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Neonatal manipulation of oxytocin prevents lipopolysaccharide-induced decrease in gene expression of growth factors in two developmental stages of the female rat ☆

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ABSTRACT

Oxytocin production and secretion is important for early development of the brain. Long-term consequences of manipulation of oxytocin system might include changes in markers of brain plasticity – cytoskeletal proteins and neurotrophins. The aim of the present study was (1) to determine whether neonatal oxytocin administration affects gene expression of nestin, microtubule-associated protein-2 (MAP-2), brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the brain of two developmental stages of rat and (2) to evaluate whether neonatal oxytocin administration protects against lipopolysaccharide (LPS) induced inflammation. Neonatal oxytocin did not prevent a decrease of body weight in the LPS treated animals. Oxytocin significantly increased gene expression of BDNF in the right hippocampus in 21-day and 2-month old rats of both sexes. Gene expression of NGF and MAP-2 significantly increased in males treated with oxytocin. Both, growth factors and intermediate filament-nestin mRNA levels, were reduced in females exposed to LPS. Oxytocin treatment prevented a decrease in the gene expression of only growth factors. In conclusion, neonatal manipulation of oxytocin has developmental and sex-dependent effect on markers of brain plasticity. These results also indicate, that oxytocin may be protective against inflammation particularly in females.

1. Introduction

Neuropeptides, including oxytocin, play a role in the growth and development of the brain in various animal species and humans (Boer and Swaab, 1985; Carter, 2003; Shen et al., 2013; Zaben and Gray, 2013). Exogenous oxytocin exposure during neonatal period may have long-term consequences on behavior (Perry et al., 2009). It was demonstrated that neonatal administration of oxytocin increased the number of oxytocin neurons in the hypothalamic nuclei (Yamamoto et al., 2004) and changed organization of the brain (Kramer et al., 2006). Moreover, a different study involving neonatal manipulation of oxytocin revealed changes of gene expression of estrogen receptors in the hypothalamus and hippocampus (Pournajafi-Nazarloo et al., 2007). Another study has suggested that oxytocin locally released from neuronal somata, axons and dendrites, (Ludwig, 1998; Jin et al., 2007; Bergquist and Ludwig, 2008; Neumann and Landgraf, 2012) contributes to reorganization of hypothalamic-neurohypophysial system, especially under conditions of lactation (Theodosis et al., 2006). Despite the fact that neuropeptides can act as trophic and differentiation factors, their exact role in development of the brain is not clear.

The oxytocin receptor belongs to the classical transmembrane G-protein coupled receptor family; its stimulation leads to activation of phosphatidylinositol-calcium system and proteinkinase C pathways (van den Burg and Neumann, 2011). Proteinkinase C participates in cytoskeleton reorganization, regulation of expression of actin-binding proteins and cell cycle changes (Uberall et al., 1999; Korulu et al., 2013). Neurons, glia, and overall extracellular matrix of the brain undergo dynamic transformations accompanied by alterations in levels of cytoskeletal proteins and neurotrophins. Cytoskeletal assembly and actin reorganization is strongly dependent on interactions with intermediate filaments: nestin and microtubule-associated protein-2 (Dehmelt et al., 2003; Chou et al., 2003, 2009). Nestin is a neuronal marker associated with cell

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differentiation that is extensively expressed in neural progenitor cells (Hendrickson et al., 2011). Microtubule-associated protein-2 (MAP-2) is an important component in the regulation of growth, differentiation, and plasticity of neurons (Johnson and Jope, 1992; Li et al., 1998; Kawashita et al., 2013). Modification and rearrangement of nestin and MAP-2, together with neuronal responses to growth factors, may comprise processes of brain plasticity.

Circulating oxytocin was suggested to have a protective role against inflammation and consequences of oxidation stress (Işeri et al., 2008). Clodi et al. (2008) demonstrated that peripheral oxytocin decreases the cytokine activation caused by bacterial endotoxin in humans (Clodi et al., 2008). Bacterial endotoxin, lipopolysaccharide (LPS), negatively affects brain development and is widely used to induce an inflammatory response in animal models of neonatal brain injury (Hagberg et al., 2002; Cai et al., 2003; Brehmer et al., 2012). LPS-induced brain-inflammation is characterized by pro-apoptotic cytokine release and results in selective white matter injury. In animal models, varying doses are used (0.3 mg/kg up to 100) with consequences ranging from hypotension, decrease of cerebral blood flow to marked systemic inflammation (Hagberg et al., 2002). To our best knowledge, no research has previously examined the role of neonatal administration of peptide hormone on expression of nestin and MAP-2. There is also a considerable lack of studies with manipulation of oxytocin system in relation to brain development and evaluation of markers of brain plasticity.

This study was performed (1) to determine whether neonatal oxytocin administration affects gene expression of nestin, microtubule-associated protein-2 (MAP-2), brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the brain of two developmental stages of rat and (2) to evaluate whether neonatal oxytocin administration is protective against lipopolysaccharide (LPS) induced inflammation.

2. Experimental procedures

2.1. Animals

Pregnant Wistar rats (AnLab Ltd., Czech Republic) were housed under controlled conditions (22 ± 2 C, 12:12 h light/dark cycle with lights on at 0600–1800 h) with access to standard pelleted diet and tap water *ad libitum*. Day of birth was considered as day 0. Litter size was adjusted to 10–12 pups per mother. Pups, both sexes, were divided to 4 experimental groups: (1) Control (saline treated on 2–6 postnatal day, i.p., 50 μl/pup), (2) oxytocin (1 mg/ml, treated on 2–6 postnatal day, i.p., 50 μl/pup) (3) lipopolysaccharide (1 mg/ml LPS, treated on 2–3 postnatal day, i.p., 50 μl/pup), (4) oxytocin + LPS. In each group, pups were intraperitoneally injected one time per day. Dose of oxytocin was selected on the basis of previous studies (Olausson et al., 2003; Bales et al., 2007). LPS exposure was modified on the basis of other neonatal model of inflammation (Cai et al., 2011). Experimental protocol represented 2 separate experiments performed with same design. Neonatal rats were kept with mother until 21st postnatal day. Animals from the first experiment were sacrificed immediately after weaning. Animals from the second experiment were sacrificed at 2 months of age. A total of 70 pups (33 males, 37 females) were included in first experiment and 78 pups (46 males, 32 females) in second experiment. State Veterinary and Food Administration of the Slovak Republic approved all experimental procedures in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC) and relevant legislation.

2.2. RNA isolation, reverse transcription and qPCR

Following decapitation, brains were quickly removed. Bilateral right and left hippocampi were separated, deeply frozen and stored at −80 °C. Total RNA was extracted and purified from homogenates of right hippocampi using NucleoSpin RNA II kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. The quantity, integrity and purity of RNA were determined by Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Rockford, IL). 1 μg of RNA was reverse transcribed to cDNA using the DyNAmo cDNA Synthesis kit (Finnzymes, Finland) according to manufacturer protocol in a volume of 20 μl by incubation at 25 °C for 10 min, 37 °C for 30 min, 85 °C for 5 min followed by a hold at 4 °C. First strand cDNA was stored at −20 °C until use. Quantitative RT-PCR (qPCR) was performed on the AB7900 (Life technologies-Applied Biosystems, Slovakia) using DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Finland). Reaction volume was 20 μl containing master mix, 0.3 μM each forward and reverse primer (Primer sequences in Table 1), 2 μM ROX reference dye, and 10 ng of cDNA template. PCR was performed for 40 cycles according to following protocol: activation of the Taq polymerase at 95 °C for 15 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min followed by fluorescence measurement (SYBR Green and ROX, respectively). To identify reaction products melting curve analysis was performed. Relative mRNA expression was calculated by Livak method (Livak and Schmittgen, 2001) for relative gene expression analysis with $2^{-\Delta\Delta Ct}$ comparative threshold.

2.3. Determination of cytokines in plasma

Trunk blood was collected in cooled polyethylene tubes containing EDTA as anticoagulant and centrifuged immediately at 4° C to separate plasma, which was then stored at −20° C until use. Proinflammatory cytokines – Interleukin-1-beta (IL-1β), Tumor necrosis factor-alpha (TNF-α) were measured in 100 μl plasma using ELISA kits (Invitrogen Co., Camarillo, California).

2.4. Statistics

The data of gene expression analyses are expressed as a ratio of the target gene in the test sample to the calibrator sample (control group), normalized to the expression of the reference gene – GAPDH. Baseline expression levels of the control group was set to 1. Unpaired *t*-test for body weights and values of relative gene expression was used to reveal significant differences in oxytocin, LPS treatment, or their combination in comparison with control

Table 1
List of primer sequences used for qPCR.

Gene	Forward primer	Reverse primer	Gen Bank	References
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	NM_017008.4	Horii et al. (2002)
BDNF	AAAACCATAAGGACGCGGACTT	AAAGAGCAGAGGAGGCTCCAA	NM_001270634.1	Rapanelli et al. (2010)
NGF	CCAAGCACTGGAACTCATACTGC	CTGCTGAGCACACACACGCAG	NM_001277055.1	Yu et al. (2011)
MAP-2	TGTTGCTGCCAAGAAAGATG	ACGTGGCTGGACTCAATACC	NM_013066.1	Bolognin et al. (2012)
Nestin	AAGGTGGGCAGCAACTGGCA	GGCTTCAGCTTGGGGTCCAGAA	NM_012987.1	