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Mechanisms involved in the regulation of neuropeptide-mediated neurite outgrowth: a minireview

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The present knowledge, regarding the neuronal growth and neurite extension, includes neuropeptide action in the central nervous system. Research reports have brought much information about the multiple intracellular signaling pathways of neuropeptides. However, regardless of the differences in the local responses elicited by neuropeptides, there exist certain functional similarities in the effects of neuropeptides, mediated by their receptors. In the present review, data of the relevant studies, focused on G protein-coupled receptors activated by neuropeptides, are summarized. Particularly, receptors that activate phosphatidylinositol-calcium system and protein kinase C pathways, resulting in the reorganization of the neuronal cytoskeleton and changes in the neuronal morphology, are discussed. Based on our data received, we are showing that oxytocin increases the gene expression of GTPase cell division cycle protein 42 (Cdc42), implicated in many aspects of the neuronal growth and morphology. We are also paying a special attention to neurite extension and retraction in the context of neuropeptide regulation.

Key words: oxytocin, neurite outgrowth, phosphatidylinositol-calcium system, Cdc42

Present understanding of the neuronal growth and neurite extension includes time and site specific effects of neuropeptides on the cells in the central nervous system. Depending on the neuronal type, local regulation of neurite extension may be very distinct and variable. Nevertheless, it is becoming clear that neurite outgrowth is controlled by similar or analogous mechanisms. How neurite extension may depend on the chemoattractants and which neuronal cell types may have a capacity to grow neurites far over the brain tissue is broadly discussed in the available studies.

The number of neurotransmitters and neuropeptides may serve as specific chemoattractants for the neurite growth cone guidance (Zheng et al. 1994; Cibelli et al. 2001; Zhong et al. 2013). Various neuropeptides may influence the neurite extension or retraction. It has been shown that Neuropeptide Y promotes axonal growth and affects the growth cone turning (Sanford et al. 2008). Another functionally related neuropeptides, galanin and galanin-like peptides, may also affect the growth and turning of the neurite (Hawes et al. 2006; Sanford et al. 2008; Hobson et al. 2013). Corticotropin-releasing hormone has been shown to induce extension of neurites with prominent growth cones (Cibelli et al. 2001). In the recent study, we have revealed a stimulating role of oxytocin on the neurite growth (Lestanova et al. 2016).

Literature data have brought many reports about the multiple intracellular signaling pathways of neuropeptides. However, regardless of the differences in local responses elicited by neuropeptides, functional

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similarities in the effects of neuropeptides mediated by their receptors have been found. According to our assumption, certain neuropeptides can contribute to the differentiation of neuronal cells by sharing their intracellular signaling pathways. The present review is focused on the G protein-coupled receptors (GPCRs) activated by neuropeptides that share their effects on the neuronal cytoskeleton. Particularly, the receptors that may activate phosphatidylinositol-calcium (PI-Ca^{2+}) system and protein kinase C (PKC) pathways, resulting in reorganization of neuronal cytoskeleton and alteration of the neuronal morphology, are discussed. PKC has been found to be involved in the neuronal differentiation. It can induce phosphorylation of several proteins related to the neurite outgrowth. Furthermore, cytosolic calcium (Ca^{2+}) that may act as a second messenger in the cytoplasm of the neuronal cell and initiate the neurite formation and extension, is discussed. Finally, Rho family of GTPases that are involved in many aspects of the neuronal growth, as also shown by our previous data, are also discussed. A special attention is paid to the neurite extension and retraction in the context of the neuropeptide regulation.

Origin of neurites

New-born neurons and neural progenitors represent immature spherical cells without neurites. Typical neuronal morphology is continuously formed by the process of polarization (Figure 1). Neurons develop axons and dendrites, structurally and morphologically distinct neurites, in a sequence of well-defined developmental stages (Figure 2). *In vitro* cultured hippocampal neuron polarization starts by producing lamellipodia (filopodia), spreading around the cell body – stage 1 (Figure 2). Subsequently, round-shaped neuron is transformed into a cell, surrounded by a number of short uniform immature processes – stage 2 (Figure 2). Several hours later, only one of these processes starts to elongate rapidly and becomes an axon – stage 3 (Figure 2). After axon differentiation, the remaining short processes begin to elongate and differentiate into dendrites – stage 4 (Figure 2). The process of polarization is terminated by maturation of formed neurites, dendritic spines morphogenesis, and synapse formation – stage 5 (Figure 2) (Dotti et al. 1988; Tahirovic and Bradke 2009). In another *in vitro* model, cultured cerebellar neurons are developing by the same way, until the end of the stage 1. Immature neurons form one elongated process (stage 2) and subsequently, another process on the opposite side of the cell body (stage 3). One of these processes continues in extension, starts branching, and forming axon (stage 4), while the other one retracts and forms 4 or 5 dendrites around the cell body (stage 5) (Powell et al. 1997; Tahirovic and Bradke 2009). *In vivo*, cerebellar neurons terminate their development few weeks after the birth and that is why they may represent an experimental model of postnatal development and axon regeneration (Erturk et al. 2007). *In vitro* models for evaluation of neurite outgrowth also include the neuroblastoma cell lines (Cotta-Grand et al. 2009; Sarma et al. 2015; Lestanova et al. 2016). Several methods for evaluation of neurite outgrowth, neurite length, and neurite branching, have been de-

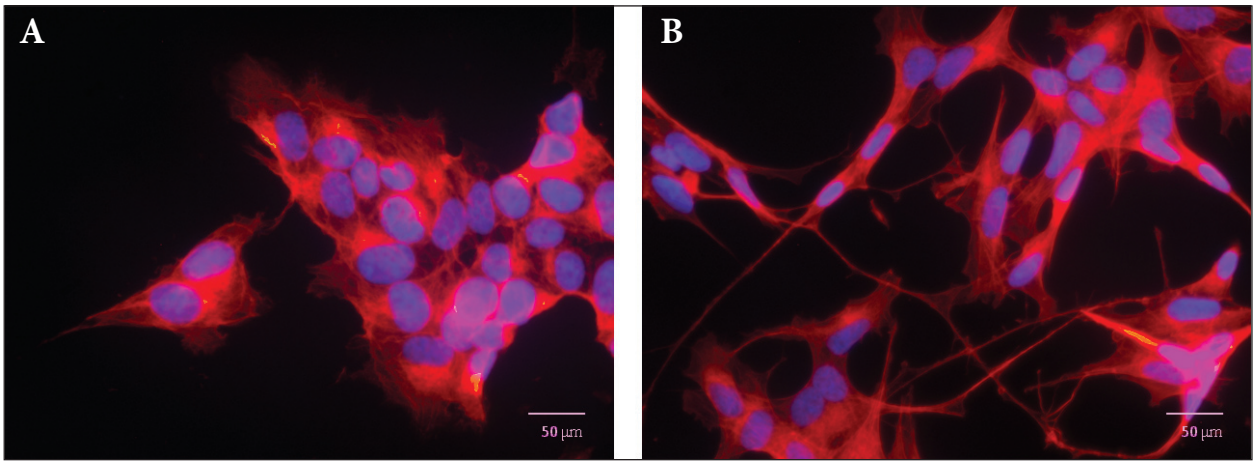

Figure 1. Undifferentiated (A) and differentiated (B) neuroblastoma SH-SY5Y cells. Fixed cells were stained for F-actin with phalloidin-tetramethylrhodamine-b isothiocyanate. Nuclei of the cells were stained with 4',6-diamidino-2-phenylindole.

veloped (Das et al. 2004; Li and Hoffman-Kim 2008; Krug et al. 2013). Quantitative assessment of the neurite outgrowth in these assays includes parameters, such as the number of neurites, neurite orientation, and neurite length. Recently, neurite outgrowth in 3D hydrogel-based environments has also been established (Assuncao-Silva et al. 2015), allowing to culture neuronal progenitors under different conditions and analyze survival, differentiation, and neurite outgrowth (Cruz Gaitan et al. 2015).

Neuropeptide action on G protein-coupled receptors

Neuropeptides activate a wide spectrum of physiological processes, most of them mediated by G protein-coupled cell surface receptors. Nowadays, it is almost clear that the subcellular effects of neuropeptides overlap in the neuronal cells. However, G protein specific action of neuropeptides on the neuronal cells underlies their effects on the neurite extension. Classical understanding of the neuropeptide binding to the specific receptor includes association of the receptor with multiple isoforms of distinct Gα, β and γ subunits. With regard to the neuropeptides, it is important to distinguish G protein subunits with different functional consequences. Based on the amino acid sequence homologies of Gα subunit, G proteins are classified into Gαs (Gs), Gαi/o (Gi/o), Gαq (Gq) (Miyano et al. 2014). Gs protein activates adenylate cyclase (AC) that induces an increase in the intracellular concentration of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA). Gi/o proteins inhibit the activity of AC and decrease the levels of cAMP. Gq proteins activate phospholipase C (PLC), which results in a production of diacylglycerol (DAG) and inositol-triphosphate from phosphatidylinositol-4,5-bisphosphate (PIP2). DAG activates PKC. Inositol-triphosphate binds to its receptors on endoplasmic reticulum and results in an increase of the intracellular Ca^{2+} levels. Availability of the PIP2 is an important factor that allows the neuronal cells to initiate several signaling pathways (An et al. 2011). Therefore, Gq protein-dependent activation of PLC and synthesis of PIP2 act synergistically to mediate the effects of neuropeptides.

Most of the studies are suggesting that a tightly bound dimeric protein complex is composed of one Gβ and one Gγ subunit, although various isoforms have been developed in mammals serving to broader roles, beyond their canonical roles in the cellular signaling (Khan et al. 2013). In general, following neuropeptide binding to the receptor, activation of

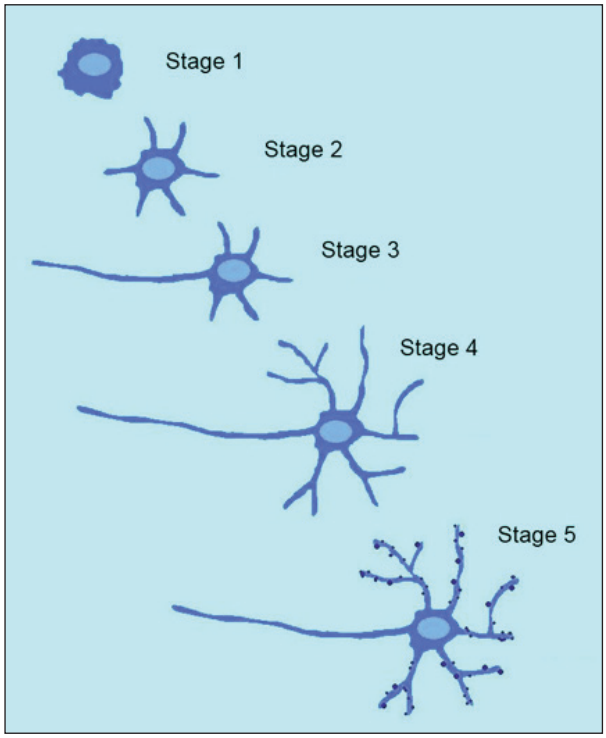

Figure 2. The process of neuron polarization. A typical *in vitro* cultured neuron polarization includes 5 stages. Process of polarization is terminated with the maturation of formed neurites, dendritic spine morphogenesis, and synapse formation.

G protein-coupled receptor occurs and the subunits undergo conformational changes, resulting in a functional dissociation of Gα and Gβγ subunits allowing them to regulate downstream effectors, such as phospholipase, AC or ion channels. GPCRs play a role in the neurite extension. Gα subunit has been shown to activate the GTPase activity of tubulin consequently modulating the dynamics of the cytoskeleton (Roychowdhury et al. 1999). It has been shown that ectopic expression of Gβγ subunits promotes neurite outgrowth (Sachdev et al. 2007).

G protein-coupled receptors mediate neurite growth

Contribution of GPCRs activation is undoubted and related to the neurite formation, development, and outgrowth. GPCRs mediate changes of the cytoskeleton and polymerization of the actin and microtubule as well (Figure 3).

Modulating the activity of all three major heterotrimeric G proteins – Gi/o, Gq, and Gs has been

shown to be associated with the neurite extension and retraction (Karunarathne et al. 2013). Recent study has suggested Gq-dependent Ca^{2+} mobilization in neurite outgrowth (Peterson et al. 2013). Many other studies have evidenced that activation of the Gq pathway is necessary for the neurite growth (Nordman and Kabbani 2014). Moreover, extensive reviews on G protein subunits assembly and trafficking have been published (Marrari et al. 2007; Smrcka et al. 2008). One study has demonstrated that mutation in G protein-coupled receptor 37 causes dendritic alterations in neurons (Tanabe et al. 2015). Activated Gα, released from the plasma membrane traffics into the cytosol, regulates the microtubules stability via direct interaction with them (Yu et al. 2009). These authors have suggested that activated Gαs is able to stimulate the intrinsic GTPase of tubulin, which can decrease the pool of the stable microtubules in cells. Consequently, translocated Gα can increase the microtubule dynamic instability and contribute to neurite outgrowth (Yu et al. 2009). It has been demonstrated that activation of Gαs enhances the microtubule dynamics and promotes neurite outgrowth in PC12 cells and hippocampal neurons (Sarma et al. 2015). It is known that interactions of GPCRs with other membrane proteins are

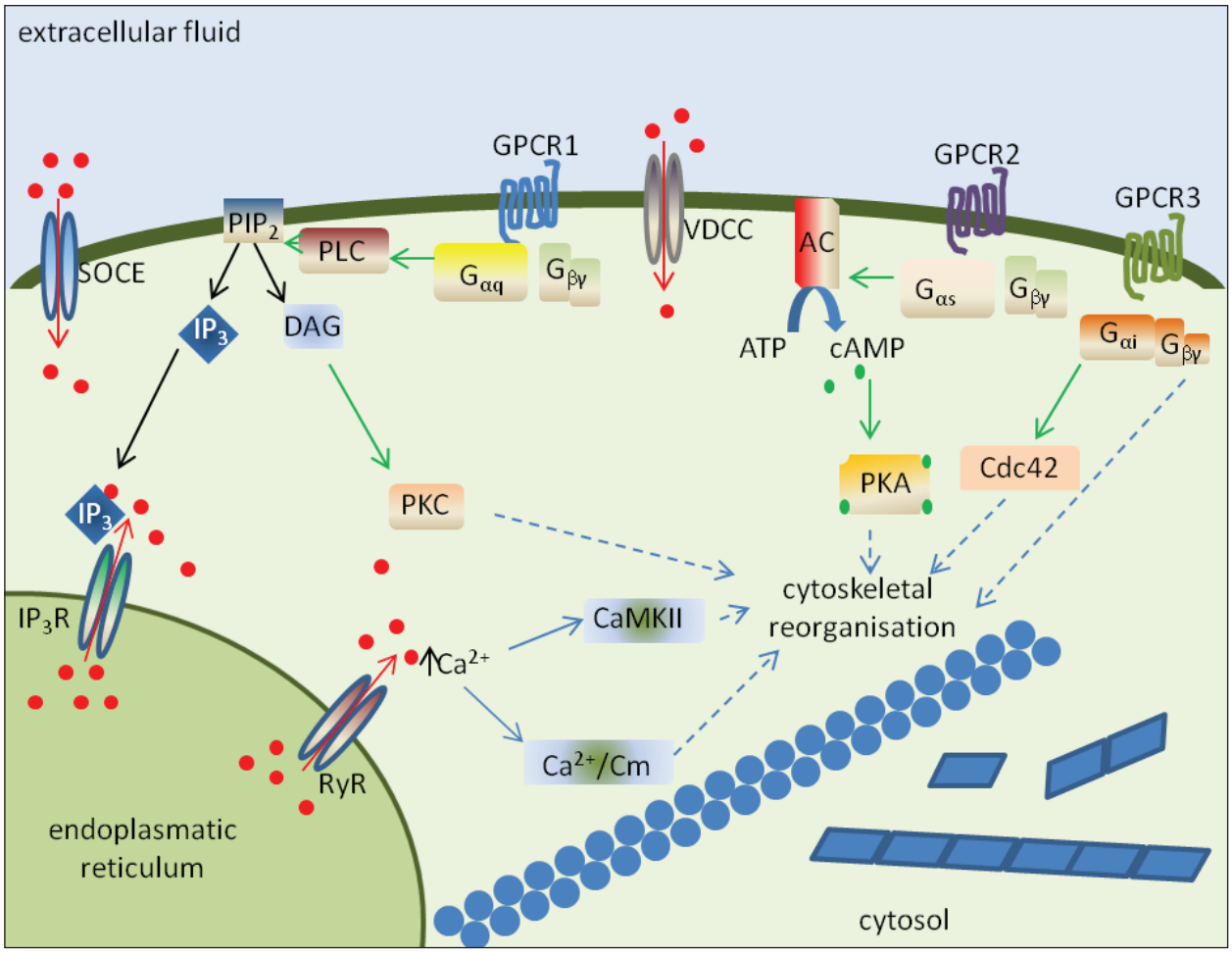

Figure 3. The role of G protein-coupled receptors (GPCRs) in cytoskeletal reorganization. Gs, Gi/o, and Gq protein-coupled receptors mediate changes in the cytoskeleton and polymerization of the actin and microtubules. The role of calcium release is depicted.
AC – adenylate cyclase; cAMP – cyclic adenosine monophosphate; ATP – adenosine triphosphate; Cdc42 – cell division cycle protein 42; DAG – diacylglycerol; GPCRs – G protein–coupled receptors; IP3 – inositol triphosphate; PLC – phospholipase C; PKA – protein kinase A; PKC – protein kinase C; SOCE – store-operated calcium entry; VDCCs – voltage-dependent calcium channels

crucial for the activation of one signaling pathway. The localization of receptors in the cell surface and particularly presence of receptors on neurites may play a role in transferring of the intracellular information, resulting in neurite outgrowth. Recent studies have also suggested that GPCRs are influenced by lipid modifications, local membrane environment, accessory binding partners etc. (Hepler 2014). Complexity of the neuropeptide G protein-coupled cell receptors is thus enormous. Several studies have evidenced that GPCRs are involved in the pathogenesis of diseases ranging from Alzheimer to autism (Thathiah et al. 2011; Tanabe et al. 2015). There are many theories dealing with the question that what level of regulation is crucial for the triggering of the pathogenic processes resulting in alterations of the neurite growth.

The role of calcium in neurite growth

The localization, concentration, and temporal aspects of the cellular Ca^{2+} signal play a complex role in the regulation of the neurite growth. It is known that intracellular Ca^{2+} is an important secondary signaling messenger in the neurodevelopmental signaling pathways, including gene transcription (West et al. 2001), axonal and dendritic outgrowth (Gomez and Spitzer 1999; Redmond et al. 2002), and neuronal migration (Komuro and Rakic 1996). Ca^{2+} concentration may be increased from extracellular and intracellular sources. Methods of entry include voltage-dependent calcium channels (VDCCs), release of intracellular Ca^{2+} stores, and store-operated calcium entry (SOCE) from extracellular stores. Although surface of the neuronal cells contains many VDCCs, the L-type of Ca^{2+} channels are the most important for the growth cone turning (Nishiyama et al. 2003). Ca^{2+} release from the intracellular stores can be triggered by inositol triphosphate (IP_3)-induced Ca^{2+} release (Akiyama et al. 2009) or Ca^{2+}-induced Ca^{2+} release (Gasperini et al. 2009).

A calmodulin-dependent protein kinase II-calcineurin switch initiates either an attractive or a repulsive response to these intracellular elevations (Sutherland and Goodhill 2015). These responses are mediated via the regulation of cytoskeletal components, such as microtubules and actin filaments and membrane dynamics, including vesicle trafficking (Sutherland et al. 2014).

Hypothesis, formed in 1991, states that the growth cone is motile only when an optimal range of intracellular Ca^{2+} concentration is present (Kater and Mills 1991). Lower levels of Ca^{2+} stabilize growth cones and higher levels stall them, in both cases preventing extension. Dynamic Ca^{2+} changes have been reported in the developing cortex (Yuste et al. 1992) and these Ca^{2+} transients could be responsible for the axonal outgrowth. The incidence of Ca^{2+} transients highly correlated with axonal growth cone morphologies and behaviors (Tang et al. 2003).

Signaling cascades of many different neuropeptides include increase of intracellular Ca^{2+} concentration. Activation of neuropeptide receptor coupled with Gαq proteins stimulates PI-PLC/IP3/Ca^{2+} signaling pathway (Liu et al. 2015). Ca^{2+} release from the endoplasmic reticulum, via IP3 and ryanodine receptors, is followed by the activation of Ca^{2+} channels at the plasma membrane, known as SOCE (Alswied and Parekh 2015; Erdmann et al. 2015). Thus, increased Ca^{2+} concentration contributes to the neurite outgrowth.

Neuropeptide signaling pathways related to neurite growth

Recent study has demonstrated that neuropeptide orexin A modulates the neurite growth via activation of the phospholipase D and phosphorylation of PKCε (Bjornstrom et al. 2014). Orexin A binds to G protein-coupled receptor. Authors have explained their observations on neurite retraction that signaling cascade involves RhoA GTPase, causing myosin light-chain phosphorylation, followed by actin and myosin contraction that retract the neurite. Findings that PKC is important for neurite growth, changes are not exclusive in the context of neuropeptides effects. In addition, the oxytocin receptor belongs to the classical G protein-coupled receptor family, involved in the activation of PKC pathways (van den Burg and Neumann 2011). PKC participates in the cytoskeleton reorganization, regulation the expression of actin-binding proteins, and cell cycle changes (Uberall et al. 1999; Korulu et al. 2013). Nevertheless, the PKC family consists of different isoforms, whose activation requires Ca^{2+} and DAG. Thus, Ca^{2+} signaling and phosphoinositide-specific PLC is involved in neurite extension as well (Kiryushko et al. 2006). Many studies have demonstrated that neuropeptide Y induces increase in neurite outgrowth (White 1998; Sanford et al. 2008). Furthermore, neuropeptide melanin-concentrating hormone is also involved in regulation of neurite outgrowth. Melanin-concentrating hormone receptor via a Gαq interaction stimulates IP3 production and induces an increase in intracellular free Ca^{2+} levels (Saito et al. 1999; Cotta-Grand et al. 2009). Another neuropeptide enkephalin activates

opioid receptors coupled to Gi/Go proteins and triggers the neurite outgrowth (Georganta et al. 2013). Several Gi/o-coupled receptors have been shown to play an important role in controlling the neurite outgrowth (He et al. 2006).

The role of phosphoinositide 3-kinase in neurite growth

Phosphoinositides (phosphatidylinositol lipids) in the cell membrane are dynamically regulated. They represent precursors of substrates of many signaling pathways. It is known that phosphorylation of phosphatidylinositol lipids contributes to the various local responses, including polymerization of actin, assembly of signaling complexes, and priming of protein kinase cascades. Phosphoinositide 3-kinase (PI3K) is considered to be the key signal and regulatory factor in several different cell survival pathways, including axons and dendrites growth (Cantley 2002). PI3K induces and promotes both neurite outgrowth and elongation, while its inhibition causes defects in neuronal polarity (Shi et al. 2003). There are two essential PI3K signaling cascades in neuronal polarization (Figure 4). PI3K/Akt kinase/glycogen synthase kinase-3β (GSK-3β) and the positive feedback loop PI3K/Rho GTPases/partitioning-defective proteins 3 and 6/atypical PKC (Par3-Par6-aPKC complex) (Yoshimura et al. 2006). PI3K is responsible for the synthesis of phosphatidylinositol-3,4,5-triphosphate (PIP3), which is then accumulated in the growth cones of developing axons (Shi et al. 2003). PI3K/Akt/GSK-3β signal pathway starts by PI3K activation of Akt kinase by phosphorylation via PIP3. Then activated Akt-kinase inactivates GSK-3β. Active GSK-3β can phosphorylate and inactivate collapsin response

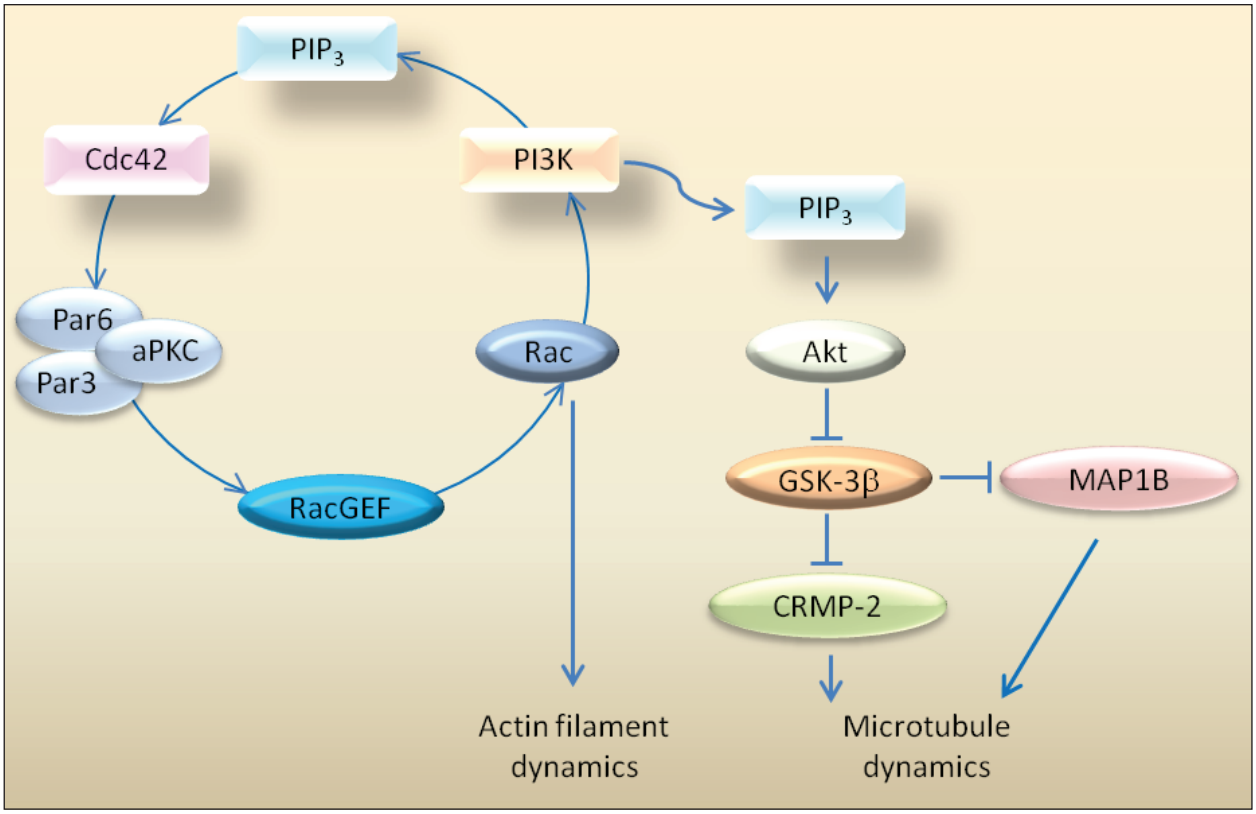

Figure 4. The role of phosphoinositide 3-kinase in the cytoskeletal reorganization. Actin and microtubule dynamics are dependent on the activation of phosphoinositide 3-kinase. Two pathways are depicted.
PI3K – phosphoinositide 3-kinase; PIP3 – phosphatidylinositol-3,4,5-triphosphate; Akt – kinase; GSK-3β – glycogen synthase kinase-3β; CRMP-2 – collapsin response mediator protein-2; MAP1B – microtubule-associated protein 1B; Cdc42 – cell division control protein 42, Par3-Par6-aPKC complex – partitioning-defective proteins 3 and 6/atypical protein kinase C; Rac – small G protein; RacGEF – guanin nucleotide exchange factor for Rac protein

mediator protein-2 (CRMP-2), which in its active form promotes tubulin polymerization and inhibits retraction of new polarized tubulin filaments (Yoshimura et al. 2006). Similarly, activated GSK-3β negatively regulates the function of microtubule-associated protein 1B (MAP1B), which interacts with microtubules and supports microtubule stability in growing axon (Trivedi et al. 2005). A constitutively active GSK-3β mutants, which inhibited axon formation and decreased activity of GSK-3β, are important assumptions for the axon elongation (Jiang et al. 2005). Hypothesis of a positive feedback loop is based on the theory that PI3K activates via PIP3 several small GTPases belonging to Rho GTPase family, which are able to activate PI3K by an indirect positive feedback in cooperation with Par3-Par6-aPKC complex (Yoshimura et al. 2006; Yang et al. 2012). Downstream intracellular signalization of PI3K is well known, however, upstream signalization from extracellular environment is indistinct and widely discussed. While some studies consider tyrosine kinase receptor (Trk)/growth factor's signalization to be an extracellular trigger mechanism of PI3K activation, others support theory about the laminin or neuron–glia cell adhesion molecule and lamellipodia interaction (Da Silva et al. 2005; Menager et al. 2004; Yoshimura et al. 2006; Tahirovic and Bradke 2009).

Neuropeptide actions depend on phosphoinositide 3-kinase

Many neuropeptides are directly or indirectly associated with the activity of PI3K (Ramirez et al. 2015; Liu et al. 2016). Among them, melanocortins are involved in the activation of signaling pathways dependent on PI3K and Src kinase in neuronal cells (Ramirez et al. 2015). Recent study has demonstrated that PI3K signaling participates in the regulation of neuropeptide Y- and proopiomelanocortin-mediated appetite suppression (Chu et al. 2014). Furthermore, involvement of the PI3K/Akt pathway in the appetite regulation has been suggested in different study (Gong et al. 2015). Activation of oxytocin receptor is associated with PI3K and extracellular signal-regulated kinase signaling pathways (Lin et al. 2012). Recent study has shown contribution of PI3K activation to the oxytocin receptor dependent modulation of intracellular Ca^{2+} (van den Burg et al. 2015). Thus, PI3K activation is an important step in mediating the neuropeptide effects on signaling pathways downstream of PI3K, which can affect the cell growth and development.

Small GTPases in relation to the neurite growth

It looks very likely that neuropeptide signaling network includes the small GTPases involved in neurite outgrowth. It has long been known that activation of GPCRs is associated with changes of microtubule dynamics and cytoskeleton dynamics. These receptors initiate a large number of signaling cascades that include the Rho family of small GTPases. The complex view includes activation of the PKC dependent Rho associated kinase pathway promoting neurite outgrowth (Tanabe et al. 2012).

Rho and Ras GTPases play a specific role during neuronal polarization. These small GTPases are cyclically switched between active GTP-bound state and inactive GDP-bound state. Guanin nucleotide exchange factors (GEFs) catalyze exchange of GDP for GTP, while GTPase-activating proteins (GAPs) promote intrinsic GTPase activity and increase hydrolysis of GTP to GDP and Pi. Rho GTPases play a key role in a large number of cell vital processes, including polymerization, depolymerization, and reorganization of microfilaments and microtubules as well as in outgrowth, elongation, guidance, and branching of neurites (Govek et al. 2005, Lee and Dominguez 2010). Ras GTPases, activated by plasma membrane receptors, transport the signal downstream pathway to several intracellular molecules, including PI3K (Hall and Lalli 2010). Main members of Rho GTPase family, involved in neuronal polarization, are Rac1, Cdc42 and RhoA. Rac1 and Cdc42 proteins stimulate actin nucleation and polymerization, microtubule growth and Cdc42. Additionally, it increases the actin filaments assembly (Govek et al. 2005). Neuronal cells of the Cdc42 null animals exhibit multiple abnormalities as disrupted cytoskeletal organization or inhibition of filopodial dynamics (Garvalov et al. 2007). In general, Rac1 and Cdc42 support neurite elongation, whereas Rho1 inhibits neurite initiation and induces neurite retraction (Govek et al. 2005). Our preliminary results have shown that oxytocin can contribute to the regulation of expression of Cdc42 (Figure 5). Although, it has to be carefully interpreted, oxytocin can mediate neuritogenesis via activation of Rho GTPases.

Conclusions and perspectives

During the recent years, research has shown that neuropeptides participate in the regulation of neurite growth through the activation of GPCRs. Various neuropeptides may activate pathways that are associated with the activity of PI3K, resulting in the

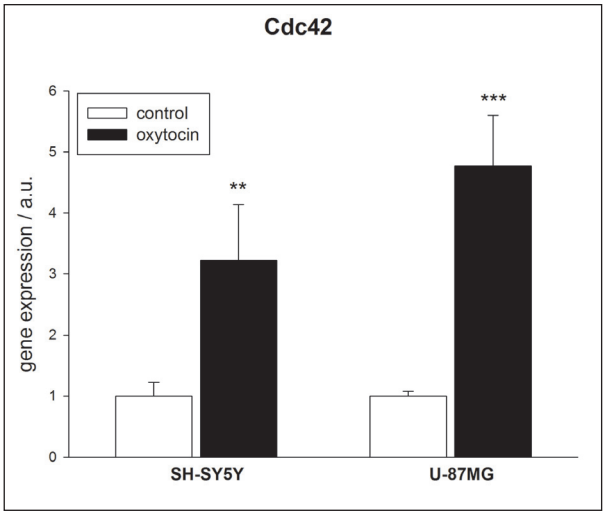

Figure 5. The effect of 48 h oxytocin (OXT) treatment on Cdc42 mRNA levels in SH-SY5Y and U-87MG cells. Graphs show relative mRNA expression, measured by qPCR, normalized to GAPDH, and calculated by $2^{-\Delta\Delta Ct}$ Livak method (Livak and Schmittgen 2001). The white bar represents control group (cells without treatment), the black bar represents cells treated with 1 μM OXT. The data represent means ± SEM (n=5–6). Significantly different values are marked with **p<0.01; ***p<0.001, compared with the control group.

changes in actin and microtubule dynamics. In this context, it is important to understand the role of specific GTPases, activated by plasma membrane receptors and transport the signal downstream pathway. Main members of Rho GTPase family, involved in neuronal remodeling, are Rac1, Cdc42, and RhoA. Our preliminary results have supported the role of oxytocin-induced Cdc42 expression in the actin synthesis. We have also found that oxytocin increases mRNA levels of Cdc42, at least in certain neuronal cells, resulting in a neurite outgrowth. Our preliminary data should be carefully interpreted, as many complex processes come into the play. However, the role of neuropeptides, particularly oxytocin, in PI3K-dependent and extracellular signal-regulated kinase dependent manner is very likely. Therefore, further research should be devoted to the clarification of the role of intracellular signaling pathways, particularly the PI3K/Akt one. In this context, necessary balance in components of signaling pathways that control cell morphology is very important. We suppose that Cdc42 protein plays a role in the oxytocin-mediated changes of neuronal cytoskeleton. It can be concluded that dynamic changes in neural differentiation, neurite outgrowth, and neuronal cytoskeleton are regulated by wider spectrum of small hypothalamic neuropeptides. Alteration in the neuropeptide-mediated signaling and consequently altered actin and microtubule polymerization may represent a mechanism of neurodevelopmental disorders. Analysis of expression profiles of GTPases, including Cdc42 in autistic subjects, may bring new insights into the origin of the developmental disorders, especially in the context of altered neuritogenesis. Nevertheless, complex methodology and techniques, including incubation of cells in the presence of oxytocin, blockage of oxytocin receptors, and specific knockdown of oxytocin receptors with consequent visualization of cell morphology, can lead to the progress in the field. Understanding of the mechanisms, involved in the regulation of neuropeptide-mediated neurite outgrowth, represents a part of a complex genetic and molecular view of the diseases. Further studies are needed for better clarification of the role of oxytocin and other neuropeptides in the neurite growth processes.

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