Neuroscience Letters 506 (2012) 214–219

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Impact of the *COMT* Val$^{108/158}$Met polymorphism on the mu-opioid receptor system in the human brain: Mu-opioid receptor, met-enkephalin and beta-endorphin expression

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ARTICLE INFO

Article history:
Received 13 August 2011
Received in revised form 1 November 2011
Accepted 3 November 2011

Keywords:
COMT polymorphism
Human brain
Mu-opioid receptor expression
Enkephalin
Beta-endorphin

ABSTRACT

The Val$^{108/158}$Met polymorphism of the catechol-*O*-methyltransferase gene (*COMT*) is known to interact with the function of various neuroreceptor systems in the brain. We have recently shown by post-mortem receptor autoradiography that the number of mu-opioid (MOP) receptor binding sites depends on the number of *COMT* Met$^{108/158}$ alleles in distinct human brain regions. We now investigated *COMT* Val$^{108/158}$Met related levels of the MOP receptor protein and its endogenous ligands met-enkephalin and beta-endorphin in the human frontal cortex, thalamus and basal ganglia. Semiquantitative immunostaining and *in situ* hybridization were applied in a cohort of 17 human brain tissues from healthy donors. MOP receptor protein levels paralleled previous ligand binding results with a significantly higher MOP receptor expression in the mediodorsal nucleus of the thalamus of *COMT* Met$^{108/158}$ allele carriers. Also met-enkephalin peptide levels correlated with the genotype in this structure, with the lowest expression in *COMT* Met$^{108/158}$ homozygous individuals. Beta-endorphin was not detectable in the cortex, basal ganglia or thalamus, and therefore is unlikely to contribute to changes of the MOP receptor system. These results confirm the impact of the *COMT* Val$^{108/158}$Met polymorphism on the MOP receptor system and may support the hypothesis of an enkephalin related turnover of MOP receptors at least in some brain structures.

Introduction

The mu-opioid (MOP) receptor system is involved in a variety of neural functions including pain perception, drug reward and tolerance as well as neuroendocrine regulation, immune response and memory. High densities of MOP receptors are found in the caudate-putamen, thalamus, interpeduncular nuclei, locus coeruleus, nucleus tractus solitarius and the dorsal horn of the spinal cord [1,15,16].

Using positron emission tomography, it has been demonstrated *in vivo* that the amount of MOP receptor binding sites in the ventral basal ganglia and the thalamus is significantly influenced by the Val$^{108/158}$Met polymorphism of the catechol-*O*-methyltransferase gene (*COMT*) [18]. The *COMT* Val$^{108/158}$Met polymorphism results in a Val to Met substitution at position 158 of the membrane-bound (MB-COMT) enzyme or at position 108 of the shorter soluble isoenzyme (S-COMT) respectively. This substitution leads to an almost four times higher enzyme activity in Val$^{108/158}$ homozygous than Met$^{108/158}$ homozygous individuals. COMT is a pivotal enzyme of catecholamine catabolism [13].

However, it is not known how the *COMT* Val$^{108/158}$Met polymorphism translates into changes of MOP receptor availability. One major hypothesis is that they are secondary due to changes in the dopamine level and its interplay with endogenous opioidergic ligands. In the basal ganglia, enkephalin is colocalised with the dopamin D2 receptor [2] and negatively feedbacks dopaminergic neurotransmission [14]. Several studies have shown that a decrease of dopamine stimulation results in an increased enkephalin expression [17]. By analogy, the *COMT* Val$^{108/158}$ allele, which decreases

Abbreviations: MOP, mu-opioid; PPE, preproenkephalin; COMT, catechol-*O*-methyltransferase; MB-COMT, membrane-bound catechol-*O*-methyltransferase; S-COMT, soluble catechol-*O*-methyltransferase; POMC, proopiomelanocortin; Val, valine; Met, methionine; PBS, phosphate-buffered saline; DAB, diaminobenzidine.

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doi:10.1016/j.neulet.2011.11.008

dopamine levels by increased enzymatic activity, may lead to increased enkephalin levels. Assuming a use-dependent interaction between receptor and its ligand, this increase in enkephalin may decrease MOP receptor availability by downregulation. However, this hypothesis is based on experimental animal data mainly from short term manipulation of dopaminergic innervation and – second – the close interaction between dopamin receptors and enkephalin is not so clear cut outside the basal ganglia.

In a recent study on human post-mortem brain tissue, we were able to confirm the impact of the *COMT* Val$^{108/158}$Met polymorphism on MOP receptor availability. By means of saturating [^{3}H] [D-Ala^2, N-Me-Phe^4, Gly^5-ol] enkephalin ([^{3}H] DAMGO) binding we demonstrated increased amounts of MOP binding sites in the caudate nucleus, nucleus accumbens and mediodorsal nucleus of the thalamus in *COMT* Met$^{108/158}$ homo- and heterozygous individuals [4]. Notably, regulation of MOP receptor expression as well as functional downregulation can contribute to the availability of binding sites. We further investigated the *COMT* Val$^{108/158}$Met related expression of preproenkephalin (PPE). We found that PPE mRNA levels in the striatum were significantly higher in *COMT* Met$^{108/158}$ homozygous cases indicating that MOP binding sites and enkephalin expression are regulated in parallel [4]. We reasoned that this renders the hypothesis of MOP receptor downregulation by enkephalin unlikely. Moreover, PPE mRNA was not detectable in the thalamus.

Thus, in the present study we addressed the following questions: Since we do not know whether the *COMT* Val$^{108/158}$Met related differences in MOP receptor binding sites are due to true changes in receptor expression or due to functional downregulation, we evaluated whether these *COMT* Val$^{108/158}$Met related changes in MOP receptor availability are reflected on MOP receptor protein levels. Second, we wanted to evaluate whether the observed changes in PPE mRNA expression translate into equivocal changes of enkephalin peptide expression. Third, we wanted to evaluate whether beta-endorphin might be an alternative candidate for this interaction, especially in the thalamus. To do so, we performed semiquantitative immunohistochemistry and *in situ* hybridization experiments on the human brain tissues which have been used in our previous study.

Materials and methods

Tissue acquisition and preparation

Human brain tissues ($n = 17$) were obtained from Caucasian patients (11 male, 6 female) at medicolegal autopsy as described previously [4]. Patient's age at death was 59.3 years (mean; range 29–86 years), all females included were in postmenopausal state. None of the donors had a history of neurological or psychiatric disease. Post-mortem interval until tissues were removed mediated 16.9 h (±7.5 h SD). Six of the donors were *COMT* Met$^{108/158}$ homozygous, eight *COMT* Val$^{108/158}$Met heterozygous and three *COMT* Val$^{108/158}$ homozygous. For the evaluation of beta-endorphin peptide expression, we investigated various regions from three of these brains and two pituitary glands (both *COMT* Met$^{108/158}$ homozygous). Sections (20 μm) of brain tissue were cut on a cryostat, mounted on poly-L-lysine-coated slices and stored at −80 °C. For *in situ* hybridization, sections were fixed in 4% paraformaldehyde; for immunohistochemistry, sections were fixed in acetone (for 8 min).

Immunohistochemistry

Sections were preincubated (60 min) with phosphate buffered saline (PBS) containing 2% normal goat serum. Subsequently sections were incubated overnight at 4 °C using the following primary antibodies: rabbit polyclonal anti mu-opioid receptor (recognizes C-terminus) (AB5511, Chemicon/Millipore, Billerica, MA, USA; diluted 1:1000), rabbit polyclonal anti met-enkephalin (AB5026, Chemicon/Millipore; diluted 1:1000) and mouse monoclonal anti beta-endorphin (CBL 98, Chemicon/Millipore; diluted 1:500). Sections were then incubated (60 min) with a goat anti-rabbit or anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and thereafter incubated (60 min) with the avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories). Finally sections were stained with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) for 6–8 min. After staining, sections were dehydrated in ethanol, fixed in Roti-Histol (Roth, Karlsruhe, Germany) and coverslipped using Roti-Histokitt (Roth). Except the incubation with the primary antibody, all incubations were done at room temperature, and PBS (pH 7.4) was used for washing after each step (3 × 10 min).

Additional tissue sections of each brain and brain area were incubated without the respective primary antibody. Of each region of interest, all slices of the entire cohort and their corresponding negative controls were processed together to ensure identical experimental conditions.

To evaluate the specificity of primary antibodies, we blocked each antibody with the specific target protein and related antigens on additional sections (*mu opioid receptor peptide* (AG375, Chemicon/Millipore, 50 μg/ml), delta opioid receptor peptide (ab38195, Abcam, Cambridge, UK; 40 μg/ml), kappa opioid receptor peptide (ab38193, Abcam; 40 μg/ml); *met-enkephalin* (M6638, Sigma, St. Louis, MO, USA; 250 μg/ml), leu-enkephalin (1889, Tocris, Ellisville, MI, USA; 250 μg/ml), dynorphin A and B (3195 and 3196, Tocris; 250 μg/ml); *beta-endorphin* (E6261, Sigma, 50 μg/ml)). For the specific antigen, tissue staining was greatly reduced whereas related antigens did not abolish the respective antibody signals. Beta-endorphin expression by the monoclonal beta-endorphin antibody CBL 98 was further validated using the polyclonal antibody AB5028 (Chemicon/Millipore), which rendered identical staining patterns. To allow for semiquantitative analyses of immunoreactivity, antigens for each antibody were blotted in different concentrations (50 μg, 25 μg, 10 μg, 7.5 μg, 5 μg, 2.5 μg, 1 μg, 0.75 μg, 0.5 μg, 0.25 μg) on a membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare, Piscataway, NJ, USA) and stained. Competition experiments and blotted proteins were processed the same way as all other tissue sections.

In situ hybridization

In situ hybridization of POMC mRNA was performed as described previously [5]. The oligonucleotide probe was designed to be complementary to the exon 2/3 transition of both published human POMC mRNA sequence splice variants (variant 1 NM_001035256, variant 2 NM_000939.2): 5′-TGC AGG CCC GGA TGC ACT CCA GCA GGT TGC TTT CCG TGG T-3′. We used [α^{35}S]dADP (NEN Perkin Elmer, Bruxelles, Belgium) to radiolabel the oligonucleotide at the 3′-end. Sections were than incubated with the oligonucleotide for 48 h at 42 °C. To assess unspecific background signal, slices were also hybridized in the presence of unlabeled probe in 100-fold excess. Sections were dipped in Kodak NTB 2 nuclear track emulsion, exposed for 8 weeks, developed with Kodak D 19 developer and counterstained with thionin.

Quantification of immunohistochemistry

Stained sections, their corresponding controls and the blotted proteins were digitized with a calibrated scanner (ArtixScan 2500f, Microtek, Hoogvliet, Netherlands) and analysed using a computer assisted image analysis system (Object-Image 2.10).