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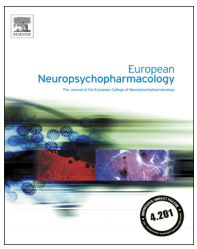

Life-long norepinephrine transporter (NET) knock-out leads to the increase in the NET mRNA in brain regions rich in norepinephrine terminals

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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