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Norepinephrine transporter (NET) knock-out upregulates dopamine and serotonin transporters in the mouse brain

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ABSTRACT

The noradrenaline, serotonin and dopamine transporters are three main transporters, which are the target of the antidepressant drugs. In the present study we demonstrate that the life-long deletion of the noradrenaline transporter (NET) induced up-regulation of two other monoamine transporters, dopamine and serotonin (DAT and SERT, respectively). An increase in the binding of [^{3}H]paroxetine to the SERT and [^{3}H]GBR12935 to the DAT was observed in various brain regions of NET-KO mice, without alterations of mRNA encoding these transporters, as measured by in situ hybridization. This important finding impacts the interpretation of previous data indicating the supersensitizity of NET-KO mice for psychostimulants or stronger effect of citalopram in behavioral tests. While using the NET-KO mice in various psychopharmacological studies is very important, one has to be aware that these mice lack NET from the earliest period of their existence, thus compensatory alterations do take place and have to be considered when it comes to interpretation of the obtained results.

1. Introduction

The introduction of norepinephrine transporter knock-out mice (NET-KO) (Wang et al., 1999; Xu et al., 2000), led to many behavioral and biochemical experiments, to analyze mechanisms of action of antidepressant drugs (Dziedzicka-Wasylewska et al., 2006; Perona et al., 2008; Xu et al., 2000). Disruption of the NET gene in mice elevated extracellular levels of norepinephrine (Xu et al., 2000), accumulation and release of this catecholamine in serotonergic varicosities (Vizi et al., 2004). The NET-KO mice displayed a significantly shortened immobility time both in the forced swim and the tail suspension tests, indicative of "depression resistant behavior". Antidepressant drugs selective for the norepinephrine transporter, failed to further alter immobility in these tests (Dziedzicka-Wasylewska et al., 2006).

NET-KO mice were also less sensitive to stress – as shown by measurements of corticosterone levels following the forced swim test (Solich et al., 2008) or in the studies of sucrose preference after restraint or social defeat stress (Haenisch et al., 2009). In contrast, the autonomic cardiovascular response to anxiety and fear was increased in these mice (Keller et al., 2006). Surprisingly, these mice are also less vulnerable to seizures induced by various means (Ahern et al., 2006; Kaminski et al., 2005; Martillotti et al., 2006).

The NET-KO mice have also been shown to be more sensitive to the behavioral effects of psychostimulants, although no differences in the density of dopamine receptors were found (Jayanthi and Ramamoorthy, 2005; Xu et al., 2000). On the other hand alterations in the adrenergic systems were indicated by down-regulation of alpha1- and beta-adrenergic receptors in the brain of NET-KO mice (Dziedzicka-Wasylewska et al., 2006), together with up-regulation of alpha2A/C-adrenergic receptors (Gilsbach et al., 2006).

Interestingly, deletion of the NET gene has been found to result in a differential gene expression profile in the neural crest stem cells using long serial analysis of gene expression (Hu et al., 2009). The differentially expressed genes include those important for neural crest formation and differentiation of noradrenergic cells.

The present study addressed the question whether the lack of the functional product of the gene encoding NET will change the main monoaminergic transmitter reuptake mechanisms. We used the in situ hybridization technique to measure mRNA encoding NET, SERT and DAT in the brains of NET-KO mice, as well as autoradiography using transporter specific ligands. The obtained results reveal an increase in the binding of radioligands specific toward SERT and DAT in various brain regions of NET-KO mice, without alterations of mRNA encoding there transporters.

2. Materials and methods

2.1. Animals

Heterozygous mice were generated by Xu and co-workers in Duke University, Medical Center, Durham, NC, USA (Xu et al.,

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2000). The vector containing the targeted construct was linearized and electroporated into 129/SvJ mouse AK7 ES cells. Positive ES cells were microinjected into C57BL/6J E3.5 blastocysts. The chimeric males were mated with C57BL/6J wild-type females to produce heterozygous mice. Heterozygous mice, obtained from Dr. M. Caron (Duke University, Medical Center, Durham, NC, USA) were mated to produce F2 and F3 generation. Homozygous WT and NET-KO (Slc6a2^{tm1Mca}) mice were bred as congenic lines for no more than 10 generations. For the experiments, we used age-matched adult (ca 3–5 months) males. The genotypes were confirmed by PCR and usage of the primers m NETEx2s (5′-GCT TTA TGG CAT GTA GTG TGC AC-3′), mNETEx2as (5′-GCT TTC TGC TTG AAC TTG AAG GC-3′), and EGFPas (5′-GCC GGA CAC GCT GAA CTT GTG-3′) to amplify a 700 and 500 bp PCR product in case of WT and NET-KO mice respectively.

The animals had free access to food and water and were kept at a constant room temperature (24 °C), under 12-h light/dark cycle. Animals were kept according to the decision of Environment Minister (No. 01–49/2009).

2.2. Autoradiography studies

2.2.1. [^{3}H]nisoxetine binding to the NET

The six males WT and six males NET-KO were killed, their brains were quickly removed, frozen on dry ice and then were stored at −80 °C until use. The frozen brains were cut in 14 μm slices using Jung CM 3000 cryostat (Leica, Germany). [^{3}H]nisoxetine (3 nM) binding to the NET was carried out as described by Hébert et al. (2001), with 1 μM mazindol (Sigma) used to define non-specific binding. Thereupon, the slices were exposed with [^{3}H]microscales (Amersham) to tritium-sensitive screens (FujiFilm, Germany) for 5 days. The images were obtained using FujiFilm BAS 5000 Phosphoimager and were analyzed using FujiFilm software (Image Gauge, V4.0). Specific binding was determined by subtracting non-specific binding from total binding images and the results are expressed as fmol of specific radioligand binding per mg protein. The identification of brain structures was defined according to the Mouse Brain Atlas (Paxinos and Franklin, 2001).

Statistical analysis was carried out by Student's *t*-test to compare brain structures between WT and NET-KO. A value of $p < 0.05$ was considered to be significant.

2.2.2. [^{3}H]GBR12935 binding to the DAT

The slices were prepared as described above and then were preincubated for 15 min in 50 mM Tris–HCl in RT and further incubated in 50 mM Tris–HCl (containing 120 mM NaCl and 0.1% bovine albumin) with 2 nM [^{3}H]GBR12935 (PerkinElmer), specific for the DAT or with [^{3}H]GBR12935/50 μM mazindol (Sigma) to define non-specific binding for one hour at RT (Kimmel et al., 2000). After that the slices were proceeded as described above.

2.2.3. [^{3}H]paroxetine binding to the SERT

The slices were prepared as described above. Then, the slices were preincubated for 15 min in 50 mM Tris–HCl at RT, and further incubated in 50 mM Tris–HCl (containing 120 mM NaCl and 5 mM KCl) with 0.5 nM [^{3}H]paroxetine (PerkinElmer) – a selective serotonin reuptake inhibitor – or [^{3}H]paroxetine/30 μM fluoxetine hydrochloride (Lilly) to define non-specific binding, for one hour at RT. Then slices were bathed two times for 20 min in 50 mM Tris–HCl buffer at 37 °C, washed in distilled water at RT (Hébert et al., 2001), dried and exposed as described above.

2.3. In situ hybridizations studies

The eight males WT and seven males NET-KO were killed. Their brains were frozen on dry ice and then stored at −80 °C. The frozen brains were cut in 14 μm slices using a cryostat.

The oligonucleotide probe complementary to nucleotides 831–870 of NET mRNA was designed in Nucleotide (NCBI), checked in BLAST (NCBI) (Table 1) and synthesized (Genomed, Poland). The oligonucleotide probes complementary to SERT and DAT mRNA were synthesized as described by others (Blakely et al., 1991; Collin et al., 2000; de Jong et al., 2008; Shimada et al., 1991) and are presented in Table 1. The probes were labeled with [^{35}S]dATP (Hartman Analytic) at 3′-end using terminal transferase (New England Biolabs).

Following the preincubation the brain slices were incubated with hybridization buffer containing the labeled probes (10000 CPM/μl) for 18 h at 37 °C, then were washed in 1xSSC solution, and three times for 15 min with 2×SSC containing formamide (MERCK) at 40 °C. The images were obtained using FujiFilm BAS 5000 Phosphorimager and they were analyzed using FujiFilm software (Image Gauge, V4.0). The identification of brain structures was defined according to the Mouse Brain Atlas (Paxinos and Franklin, 2001).

Statistical analysis was carried out by Student's *t*-test to compare brain structures between WT and NET-KO. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Autoradiography studies

In the present experiment the radioligand binding to the noradrenaline, dopamine and serotonin transporters (NET, DAT and SERT, respectively) were compared in various areas of the brains of NET-KO and WT mice.

3.1.1. [^{3}H]nisoxetine binds to different brain regions of WT mice but not to NET-KO mice

As can be seen in Fig. 1, the obtained results indicate the highest specific binding of [^{3}H]nisoxetine to the NET in the locus coeruleus (LC) of WT mice (Fig. 1B). Considerable binding was also observed in the frontal (FrA) and cingulate cortex (Cg), CA3 area of hippocampus (CA3), basal-lateral amygdala (BL), substantia nigra (SNC), paraventricular thalamic nucleus (PVA), hypothalamic area (LH) and nucleus accumbens shell (AcbSh) but not in the nucleus accumbens core (AcbC), what probably is derived from only sparse noradrenaline fibres, which innervate this region (Berridge et al., 1997).

There was no binding of [^{3}H]nisoxetine to the NET in different areas of NET-KO mice brain.

Table 1
The oligonucleotide probes complementary to NET, SERT and DAT used in the in situ hybridizations studies.

Transporter	Oligonucleotide probe	Accession No.
NET	CTCGTGCAAGTGCAGGACTCCGCGTTCATAAAACTCCGCA	NM_009209
SERT	AGCAGGACAGAAAGGACAATGTAAGGGAAGGTGGCTGTCACCCACACC	NM_010484
DAT	CTTGTCCTCCGTGGCTCAGAACAGACCCTGCGTGTGTGTAATA	NM_010020