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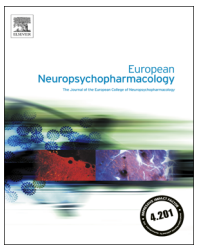

Life-long norepinephrine transporter (NET) knock-out leads to the increase in the NET mRNA in brain regions rich in norepinephrine terminals

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Abstract
These studies aimed to identify the genes differentially expressed in the frontal cortex of mice bearing a life-long norepinephrine transporter knock-out (NET-KO) and wild-type animals (WT). Differences in gene expression in the mouse frontal cortex were studied using a whole-genome microarray approach. Using an alternative approach, i.e. RT-PCR (reverse transcription polymerase chain reaction) with primers complementary to various exons of the NET gene, as well as TaqMan arrays, the level of mRNA encoding the NET in other brain regions of the NET-KO mice was also examined. The analyses revealed a group of 92 transcripts (27 genes) that differentiated the NET-KO mice from the WT mice.
Surprisingly, the studies have shown that the mRNA encoding NET accumulated in the brain regions rich in norepinephrine nerve endings in the NET-KO mice. Because there is no other source of NET mRNA besides the noradrenergic terminals in the brain regions studied, these

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results might speak in favor of the presence of mRNA in axon terminals. RNA-Binding Protein Immunoprecipitation approach indicated that mRNA encoding NET was detected in the Ago2 protein/mRNA complex. In addition, the amount of Ago2 protein in the frontal cortex was significantly higher in NET-KO mice as compared with that of the WT animals.
These results are important for further characterization of the NET-KO mice, which – besides other merits – might serve as a good model to study the fate of truncated mRNA in neurons.

1. Introduction

The identification of the sequence encoding the norepinephrine transporter allowed the study of this transporter's structure and function (Pacholczyk et al., 1991). Knowledge of the sequence permitted the creation of mice lacking the norepinephrine transporter (NET-KO) and the investigation of its biological role as well as its role in the mechanism of action of some drugs, especially antidepressants.

The gene encoding the human norepinephrine transporter (hNET) is located on chromosome 16 and consists of 45 kbp (Bönisch and Brüss, 2006), while in mice, this gene (mNET) is located on chromosome 8; however, the sequence and organization of the mNET gene are very similar to the hNET gene (Fritz et al., 1998). This gene contains 14 exons separated by 13 introns (Pörzgen et al., 1995). Both genes, the human and the mouse, consist of longer introns between exons 1–2 and 3–4, and the C-terminus of one gene corresponds that of the other (Pacholczyk et al., 1991). An analysis of the promoter regions of the human, mouse and rat NET genes identified several putative transcription factor binding sites, such as SP1, AP1, NRE, TATA box and CRE (Fritz et al., 1998; Meyer et al., 1998).

The activity of the norepinephrine transporter is dependent on the concentration of Na^+/Cl^- ions (Shafqat et al., 1993). The main substrate for the NET is norepinephrine, but dopamine and serotonin can also be captured by this transporter (Bönisch and Brüss, 2006; Carboni et al., 2006; Daws et al., 2005). The NET is regulated by many protein kinases (Mandela and Ordway, 2006). This transporter serves as the main target of antidepressant drugs such as reboxetine, desipramine, amitriptyline and nortriptyline, which also regulate the level of the NET (Benmansour et al., 2004; Zhao et al., 2009), and cocaine (Mash et al., 2005). Many diseases, such as dysautonomia, hypertension, myocardial ischemia, obesity, anorexia nervosa, ADHD, addiction, epilepsy and depression, are linked to NET dysfunction (Bönisch and Brüss, 2006). Therefore, mice lacking the norepinephrine transporter (NET-KO mice) serve as a good model to study certain aspects of these disorders (Bönisch and Brüss, 2006). In particular, these mice have been used to study the mechanisms of action of antidepressant drugs (Xu et al., 2000; Dziedzicka-Wasylewska et al., 2006; Vizi et al., 2004). It has been shown that the NET-KO mice display "depressive-resistant" behavior because they manifest significantly shorter immobility times in both the forced swim test (FST) and the tail suspension test (TST). They are also resistant to stress, as measured by the plasma corticosterone concentration (Solich et al., 2008).

It has been shown that NET mRNA and protein are located in the pons (locus coeruleus) and in the medulla oblongata where the bodies of norepinephrine neurons are found. In addition, NET protein itself has also been detected in the brain cortex, the hippocampus, the thalamus, the hypothalamus and the amygdala – brain regions rich in norepinephrine terminals (Bönisch and Brüss, 2006; Lorang et al., 1994; Schroeter et al., 2000).

Recently, it has been shown that a life-long deletion of the NET induced the up-regulation of other monoamine transporters, dopamine and serotonin transporters (DAT and SERT, respectively), similar to what was observed after the chronic pharmacological blockade of this transporter by desipramine in wild-type (WT) animals (Solich et al., 2011). Such adaptive changes must be taken into account when interpreting the results of various behavioral and biochemical experiments that involve the use of transgenic animals. To further assess the possible differences induced by a NET gene knock-out, we used microarray technology to study gene expression in the frontal cortex of NET-KO mice. The results were puzzling because the data indicated a strong up-regulation of the mRNA encoding the NET in the frontal cortex of the NET-KO mice. This finding was further confirmed with RT-PCR in the frontal cortex as well as in the locus coeruleus. In addition, custom made TaqMan arrays were used to study the expression of gene sets in four other brain regions (the frontal and cingulate cortices, the dentate gyrus of the hippocampus and the basal-lateral amygdala), which are rich in noradrenergic axon terminals, and this approach again revealed the up-regulation of the mRNA encoding the NET in those brain regions.

2. Experimental procedures

2.1. Animals

Heterozygous mice were generated by Xu et al. (2000) at Duke University, Medical Center, Durham, NC, USA. As can be read in the orginal work: 'A 14 kb clone from a 129/SvJ mouse genomic library (Stratagene, La Jolla, California) was isolated using a PCR-derived probe from exons 2-3 of the human NET gene. The targeting construct contained a cassette in which the enhanced green fluorescent protein (EGFP) cDNA (Clontech, Palo Alto, California) was inserted inframe into the *Bgl II* site of exon 2 and the PGK-neomycin-resistance gene was inserted in the reverse orientation following the EGFP gene. A thymidine kinase (TK) cassette was inserted 2 kb upstream of exon 2 and used as an negative selection marker'. 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. The heterozygous mice, obtained from Dr. M. Caron (Duke University, Medical Center, Durham, NC, USA), were mated to produce F2 and F3 generations. 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 with PCR using the primers mNETEx2s (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 from 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 a 12-h light/dark cycle. The animals were kept according to the decision of the Minister of Environment (no. 01-49/2009).

2.2. Microarray studies

The WT and NET-KO males mice (ca. 3-5 months) were injected with saline (i.p). Following 7 days of injections (24 h after last injection) the mice were sacrificed, their brains were dissected, frozen on dry ice and stored at −80 °C until use. The frontal cortex was homogenized in TRI Reagent (Sigma, USA) with a Tissue Lyser (Qiagen, Germany). The RNA was purified with TRI Reagent and an RNeasy Plus Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. The amount of purified RNA was determined with a Nano-Drop Spectrophotometer ND-1000 (Thermoscientific, USA) and the quality was checked with an Automated Electrophoresis Station - Experion (Bio-Rad, USA) using an Experion RNA StdSens Analysis Kit (Bio-Rad) according to the provided procedure. The reverse transcription, labeling, and hybridization to the GeneChip Mouse Genome 430 2.0 Array were performed using the standard procedures provided by Asuragen (Austin, USA). The CEL files resulting from the Affymetrix Chip scanner were analyzed using ChipInspector (version 2.1, Genomatix). This program analyzes the expression level of a single probe. The statistical algorithm in ChipInspector is a *t*-test with a permuted artificial background and is based on the enhanced original SAM (Significance Analysis of Microarrays) algorithm. The analysis was performed according to the manufacturer's recommendations. Positive delta was set 0.51 while negative delta was set −0.42. Positive FDR and negative FDR was set <1%. Three significant probes were used to detect a transcript as significant. Upper fold change cutoff was 1.0 while lower fold change cutoff was −1.0. The BiblioSphere Pathway Edition (Genomatix) was used to create a gene relationship network, and The Gene Ontology was used to characterize the genes and gene products.

2.3. RT-PCR studies

2.3.1. Frontal cortex

The aim of the RT-PCR study was to validate the microarray study. The mice (3 WT males and 3 NET-KO males; ca. 3-5 months) were injected with saline (i.p.) once a day for seven consecutive days. Twenty-four hours after the last injection, the animals were killed, and their brain tissues were frozen on dry ice and stored at −80 °C until use. The frontal cortex was homogenized with the Tissue Lyser (Qiagen, Germany). The RNA was purified with TRI Reagent (Sigma, USA) and the RNeasy Plus Micro Kit (Qiagen, Germany) according to the provided manuals. The amount and the quality of the purified RNA were checked as described above. The RNA was reverse transcribed with a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

The primers complementary to different regions of the NET mRNA were designed and synthesized (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland). The primers were complementary to the NET gene exons numbered 1, 4, 8, 12 and 14 in the nucleotide sequence (NCBI) and were checked in BLAST (NCBI). The primers complementary to the reference genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin] were synthesized as described by Haenisch et al. (2008). All the sequences are presented in Table 1. For the RT-PCR reaction, a volume of 20 μl of total mixture (Fast SYBR Green Master Mix, Applied Biosystems) was used, which contained 75 ng of cDNA and 200 nM of each primer. The reactions were run in duplicates on a Chromo4 System for RT-PCR detection (Bio-Rad, USA) with the following cycles: initial activation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 33 s and subsequent annealing/elongation at 60 °C for 30 s. The PCR products were confirmed with a melting curve. The results were analyzed using MJ Opticon Monitor analysis software (Version 3.1, Bio-Rad). The relative mRNA expression was calculated from the ratio of WT/NET-KO according to Pfaffl (2001), and the results for each gene were normalized according to the results of the reference genes. The statistical analysis was conducted using Student's *t*-test to compare different genes expression levels between WT and NET-KO mice. A value of $p \leq 0.05$ was considered to be significant.

2.3.2. Locus coeruleus

The structure was microdissected out of the 50 μm brain slices (Bregma −5.34 mm) from the brains of 3 males WT and 3 males NET-KO mice (ca. 3-5 months) and stained by cresyl violet (LCM Staining Kit, Applied Biosystems). The RNA from the tissue samples was purified with the RNeasy Plus Micro Kit (Qiagen, Germany) according to the provided manual. The amount and the quality of the purified RNA were checked as described above. The mRNA was reverse transcribed as described previously. The primers complementary to different exons (1, 3/4, 4, 8, 12 and 14; Table 1) of the NET gene and the reference genes were designed and synthesized as described above (Table 1). For the RT-PCR reaction, a volume 20 μl of total mixture (Fast SYBR Green Master Mix, Applied Biosystem) was used, which contained 2.5 ng of cDNA and 200 nM of each primer. The reactions were run in duplicates and analyzed as described above.

2.4. TaqMan low density array studies

The TaqMan low-density array studies were performed on brain tissues from 3 males WT and 3 males NET-KO mice (ca. 3-5 months). The brains were frozen on dry ice and were stored at −80 °C until use. The brains

Table 1 The primers complementary to different parts of NET and house-keeping genes used in RT-PCR studies.

Target gene	Primers sequence forward	Primers sequence reverse
NET; exon 1	5′ACTCAGGCGCTCCTTTTCTT3′	5′GCACCTGCGGATTCATTC3′
NET; exon 3/4	5′GCCCTGGACCAACTGCGGAC3′	5′CTGCCATTGAGGCAGGCCGA3′
NET; exon 4	5′TGTCTGATGGTCGTCATCGT3′	5′TCAAGACTCCAAATCCAGCC3′
NET; exon 8	5′TTCTTGATGCTCCTGGCTCT3′	5′GCTCACGAACTTCCAACACA3′
NET; exon 12	5′TCAACTTCAAGCCGCTTACC3′	5′CTCCTTCCAGCCTCTTCCTT3′
NET; exon 14	5′CATCTCATGCCAGAAAGCCT3′	5′GTAGAGCAAGGACAGGTGCC3′
GAPDH	5′TGCACCACCAACTGCTTAGC3′	5′GGCATGGACTGTGGTCATGAG3′
β-actin	5′TCCATCATGAAGTGTGACGT3′	5′GAGCAATGATCTTGATCTTCAT3′

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were cut into 50-μm slices using a cryostat and were placed on membrane slides (Leica, Germany). The slices were then stained with cresyl violet using an LCM Staining Kit (Applied Biosystems), and the appropriate brain regions (the frontal cortex, the cingular cortex, the dentate gyrus of the hippocampus and the basal-lateral amygdala) were microdissected using a laser microdissection system (Leica, Germany). The RNA was purified with the RNAsy Plus Micro Kit (Qiagen, Germany) according to the provided manual. The Experion RNA HighSens AnaLysis Kit (Bio-Rad) was used to check the quality of RNA. Next, the RNA was reverse-transcribed with a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The RT-PCR was performed using, on average, 50 ng cDNA per reservoir loaded onto a TaqMan low density array (Applied Biosystems) in a custom format that covered 48 assays, including *Slc6a2* (Mm00436661_m1), *Slc6a3* (Mm00438396_m1), and *Slc6a4* (Mm00439391_m1) and was run on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, USA).

The results obtained from triplicate samples were analyzed using the SDS Relative Quantitation software (Applied Biosystems), and the relative mRNA expression level was calculated from the ratio of WT/NET-KO according to Pfaffl (2001); the results for each gene were normalized according to the result of the reference genes (*Gapdh* – Mm99999915_g1 and *Actb* (β-actin) – Mm01205647_g1).

2.5. Western-blot studies

The rabbit anti-Staufen polyclonal antibody (ab78533), rabbit anti-Dcp1a polyclonal antibody (ab66009), and goat anti-rabbit secondary antibody (ab6721) were purchased from Abcam (Cambridge, UK). Mouse anti-β-actin monoclonal antibody and goat anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Protein mixtures were extracted from the frontal cortex. Samples containing 30 μg of protein for the Staufen assay and 60 μg of protein for the Dcp1a assay were resolved with 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). In the case of Dcp1a, the extraction of the protein mixture was conducted immediately after the tissue isolation to avoid possible protein degradation. The membranes were blocked overnight at 4 °C in Tris-buffered saline containing 5% non-fat milk powder for Staufen or 3% BSA for Dcp1a. The blocked membranes were incubated for 2 h at room temperature with anti-Staufen rabbit polyclonal antibody (dilution 1:1000) or anti-Dcp1a rabbit polyclonal antibody (dilution 1:1000) in Tris-buffered saline with 0.1% Tween-20 (TNT). After four TNT buffer washes, anti-rabbit IgG conjugated with horseradish peroxidase (dilution 1:8000 and 1:15,000 for Staufen and Dcp1a, respectively) was applied to the membranes for 1 h. The membranes were then washed with TNT buffer and subjected to an enhanced chemiluminescence reaction (ECL, Santa Cruz Biotechnology) before visualization with a Fusion FX (Vilber Lourmat Deutschland GmbH) for Dcp1a or exposure to X-ray films for Staufen. The same blot was re-probed for β-actin, which served as a loading control, using anti-β-actin mouse monoclonal antibody (dilution 1:2000) and anti-mouse IgG conjugated with horseradish peroxidase (dilution 1:5000). The intensity of each band on the Western blot was quantified with Image Quant TL Version 7.0 (GE Healthcare). During densitometric analysis the signal of background was subtracted. The ratios of intensity between interesting protein band and actin band were calculated. The averages and standard deviation for three biological replicates in both the knockout and wild type group was determined. The results are depicted as a percentage of changes (in relation to the sum of the ratio values for all replicates in group).

2.6. Magna RIP (RNA-binding protein immunoprecipitation) studies

Magna RIP (Millipore) studies were performed using anti-Staufen antibody (ab78533; Abcam), anti-Dcp1a antibody (ab66009; Abcam), anti-FMRP antibody (ab17722; Abcam), anti-GW182 antibody (ab84403; Abcam), anti-RISC antibody (ab13716; Abcam) and anti-Ago2/eIF2C2 antibody (ab32381; Abcam). Each antibody was used in a separate experiment which was carried out on 3 males WT and 3 males NET-KO mice (ca. 3-5 months). Experiments were performed according to the instruction attached to Magna RIP Kit (Millipore). The lysates were prepared from freshly collected frontal cortices, which were homogenized with the Tissue Lyser. The magnetic beads were coated of 5 μg of the antibody. The lysate containing magnetic beads-antibody complex was incubated overnight at 4 °C, then the RNA was purified and kept at −80 °C for 3.5 h (precipitation step).

In next step, the Western blots were performed to verify the efficiency of immunoprecipitation and to check the amount of protein in the lysate, according to the iBlot2 Dry Blotting System (Life Technologies) and iBind Western System (Life Technologies) instructions. Samples contained 20 μg protein for each antibody used. The dilution 1/100 was used for all primary antibodies and 1/600 for secondary antibodies (Goat anti-rabbit IgG H&L (HRP); ab6721). The results were normalized according to the result of β-actin protein, which was detected in the same blot, using monoclonal anti-β-actin antibody produced in mouse (A5441; Sigma-Aldrich; dilution 1/2000) and anti-mouse IgG-HRP produced in rabbit (A9044; Sigma-Aldrich; dilution 1/10,000). The intensity of each band on the Western-blot was quantified with Image J version 1.48 (http://imagej.nih.gov).

The quality and quantity of purified RNA were checked as described above. The RNA was reverse transcribed with a high capacity cDNA Reverse Transcription Kit and used for the RT-PCR reaction of volume 10 μl of total mixture (Fast SYBR Green Master Mix), which contained 20 ng of cDNA and 200 nM of each primer (NET 3/4 primer sequence; Table 1). The reactions were run in duplicates on a CFX96 Touch System (Bio-Rad). The PCR products were confirmed with a melting curve. The results for each gene were normalized according to the results of the reference genes (*Gapdh* and *β-actin*; Table 1). The results were analyzed with the CFX Manager Software (Version 3.1; Bio-Rad). A value of $p \leq 0.05$ was considered as significant.

3. Results

3.1. Microarray studies

Microarray technology was used to find an interesting group of genes that could differentiate the two genotypes of the mice under study. The frontal cortex was selected for the microarray studies because it is rich in neuronal terminals containing the NET (Schroeter et al., 2000), and dysfunctions of this area have been related to depressive disorders (Canbeyli, 2010).

The results that were obtained were analyzed with ChipInspector (Genomatix), and a statistically significant group of 92 transcripts was selected, which corresponds to 27 genes differentiating the NET-KO mice from the WT mice (Figure 1A). Some of the genes were unidentified; therefore only 19 genes were further analyzed using the BiblioSphere Pathway Edition (Genomatix). Some of the genes were connected in the gene relationship network according to the literature and promoter analyses as well as experimental resources. Genes such as *Slc6a2*, *Fos*, *Ang*, *Arc*, *Paip1*, *Gadd45gip1*, *Nr4a1*, *Prdx2* and *Ier5* differentiate the NET-KO mice from WT mice, and these genes are involved in the regulation of monoamine transport, the regulation of gene expression and translation, and protein binding (Figure 1B).

However, the most interesting finding in this area of the studies (the microarray) was the significant up-regulation of the level of mRNA expression of *Slc6a2* (the gene encoding the

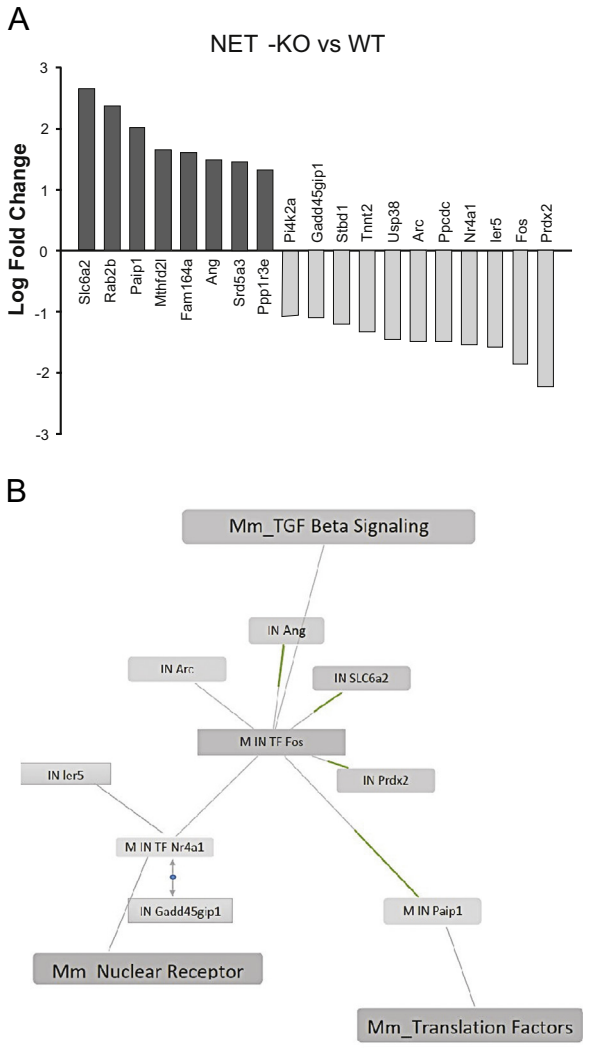

Figure 1 The genes differentiating the wild-type animals (WT) from the norepinephrine transporter knock-out mice (NET-KO) (A). The changes in gene expression were analyzed with the ChipInspector program and are expressed on a logarithmic scale. The network between some of the genes differentiating the WT mice from the NET-KO mice (B). The network was created with the BiblioSphere Pathway Edition program; $n=3$. "IN" symbols marked the genes; "TF" symbols marked transcription factors; and "M" symbols marked metabolic pathway. Functional relationships between genes are visualized by the lines: black - connection with no expert curated annotations; gray - transcription factor binding site match in target promoter; arrow - an interaction between genes with an expert curated connection with a disc in the center of the interaction.

norepinephrine transporter) in the frontal cortex of the NET-KO mice in comparison to the WT mice, while the expression of the genes encoding the DAT and SERT proteins was not altered.

3.2. RT-PCR studies

We validated this interesting, however puzzling, finding obtained from the microarray studies with the RT-PCR reactions.

3.2.1. Frontal cortex

The primers complementary to the different regions of the NET mRNA were designed bearing in mind that the knock-out in this particular model was introduced into exon 2 of the NET gene.

The RT-PCR reactions were performed on cDNA obtained from the frontal cortex of the NET-KO and WT mice. We observed significant increases in the expression of all of the examined exons of the NET gene in the frontal cortex of the NET-KO mice in comparison to the WT animals (Figure 2A). The results of the RT-PCR were validated with a melting curve, and the melting temperatures of each of the products were determined (dI/dT max. tm.: NET1 - 82; NET2 - 80; NET3 - 80; NET4 - 83; NET5 - 83).

3.2.2. Locus coeruleus

The RT-PCR reactions were also performed with cDNA obtained from the locus coeruleus, where the cell bodies of the noradrenergic cells are located (Schroeter et al., 2000). In addition to the primers used in the frontal cortex experiments, the primers were designed to measure the formation of an amplicon at the boundary of exons 3 and 4. There was significantly lower expression of all parts of the NET gene in the locus coeruleus regions of the NET-KO mice, with the exception of the amplicon complementary to exon 1, which was most likely due to the site of the knock-out

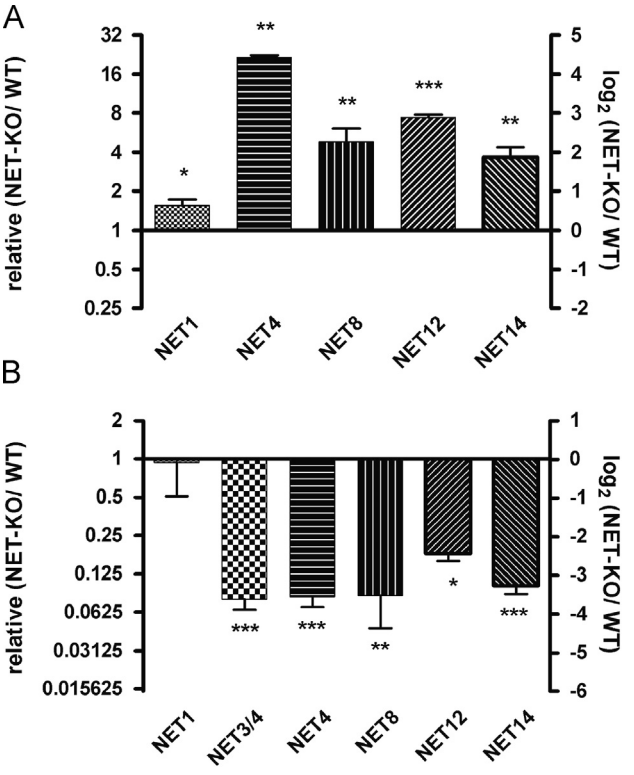

Figure 2 The relative expression of different regions of the NET mRNA complementary to exon 1 (NET1), exon 3/4 (NET 3/4), exon 4 (NET4), exon 8 (NET8), exon 12 (NET12) and exon 14 (NET14) measured by RT-PCR in the mouse frontal cortex (A) and the locus coeruleus (B). The data represent the mean ± SEM. Student's t-test was used to compare NET-KO vs. WT; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$; $n=3$.

introduction within the gene (Figure 2B). However, the mRNA encoding the NET was still detectable in the locus coeruleus area of the brains of the NET-KO mice.

3.3. TaqMan low density array studies

Simultaneously, we performed the TaqMan low-density array analysis to characterize the expression of transporter genes in four brain regions [the frontal (FrA) and the cingulate (Cg) cortices, the dentate gyrus of the hippocampus (DG) and the basal-lateral amygdala (BL)] in the brains of the NET-KO mice in comparison to the WT brains.

The expression of the *Slc6a2* gene, encoding the norepinephrine transporter, was significantly higher in all the examined brain structures of the NET-KO mice compared with the WT animals (Figure 3A), while the expression of the genes for the dopamine (*Slc6a3*) and serotonin (*Slc6a4*) transporters did not differ in any of the brain structures when the NET-KO and WT mice were compared (Figure 3B and C).

3.4. Western-blot studies

To confirm our hypothesis that untranslated NET mRNA may be stacked in GW/P-bodies or RNP complexes, we performed Western blot studies.

RNP complexes can be identified by the presence of the Staufen protein. A larger amount of this protein was identified in the frontal cortex of the NET-KO mice (Staufen protein content relative to β-actin; Figure 4A), but this result was not significant. The amount of the Dcp1a protein, a marker of GW/P bodies, was also greater in this structure of the brains of NET-KO mice (Dcp1a protein content relative to β-actin; Figure 4B) and this alteration was statistically significant. The amount of Dcp1a was verified in one protein band recognized by the antibodies.

3.5. Magna RIP (RNA-binding protein immunoprecipitation) studies

We performed Magna RIP studies to prove directly which complex binds untranslated NET mRNA in the frontal cortex of NET-KO mice. All antibodies used (anti-Staufen, anti-Dcp1a, anti-FMRP, anti-GW182, anti-RISC and anti-Ago2/eIF2C2) showed positive results when the efficiency of immunoprecipitation was verified by Western blots but mRNA encoding NET was detected only in the Ago2 protein complex (Figure 5 A and B). Markers of P-body, RNP granule and Stress granule did not bind the NET mRNA. In addition, the amount of Ago2 protein in the frontal cortex lysate was checked, and the results indicate that the level of this protein was significantly higher in NET-KO mice as compared with the WT animals (Figure 5C and D).

4. Discussion

The study of the frontal cortex using microarray technology identified 27 genes that differentiate the NET-KO mice from the WT animals. Some of these genes (*Slc6a2*, *Fos*, *Ang*, *Arc*, *Paip1*, *Gadd45gip1*, *Nr4a1*, *Prdx2* and *Ier5*) were identified

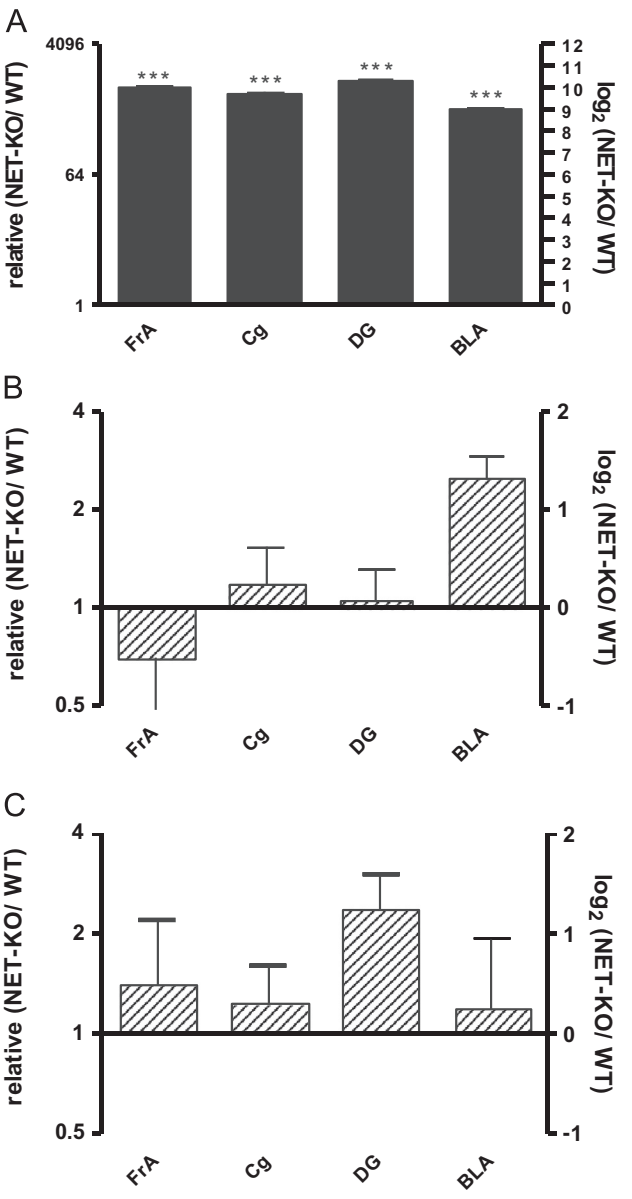

Figure 3 The relative expression of NET (A), DAT (B) and SERT (C) mRNA measured with RT-PCR (TaqMan low-density array) in various regions of the mouse brain: frontal (FrA) and cingulate (Cg) cortices, dentate gyrus of hippocampus (DG) and basal-lateral amygdala (BLA). The data represent the mean ± SEM. Student's *t*-test was used to compare NET-KO vs. WT; $^{***}p \leq 0.001$; $n=3$.

with the BiblioSphere Pathway Edition program (Genomatix) and linked to a common network.

According to the results, the genes such as *Nr4a1*, *Ier5*, *Gadd45gip1*, *Fos* and *Arc* form a common functional group that mediates the early response induction after stimulation by growth factors or stress (Williams et al., 1999; Wu et al., 2011; Yang et al., 2009). The products of these genes protect the cell from DNA damage (*Gadd45*) and take part in the modification of synapses (*Arc*). The expression of all of the genes belonging to this group was reduced in the brains of the NET-KO mice. This effect may be associated with the disturbance of the expression of the NET gene,

leading to the overall increase in the synaptic level of noradrenaline (Xu et al., 2000).

The effects of antidepressant drugs and depression on the expression of the immediate early genes (IEGs) *Fos*, *Arc*, and *Nr4a1* have previously been described. The *Arc* gene encodes a protein that regulates the activity of the cytoskeleton. This protein is important for transport and neuroplasticity. It is believed that *Arc* expression is up-regulated by chronic treatment (14 days) with paroxetine (a SERT inhibitor), as well as desipramine (a NET inhibitor) (Pei et al., 2003). However, SERT-KO rats exhibited a decrease in *Arc* mRNA levels in the frontal cortex and hippocampus (Molteni et al., 2009). Therefore, it appears that changes in gene expression after pharmacological interventions are sometimes not consistent with the changes obtained by genetic manipulation.

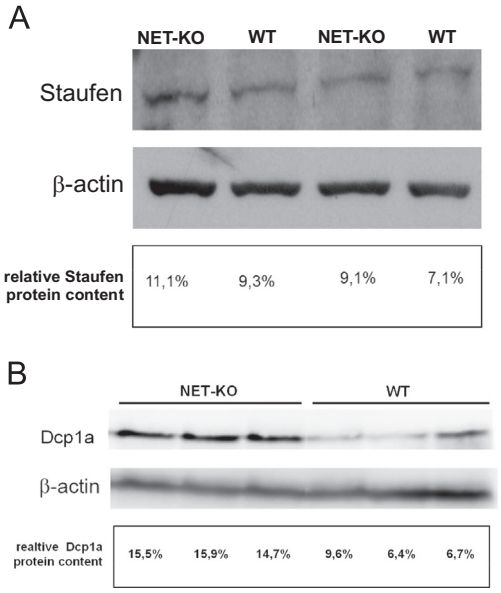

Figure 4 The differences in Staufen (A) and Dcp1a (B) protein levels when the norepinephrine transporter knock-out mice and wild-type mice were compared; $n=4$.

The level of mRNA encoding the *Nr4a1/Nur77/N/NGFI-B* gene (nuclear receptor subfamily 4, group A, member 1) was also decreased in the frontal cortex of patients with depression, as demonstrated in postmortem studies (Xing et al., 2006). Likewise, 21 days of administration of drugs that do not impact directly on the noradrenergic system (buspirone, fluoxetine, 8-OH-DPAT) did not cause any changes in *Nr4a1* mRNA expression in the cortex and different regions of the rat hippocampus (Bjartmar et al., 2000), while the repeated subjection of rats to immobilization stress (15 days, 2 h a day) increased *Nr4a1* mRNA expression in the BNST (bed nucleus of the stria terminalis). Furthermore, it has been shown that desipramine administration resulted in a significant decrease in the level of this mRNA compared with the stress control (Campos-Melo et al., 2011; Gąska et al., 2012).

A decrease in *Fos* mRNA expression was also observed after desipramine injection, but this expression had been increased by an earlier forced swim test (Jama et al., 2008). The *Fos* gene is associated with other interesting genes; however, their expression was variable. One of these genes

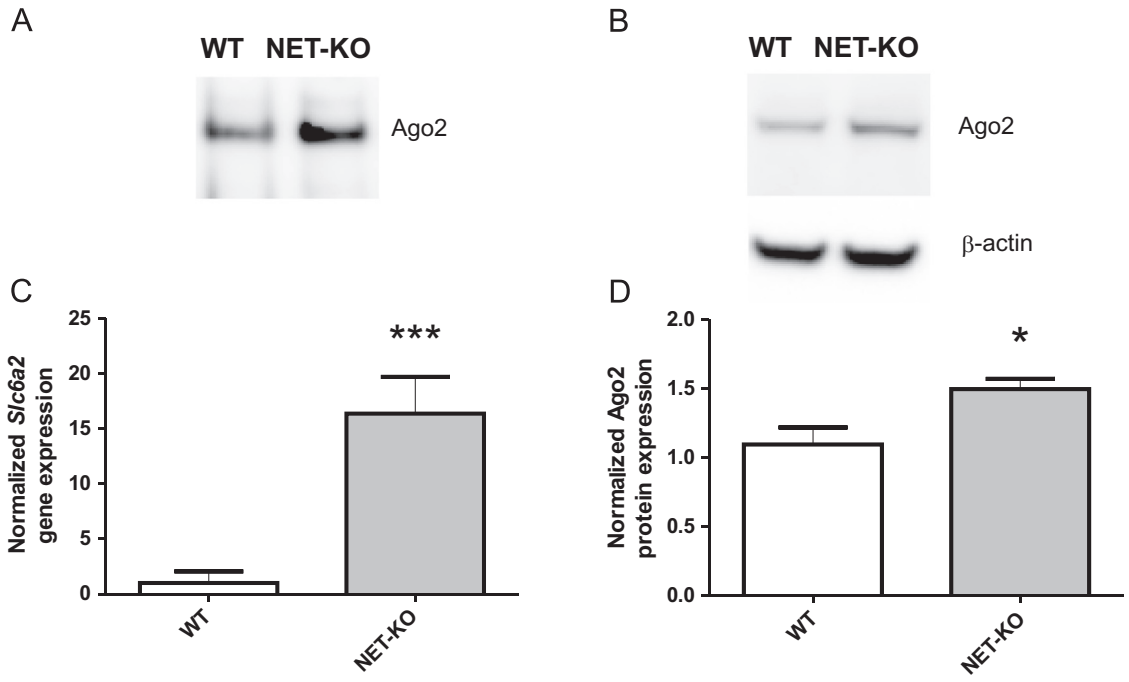

Figure 5 The results of Magna RIP (RNA-binding protein immunoprecipitation) studies. The result of the Western-blot showing the efficiency of immunoprecipitation with anti-Ago2/eIF2C2 antibody (A). The level of *Slc6a2* mRNA expression in the frontal cortex of WT and NET-KO mice, normalized by *Gapdh* and *β-actin* (B). The result of the Western-blot showing the detection of Ago2 protein in the frontal cortex of WT and NET-KO mice (C). The level of Ago2 protein in the frontal cortex of WT and NET-KO mice, normalized by β-actin protein (D). The data represent the mean ± SEM. Student's *t*-test was used to compare NET-KO vs. WT; $^{***}p \leq 0.001$; $^{*}p \leq 0.05$; $n=3$.

is the *Ang* gene, encoding angiogenin, which is an endoribonuclease – RNase A family member (Li et al., 2010). The expression of this gene was increased in the brains of the NET-KO mice.

Likewise, *Paip1* gene expression was higher in the frontal cortex of the NET-KO mice. This gene encodes a translation factor protein that mediates the interaction of PABPA proteins binding to the mRNA Poly-A tail and the elF3 or elF4A translation factor binding to the 40S ribosomal subunit, which allows for the control of the translation process (Martineau et al., 2008).

Other genes up-regulated in the NET-KO mice, which were not related according to the BiblioSphere Pathway Edition program, are important for cell functions. The protein encoded by *Rab2b* is a small GTPase that controls vesicular transport from the endoplasmic reticulum to the Golgi apparatus by binding to different proteins in eukaryotic cells (Ni et al., 2002). The product of another gene, *Srd5a3*, belongs to the steroid 5α-reductase family and plays a key role in the N-glycosylation of proteins.

Lower level of mRNA encoding the phosphatidylinositol 4-kinase type 2 isoform A (*Pi4k2a*), which phosphorylates phosphatidylinositol, was observed in the frontal cortex of the NET-KO mice. The product of this gene, PI4K2α, is an isoform of the PI4K family (phosphatidylinositol 4-kinases) found in synaptic vesicles and is involved in exocytosis, which is important for the release of neurotransmitters from synaptosomes. The pharmacological inhibition of PI4K causes a significant decrease in norepinephrine release, but does not affect the release of GABA or glutamate (Khvotchev and Südhof; 1998). These studies suggest that the *Pi4k2a* gene is associated with noradrenergic neurons. The expression of the *Prdx2*/*Prx2* gene, which encodes peroxiredoxin, a protein that plays a protective role during oxidative stress, was also reduced in the NET-KO mice (Chevallet et al., 2003).

Therefore, it appears that the genes described above are involved in mRNA transport, posttranslational processing, vesicular transport or "protect" cells against stress. A few of these genes have been described as being involved in the effects of antidepressant drugs.

Hu et al. (2009) studied gene expression in neural crest cells using LongSAGE and identified the genes differentiating the NET-KO mice from WT animals. Our results differ from those of Hu et al. (2009) most likely because of the different research specimens utilized (neural crest cells vs. the brains of adults animals).

The most surprising result of the present study concerns the NET gene (*Slc6a2*) itself: its mRNA level was significantly increased in the frontal cortex of the NET-KO mice, the animals with no functional product of this gene (Solich et al. 2011). This surprising finding was possible only because of the use of the whole-genome microarray technology – otherwise, one would not have checked the expression of this gene in the knock-out mice. A precise analysis of the probe sets used in the microarray indicated that they were complementary to the 3′-UTR of the NET gene. Therefore, we decided to check this result with another approach. We designed primers complementary to the coding regions of the NET gene. The RT-PCR reactions allowed us to observe a significant increase of different exons of the NET gene in the frontal cortex of the NET-KO mice.

These data confirmed the results obtained by both microarray studies, as well as the TaqMan low-density arrays, performed in the other brain regions (the frontal and cingulate cortices, as well as the hippocampus and the basal-lateral amygdala), where the increased NET mRNA levels in the NET-KO mice were also observed. These brain areas are rich in the norepinephrine axons containing the NET (Schroeter et al., 2000).

From these results, it may be concluded that the gene encoding the NET that was truncated in the NET-KO mice was transcribed but not translated, although the NET mRNA was transported to norepinephrine terminals. This is the most probable explanation of the results that were obtained. However, this effect was not observed in the locus coeruleus, where the cell bodies are located.

This hypothesis seems reasonable because no other cell type that was present in the examined brain structures can be the source of the NET mRNA – NET mRNA has not been demonstrated in non-noradrenergic neurons or in glial cells (Lorang et al., 1994).

Studies by Parker and Sheth (2007) indicated that many types of non-translated mRNA are stored in specific structures called processing bodies (GW/P-bodies). These protein complexes were recently extensively studied (Moser and Fritzler, 2010). It has been shown that transcripts associated with GW/P bodies can be degraded or translated again (Meyer et al., 2004; Parker and Song, 2004).

The mRNA localized in the GW/P-bodies of dendrites can be translated during synapse activation (Bramham and Wells, 2007; Cougot et al., 2008; Zeitelhofer et al., 2008). These processes can also occur in the developing axon cone in response to directing signals (Lin and Holt, 2007). Furthermore, the data provided by Taylor et al. (2009) demonstrate the presence of more than 300 types of mature mRNA in the axons of the cortex and hippocampus. Local translation processes were demonstrated during neuronal regeneration after the damage of the axons of vertebrates (Willis and Twiss, 2006). In addition, it has been demonstrated that injection of mRNA encoding the G protein-coupled conopressin receptor into an isolated invertebrate axon resulted in the detection of receptor protein on the axonal membrane, which suggests that translational processes take place in axon terminals (Spencer et al., 2000). Our hypothesis suggesting the storage of the disrupted NET gene mRNA in the GW/P-bodies seems further justified because the genes differentiating the NET-KO mice from the WT animals, according to the results obtained in the present study, were related to post-translational regulation and mRNA transport.

The significantly higher levels of mRNA encoding the NET that were observed in our studies using the microarray and TaqMan methods, as well as RT-PCR, most likely resulted from the dissociation of nucleic acid from the different proteins within the RNP complexes/GW/P-bodies during the extraction of RNA from the tissue. We have previously shown that noradrenergic transporter mRNA was not detected in the locus coeruleus of NET-KO mice by in situ hybridization (Solich et al., 2011). However, more precise techniques, such as RT-PCR, allowed the detection of the presence of NET mRNA in this region of the brain, although its level was much lower in the NET-KO mice compared to the WT animals.

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Using the same experimental approach to study the expression of the genes encoding the SERT and DAT proteins did not reveal any changes in the levels of the mRNAs encoding these transporters in the examined brain regions of the NET-KO mice compared to the WT animals. This result is most likely because the genes encoding the serotonin and dopamine transporters were not disturbed by any genetic manipulations, and the translation of these mRNAs could have been precisely regulated by the widely known mechanisms described recently by Besse and Ephrussi (2008).

Trials undertaken to prove our hypothesis involved measurements of the Staufen (a component of RNP complexes) and Dcp1a (a component of the GW/P-bodies) proteins using the Western blot technique. Indeed, a greater amount of these proteins was found in the frontal cortex of the NET-KO mice, which indirectly confirms our hypothesis.

However, additionally performed RNA-Binding Protein Immunoprecipitation studies revealed that mRNA encoding NET was bound to Ago2 protein, not to markers of P-body, RNP granule or Stress granule; moreover, the amount of Ago2 protein in the frontal cortex was significantly higher in NET-KO mice as compared with the WT animals.

These results indicate that one must be careful when interpreting the data obtained with the use of transgenic animals – both behavioral as well as molecular. When using NET-KO mice as a model in psychopharmacological research, one should be aware of a number of important compensatory alterations that take place in the brains of these animals, e.g., at the level of the other two main amine transporter proteins, as shown in previous studies (Solich et al., 2011). Possible variations in the results obtained might be due to different techniques used for the measurement of mRNA levels. Nucleic acid extraction – especially from brain regions rich in axon terminals – might reveal an increase in the level of non-active mRNA associated with GW/P-bodies, which is not detectable by in situ hybridization.

The results obtained in the present studies are important not only as a contribution to the further characterization of NET-KO mice but also as a contribution to further understanding the consequences of the life-long knock-out of a gene – the NET gene in this case. In addition to serving as a useful model to study various functions of the NET, the NET-KO mice might also be a good model to study the fate of truncated mRNA in neurons.

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Contributors

All the authors have been sufficiently involved in the submitted study and have approved the final paper.

Conflict of interests

There is no conflict of interests.

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