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Oxytocin alters the morphology of hypothalamic neurons via the transcription factor myocyte enhancer factor 2A (MEF-2A)☆

Magdalena Meyer[a,*], Ilona Berger[a,b], Julia Winter[a], Benjamin Jurek[a]

[a] Department of Behavioral and Molecular Neurobiology, University of Regensburg, Regensburg, Germany
[b] Technische Universität Dresden, University Hospital, Department of Internal Medicine III, Dresden, Germany

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ABSTRACT

Oxytocin (OT) has gained attention not only as anxiolytic drug and as potential treatment option for autistic children; it also acts as a growth and differentiation factor in neuronal cells. While behavioral effects of OT have been studied in detail, knowledge about the cellular effects of OT is relatively sparse. In this study, we present evidence for three hypotheses: 1) OT leads to neurite retraction in hypothalamic neurons via the OT receptor (OTR) 2) The transcription factor MEF-2A is a central regulator of OT-induced neurite retraction, and 3) The MAPK pathway is critical for OT-induced MEF-2A activation.

Incubation of rat hypothalamic H32 cells with 10 nM to 1 μM OT, vasopressin, and the specific OTR agonist TGOT, over the course of 12 h resulted in a time-dependent, significant retraction of neurites. In addition, the size of the nuclear compartment increased, whereas the overall cell size remained unchanged. OT treatment for 10 h increased the cellular viability significantly, and this effect could be blocked by a specific OTR antagonist, providing evidence for a specific and pro-active effect of OT on neurite retraction, and not as an unspecific side effect of apoptosis.

The molecular mechanism that controls OT-induced neurite retraction includes a reduced phosphorylation of the transcription factor MEF-2A at Serine 408 (S408). This dephosphorylation is under the control of the OTR-coupled MAPK pathway, as blocking MEK1/2 by U0126 inhibited MEF-2A activation and subsequent neurite retraction. The siRNA-mediated knockdown of MEF-2A prevented the OT-induced neurite retraction, providing direct evidence for a role of MEF-2A in morphological alterations induced by OT treatment.

In summary, the present study reveals a previously unknown OTR-coupled MAPK-MEF-2A pathway, which is responsible for OT-induced neurite retraction of hypothalamic neurons.

1. Introduction

The neuropeptide oxytocin (OT) is a central modulator of complex socio-emotional behavior, such as anxiety, affective behavior, and aggression (Jurek and Neumann, 2018; Neumann and Landgraf, 2012; Kosfeld et al., 2005; Kumsta and Heinrichs, 2013). The molecular underpinnings of these behavioral effects are largely unknown. The OT receptor (OTR) is almost ubiquitously expressed in the central nervous system (Jurek and Neumann, 2018; Grinevich et al., 2016), most notably in the hypothalamus, the site of OT synthesis. The OTR is a G-protein coupled receptor, whose activation by its ligand leads to promiscuous coupling to various Gα-proteins (Busnelli and Chini, 2017), Ca^{2+} release from intracellular stores (Tobin et al., 2011) and subsequent influx of Ca^{2+} from the extracellular space through TrpV2 channels (van den Burg et al., 2015). These G-proteins in combination with the Ca^{2+} influx activate several signaling cascades, most prominently the MAP kinase pathway, with its main kinases MEK1/2 and ERK1/2 (Blume et al., 2008; Jurek et al., 2012). MAP kinase pathways have been associated with neurite outgrowth (Won et al., 2015; Kang et al., 2011; Xu et al., 2015), anxiety (Blume et al., 2008; Jurek et al., 2012; Borges et al., 2015), and memory formation (Tomizawa et al., 2003). Downstream effectors of ERK1/2 are the transcription factors CREB (Tomizawa et al., 2003; Jurek et al., 2015) and myocyte enhancer factor 2 (MEF-2) (Devost et al., 2008). MEF-2 was originally defined as a muscle-specific factor that binds an A/T-rich element in the promoter of target genes (Gossett et al., 1989), and was later found to be

List of abbreviations: OT, oxytocin; OTR, oxytocin receptor; Ca^{2+}, Calcium; MEK1/2, Mitogen activated ERK kinase 1/2; ERK1/2, Extracellular signal regulated kinase 1/2; CREB, cyclic AMP responsive element binding protein; MEF-2, myocyte enhancer factor 2; TGOT, Thr^4, Gly^7-OT; VP, Vasopressin; VEH, Vehicle; scrRNA, scrambled siRNA

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* Corresponding author.
E-mail address: magdalena.meyer@biologie.uni-regensburg.de (M. Meyer).

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ubiquitously expressed in all types of tissue, especially in the brain (Dietrich, 2013). One central function of MEF-2 is the regulation of neuronal morphology, i.e. neurite outgrowth (Flavell et al., 2006), either *via* gene inhibition or activation. Among the MEF-2-regulated genes are immediate-early genes such as c-JUN and NUR77, as well as regulators of neuronal activity, such as ARC, SYNGAP, HOMER1A, and BDNF (Flavell et al., 2006). Consequently, MEF-2 represents a central factor for the regulation of neurite outgrowth, synapse formation, and, if dysregulated, was found to play a key role in the development of autism spectrum disorder (Potthoff and Olson, 2007). Here, the functions between OT and MEF-2 may overlap, as both are implicated in autism spectrum disorder (Parker et al., 2014; Bales et al., 2013) and neurite outgrowth (Lestanova et al., 2016; Lestanova et al., 2017). However, the literature describes inconsistent effects of OT on cellular morphology. For instance, OT bursts during milk letdown lead to retraction of glial cells in rat dams (Langle et al., 2002; Theodosis, 2002), and *in vitro* application of synthetic OT leads to neurite retraction in hippocampal neurons (Ripamonti et al., 2017); however, when tested in human SH-SY5Y neuroblastoma cells and glial U-87MG cell lines, OT leads to neurite outgrowth (Lestanova et al., 2016, 2017). This outgrowth seems to be executed by the rearrangement of actin filaments to the apical part of neuronal cones and paralleled by changes in expression of the intermediate filament protein nestin, which is implicated in axon growth (Bakos et al., 2012). It is unknown so far, whether the differential effects of OT on neuronal and glial cells, i.e. neurite retraction (Ripamonti et al., 2017) or neurite outgrowth (Lestanova et al., 2016), are caused by the use of different doses of OT, by the use of different cell types (primary neurons or cell lines), different species (mouse, rat, human cells), or even the sex of the cell donor (Li et al., 2016). Therefore, it is of paramount importance to choose a physiological relevant model to investigate the effects of OT on cell morphology.

As OT and the OTR are expressed in the hypothalamus of the brain (Jurek et al., 2015; Freund-Mercier et al., 1994; Dabrowska et al., 2011), rat hypothalamic neurons (H32 cells) provide a suitable *in-vitro* model to provide a full characterization of the cellular response to OT. A full characterization of the cellular response is pivotal, especially since the OT molecule is known to bind the receptor of the related nonapeptide vasopressin as well at higher concentrations, with the result of mixed OTR and vasopressin receptor-mediated cellular effects. In turn, vasopressin can also bind the OTR, which could result in a similar effect of both peptides on cellular morphology.

In order to circumvent any non-OTR-mediated effects we made use of specific antagonists and agonists of the OTR such as Thr^4, Gly^7-OT (TGOT), an OT agonist with a 16.000 times higher affinity for the rat OTR than for the rat vasopressin receptor (Chini et al., 2008; Manning et al., 2008); however, this OTR-specificity is lost in cells of human origin (Chini et al., 2008). The advantage of OTR-specificity of TGOT in rat cells, in combination with the hypothalamic origin, convinced us to conduct comprehensive morphological studies regarding OT's cellular effects in H32 cells.

In summary, we aim to contribute to the clarification of the effects of OT on cellular morphology and identify the intracellular signaling cascades that control the observed morphological alterations. In order to do so, we tested the hypothesis that the transcription factor MEF-2 is a key regulator of neurite retraction in hypothalamic neurons, and that its activation is dependent on the OTR-coupled MEK1/2-ERK1/2 pathway.

2. Material & methods

2.1. Cell culture

Rat hypothalamic H32 cells (passages 15–30) were cultured in 1:1 Dulbecco's Minimum Essential Medium/Ham F12 Medium (growth medium). The growth medium was supplemented with 10% heat inactivated fetal bovine serum (Capricorn, Germany), 0.1% nonessential amino acids, 100U/ml gentamycin (both Invitrogen, Germany) in humidified atmosphere containing 5% CO_2 at 37 °C. Passaging was performed at least once a week by gentle trypsination.

2.2. Cell viability assay

Cellular viability was tested using the PrestoBlue Cell Viability Assay (A13261, Invitrogen) according to manufacturer's protocol. Briefly, 20×10^3 cells per well were seeded the day before the test in a 96-well-plate in growth medium. The volume of the treatment and serum-free medium (DMEM/F12 + 0.1% BSA, sterile filtered) for the stimulation was calculated to a total of 90 µl per well. 10 µl of PrestoBlue Reagent were added directly to the cells, incubated for 30 min to 2 h, before reading the fluorescence intensity with a FluoStar Plate reader (BMG). Optimal DMSO concentrations as solvent for the U0126 MAPK inhibitor (Sigma Aldrich) were determined by a separate dose response experiment.

2.3. Protein isolation

For the extraction of proteins from adherent H32 cells, medium was removed and cells were washed with PBS supplemented with Protease and Phosphatase inhibitors (PI, A32959, Thermo Fisher). Cells were scraped, centrifuged, and the cell pellet was resuspended in 100 µl RIPA lysis buffer (R0278, Sigma Aldrich) with HALT Inhibitor and EDTA (78444, Thermo Fisher) to extract whole cell lysate.

2.4. Western blot

Between 5 and 30 µg of whole cell extract and an equivalent volume of 4X loading buffer (2.4 ml TRIS, 0.8 g SDS, 4 ml 100% Glycerol (AppliChem), 0.01% Bromphenol Blue (Sigma Aldrich), 1 ml Mercaptoethanol and 2.8 ml H_2O) were applied to 12% Mini and Midi PROTEAN or Criterion TGX Stain-free gels (456–8044 and 5678045, BioRad). Western blot analysis was performed using the Stain-free total protein method (BioRad) as loading control, according to the manufacturer's protocol. Stain-free gels contain trihalo compounds that covalently bind to tryptophan residues in proteins when exposed to UV light. By that, the proteins are made fluorescent directly in the gel, allowing the immediate visualization of proteins at any point during electrophoresis or blotting and permitting the user to normalize bands to total protein in each lane. This technology circumvents the problematic use of housekeeping proteins as loading controls on western blots. In order to detect activated MEF-2, a phospho-specific MEF-2A S408 (CSB-PA000728, Flarebio Biotech) antibody was used at 1:5000 in 5% BSA concentration. A MEF-2A total antibody (#TA307807, OriGene, 1:5000 in 5% BSA) was used to detect total MEF-2A levels. Changes in expression levels were analyzed measuring grey density in a semi-automatic manner by Image Lab Software (Version 6.0, BioRad).

2.5. Cell stimulations

Cells were grown in the presence or absence of 10 nM, 100 nM, or 1 µM of OT (Bachem, Germany) for 0, 2, 4, 8, 10, and 12 h in cell culture dishes or 3-part chamber slides (BD Falcon, Germany). A separate experiment was performed using cells incubated with [Thr^4, Gly^7]-oxytocin (TGOT, 100 nM, Bachem) or vasopressin (100 nM, Bachem). When inhibitors (U0126, Sigma Aldrich) or OTR antagonist des-Gly-NH_2*d*$(CH_2)_5$[Tyr(Me)$_2$Thr$_4$]-OVT (kindly provided by M. Manning) were used, cells were incubated in serum free medium for 1 h, pretreated with the inhibitor or VEH (H_2O or DMSO) for 10min, and stimulated with the according treatment. To assess the role of MEF2A, H32 cells were transfected with small interfering RNA (siRNA). 3 unique 27mer MEF2A siRNA duplexes (Origene, SR504191; for sequences see Table 1) and Lipofectamine RNAiMAX Reagent (Invitrogen), diluted in OptiMEM were added for 72 h in a concentration