<0.01$), with significant reduction in the number of entries in unprotected zones in the third session ($F(1,47)=5.864$ $P<0.05$) and in the time spent in these zones in the first ($F(1,47)=7.758$ $P<0.01$) and third ($F(1,47)=9.047$ $P<0.01$) sessions in TgNTRK3 mice, suggesting a degree of sensitization in these mice. In the light–dark box the latency to visit the lit compartment the first time was significantly increased in TgNTRK3 ($F(1,47)=9.021$ $P<0.01$; Fig. 3d). Due to this reason TgNTRK3 mice showed an increase in the time spent in the dark compartment ($F(1,47)=3.111$ $P=0.048$), although this effect barely reached statistical significance. No significant differences in the number of entries in the lit ($F(1,47)=0.697$ $P=0.408$) or the dark compartment ($F(1,47)=2.345$ $P=0.133$) were observed in TgNTRK3 as compared to wild type mice. TgNTRK3 did not show modifications in locomotor activity in this test since no differences were observed in the number of squares crossed ($F(1,47)=2.631$ $P=0.112$).

TgNtrk3 mice show increased panic reaction in the mouse defense test battery

The preliminary study of wild type mice showed values similar to those described by Griebel et al. (1995b, 1996) as shown in

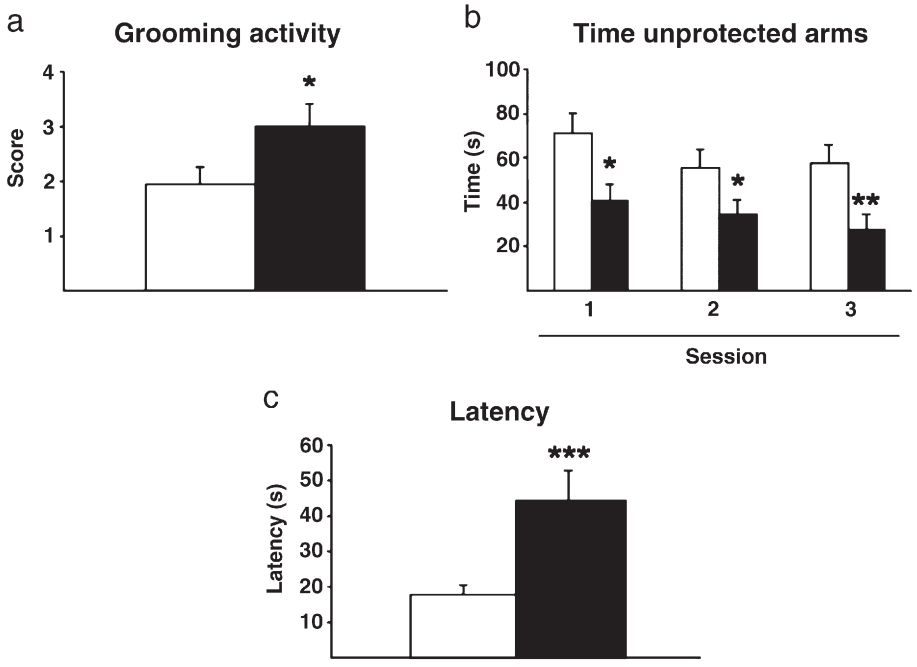

Fig. 3. Anxiety-like behavior in TgNTRK3 mice. (a) Open field test. Increase in grooming activity in TgNTRK3 (filled bars) mice vs. control mice (open bars); (b) Zero-maze. Reduction in the time spent in the unprotected zones in TgNTRK3 mice; (c) Light–dark box test. Significantly longer latency to enter the lit box in TgNTRK3. Data are expressed as mean±SEM. *$P<0.05$ **$P<0.01$ ***$P<0.005$.

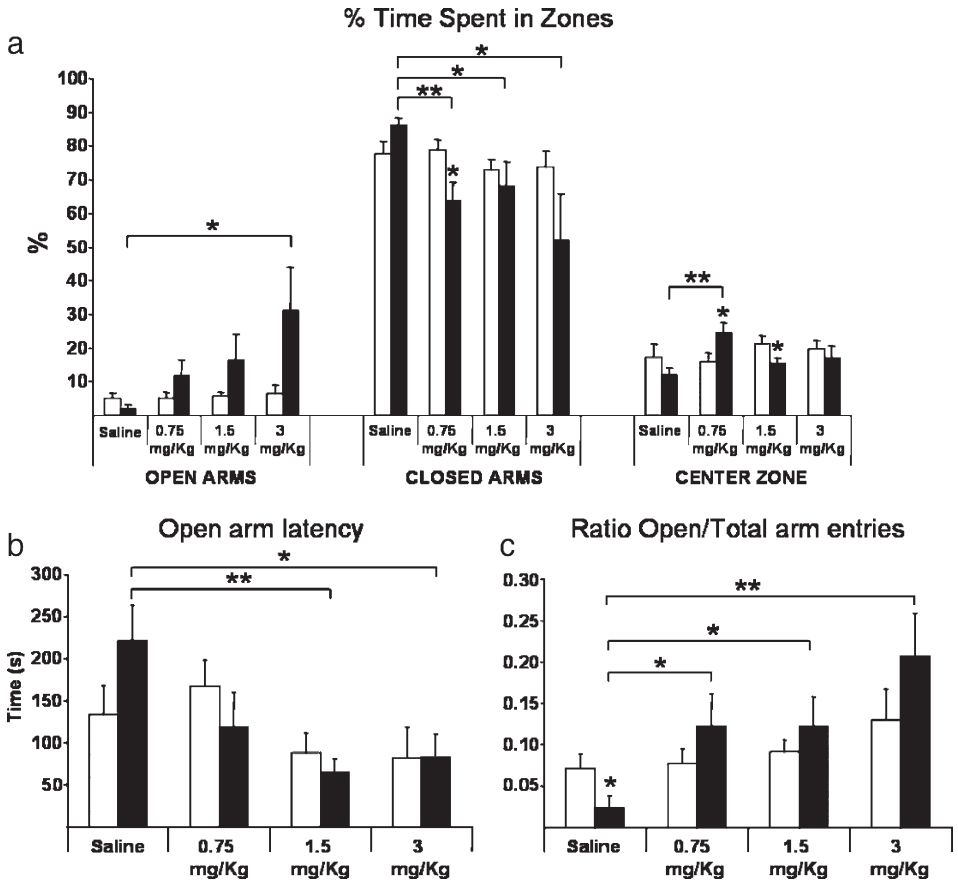

Fig. 4. Pharmacological studies in TgNTRK3. The effects of increasing doses of diazepam (0.75, 1.5 and 3 mg/kg) were tested in the elevated plus maze in wild type (open bars) and TgNTRK3 (filled bars). (a) Percentages of time spent in open and enclosed arms and in the center area of the plus maze. A reduction in time spent in open arms was observed in saline-injected TgNTRK3 mice, that was reversed upon diazepam treatment in a dose-dependent manner. (b) Open arm latency in the elevated plus maze. A tendency to a higher open arm latency was observed in saline-injected TgNTRK3 mice, that was reduced upon diazepam treatment. (c) Percentage of open arm entries/total entries in the elevated plus maze. A reduction in the ratio of open/total arm entries was observed in saline-injected TgNTRK3 mice, that was reversed upon diazepam treatment. Data are expressed as mean±SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 2. In the pre-test familiarization period of the mouse defense test battery (MDTB), prior to confrontation with the rat, the distance traveled ($F(1,23)=3.053$ $P=0.095$) and the mean speed ($F(1,23)=3.846$ $P=0.084$) were not affected by genotype, thus indicating a similar reaction to the apparatus.

In the chase/flight test a one-way ANOVA revealed a significant reduction in the number of orientations in TgNTRK3 mice ($F(1,23)=6.455$ $P<0.01$; Fig. 5a) but speed, reversals, and number of stops were similar between genotypes. In the predator avoidance test TgNTRK3 showed a significant increase in flight speed ($F(1,23)=5.662$, $P<0.05$) along with a tendency to increase avoidance distance and frequency that did not reach statistic significance. Moreover, the number of flight responses, a measure that is modified by administration of panicogenic drugs, was increased in TgNTRK3 mice ($F(1,20)=6.681$ $P=0.018$; Fig. 5b), and the percentage of subjects performing escape responses was also higher (90% in transgenic vs. 60% in wild type mice ($F(1,23)=12.301$ $P=0.048$) than in the control group. In the straight alley test a one-way ANOVA revealed a significant reduction of the number of approaches to the rat in TgNTRK3 mice ($F(1,23)=4.871$ $P=0.03$; Fig. 5c), with no differences in the closest distance to the rat or in the immobility time. Finally, in the forced contact test a one-way ANOVA revealed no genotype effect on number of bites ($F(1,23)=0.018$ $P=0.895$), vocalizations ($F(1,23)=0.321$ $P=0.576$) and upright postures ($F(1,23)=0.302$ $P=0.588$) but

Table 2
Behavioral parameters obtained in wild type mice of the B6SJL strain in the Mouse Defense Test Battery

	Swiss–Webster Mean±SEM	B6SJL Mean±SEM
Line crossings	162.7±3.57	149.0±12.04
Rearings	7.41±0.55	6.75±0.85
Head orientations	3.84±0.34	1.29±0.20
Reversals	2.21±0.27	2.31±0.50
Aproaches/Withdrawals	3.49±0.19	1.99±0.43
Vocalizations	2.88±0.06	3.20±0.35
Jump attacks	1.34±0.12	1.60±0.41
Upright postures	2.50±0.08	2.53±0.47
Bitings	2.84±0.07	3.40±0.46
Jump escapes	2.15±0.38	2.33±0.29
Stops	10.00±0.52	14.00±0.76
Flight speed (m/s)	1.08±0.03	0.75±0.10

For comparison data on the same test in Swiss–Webster mice reported by Griebel et al. (1996) are given in the left column.

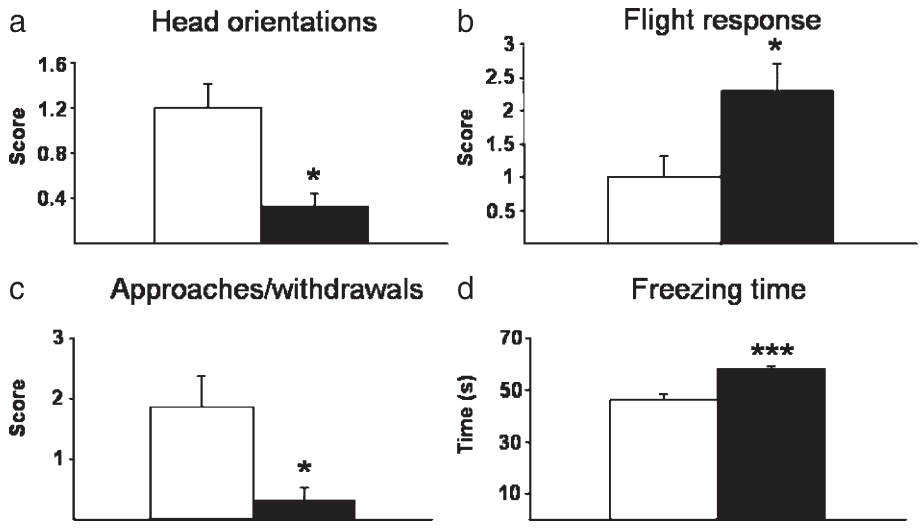

Fig. 5. Mouse Defense Test Battery in TgNTRK3 mice. (a) Chase/Flight test. Significant reduction in the number of orientations in TgNTRK3 mice; (b) Predator Avoidance test. Increases in flight response in TgNTRK3; (c) Straight Corridor test. Reduction in the number of approaches in TgNTRK3; (d) Increase in immobilization time in TgNTRK3 mice (filled bars) with respect to wild type littermates (open bars) after confrontation with the rat. Data are expressed as mean±SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

the number of jump escapes was significantly increased in TgNTRK3 ($F(1,23)=4.871$ $P<0.038$).

In the MDTB, the pre-versus post-test comparison allows to evaluate the contextual defense behaviors. In our experiments, after removal of the rat (post-test), significant differences between genotypes were observed in the runway activities. Transgenic mice traveled significantly less distance ($F(1,23)=5.585$ $P<0.027$), and present a significant reduction of mean ($F(1,23)=5.070$ $P<0.035$) and maximum speed ($F(1,23)=15.272$ $P<0{,}001$) in comparison to control mice. This paradoxical reduction in activity was due to a marked increase in duration of immobilization in transgenic ($F(1,23)=18.996$ $P<0.001$; Fig. 5d) vs. control mice. For this reason, comparison of pre- vs. post-exposure data for each group revealed a significant increase in distance traveled ($P=0.012$, two-tailed Wilcoxon matched pair test), mean and maximum speed ($P<0.01$ and $P<0.05$, respectively; two-tailed Wilcoxon matched pair test) in the post-test with respect to the pre-test in control mice, whereas transgenic mice showed no significant changes in their activity between pre- and post-test.

Discussion

In this study we have examined the effects of TrkC overexpression in the central nervous system by generating transgenic mice (TgNTRK3). The results presented here suggest the importance of the NT3–TrkC system on the prenatal development of catecholaminergic nuclei in vivo and indicate a role for TrkC in anxiety related behavior, as supported by the following findings: first, TgNTRK3 mice present significant changes in the number and density of NA neurons in catecholaminergic nuclei such as SNc and LC, a brain region that plays an essential role in anxiety and fear mechanisms; second, and most important, overexpression of TrkC in mice leads to an increased anxiety-related behavior in different tests and enhanced panic reaction in the MDTB; and third, response to benzodiazepam, reversed the anxiety-related behaviors in TgNTRK3. Phenotypes were identical regarding to general growth, locomotor activity and coordination, neurological/sensory phenotypes and spontaneous exploratory activity. Taken together these results support the idea of a developmental effect of TrkC on catecholaminergic circuits that may be responsible for the increase in anxiety-related behavior and panic reaction in these mice (Fig. 5).

In the central nervous system, the role of neurotrophins has been addressed by genetic engineering but most of the work so far has focused on the role of BDNF and of its receptor TrkB. Transgenic mice overexpressing BDNF under a β-actin promoter from the early development exhibit passive avoidance deficit, increased seizure severity, and increased dendritic complexity (Tolwani et al., 2002). Some authors found that BDNF mouse mutants present higher anxiety levels when evaluated using the light/dark test and are hyperactive after exposure to stressors (Rios et al., 2001), although these results could not be confirmed (Chourbaji et al., 2004). Moreover, transgenic mice overexpressing the full-length neurotrophin receptor TrkB exhibit reduced anxiety (Koponen et al., 2004). It thus may be argued that the BDNF–TrkB system may not play a significant role in anxiety-related phenotypes. However, relatively little is known about the function of the NT3–TrkC system in the central nervous system. Mice with a central nervous system-wide conditional ablation of NT-3 show attenuation of functional aspects regulated by NA signaling, such as opiate withdrawal symptoms, and were restored by transgene-derived NT-3 expressed by NA neurons of these conditional mutants (Akbarian et al., 2001). It has been reported that endogenous NT-3 expression is up-regulated in NA projection areas of the ventral forebrain after manipulations inducing neural plasticity in LC, such as chronic morphine exposure. However, the paradigm of overexpressing the neurotrophin or its receptor has been less studied. In our study the use of the PDGFB promoter directs the expression into neuronal cells thus locating the overexpression to where TrkC is endogenously expressed and activates from early embryonic stages, thus giving the opportunity to examine the effects of excess TrkC in crucial steps of LC development (Fig. 6).

TgNTRK3 presented a consistent increase in anxiety-related behavior that was observed across several tests. In the open field,

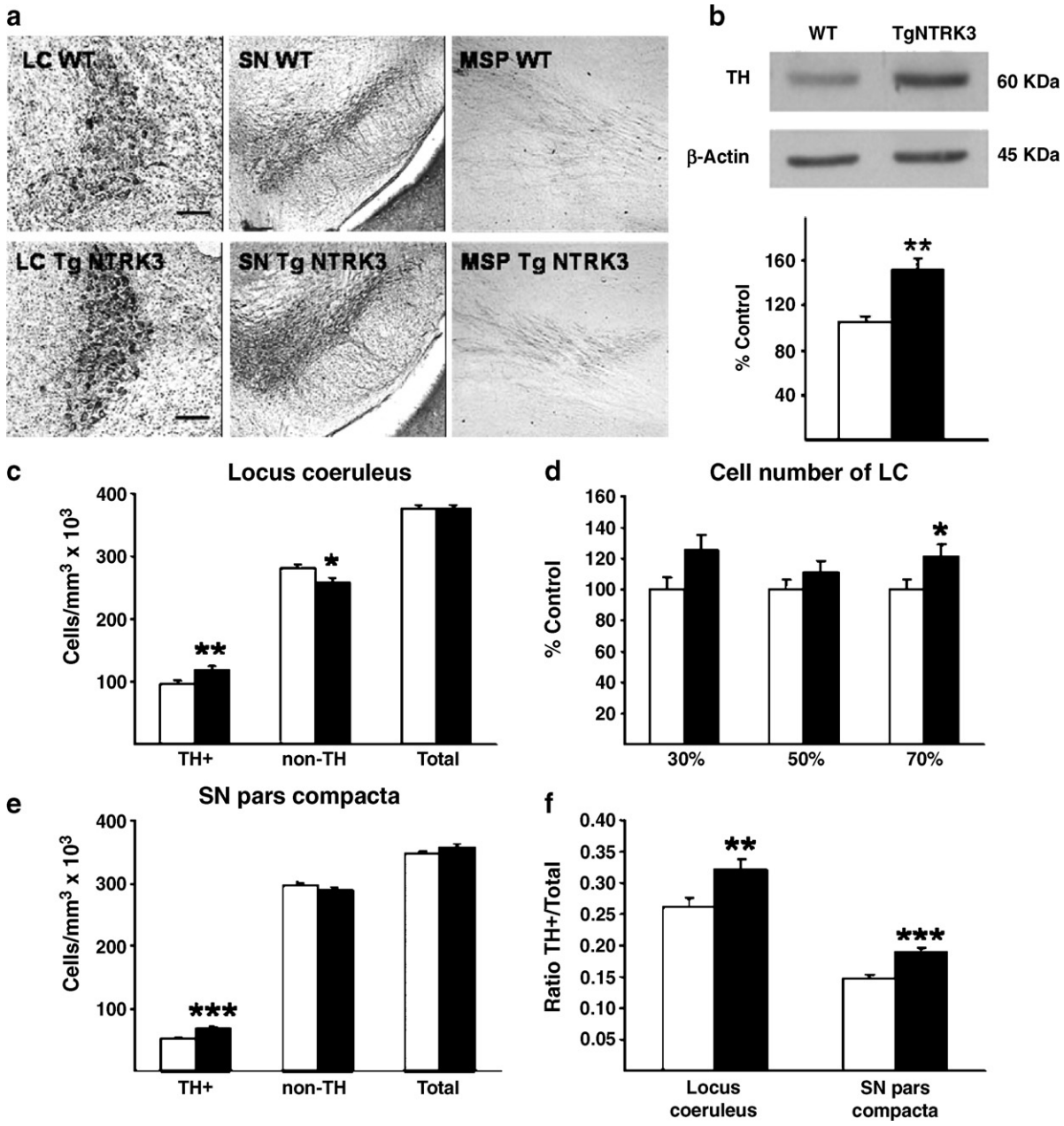

Fig. 6. Stereological analysis in TgNTRK3 (a) Photomicrographs of 50 μm coronal sections illustrate the increased TH-immunoreactivity in locus coeruleus (LC), substantia nigra (SN) and mesostriatal projections (MSP) of control (upper panel) and TgNTRK3 (lower panel). Scale bar = 100 μm. (b) Representative Western blot showing TH-immunolabeling in the bregma −4.60 to −7.56 region of wild type (open bars) and TgNTRK3 (filled bars) mice. β-actin was used as loading control. Relative TH immunoreactivity was determined densitometrically. Notice the increase in TH expression in TgNTRK3. Ratios are expressed as mean percentage of control ± SEM. (c, d, e, f) Stereological studies in LC and SN. A significant increase in TH-positive cell density (c) and in the proportion of TH-positive cells respect to the total neuronal population in LC (f) was observed in TgNTRK3. (d) The number of TH positive neurons in the LC at the 30%, 50%, and 70% levels of control and TgNTRK3 mice are given. (e, f) Stereological studies of SN-pars compacta. TH-positive cell density (e) and the proportion of TH-positive cells respect to the total neuronal population (f) were increased in TgNTRK3. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. TH+ = TH-positive cells; non-TH = non-TH stained cells; total = TH-positive plus non-TH cells.

an increase in grooming activity, which is dependent on emotionality-related factors, was detected in TgNTRK3 with no differences in locomotor activity. In the light and dark paradigm a significantly higher latency to enter the lit compartment and a reduced number of entries in this area were observed in TgNTRK3 mice. Finally, the most marked anxiety-like behavior was observed in the elevated plus maze and in the elevated zero-maze, two tests that share some characteristics. In both situations, TgNTRK3 mice showed a significant tendency to avoid unprotected zones of the apparatus, again indicating an enhanced anxiety-like behavior in these experimental conditions that was reversed by diazepam in a dose-dependent manner in TgNTRK3 mice. The primary indices of plus maze anxiety (i.e. open arm entries and time spent in the open arms) reflect the natural

avoidance of rodents for open spaces and are bi-directionally sensitive to anti and pro anxiety manipulations. In TgNTRK3 mice, diazepam increased the percentage of time in open arms and the percentage of open/total arm entries. The absence of statistically significant effect on percentage open time in wild type mice at low doses of diazepam indicates a somewhat milder anxiolytic profile for diazepam than that observed in TgNTRK3 mice, that could be related to the strain used, since previous studies (Griebel et al., 2000) have suggested that the SJL strain is almost unresponsive to the diazepam effect.

The tests described before are good measures for anxiety, but do not allow the evaluation of panic-like behavior in mice. To specifically address the panic reactions in TgNTRK3 mice, we used the MDTB, a test that measures a full range of specific defensive behaviors in response to a discrete, highly discriminative threat source (i.e. a rat), and has been extensively used to evaluate the panicogenic/panicolytic properties of drugs (Griebel et al., 1995a,b, 1996, 1999; Blanchard et al., 2001). In the present study, the baseline levels of defensive behaviors of control animals were qualitatively similar to those reported in previous MDTB studies (Griebel et al., 1995a,b; 1996). Prior to confrontation with the rat spontaneous locomotor activity (Griebel et al., 1999; Blanchard et al., 2001) was not affected by genotype. TgNTRK3 mice showed significantly increased flight responses (avoidances and flight speed) being the percentage of subjects presenting escape responses also increased with respect to wild type animals. This escape behavior is highly sensitive to panicogenic and panicolytic agents and is considered a measure of panic reaction in rodents (Blanchard et al., 1997, 2001).

Moreover, both cognitive and affective oriented behaviors were also altered in TgNTRK3 mice, as indicated by the significantly reduced risk assessment behaviors in the chase-flight test and the lower number of approaches in the straight alley test (Blanchard et al., 1997, 2001). 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 in TgNTRK3 mice. Finally, a marked increase in immobility time and in freezing behavior was observed in transgenic mice in the straight alley test that continued in the post-test. 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 our study, there was an interesting effect of TrkC overexpression on the number and density of TH-positive neurons in two catecholaminergic nuclei (LC and SNc) that was increased in TgNTRK3 mice without changes in size or shape of the TH-positive neurons, thus indicating that cell shape/size were not confounding the cell counts. This effect was accompanied by a significant increase of TH levels in TgNTRK3 as measured by Western blot analysis. Double-labeling immunofluorescence demonstrated that almost all of the TH-containing neurons in the LC also expressed TrkC.

The localization of TrkC positive neurons within the peri-LC dendritic field (Jones, 1991) raises the possibility that expression of TrkC in this region contribute to the functional modulation of LC activity. Our observations suggest that NT-3-TrkC input to LC neurons from these local neurons in the peri-LC zone might represent a potentially significant element in the integration of afferent inputs that regulate activity in the LC–NE system, altered in panic disorder.

The increased cellularity observed in catecholaminergic nuclei in TgNTRK3 could have taken place during the developmental stages, where the levels of TrkC observed were higher. In vitro studies in fetal tissue have demonstrated that NT3 increases NA neuron survival (Friedman et al., 1993; Reiriz et al., 2002) and up-regulates the expression of NA markers (Sklair-Tavon and Nestler, 1995), suggesting that NT3 may play a role in LC development. Taking into account our findings of increased cellularity in LC and other catecholaminergic nuclei by overexpressing the TrkC receptor, it is tempting to speculate that increased NT3 signaling may be one of the key features for the development of the catecholaminergic system and could thus be contributing to express an anxiety phenotype as inappropriate activation of the LC has been involved in panic attacks and may participate in the exaggerated stimulus-responsiveness and increased emotionality seen in patients with stress or anxiety disorders (Aston-Jones et al., 1996; Goddard and Charney, 1997). However, it must be considered that trophic requirements for each neuronal population depend on a cross-talk between different neurotrophic factors. Thus, in a recent study, TrkB−/−, but not TrkC−/−, mice showed 30% decrease in the number of TH-positive LC neurons at P0, indicating that the TrkB receptor activation is also required for normal development of LC (Holm et al., 2003). Another interesting issue is the regulation of the NT-3/TrkC system. In our transgenic model we detected increases in NT3 levels in SN but not in LC that could also act on TrkB receptors. This may be explained that it could be a differential regulation between the ligand and its receptor as it has been described in other brain areas (Canals et al., 1998, 1999). Although we cannot rule out that the lack of a fine dissection of the LC did not allow detecting small changes in NT-3 levels. In fact, we found that TrkC overexpression resulted in constitutively increased tyrosine phosphorylation of TrkC in this transgenic mouse suggesting that NT-3/TrkC activity is modified in this model.

In conclusion, overexpression of TrkC in mice leads to an increase in number of catecholaminergic neurons in the LC and SN, possibly by specifically promoting TH-positive neurons. We propose that this trophic effect of TrkC in specific brain regions related to anxiety and panic may be involved in the anxiety-related phenotype and in the increased response to panicogenic agents observed in these mice and may thus play a role in the pathogenesis of anxiety disorders. Thus, we propose TgNTRK3 mice as a model for the development of therapeutic strategies for anxiety and panic disorder.

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