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Elevated Hippocampal Cholinergic Neurostimulating Peptide precursor protein (HCNP-pp) mRNA in the amygdala in major depression

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ABSTRACT

The amygdala is innervated by the cholinergic system and is involved in major depressive disorder (MDD). Evidence suggests a hyper-activate cholinergic system in MDD. Hippocampal Cholinergic Neurostimulating Peptide (HCNP) regulates acetylcholine synthesis. The aim of the present work was to investigate expression levels of HCNP-precursor protein (HCNP-pp) mRNA and other cholinergic-related genes in the postmortem amygdala of MDD patients and matched controls (females: N = 16 pairs; males: N = 12 pairs), and in the mouse unpredictable chronic mild stress (UCMS) model that induced elevated anxiety-/depressive-like behaviors (females: N = 6 pairs; males: N = 6 pairs). Results indicate an up-regulation of HCNP-pp mRNA in the amygdala of women with MDD ($p < 0.0001$), but not males, and of UCMS-exposed mice (males and females; $p = 0.037$). HCNP-pp protein levels were investigated in the human female cohort, but no difference was found. There were no differences in gene expression of acetylcholinesterase (AChE), muscarinic (mAChRs) or nicotinic receptors (nAChRs) between MDD subjects and controls or UCMS and control mice, except for an up-regulation of AChE in UCMS-exposed mice (males and females; $p = 0.044$). Exploratory analyses revealed a baseline expression difference of cholinergic signaling-related genes between women and men ($p < 0.0001$). In conclusion, elevated amygdala HCNP-pp expression may contribute to mechanisms of MDD in women, potentially independently from regulating the cholinergic system. The differential expression of genes between women and men could also contribute to the increased vulnerability of females to develop MDD.

1. Introduction

Major Depressive Disorder (MDD) is a severe mental disorder that is often chronic and recurrent and that leads to substantial impairments in an individual's ability to take care of everyday responsibilities. MDD is the leading cause of disability worldwide, as measured by years lost due to disability (WHO, 2008). The World Health Organization ranked MDD as the 3rd leading cause of burden of disease as of 2004, but importantly, projected that MDD would be the number one cause for burden of disease by 2030 (WHO, 2008).

In the 1970s, Janowsky et al. first proposed a possible involvement of the cholinergic system in the etiology of MDD (Janowsky et al., 1974, 1972). They hypothesized that a given affective state may represent a balance between central cholinergic and adrenergic neurotransmitter activity in those areas of the brain that regulate affect, with depression being a disease of cholinergic dominance and mania being a disease of adrenergic dominance (Janowsky et al., 1974, 1972). This possible mechanism was recently revisited by Mineur and Picciotto (Mineur and Picciotto, 2010). Neurotransmission of the cholinergic system is carried out by acetylcholine (ACh), which is synthesized by cholineacetyltransferase (ChAT) and degraded by acetylcholinesterase (AChE). The main receptors for ACh are the nicotinic (nAChRs) and muscarinic (mAChRs) receptors. Several lines of evidence suggest

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involvement of the cholinergic system in MDD. Organophosphate poisoning inhibits AChE, resulting in increased ACh, and can cause depressive-like behavior in humans (Gershon and Shaw, 1961). Additionally, a neural nAChR antagonist reduces anxiety-like behavior in mice (Roni and Rahman, 2011) and an $\alpha_4\beta_2$nAChR partial agonist elicits antidepressant properties in the forced swim test in mice (Zhang et al., 2012). Administration of scopolamine (a mAChR antagonist) showed antidepressant properties in unipolar and bipolar patients (Drevets and Furey, 2010; Furey and Drevets, 2006; Furey et al., 2010), and MDD patients on both oral scopolamine and citalopram had better remission rates than with citalopram alone (Khajavi et al., 2012). Many MDD patients exhibit sleep disturbances, including a decrease in rapid eye movement (REM) latency. Interestingly, a cholinergic agonist produced a faster induction of REM sleep only in MDD patients and in subjects at high risk for psychiatric disorders (Palagini et al., 2013). Finally, knockdown of AChE in the hippocampus of adult mice increases anxiety- and depression-like behaviors and susceptibility to social stress, which was prevented by fluoxetine (Mineur et al., 2013). Taken together, these results support the idea that hyper-activation of the cholinergic system may be involved in MDD.

Hippocampal Cholinergic Neurostimulating Peptide (HCNP) is involved in regulating ACh synthesis in a medial septal nucleus culture system (Ojika et al., 1992) by increasing the levels of ChAT in cholinergic neurons (Uematsu et al., 2009). HCNP is an undecapeptide cleaved from the precursor protein (HCNP-pp) (Otsuka and Ojika, 1996). HCNP-pp is also known as phosphatidylethanolamine-binding protein (PEBP) and Raf kinase inhibitor protein (RKIP) (Sedivy, 2011). The release of HCNP from hippocampal culture is specifically mediated by the NMDAR (Ojika et al., 1998). Results suggest that HCNP/HCNP-pp also acts as a key regulator for differentiation of cultured hippocampal progenitor cells (Sagisaka et al., 2010).

At the neural network level, changes in the function of several cortical and subcortical brain regions are thought to underlie the mood regulation deficit in depression (Seminowicz et al., 2004). We previously found differential gene expression in the amygdala of men and women with MDD compared to controls, although with notable sex differences (Guilloux et al., 2012; Sibille et al., 2009). This is in accordance with neuroimaging studies showing reduced volume or grey matter density of the amygdala in female MDD patients compared to control subjects, with no change in male MDD (Hastings et al., 2004; Kong et al., 2013).

The amygdala receives cholinergic input from the Nucleus Basalis of Meynert (Schafer et al., 1998) and expresses both muscarinic and nicotinic receptors (Klein and Yakel, 2006; McDonald and Mascagni, 2010, 2011). However, the cholinergic system in the amygdala has not been studied in detail in MDD subjects. Here, our working hypothesis is that HCNP-pp expression in the amygdala is involved in the pathogenesis of MDD by regulating the cholinergic system through HCNP. The aim of the present work was to investigate gene expression levels of HCNP-pp and genes involved in the cholinergic system in the postmortem amygdala of MDD patients and matched controls, and in a mouse model that elicits increased anxiety-/depressive-like behaviors.

2. Materials and methods

Details of all methods are available in the Supplementary Information.

2.1. Human postmortem subjects

Brain samples were obtained after consent from next-of-kin during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA, USA) using procedures approved by University of Pittsburgh's Institutional Review Board and Committee for Oversight of Research Involving the Dead. Two cohorts of MDD subjects were examined here (male, n = 12 pairs; female, n = 16 pairs). Each MDD subject was matched with one control subject for sex and as closely as possible for age (Tables 1 and 2). See cohort details in Supplementary Information and (Guilloux et al., 2012; Sibille et al., 2009). The effects of putative confounds (age, antidepressants, death by suicide, pH, PMI, RNA ratio, RIN) were evaluated. When comparing male MDD subjects versus male controls, or female MDD subjects versus female controls, subject groups did not differ in mean age, postmortem interval (PMI), RNA integrity number (RIN), RNA ratio, or brain pH, as determined by one-way ANOVA ($p > 0.05$). When comparing men and women, pH and RNA ratio were significantly different ($p = 0.009$ and $p = 0.004$, respectively) in MDD patients but they did not differ in RIN. Importantly, RIN is a better indicator of RNA quality than pH (Stan et al., 2006) or RNA ratio (Copois et al., 2007). Subjects did not differ in mean age, antidepressants, death by suicide or PMI. Male and female control subjects did not differ by age, pH, PMI, RNA ratio, or RIN. Significant co-factors were included in the ANCOVA analyses.

2.2. Protein purification and Western blotting

Following RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), acetone precipitation of proteins was carried out (Guilloux et al., 2012). Using Western blotting, HCNP-pp signal ratios relative to actin were calculated. To reduce the within- and between-subject measurement variance, samples were processed in matched pairs on the same gel four times, and results were replicated for a total of four different Western blots, with 16 replicates per pair (Curley et al., 2011).

2.3. Mouse samples

Amygdala cDNA from a mouse cohort previously described was used (Edgar et al., 2011).

2.4. Real-time quantitative polymerase chain reaction (qPCR)

qPCR analyses were performed using specific primers for HCNP-pp, AChE, ChAT, mAChRs (1–4) and nAChRs (α3, α4, α7, and β2) and three internal controls (beta-actin, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase) on amygdala cDNA samples, as described previously (Sibille et al., 2009). In brief, small PCR products (80–120 basepairs) were amplified in quadruplet on an Opticon real-time PCR machine (BioRad, Waltham, MA, USA). Each qPCR run included one MDD subject and one matched control.

Using a similar qPCR methodology as described above for human samples, qPCR on mouse samples was performed. Each run included one UCMS mouse and one control mouse, matched for sex.

2.5. Statistical analysis

2.5.1. Human samples

Diagnosis-related expression differences in gene of interest (GOI) signal were determined by analysis of covariance (ANCOVA) using SPSS (SPSS, Inc., Chicago, IL, USA). Relevant factors showing significant differences by ANOVA were included in the ANCOVA model. The qPCR data were averaged across the four replicates and transformed into expression levels relative to the internal control genes. Variance homogeneity was tested by Levene's test. Sex-related expression differences in GOI signal were determined by analysis of covariance (ANCOVA), using a similar method. Since gene expression in sex-related comparisons did not present