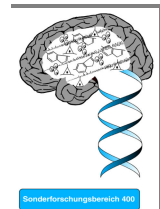


Norepinephrine transporter knockout-mediated regulation of adrenergic receptor mRNAs in mice brain

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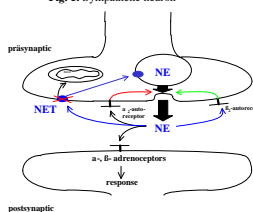


Introduction:

The noradrenaline transporter (NET) is responsible for the rapid removal of noradrenaline (NE) from the synaptic cleft. NE release of sympathetic neurons is controlled by inhibitory alpha₂-autoreceptors and partly also by facilitatory beta₂-autoreceptors (see Fig. 1). Inhibition of the NET is the primary mechanism of a major group of clinically important antidepressants (AD) such as reboxetine or desipramine. During long-term therapy these drugs lead to alterations in the density of pre- and postsynaptic adrenoceptors, which contributes to the antidepressant effect (Engelhardt S; Leonard BE).

Recently, mice with targeted disruption of the gene encoding the NET have been described; these mice behave like AD-treated mice (Xu F et al.). Little is known from these NET knockout (NET^{-/-}) mice about changes in the expression or function of adrenoceptors within the central nervous system (CNS). Therefore, we searched for differences between wild type (NET^{+/+}) and NET^{-/-} mice in mRNA expression of alpha_{1A}-, alpha_{1B}-, alpha_{1C}-, alpha_{2A}-, alpha_{2B}-, alpha_{2C}-, beta₁- and beta₂-adrenoceptors (a_{1A}-R, a_{1B}-R, a_{1C}-R, a_{2A}-R, a_{2B}-R, a_{2C}-R, β₁-R, β₂-R) in various brain regions. Relative changes in mRNA levels were determined by means of quantitative real-time PCR. In addition, various housekeeping genes were examined for stable expression.

Fig. 1: Sympathetic neuron



Methods

Brains from decapitated, adult, male (2-5 months) NET^{+/+} and NET^{-/-} mice were rapidly removed and brain regions (olfactory bulb, cerebellum, cortex, hypothalamus, hippocampus, brainstem and striatum) were rapidly dissected. RNA from brain sections or total brain was isolated using the RNA Lipid Mini Kit (Qiagen) with DNase treatment according to the manufacturer's instructions. RNA was quantified spectrometrically.

Total RNA (2 μg per sample) was reverse transcribed according to the manufacturer's instructions (Superscript II, Invitrogen; random hexamer primers, MWG). For quantitative real-time polymerase chain reaction (qPCR) 35 μl of amplification mixture (Qiagen, QuantitectSsBGreen Kit) was used containing 20 ng or in the case of r18s 0.5 mg reverse transcribed RNA, primers (Tab. 1) and 10 nM Fluorescein (Bio-Rad) according to the manufacturer's instructions. Reactions (triplicates, 10 μl) were run on an iCycler detector (Bio-Rad). The cycling conditions were: 15 min polymerase activation at 95°C and 45 cycles at 95°C for 30s, at 58°C for 15s and at 72°C for 15s. Each assay included a standard curve (5 points from 100 to 0.01 ng/35 μl; r18s: from 5 to 0.0005 ng) and negative controls (no template). The results were analysed using the Bio-Rad software (Version 3.0A). The baseline was set manually and the threshold automatically by the software. The crossing point of the amplification curve with the threshold represents the Ct. These results were exported to Excel and Prism 3.0 (GraphPad) for further analysis.

The PCR products were initially confirmed by sequencing and then after each real-time reaction by melt point analysis.

Calculations

a) The relative expression NET^{-/-}/NET^{+/+} were calculated according to Pfaffli (2001) (Eq. 1-3). The relative quantity was determined using the mean calculated efficiency (E) (Eq. 1) of all experiments. This parameter was measured with a standard curve during all experiments. The results for the house keeping genes were determined by the same method. In the next step each target result was normalized separately by the results of the house keeping genes, and the resulting values were displayed in log₂-values (Eq. 3). The arithmetic mean of these values was calculated for each experiment.

$$\text{Eq. 1: } E = 10^{-1/\text{slope}}$$

$$\text{Eq. 2: } R = E^{Ct_{\text{target}} - Ct_{\text{housekeeper}}}$$

$$\text{Eq. 3: } \log_2(\text{Ratio}) = \log_2(R_{\text{target}} / R_{\text{housekeeper}})$$

b) The expressions of the different mRNAs encoding the receptors in whole brain of NET^{-/-} mice were determined without normalization to housekeeping genes. This step could be omitted, because the same cDNA solutions were used for each gene. The results were determined using the conventional method (? Ct; Indervies T). In each group (a₁-R, a₂-R, β-R) the lowest expressed gene (highest Ct) was determined. From these values the mean Ct of the corresponding receptor subtype was subtracted.

Tab. 1

Primer	Sequence 5'→3'	Product size (bp)	Efficiency (E ± SEM)	n	Accession number
GAPD ⁺	TCACCACCACTGCTTACG GGCATGGACTGGTCAATGAC	86	2.01 ± 0.01	7	NM_008084
HRPT1 ⁺	TGACACTGGCAAAAACAATGA GGCTCTTCCACACAGAGCT	94	2.03 ± 0.01	11	BC004686
β-Actin ⁺	CACCTCCAGGCTCCCTCCTG TAGTCCGCTGAGAACGATTTGGG	112	1.97 ± 0.05	11	NM_0013461
r18s RT ⁺	TTCGATGCTGGAGGCTGAT TTTCGCTCTGGTCCCTCTTG	100	1.94 ± 0.01	9	X00686
alpha _{1A}	ATGTACTGTCGAGTCTACGTGTAG TGGATACGAGGCTCACCTGCT	104	2.04 ± 0.03	3	NM_013461
alpha _{1B}	GACACCGCCACAACACATCA CGAGTGTGGAGTGTCTCGAG	97	1.93 ± 0.03	3	NM_007416
alpha _{1C}	AGCACACCGCCACAACACATCA GACGAGTGTGGAGTGTCTCGAG	153	2.19 ± 0.04	3	XM_205022
alpha _{2A}	GACGAGTGTGGAGTGTCTCGAG CAGAGTCAACGACGAGAGT	120	1.96 ± 0.04	3	NM_007417
alpha _{2B}	GTCGAGGCTGTGGAGTGTCTCGAG GGAGGAGTGTGGAGTGTCTCGAG	130	1.83 ± 0.04	4	NM_009633
alpha _{2C}	GGCCTCCGACAGAGAGATA GTGGGGCTCAACGATGA	116	2.02 ± 0.04	3	NM_007418
beta ₁	CGCCATCAGCTCGCCCTT CGACGGGCGGCTTCTGT	161	1.92 ± 0.06	4	NM_007419
beta ₂	GAGGAGTACCAACCGTCA TCCGATGCTGGCTGTGACGCA	182	2.04 ± 0.04	3	XM_176880

⁺ house keeping genes: β-Actin, Glyceraldehyd-3-phosphat dehydrogenase (GAPD), Hypoxanthine phosphoribosyl-transferase 1 (HPRT1), ribosomal 18s RNA (r18s)

* sequence of primers taken from: RTPPrimerDB (<http://medgen31.ugent.be/primedatabase/index.php>) submitted by J. Vandromme (Ghent, Belgium)

Examination of housekeeping genes (for stable expression)

The relative (NET^{-/-}/NET^{+/+}) mRNA expression of the housekeeping genes β-actin, r18s, GAPD and HRPT1 was studied in various brain regions to find those genes, which were stably expressed. Each housekeeping gene was normalized against the remaining three genes. A typical result is shown for β-Actin in Fig. 2. Only normalization to β-actin, GAPD and HPRT1 were not significantly different. This indicates that these three are useful housekeeping genes for our experiments.

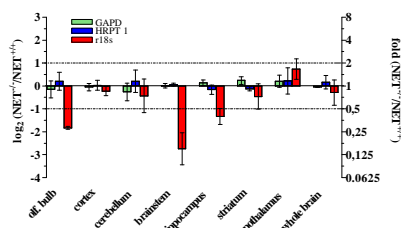


Fig. 2: β-actin mRNA expression (NET^{-/-}/NET^{+/+}) normalized to three housekeeping genes (GAPD, HRPT1, r18s) Shown are means (± SEM) of 5 independent experiments.

Differences between NET^{-/-} and NET^{+/+} mRNA levels

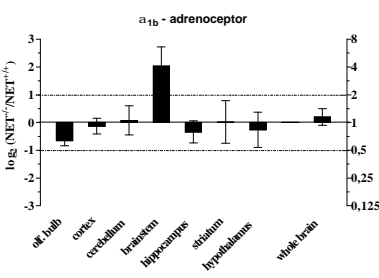


Fig. 3: relative (NET^{-/-}/NET^{+/+}) mRNA expression of the α_{1B}-R in mice brain Shown are means (± SEM) of 5 independent experiments; For normalization the following housekeeping genes were used: β-Actin, GAPD, HPRT1.

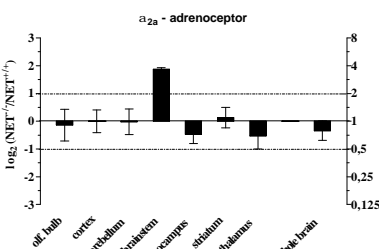


Fig. 4: relative (NET^{-/-}/NET^{+/+}) mRNA expression of the α_{2A}-R in mice brain Shown are means (± SEM) of 5 independent experiments; For normalization the following housekeeping genes were used: β-Actin, GAPD, HPRT1.

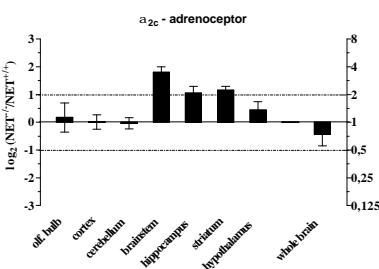


Fig. 5: relative (NET^{-/-}/NET^{+/+}) mRNA expression of the α_{2B}-R in mice brain Shown are means (± SEM) of 5 independent experiments; For normalization the following housekeeping genes were used: β-Actin, GAPD, HPRT1.

Adrenoceptor mRNA levels in NET^{+/+}

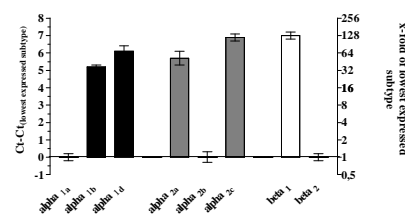


Fig. 6: mRNA expression of adrenoceptors in whole brain of NET^{+/+} mice relative to the lowest expressed subtype of the group (same colour) Shown are means (± SEM) of 10 independent experiments. (Mean Cts: α_{1A}-R = 28.8 ± 0.2; α_{1B}-R = 23.6 ± 0.12; α_{1C}-R = 22.7 ± 0.3; α_{2A}-R = 27.9 ± 0.4; α_{2B}-R = 33.6 ± 0.3; α_{2C}-R = 26.7 ± 0.2; β₁-R = 24.2 ± 0.2; β₂-R = 31.2 ± 0.2)

Results

1) Expression of housekeeping genes

The mRNA expression of r18s is upregulated by the knock-out of the NET in various brain regions (olf. bulb, brainstem and hippocampus). The mRNA densities of β-actin, GAPD and HPRT1 were not influenced by the knock-out.

2) Expression of adrenoceptors

a) α₁-adrenoceptors:

In total brain of NET^{-/-} mice mRNA expression of the α_{1B}-R and α_{1A}-R in the mouse brain is more than 30-fold higher than that of the α_{1C}-R.

The knock-out of the NET induces a brain region-specific up regulation (4-fold) of the α_{1B}-R mRNA in the brainstem. In any brain region the α_{1A}-R and α_{1C}-R mRNA levels were not influenced by the NET knock-out (data not shown).

b) α₂-adrenoceptors:

In the total NET^{-/-} mouse brain the mRNA concentration of α_{2A}-R and α_{2C}-R is more than 40-fold higher than of α_{2B}-R mRNA.

In the brainstem the mRNA encoding the α_{2A}-R and α_{2C}-R is elevated 3.5-fold. In addition, the α_{2A}-R mRNA is upregulated in the hippocampus and striatum (2-fold). α_{2B}-R mRNA expression is not significantly affected by the knock-out in any brain region (data not shown).

c) β-adrenoceptors:

In the whole mouse brain (NET^{-/-}) the mRNA of the β₂-R is about 100-fold lower expressed than of the β₁-R. Both mRNA expressions were not influenced by knock-out of the NET in any brain region (data not shown).

Summary and Conclusions

β-actin, GAPD and HPRT1 are well suitable housekeeping genes for our studies.

In the whole brain the knock-out of the NET induces no significant changes in the mRNA expression of adrenoceptors.

No changes in the examined brain regions were observed for α_{1A}-R, α_{1A}-R, α_{2B}-R and for the β-R mRNAs.

In the brainstem of NET^{-/-} mice the mRNA levels of α_{2A}-R, α_{2C}-R and α_{1B}-R were upregulated.

First experiments with clonidine indicate that also the α₂-R protein and function is elevated in the CNS of NET knock-out mice: clonidine caused significant stronger sedation in NET^{-/-} than in NET^{+/+} mice (results not shown).

References

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This study was supported by the Deutsche Forschungsgemeinschaft (SFB 400/A1).