Abstract. Neuropeptides including oxytocin belong to the group of factors that may play a role in the control of neuronal cell survival, proliferation and differentiation. The aim of the present study was to investigate potential contribution of oxytocin to neuronal differentiation by measuring gene and protein expression of specific neuron and glial markers in the brain. Neonatal and adult oxytocin administration was used to reveal developmental and/or acute effects of oxytocin in Wistar rats. Gene and protein expression of neuron-specific enolase (NSE) in the hippocampus was increased in 21-day and 2-month old rats in response to neonatal oxytocin administration. Neonatal oxytocin treatment induced a significant increase of gene and protein expression of the marker of astrocytes – glial fibrillary acid protein (GFAP). Oxytocin treatment resulted in a decrease of oligodendrocyte marker mRNA – 2’,3’-cyclic nucleotide 3’-phosphodiesterase (CNPase) – in 21-day and 2-month old rats, while no change of CD68 mRNA, marker of microglia, was observed. Central oxytocin administration in adult rats induced a significant increase of gene expression of NSE and CNPase. The present study provides the first data revealing the effect of oxytocin on the expression of neuron and glial markers in the brain. It may be suggested that the oxytocin system is involved in the regulation of development of neuronal precursor cells in the brain.

Introduction

The functional complexity of the mammalian central nervous system is based on the ability to generate diverse cell types during development. Currently, many studies are devoted to understanding the neuronal cell development (Molofsky et al., 2012). The differentiation from a neuronal progenitor state towards neurons and glial cells is based on the activation of specific cell fate genes and various intrinsic and extrinsic factors (Massirer et al., 2011). For example, neuropeptides galanin, neuropeptide Y and vasoactive intestinal peptide belong to the factors that play a role in the control of hippocampal stem cell survival, proliferation and differentiation during the postnatal development and adulthood (Zaben and Gray, 2013). Few studies have evidenced that neuropeptide oxytocin induces differentiation of mouse embryonic stem cells to cardiomyocytes (Paquin et al., 2002) and influences neural differentiation of adipose tissue-derived stem cells (Jafarzadeh et al., 2014). A direct effect of oxytocin on neonatal brain metabolism, independent of maternal physiology, has been suggested (Boksa et al., 2015). Moreover, oxytocin has been implicated in the regulation of cellular growth, differentiation or contact with other cells, and thus its potential to remodel the nervous system is very likely (Carter, 2003). In our previous studies we have proved that oxytocin increases growth and viability of neuroblastoma and glioblastoma cells, implicating effects on the cell cycle (Bakos et al., 2012). However, there is only limited information about the influence of oxytocin...
on gene and protein expression of markers of differentiation of neuron and glial cells.

Many specific markers are used to visualize and quantify neuron and glial cell bodies (Lyck et al., 2008; Zhang et al., 2013). The expression of neuron-specific enolase (NSE) is associated with the neuronal cell type, and it is used as a marker of neurons (Creus et al., 2008). Quantitative analysis of the expression of glial fibrillary acid protein (GFAP) is used to evaluate the number of astrocytes. GFAP is a primary intermediate filament in astrocytes regulating multiple functions, including maintenance of astrocyte morphology, and it is considered to be a marker for astrocyte maturation (Bonni et al., 1997; Fatemi et al., 2008). Another important glial marker is represented by 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), which is considered to be a myelination marker specific for oligodendrocytes. Microglia are the main resident immunological cells in the central nervous system, and quantification of lysosomal microglial marker CD68 is commonly used to assess microglial activation (Kobayashi et al., 2013). In the present study, we hypothesized that the expression of specific neuron and glial markers in the rat hippocampus depends on neonatal oxytocin administration. Consequently, we have conducted a second experiment where we tested whether 7-day intracerebroventricular administration of oxytocin affects neuron and glial markers in the hippocampus of adult rats.

Material and Methods

Animals

Adult and neonatal male Wistar rats (AnLab Ltd., Prague, Czech Republic) were used in the experiments. Animals were housed under controlled conditions (22 ± 2 °C, 12:12 h light/dark cycle with lights on at 06.00–18.00 h) with access to standard pellet diet and tap water ad libitum. The present study includes analysis of the brain tissue from animals used in previously published experiments (Bakos et al., 2014; Havranek et al., 2015). The State Veterinary and Food Administration of the Slovak Republic approved all experimental procedures in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and relevant legislation.

Experiment I

Oxytocin was administered in five consecutive injections to neonatal rats (N = 6) on 2nd–6th postnatal days in the dose of 1 mg/ml, i.p., 50 μl/pup. Control rats (N = 6) received saline i.p., 50 μl/pup once a day. The dose of oxytocin and the experimental protocol were the same as in experiments performed with the same design as described previously (Bakos et al., 2014). Neonatal rats were kept with their mother until 21st postnatal day. Animals from the first part of the experiment were sacrificed immediately after weaning. Animals from the second part of the experiment were sacrificed at two months of age. Following decapitation, brains were quickly removed. The cortex was peeled away, the whole hippocampus was removed and divided into the right and left halves, deeply frozen and stored at –80 °C until analysed.

Experiment II

Oxytocin was administered to adult Wistar rats via intracerebroventricular osmotic minipumps (model 1007D; Alzet, Cupertino, CA) as described previously (Havranek et al., 2015). Each minipump was positioned subcutaneously in the scapular region and attached to an infusion cannula targeting the left lateral ventricle (stereotaxic coordinates for implantation of infusion cannula: AP: –1.4 mm from bregma, ML: +2.2 mm, DV: –4.5 mm below the surface of the skull; (Paxinos and Watson 1997) via polyethylene tubing). The minipumps were filled with vehicle (saline, pH 7.4, N = 5) or oxytocin (20 ng/μl saline, N = 6). After performing behavioural evaluations (Havranek et al., 2015) not related to the present study, on the 8th day from implantation of minipumps, both control and oxytocin-treated rats were quickly moved to an adjacent room and sacrificed by decapitation.

RNA isolation, reverse transcription and qPCR

Total RNA was extracted and purified from homogenates of the right hippocampus using Trizol Reagent (Life Technologies-Invitrogen, Bratislava, Slovakia). The quantity, integrity and purity of RNA were determined by a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc, Rockford, IL). One μg of RNA was reversely transcribed to cDNA using a high capacity cDNA reverse transcription kit (Life Technologies- Applied Biosystems, Bratislava, Slovakia) according to the manufacturer’s protocol in a volume of 20 μl by incubation at 25 °C for 10 min, 37 °C for 2 h, 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 in the AB7900 apparatus (Life Technologies-Applied Biosystems) using a Power SYBR Green PCR Kit (Life Technologies-Applied Biosystems). The reaction volume was 20 μl containing master mix, 0.3 μmol/l each forward and reverse primer (Table 1), 2 μmol/l ROX reference dye, and 10 ng cDNA template. PCR was performed for 40 cycles according to the 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 the reaction products, melting curve analysis was performed. Relative mRNA expression was calculated by the Livak method (Livak and Schmittgen, 2001) for relative gene expression analysis with 2–ΔΔCt comparative threshold. The data of gene expression analyses were expressed as a ratio of the target gene in
the test sample to the calibrator sample (control group) and normalized to the expression of the reference gene – GAPDH. The baseline expression level of the control group was set to 1.

**Western blot**

Proteins were isolated from the left hippocampus using lysis buffer (137 mmol/l NaCl, 20 mmol/l Tris-HCl, 10% v/v glycerol) with protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO). Protein concentrations were measured using the BCA kit (Thermo Fisher Scientific Inc) with bovine serum albumin as a standard. In order to adjust the equal amount of loaded proteins, samples were diluted to 2 μg prot/μl with ultrapure water and sample loading buffer (1:2 solution; 10% w/v sodium dodecyl sulphate (SDS), 0.02% bromophenol blue, and 25% glycerol in 0.5 mol/l Tris-HCl, pH 6.8). Proteins were separated by 12% SDS-PAGE running gels with 5% stacking gels. Constant current 150 mA was appointed as a running parameter. The proteins were transferred to low fluorescent polyvinylidene difluoride membrane (0.45 µm pore size PVDF membranes; Immobilon-FL, Merck-Millipore, Prague, Czech Republic) using a Mini Trans-Blot® cell system (Bio-Rad, Bratislava, Slovakia). After blocking with 4% w/v bovine serum albumin (Sigma-Aldrich Corp.) for 1 h at room temperature, membranes were incubated overnight at +4 °C with rabbit primary antibody anti-NSE (1:1000) or mouse primary antibody anti-GFAP (1:1000). As a reference, the GAPDH protein (mouse anti-GAPDH, 1:1000) was used.

Next day, the membranes were incubated with goat anti-rabbit IR-Dye 800CW or goat anti-mouse IR-Dye 670CW secondary antibody for 1 h at room temperature. Antibodies were diluted in 0.1% Tween Tris buffer solution (0.1% T-TBS). All used antibodies were obtained from Sigma-Aldrich Corp. The membranes were washed three times for 10 min after each step in 0.1% T-TBS. For signal detection, an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) was used. The density of each band was quantified and normalized to GAPDH. Quantification was performed in a single channel; bands of interest were manually signposted with intra-lane background subtracted using Odyssey 2.0 analytical software (LI-COR, Lincoln, NE).

**Statistical analysis**

Unpaired two-tailed t-test for values of relative gene expression and protein levels was done to reveal significant differences in oxytocin compared with the control group. The value of P < 0.05 was considered statistically significant. Results are shown as means ± SEM.

**Results**

The gene expression of NSE in the hippocampus increased in 21-day (P < 0.001, df = 10, T = 5.91) and 2-month (P < 0.01, df = 10, T = 3.95) old rats (Fig. 1A) in response to neonatal oxytocin administration. The same trend was found for protein levels of NSE in 21-day (P < 0.05, df = 6, T = 3.49) and 2-month (P < 0.05, df = 6, T = 3.66) old rats (Fig. 1B) in response to oxytocin treatment. Neonatal oxytocin administration induced a significant increase of the GFAP gene ((P < 0.001, df = 10, T = 4.97) and protein (P < 0.01, df = 14, T = 3.16) levels in the hippocampus of 2-month old rats (Fig. 1C, 1D). The opposite trend was observed for CNPase. Oxytocin treatment resulted in a decrease of CNPase mRNA in 21-day (P < 0.05, df = 10, T = 2.45) and 2-month (P < 0.05, df = 9, T = 2.61) old rats (Fig. 2). No differences were found in the CD68 gene expression. Central oxytocin administration (Fig. 3) in adult rats induced a significant increase of gene expression of NSE (P < 0.001, df = 9, T = 5.76) and CNPase (P < 0.01, df = 10, T = 4.08).

**Discussion**

The data obtained in the present study support the hypothesis that oxytocin contributes to neuronal differentiation. Both, neonatal and adult administration of oxytocin resulted in upregulation of the gene and protein expression of NSE, marker of neurons in the hippocampus. Moreover, alterations in GFAP and CNPase expression implicate changes of the ratio of glial cells observed under the effect of oxytocin.

The stimulation of gene expression of NSE in the hippocampus after neonatal application of oxytocin corresponds with our previous results, which have shown upregulation of neurotrophins under the influence of oxytocin (Bakos et al., 2014). Furthermore, we have observed that both neurotrophins and synaptic proteins
rise in response to central administration of oxytocin in adult rats (Havranek et al., 2015). The neurotrophins are crucial for survival and development of neuronal cells (Alderson et al., 1990). A recent study has evidenced that isolation of hippocampal and hypothalamic progenitor cells includes the cell phenotype producing oxytocin (Markakis et al., 2004). This suggests that early development of the brain may be dependent on the oxytocin secretion. The direct effects of oxytocin on NSE expression implicate faster rate of proliferation of neurons and/or growth of neuronal progenitors. On the other hand, the dynamics of NSE expression is potentially more complexly regulated as an elevation of NSE has been observed after neuronal damage, and it could play a role in some pathological states (Haque et al., 2016). Oxytocin may play an important role in the regulation of cell cycle and its proliferative effects were revealed in neuroblastoma cells in another study (Bakos et al., 2012). Moreover, recent findings of Leuner et al. (2012) stress a role of oxytocin in neurogenesis in vivo. It looks likely that oxytocin may contribute to generation of new neurons in the hippocampus, rather than in the other re-

Fig. 1. Effects of oxytocin on mRNA (A, C) and protein (B, D) levels of neuron-specific enolase (NSE) and glial fibrillary acid protein (GFAP)
Pups with different treatments were intraperitoneally injected on 2nd–6th postnatal days, once a day: 1. Control (saline treated, i.p., 50 µl/pup), 2. Oxytocin (1 mg/mL i.p., 50 µl/pup). The first part of animals were sacrificed on 21st day and the second part at the age of two months. The mRNA data are expressed as relative mRNA expression calculated by the $2^{-\Delta\Delta Ct}$ method by Livak (Livak and Schmittgen, 2001). Relative changes in protein levels were measured by Western blot, where GAPDH served as an endogenous control. Results are shown as means ± SEM (N = 6). Significant differences compared to control are marked with * P < 0.05, ** P < 0.01, *** P < 0.001.
regions of the brain. The subventricular zone and dentate gyrus of the hippocampus are the most active known regions of neurogenesis in the brain (Spalding et al., 2013). Further immunohistochemistry studies may shed light on the region-specific generation of neurons under the effect of oxytocin.

The neonatal oxytocin treatment resulted in higher levels of mRNA and protein for GFAP, suggesting an altered ratio of neuronal cells in favour to astrocytes. Neuron-to-astrocyte transition was suggested in differentiating neurospheres \textit{in vitro} (Laywell et al., 2005) and the existence of proliferative precursor cells is discussed in the transition between glia-like states (Steiner et al., 2006). Although it is difficult from the results of the present study to completely rule out the role of oxytocin in maturation of neuronal cells, it is clear that the fate of neural progenitor cells may be dependent on neuropeptides. It has been demonstrated that oxytocin treatment promotes neural differentiation (Jafarzadeh et al., 2014). Moreover, it has been suggested that astrocyte growth is regulated by neuropeptides through immediate early genes (Hu and Levin, 1994). Another neuropeptide, reelin, modulates neuronal progenitor migration in the hippocampus (Gong et al., 2007) and neuropeptide \textit{Y} stimulates proliferation and migration of neuronal precursors in the subventricular zone (Decressac et al., 2009).

In the present study, the dynamics of glial cell transition is demonstrated by the decrease of CNPase mRNA, marker of oligodendrocytes in 21-day and 2-month old rats exposed to neonatal oxytocin. The changes of expression of oligodendrocyte-specific CNPase in the hippocampus have been observed in different experimental models during the development (Mustapha et al., 2014; Tiwari et al., 2015). The downregulation of CNPase in our model after neonatal administration of oxytocin may relate to the stimulation of gene expression of NSE in the hippocampus. In contrast to the neonatal manipulation, central administration of oxytocin in adult rats stimulated expression of CNPase together with NSE. Differentiated neurons express NSE (Mistry et al., 2002); nevertheless, the hippocampal levels of NSE vary across the oestrous cycle and they are hormonally dependent (Diao et al., 2008). Thus, differentiation from

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Effects of oxytocin on mRNA levels of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, A) and CD68 (B) for experimental group description – see legend to Fig. 1. The data are expressed as relative mRNA expression calculated by the 2$^{-\Delta\Delta C_t}$ method by Livak (Livak and Schmittgen, 2001). Relative changes in protein levels were measured by Western blot, where GAPDH served as an endogenous control. Results are shown as means ± SEM (N = 6). Significant differences compared to control are marked with * P < 0.05.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Effects of intracerebroventricular administration of oxytocin on mRNA levels of NSE, GFAP, CNPase, CD68, in the hippocampus
Gene expression data are expressed as relative mRNA expression calculated by the 2$^{-\Delta\Delta C_t}$ method by Livak (Livak and Schmittgen, 2001). Means are represented on bars ± SEM (N = 6). Significant changes are marked with ** P < 0.01, *** P < 0.001 compared to control animals.}
\end{figure}
a neuronal progenitor state towards neurons and glial cells may be different in a short-term paradigm compared to long-term developmental effects. The conclusion should be taken carefully, and more specific in vitro studies should be performed to reveal activation of specific cell fate genes. No change of CD68 expression after neonatal administration of oxytocin supports the theory that there is no change of the number of microglia and/or their inflammatory activity.

Previous studies have shown that mRNA expression of CD68 is augmented with an accompanying increase of the total number of microglia in cortical regions in a model of neuronal injury (Zhou et al., 2013). In a different model, the increase in the microglia number has been associated with an increase in apoptotic cells and axonal injury (Supramaniam et al., 2013). A recently published in vitro study has shown an immunomodulatory effect of oxytocin on activated macrophages (Oliveira-Pelegrin et al., 2013). Direct or indirect effects of oxytocin on microglia needs further studies. Based on the gene and proteins expression of markers of neuron and glial cells, our results provide evidence supporting the view of oxytocin as a regulator of neuronal cell fate. It may be suggested that the oxytocin system is involved in the regulation of development of neuronal precursor cells in the brain. Thus, a deficit in oxytocin signalling in certain developmental stages may result in pathogenesis of neuropsychiatric diseases, such as the autism spectrum disorders.

References


