Published in final edited form as:

J Neuroendocrinol. 2013 December ; 25(12): 1241–1254. doi:10.1111/jne.12112.

Annexin A1 Complex Mediates Oxytocin Vesicle Transport

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Abstract

Oxytocin is a major neuropeptide that modulates the brain functions involved in social behavior and interaction. Despite of the importance of oxytocin for neural control of social behavior, little is known about the molecular mechanism(s) by which oxytocin secretion in the brain is regulated. Pro-oxytocin is synthesized in the cell bodies of hypothalamic neurons in the supraoptic and paraventricular nuclei and processed to a 9-amino-acid mature form during post-Golgi transport to the secretion sites at the axon terminals and somatodendritic regions. Oxytocin secreted from the somatodendritic regions diffuses throughout the hypothalamus and its neighboring brain regions. Some oxytocin-positive axons innervate and secrete oxytocin to the brain regions distal to the hypothalamus. Brain oxytocin binds to its receptors in the brain regions involved in social behavior. Oxytocin is also secreted from the axon terminal at the posterior pituitary gland into the blood circulation. We have discovered a new molecular complex consisting of annexin A1 (ANXA1), A-kinase anchor protein 150 (AKAP150), and microtubule motor, that controls the distribution of oxytocin vesicles between the axon and the cell body in a protein kinase A (PKA)- and protein kinase C (PKC)-sensitive manner. ANXA1 showed significant co-localization with oxytocin vesicles. Activation of PKA enhanced the association of kinesin-2 with ANXA1, thus increasing the axon-localization of oxytocin vesicles. Conversely, activation of PKC decreased the binding of kinesin-2 to ANXA1, thus attenuating the axon-localization of oxytocin vesicles. Our study suggests that ANXA1 complex coordinates the actions of PKA and PKC to control the distribution of oxytocin vesicles between the axon and the cell body.

Keywords

Oxytocin; Annexin A1; Kinesin-2; AKAP150; PKA; PKC

Introduction

Pro-oxytocin is synthesized in the cell bodies of hypothalamic neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus and is processed to a 9-amino-acid mature form during post-Golgi transport to the secretion sites at the axon terminals and somatodendritic regions (1, 2). The axon terminals of PVN neurons innervate the posterior

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pituitary gland (neurohypophysis) and secrete oxytocin directly into the blood stream. Plasma oxytocin facilitates lactation, parturition, and natriuresis, and reduces blood pressure and heart rate (3–6). Oxytocin secreted from the soma and dendrites diffuses throughout the hypothalamus and the neighboring brain regions of the hypothalamus. Oxytocin-positive axons also innervate and secrete oxytocin into the amygdala, septum, substantia nigra, stria terminalis, olfactory bulb, brainstem (nucleus tractus solitarius), and spinal cord (7–14). Oxytocin secreted inside the brain affects brain functions involved in behavior, anxiety, cognition, and autonomic nerve activity (15).

Acute oxytocin secretion at the axon terminal differs from that at the somatodendritic region with respect to its sensitivity to membrane depolarization and calcium. Plasma membrane depolarization by high K^+ induces axonal, but not somatodendritic, oxytocin secretion (16). Conversely, somatodendritic, but not axonal, oxytocin secretion is induced by increased cytoplasmic calcium ($[Ca^{2+}]_i$) (16, 17). In addition to the differential sensitivity to membrane depolarization and Ca^{2+} influx, axonal and somatodendritic oxytocin secretion appears to be affected differently by protein kinase A (PKA) and protein kinase C (PKC). Activation of the serotonergic receptors increases cyclic AMP (cAMP), a PKA activator, and enhances axonal oxytocin secretion to the neurohypophysis (18, 19). Glucagon-like peptide-1 is released from the neurons of the nucleus of the solitary tract that innervate into the PVN and causes accumulation of oxytocin vesicles at the axon terminal in the pituitary gland in a PKA-dependent manner (20). Norepinephrine and prolactin releasing peptide released from neurons of the medulla oblongata innervating into the PVN also increase axonal oxytocin secretion from the neurohypophysis (21).

Conversely, signaling pathways that increase $[Ca^{2+}]_i$ and activate PKC facilitate somatodendritic oxytocin secretion. α -Melanocyte stimulating hormone (α -MSH) that increases $[Ca^{2+}]_i$ and activates PKC via the G_q receptor enhances somatodendritic oxytocin secretion (16). Simultaneously, α -MSH decreases axonal oxytocin secretion by reducing cAMP via the $G_{i/o}$ receptor and attenuating the membrane potential of oxytocin neurons (16). Endocannabinoids have a similar effect on the secretion of oxytocin (22). Galanin, an activator of the $G_{i/o}$ receptor, appears to decrease axonal oxytocin secretion by inhibiting PKA (23).

All of the foregoing studies demonstrate the effects of extracellular stimuli on the exocytosis of oxytocin vesicles from secretion sites at the axon terminals and somatodendritic regions. However, little is known about the molecular mechanism by which the distribution of oxytocin vesicles between the axon and the somatodendritic region is regulated by extracellular stimuli. We have discovered a molecular complex consisting of annexin A1 (ANXA1), A-kinase-associated protein 150 (AKAP150), and kinesin-2 that coordinates the actions of PKA and PKC to enhance and attenuate, respectively, the targeting of oxytocin vesicles to the axon terminals.

A-kinase anchor protein 5 (AKAP5 = rat AKAP150 = human AKAP79) binds to PKA and PKC and coordinates their phosphorylation actions (24–28). AKAP5 targets the phosphorylating action of PKA to 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptor (29, 30). AKAP5 also coordinates PKC-mediated phosphorylation of voltage-gated M-type K^+ channels (31, 32). In contrast, little is known about the function of AKAP5 in hormone secretion. AKAP5 coordinates glucagon-like peptide-1-induced enhancement of insulin secretion (33, 34). In melanocytes, AKAP5 appears to form a complex with PKA, kinesin-2, cytoplasmic dynein, and dynactin on melanosomes, thus affecting the direction of melanosome transport (35).

ANXA1, a phospholipid/Ca²⁺-binding protein (36), is abundant in the pituitary gland and, to lesser extent, in the hypothalamus (37). ANXA1 mediates some of the steroid-mediated stress responses (38, 39). Glucocorticoids increase the *de novo* synthesis of ANXA1 (40) and cause the extracellular secretion of ANXA1 (41). In pancreatic beta cells, ANXA1 was almost exclusively observed on most of the insulin-containing vesicles (~90%) (42), suggesting that ANXA1 may mediate the interaction of vesicles with cytoplasmic machinery. According to a recent finding in which knockout of ANXA1 blocked the anterograde transport of Shiga toxin (43), one of the ANXA1-interacting cytoplasmic machineries appears to be microtubule-based transport system. Hence, ANXA1 may be involved in intracellular trafficking of hormone-containing vesicles.

Here, we demonstrate how ANXA1 mediates the anterograde transport of oxytocin vesicles via its interaction with kinesin-2 and AKAP150 in PKA- and PKC-sensitive manners.

Results

The antibody 4660 specifically recognizes ANXA1 in mouse hypothalamus and immortalized oxytocin neurons

We have studied about the role of snapin (44) in microtubule-based transport of vesicles containing pro-opiomelanocortin (POMC), the precursor of the stress hormone, adrenocorticotrophin (ACTH), in the anterior pituitary AtT20 cells (in preparation). We generated two different batches of chicken antibodies (4659 and 4660) against the recombinant protein of full-length snapin. The antibody 4659 recognized 15kD snapin and other proteins in the cytosols of AtT20 cells. Snapin in AtT20 cells was also recognized by rabbit anti-snapin antibody (PTG10055-2-AP, Proteintech Group, Inc.) (Fig. 1A). Conversely, the antibody 4660 recognized only the 38kD protein in the cytosols of AtT20 cells. To examine whether snapin exists in the hypothalamus and oxytocin neurons, we probed the cytosols extracted from mouse hypothalamus and immortalized oxytocin neurons (N11 cells) with the antibodies 4659 and 4660. The antibody 4659 did not recognize either 15kD snapin or 38kD protein in mouse hypothalamus and N11 cells while the antibody 4660 recognized only the 38kD protein (Fig. 1B). We precipitated the 38kD protein in the cytosol of N11 cells by immunoprecipitation using antibody 4660 and sent the 38kD protein to the Department of Chemistry at the University of Kentucky for peptide sequencing. The amino acid sequence of the 38kD protein matched 100% to that of ANXA1 but <13% to snapin according to ExPASy alignment program 'lalign.' We confirmed the identity of the 38kD protein band in the cytosol of N11 cells using mouse anti-ANXA1 antibody (sc-12740) from Santa Cruz (Fig. 1B, C). Of note, mouse anti-ANXA1 antibody also recognized two other unknown proteins of ~40kD and ~15kD in addition to 38kD ANXA1 in the cytosol extracted from the mouse hypothalamus. We also confirmed that the 4660 antibody specifically recognized recombinant ANXA1 protein tagged with glutathione-S-transferase (GST-ANXA1: molecular weight = 64.6: 26.6 [GST] + 38 [ANXA1]) but not GST tag alone (Fig. 1D). The 4660 antibody also recognized a cleavage product (~58kD) of GST-ANXA1 that might be generated during GST pulldown experiment. Mouse anti-ANXA1 antibody recognized GST-ANXA1 well but not GST alone (Fig. 1D). Although it is possible that both 4659 and 4660 antibodies may react with an epitope of the amino acid sequence that is similar to snapin and ANXA1, the antibody 4660 could not maintain its binding to the amino acid sequence of snapin with our stringent washing conditions using 0.1% Tween 20. We used antibody 4660, which recognized only ANXA1 in the mouse hypothalamus and immortalized oxytocin neurons to detect and precipitate endogenous ANXA1 in N11 cells and mouse hypothalamic neurons for our study.

ANXA1 colocalizes with oxytocin-containing vesicles in the processes of N11 cells and along the axons of primary oxytocin neurons

Given that the majority of ANXA1 associates with large dense core vesicles (LDCVs) in endocrine cells (42), it is possible that ANXA1 associates with oxytocin-containing LDCVs in hypothalamic neurons. We examined this possibility by immunostaining N11 cells and primary mouse oxytocin neurons (E17, DIV14) cultured on poly-lysine-coated coverslips with primary antibodies to ANXA1 and oxytocin. In N11 cells, ANXA1 co-localized with oxytocin-containing vesicles accumulated at the process tips (Fig. 2A). We quantified the correlation co-efficient of co-localization (CCC) (45) between ANXA1 and oxytocin vesicles using 'Metamorph' software. The CCC between ANXA1 and oxytocin vesicles in the processes reached 0.80 ± 0.01 (average $\pm$ standard error of mean [SEM], $n=40$ cells), indicating significant co-localization.

Primary oxytocin neurons formed long thin axons negative for microtubule-associated protein 2 (MAP2) and thick dendrites positive for MAP2 (Fig. 2B). In order to examine whether LDCVs containing carboxypeptidase E (CPE), a LDCV marker, co-localize with oxytocin in the axons, we stained primary hypothalamic neurons with anti-CPE and anti-oxytocin antibodies. CPE was very sparse along the axons of oxytocin neurons but rich in the cell bodies and dendrites of the neurons. At the axon terminals of untreated oxytocin neurons, a small amount of CPE partially co-localized with oxytocin vesicles (Fig. 2C). It suggests that only a subpopulation of oxytocin-containing vesicles in the axon may have CPE.

There were distinguishable individual LDCVs containing oxytocin along thin axons (Fig. 2D–F). Meanwhile, we could not find distinguishable individual oxytocin vesicles in the cell body and dendrites (Fig. 2D). The oxytocin-containing LDCVs along the axons showed significant co-localization with ANXA1 (Fig. 2E, average CCC $\pm$ SEM = 0.71 ± 0.03 , $n=40$ neurons). In order to obtain more detailed information about the co-localization between ANXA1 and oxytocin vesicles, we attempted to perform voxel co-localization analysis, a method to quantify the extent of the overlapping of volumic pixels (voxels) between two different fluorescence colors. However, we could not measure voxels because it was not possible to take multiple images along the z-axis through the thin axons of oxytocin neurons. Instead, we calculated the percent of the overlapping of the green fluorescent pixels of oxytocin vesicle with the red fluorescent pixels of ANXA1 on the vesicle by Metamorph software (Fig. 2F). The average percent $\pm$ SEM of ANXA1-colocalizing oxytocin pixels per vesicle was $70.4 \pm 1.2\%$ ($n=47$ vesicles). Thus, it is clear that ANXA1 associates with oxytocin-containing LDCVs in the axons of primary hypothalamic oxytocin neurons.

ANXA1 mediates the anterograde transport of oxytocin vesicles along the axon via its interaction with kinesin-2

A previous study showed that knockout of ANXA1 blocked the anterograde transport of Shiga toxin (43), suggesting that ANXA1 may be involved in intracellular anterograde transport. We examined whether ANXA1 is required for the anterograde transport of oxytocin-containing LDCVs by knocking down ANXA1 in primary hypothalamic neurons using lentiviral shRNA against ANXA1. Primary hypothalamic neurons (E17, DIV14) were transduced with lentiviral particles containing either control or anti-ANXA1 shRNA for 5 days and processed for immunocytochemistry with antibodies to oxytocin and ANXA1. The average intensity ($\pm$ SEM) of immunostained ANXA1 in neurons transduced with anti-ANXA1 lentiviral shRNA was decreased to $\sim 26\%$ (5.35 ± 0.37) of that (20.78 ± 1.33) in neurons transduced with control lentiviral shRNA ($n>50$ neurons, $p<0.0001$) (Fig. 3A). Similarly, the average intensity of immunostained oxytocin vesicles along the axons of neurons transduced with anti-ANXA1 lentiviral shRNA was significantly lower than that of

neurons transduced with control lentiviral shRNA (n=40 neurons) (Fig. 3B, C). These results suggest that ANXA1 is required for the anterograde transport of oxytocin vesicles along the axons.

Given that ANXA1 is required for the anterograde transport of oxytocin vesicles along the axons, it is possible that ANXA1 interacts with kinesins, microtubule-based anterograde transporters. We performed co-immunoprecipitation using the antibody 4660 and the cytosols of mouse hypothalamus and N11 cells. ANXA1 interacted with kinesin-2 (KIF3A) but not with kinesin-1 (kinesin heavy chain [KHC]) and kinesin-3 (KIF1A) in N11 cells and mouse hypothalamus (Fig. 4A, B). Thus, kinesin-2 appears to mediate the anterograde transport of oxytocin vesicles along the axons via its interaction with ANXA1. In addition to kinesin-2, ANXA1 interacted with the retrograde transporter, cytoplasmic dynein, and its activator, dynactin (Fig. 4A, B).

Activation of PKA enhances the anterograde transport of oxytocin vesicles to the axon terminals

Given that pharmacological treatments that activate PKA enhance oxytocin secretion at the axon terminals (18–21), activation of PKA may increase the anterograde transport of oxytocin vesicles to the axon terminals for secretion. In order to examine this possibility, we treated primary hypothalamic neurons with forskolin, which increases the intracellular level of cyclic AMP, an activator of PKA, and then quantified the intensity of immunostained oxytocin vesicles along the axons and at the axon terminals. Mouse hypothalamic neurons (E17, DIV14) were treated with either DMSO or 10 μ M forskolin for 10, 20, and 30 min and immunostained using antibodies to oxytocin and ANXA1 (n=40 neurons per each condition, two independent experiments). The average intensity of oxytocin vesicles in the axons was gradually increased by longer period of incubation with forskolin (Fig. 5). The average intensities of oxytocin vesicles in neurons treated with DMSO were ~20 (in arbitrary units) in the cell bodies, 10–15 in the axons between 0 and 100 μ m from the cell body, and ~7 in those between 100 and 200 μ m. After 10- and 20-min treatments with forskolin, the average intensity of oxytocin vesicles in the cell body was decreased from ~20 (0 min) to ~8 (10 min) and ~8 (20 min) (Figs. 5). Meanwhile, the average intensities of oxytocin vesicles were increased to 20–22 in the axon between 0 and 100 μ m and to 13–16 between 100 and 200 μ m (Figs. 5B, C, E). At 30 min after forskolin treatment, the average intensity of oxytocin vesicles in the axon was significantly increased to ~30, ~26, ~21, and ~20 in axons between 0–50, 50–100, 100–150, and 150–200 μ m, respectively. We specified the involvement of PKA in the enhancement of the axon-localization of oxytocin vesicles by pre-treating neurons with 10 μ M PKI-ester, a PKA inhibitor, for 1 h prior to 30-min forskolin treatment. Pre-treatment with PKI-ester blocked forskolin from enhancing the axon-localization of oxytocin vesicles (Fig. 5D). The average intensities of oxytocin vesicles in the cell bodies and the axons of neurons pre-treated with PKI and forskolin were similar to those of neurons treated with DMSO (data not shown). In addition, we assessed the extents of co-localization between oxytocin vesicles and ANXA1 along the axon during 30-min stimulation with forskolin. ANXA1 maintained high levels of co-localization with oxytocin vesicles (average CCC $\pm$ SEM = 0.73 $\pm$ 0.01, n=60) in the axons during 30-min stimulation by forskolin.

We also quantified the average intensities of oxytocin vesicles at the axon terminals of neurons treated with either DMSO or 10 μ M forskolin for 30 min. The average intensity of oxytocin vesicles at the axon terminals of forskolin-treated neurons was ~3 fold higher than that of DMSO-treated neurons (n>70 axon terminals) (Fig. 6A, B, D).

All these results suggest that activation of PKA enhances the anterograde transport of oxytocin vesicles along the axons, thus accumulating more oxytocin vesicles at the axon terminals.

Activation of PKA increases the association of kinesin-2 and AKAP150 with ANXA1

Since kinesins are the anterograde transporters that carry proteins and vesicles toward the axon terminals (46), it is possible that activation of PKA recruits more kinesins to oxytocin vesicles, thus enhancing the anterograde transport of the vesicles. Possibly, activation of PKA increases the binding of kinesins to ANXA1, a protein associated with oxytocin vesicles. We therefore performed co-immunoprecipitation using the antibody 4660 and the cytosols extracted from N11 cells treated with either DMSO or 10 μ M forskolin for 30 min. Activation of PKA did, indeed, increase the association of kinesin-2 with ANXA1 compared to control (Fig. 4C). Conversely, activation of PKA had no effect on the binding of cytoplasmic dynein, dynactin, kinesin-1, and kinesin-3 to ANXA1. We also examined the effect of forskolin treatment on the association of AKAP150, a scaffold protein that targets the action of PKA to its associated proteins, with ANXA1. In untreated N11 cells and mouse hypothalamus, there was a basal level of interaction between AKAP150 and ANXA1 (Fig. 4A, B). The basal interaction between AKAP150 and ANXA1 was also observed in the counter pulldown by anti-dynactin antibody (Fig. 4E). Upon forskolin treatment, more AKAP150 was associated with ANXA1 (Fig. 4C). Thus, activation of PKA increases the binding of kinesin-2 to ANXA1, which should increase the anterograde transport of oxytocin vesicles. Increased binding of AKAP150 to ANXA1 may concentrate the action of PKA on oxytocin vesicles, thus enhancing the anterograde transport of the vesicles.

Activation of PKC decreases the binding of kinesin-2 to ANXA1, which may attenuate the anterograde transport of oxytocin vesicles

Previous studies suggest that activation of PKC may enhance somatodendritic oxytocin secretion but attenuate axonal secretion (16, 23). Hence, we examined the effect of PKC on the axon-localization of oxytocin vesicles and the interaction of ANXA1 with kinesin-2 and other proteins. Primary hypothalamic neurons (E17, DIV14) were treated with either DMSO or 10 nM phorbol ester (PMA) and immunostained with antibodies to oxytocin and ANXA1. The average intensities $\pm$ SEMs of oxytocin (4.71 ± 0.46) and ANXA1 (6.75 ± 0.58) in the axons of neurons treated with PMA (Fig. 6F, G) were similar to those of neurons treated with DMSO (oxytocin = 7.1 ± 0.7 and ANXA1 = 6.9 ± 0.6 , $n=60$ neurons) (Fig. 6E, G). This finding suggests that PKC activation does not enhance the axon-localization of oxytocin vesicles. We also quantified the average intensity of oxytocin vesicles at the axon terminals of neurons treated with either DMSO or 10 nM PMA for 30 min. The average intensity of oxytocin vesicles at the axon terminals of PMA-treated neurons was not different from that of DMSO-treated neurons ($n>70$ axon terminals, $p>0.05$) (Fig. 6A, C, D). Next, we performed co-immunoprecipitation using the antibody 4660 and the cytosols extracted from N11 cells treated with either DMSO or 10 nM PMA. Activation of PKC did not affect the association of cytoplasmic dynein, dynactin, and AKAP150 with ANXA1 but decreased the binding of kinesin-2 to ANXA1 (Fig. 4D). The binding of kinesin-1 and kinesin-3 to ANXA1 was not affected by activation of PKC. These results suggest that activation of PKC may attenuate the anterograde transport of oxytocin vesicles along the axon by decreasing the association of kinesin-2 with oxytocin vesicles.

Activation of PKA increases the association of kinesin-2 and AKAP150 with light-density vesicles

We performed differential centrifugation to examine the effect of PKA and PKC on the association of ANXA1, AKAP150, and microtubule motors with LDCVs in N11 cells. N11 cells treated with DMSO, 10 μ M forskolin, or 10 nM PMA were processed to obtain cytosols (1 mg/mL) in 0.3 mL PMEE buffer (47) + protease/phosphatase inhibitors + 0.25 M sucrose. Post-nucleus supernatant (PNS) was obtained by spinning cell lysates at $0.6K \times g$. PNS was spun sequentially at 3K, 4.8K, 15K, 100K, and $161K \times g$ to separate different

membranous compartments. The plasma membrane (syntaxin-1), Golgi complex (p115), and endoplasmic reticulum (ER: calreticulin) were pelleted at 3K, 4.8K, and 15K (Fig. 7A). Carboxypeptidase E (CPE), a heavy-density LDCV protein that travels from the ER through the Golgi complex to the plasma membrane (48), was found in the ER, Golgi complex, plasma membrane, and heavy-density vesicle pool, and a little in light-density vesicle pool. Synaptophysin (SYN), a light-density vesicle marker, was pelleted at 161K and to some extent at 3K. Oxytocin-containing intermediates (15, 20–28, 34 kD) were pelleted at 100K and 161K, suggesting that there are two different-density LDCVs containing oxytocin. Two different-density oxytocin vesicles were also found in the mouse hypothalamus (Fig. 7D). We could not detect the mature form (9 amino acids, <1kD) of oxytocin because the nitrocellulose membrane (pore size = 0.45 μ m) that we used could not hold peptides smaller than 2kD. Given that oxytocin vesicles in the axon contain a little CPE (Fig. 2C), it appears that light-density vesicles containing oxytocin and a little CPE (Fig. 7A) are targeted to the axon.

In untreated N11 cells, most ANXA1 was found in the heavy membrane fractions pelleted at 3K and 4.8K and the cytosol while a small amount of ANXA1 was pelleted at 15K, 100K, and 161K (Fig. 7A). Conversely, in forskolin-treated N11 cells, the amount of ANXA1 that was associated with light-density vesicles was increased (Fig. 7B). In PMA-treated N11 cells, ANXA1 was found in all the fractions (Fig. 7C). Kinesin-2 was found mostly in the vesicle pools and cytosol of untreated N11 cells. Treatment with forskolin increased the association of kinesin-2 with light-density vesicles (Fig. 7B) while that with PMA decreased the association of kinesin-2 even with heavy-density vesicles (Fig. 7C). AKAP150 was found mostly in the light-density vesicles and cytosols of cells when the cells were treated with forskolin (Fig. 7B) while it was in all the fractions of cells treated with either DMSO (Fig. 7A) or PMA (Fig. 7C). The association of cytoplasmic dynein, dynactin, and kinesin-1 with heavy- and light-density vesicles was not changed by treatment with either forskolin (Fig. 7B) or PMA (Fig. 7C). These results suggest that activation of PKA increases the association of kinesin-2 with light-density oxytocin vesicles that contain oxytocin and a little CPE, thus enhancing the anterograde transport of the vesicles to the axon. Conversely, activation of PKC that decreases the association of kinesin-2 with ANXA1 on oxytocin vesicles may block the anterograde transport of oxytocin vesicles to the axon.

Discussion

Oxytocin is a major socializing neuropeptide in the brain (49). Oxytocin secreted from the somatodendritic regions of the hypothalamic neurons activates its receptors in the hypothalamus and the brain regions neighboring the hypothalamus (1, 2). Some oxytocin travels along the long axon to the distal brain regions such as septum, substantia nigra, stria terminalis, olfactory bulb, brainstem, and spinal cord (7–14). Brain oxytocin modulates brain functions involved in behavior, anxiety, cognition, and autonomic nerve activity (15). Oxytocin is also secreted from the axons that innervate the posterior pituitary gland into the blood circulation, thus increasing natriuresis and reducing blood pressure and heart rate (3–6). There appears to be a ‘yin and yang’ mechanism for controlling the distribution of oxytocin secretion between the axon and the somatodendritic region. Extracellular stimuli that activate PKA enhance the secretion of oxytocin at the axon terminals (18–21) while those that activate PKC enhance somatodendritic oxytocin secretion and attenuate oxytocin secretion at the axon terminal (16, 22, 23). Thus far, the molecular mechanism underlying the regulation of the distribution of oxytocin vesicles for secretion between the axon and the somatodendritic region is largely unknown.

We have discovered that ANXA1 forms a complex with AKAP150, a protein that interacts with PKA and PKC (24–28), and microtubule motors in the hypothalamus and immortalized

oxytocin neurons. Pixel analysis of the co-localization between ANXA1 and oxytocin vesicles clearly shows that ANXA1 associates with oxytocin-containing LDCVs in the axons of primary oxytocin neurons. The interaction of ANXA1 complex with kinesin-2, an anterograde transporter, is modified differently by PKA and PKC. Activation of PKA increases the binding of kinesin-2 to ANXA1, which appears to enhance the anterograde transport of oxytocin vesicles toward the axon terminals. Conversely, activation of PKC decreases the binding of kinesin-2 to ANXA1, which may decrease the axon-localization of oxytocin vesicles. Thus, our results indicate that PKA and PKC determine the directionality of oxytocin vesicle movements in the axon by controlling the binding of kinesin-2 to ANXA1 on oxytocin vesicles (Fig. 8).

Our finding of a new molecular mechanism by which the directionality of vesicle movements is controlled should provide new insight into peptidergic vesicle trafficking in endocrine and neuroendocrine cells. As we reviewed recently (46), even the molecular mechanism by which peptidergic vesicles are transported along microtubules in peptidergic neurons and endocrine cells are not fully understood. Kinesin-3 (KIF1A) mediates the anterograde transport of peptidergic vesicles in *C. elegans* (50). A microtubule motor complex consisting of kinesin-2, cytoplasmic dynein, and dynactin mediates the bi-directional movement of stress hormone vesicles in AtT20 cells (47). A similar complex mediates the transport of vesicles containing brain-derived neurotrophic factor (BDNF) in hippocampal neurons (51) with the assistance of Htt-associated protein-1 (HAP1) (52, 53). In premature neurons, bicaudal-D-related protein 1 (BICDR-1) mediates peptidergic vesicle transport required for neurite outgrowth by interacting simultaneously with Rab6 on LDCVs and with a motor complex consisting of dynactin, cytoplasmic dynein, and kinesin-3 (54). We speculate that ANXA1 complex may coordinate the actions of PKA and/or PKC in those transport systems, thus determining the direction of hormone vesicle movements to different secretion sites (e.g., the axon versus the cell body).

Is the binding of more kinesin-2 to vesicles strong enough to push the vesicles toward the anterograde direction? Under resting condition, bi-directional microtubule-based transport is nearly at force balance between a small group of strong torque kinesins and a large team of weak torque cytoplasmic dynein (55–57). A single kinesin competes with at least five cytoplasmic dyneins for changing the direction of microtubule-based transport (57). Thus, the directionality of microtubule-based transport is determined by either the association/dissociation of a single strong torque kinesin or the number of multiple weak torque cytoplasmic dyneins. Therefore, the increase in the association of kinesin-2 with ANXA1 on oxytocin vesicles without affecting that of cytoplasmic dynein should push oxytocin vesicles toward the axon terminals of neurons treated with PKA activator. Conversely, the dissociation of kinesin-2 from ANXA1 on oxytocin vesicles in neurons treated with PKC activator should shift the direction of vesicle movement toward the cell body. This mechanism may be how extracellular stimuli adjust the amount of oxytocin for secretion at the axon terminal versus the somatodendritic region. It is possible that most peptidergic neurons with long axons such as hippocampal neurons use ANXA1-AKAP150-kinesin-2 complex or a similar complex to refill depleted secretion sites at the axon terminal quickly in a PKA-dependent manner for release of a proper amount of hormones in response to repetitive stimulation.

In order to clarify the difference of our finding of the association of ANXA1 with peptidergic vesicles from those of a subset of previous studies (58, 59), we attempted to do immuno-electron microscopy using primary hypothalamic neurons cultured *in vitro* but could not because of technical difficulty in obtaining the sections of the intact thin axons of hypothalamic primary neurons for immune-EM. Nonetheless, our high magnification images clearly show significant co-localization of ANXA1 with oxytocin vesicles in the axon.

Given that ANXA1 is expressed as an intracellular (not extracellular) protein (36) and found mostly on LDCVs in endocrine cells (42), it may reside on the extravesicular surface of oxytocin vesicles.

We observed the presence of two different-density oxytocin vesicles in immortalized N11 cells and mouse hypothalamus. Light-density vesicles appear to contain some CPE, an LDCV enzyme that removes the C-terminal bi-basic residues of pro-hormones (48). Most oxytocin vesicles in the axons may be light-density vesicles containing a little CPE. Additional studies are needed to further characterize these light-density oxytocin vesicles.

In closing, our study demonstrates that ANXA1 forms a complex with AKAP150 and microtubule motors in the hypothalamus, specifically in oxytocin neurons. This complex coordinates the actions of PKA and PKC to control the direction of oxytocin vesicle movement by changing the association of the anterograde strong torque motor, kinesin-2, with oxytocin vesicles (Fig. 8). Our study reveals, for the first time, a novel molecular mechanism which peptidergic neurons use to control the microtubule-based transport of neuropeptide-containing vesicles to secretion sites. Further study should help identify molecular targets for improving the treatments of endocrine and psychiatric disorders that are caused by abnormal hormone secretion.

Experimental methods

Antibodies and Materials

4659 and 4660 antibodies were gifts from Dr. Y. Peng Loh (NIH). The 4659 and 4660 antibodies were generated against the recombinant protein of full-length snapin in chicken by AVES Labs, Inc. (Tigard, OR). According to the manufacturer's protocol, antigen was injected four times into the breast muscle of chicken: the first in complete Freund's Adjuvant and the remaining injections at a 1:1 dilution of complete and incomplete Freund's Adjuvants. After 1 week following the fourth injection, the IgY batches (4659 and 4660) were purified from pre-immune and immune eggs and sent to us. Anti-snapin antibody (clone L8/15) was purchased from NeuroMab Inc. (Davis, CA). Antibodies to ANXA1 (sc-12740), AKAP150 (sc-10765), dynein intermediate chain (DIC, sc-13524), kinesin-1 (kinesin heavy chain, sc-28538), kinesin-2 (KIF3A, sc-135960), CPE (sc-136252), dynactin (p150, sc-11363) and syntaxin-1 (sc-12736) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies to MAP2 (610460), kinesin-2 (KIF3A, 611508), kinesin-3 (KIF1A, 612094), p115 (612260), CPE (610758), and calreticulin (612137) were from BD Biosciences (San Jose, CA). Anti-synaptophysin antibody (S5768) was from Sigma. Anti-oxytocin antibody (ab2078) was from Abcam (Cambridge, MA). Forskolin, PKI-ester, and phorbol ester (phorbol 12-myristate 13-acetate [PMA]) were purchased from Sigma (St. Louis, MO) and dissolved in DMSO (Sigma). Control and annexin-1 shRNA lentiviral particles and Polybrene® were purchased from Santa Cruz Biotech.

GST-ANXA1 expression and purification

Glutathione-S-transferase (GST) tagged ANXA1 was generated by inserting the BamHI-XhoI digest of the PCR product of annexin-1 cDNA in GFP-ANXA1 that was purchased from Origene Co. (Rockville, MD) into pGEX4T-2. GST tag alone or GST-tagged ANXA1 was purified on 40 μ L of glutathione (GSH) beads (Novagen Co., Madison, WI) from bacterial cell cytosol in 5 mL of Bugbuster solution (EMD Chemicals Inc., San Diego, CA) containing protein inhibitor cocktails. After extensive washing in PBS, GST proteins were eluted from GSH beads in 100 μ L of 10 mM GSH + protein inhibitor cocktails. The eluate from the GSH beads was mixed with 40 μ L SDS loading buffer and loaded onto NuPAGE SDS gel for immunoblotting.

Cell culture and drug treatment

AtT20 cells that were used for our study are F2 subclone (AtT-20/D16V-F2, ATCC® CRL-1795TM, ATCC [Manassas, VA]). The F2 subclone of AtT20 cells is different from the D1 and D16 subclones of AtT20 cells that do not express endogenous ANXA1 (60). N11 cells were purchased from Cedarlane Co. (Burlington, NC). N11 cells are cloned from mouse embryonic hypothalamic primary cultures immortalized by retroviral transfer of SV40 T-Ag. N11 cells express oxytocin, steroidogenic factor 1 (SF-1), and other hypothalamic neuron markers. Both AtT20 (passage 14–16) and N11 cells (passage 2–3) were grown in Dulbecco's modified Eagle's medium (DMEM [Life Technologies, Grand Island, NY]) containing 10% heat-inactivated fetal bovine serum (FBS), 1 g/L D-Glucose, 110 mg/L sodium pyruvate, and 1 × Pen Strap (Life Technologies). To obtain primary hypothalamic neurons, female pregnant mice (BALB/C, Charles River Laboratories International, Inc., Wilmington, MA) at the gestation day 17 (E17) were anesthetized and dissected to obtain 8–9 embryos per mouse. Cortical neurons were isolated from embryonic brains and differentiated on poly-lysine-coated coverslips in B27/neurobasal medium for 14 days (DIV14). All the cell cultures were incubated at 37°C in a humidified 5% CO₂ incubator. For drug treatment, N11 cells or primary hypothalamic neurons were treated with DMSO (0.1%), 10 μM forskolin, or 10 nM PMA prior to immunocytochemistry and immunoprecipitation.

Transduction

Transduction of shRNA lentiviral particles (sc-29198-V, Santa Cruz Biotech.) was conducted according to the manufacturer's protocol (Santa Cruz Biotech.). The shRNA lentiviral particles contain four expression constructs each encoding target-specific 19–25 nt (plus hairpin) shRNA designed to knock down ANXA1. Primary hypothalamic neurons (E17, DIV14) differentiated on coverslips in 24-well plates were incubated in 0.5 mL B27/neurobasal medium containing 5 μg/mL Polybrene® and 3 μL shRNA particles (15,000 infectious units of virus [IFU]) for 24 h. On the second day of transduction, the medium containing shRNA particles was replaced with fresh medium minus shRNA particles and incubated for 5 days prior to immunocytochemistry.

Immunocytochemistry

For microscopic purpose, neurons or N11 cells cultured on poly-lysine-coated coverslips were fixed at 37°C in DMEM medium containing 3.7% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 in PBS for 30 min. After blocking with 2% BSA in T-TBS (TBS + 0.1% Tween 20), neurons or cells were immunostained with primary antibodies. Bound primary antibodies were detected using their corresponding secondary antibodies conjugated with Alexa_{488nm} or Alexa_{594nm} (Life Technologies). Coverslips were then mounted on glass slides using Fluoromount G (Fisher Scientific).

Confocal microscopy and image analysis

The images of immunostained neurons and N11 cells were taken using a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL). The confocal microscope is equipped with conventional solid state, a ti-sapphire tunable multi-photon laser (Coherent, Santa Clara, CA), and acousto optical beam splitter AOBs. Images were taken with 63 x Zeiss alpha plan fluor oil objective (1.4 NA). The Metamorph software (Life Technologies) was used to quantify the intensity of oxytocin and ANXA1 in the axons, axon terminals, and cell body. The correlation co-efficient of co-localization (CCC) between ANXA1 and oxytocin vesicles was obtained using 'Metamorph' software as described by Park et al. 2011 (45). Region of Interest was limited to either whole cell or small cell region per image. According to the developer of the Metamorph software, $CCC = \sum_i [S_{c1} \cdot S_{c2}] /$

$\text{SQRT}(\sum_i [S1_i]^2 \times \sum_i [S2_i]^2)$. i : i 'th pixel in the region of interest, S_{c1} : signal intensity of pixel involved in co-localization in the first channel, S_{c2} : signal intensity of pixel involved in co-localization in the second channel, $S1$: signal intensity of pixel, regardless of co-localization, in the first channel, $S2$: signal intensity of pixel, regardless of co-localization, in the second channel, SQRT: square root. In order to obtain data objectively, all of the image were acquired and analyzed blindly by different persons.

In-gel trypsin digestion and mass spectrometry

38-kD protein band was excised from coomassie-stained NuPAGE gel with a clean, sterilized blade and transferred to Eppendorf microcentrifuge tubes. In-gel trypsin digestion and mass spectrometry were conducted as described in (61).

Co-immunoprecipitation

For coimmunoprecipitation studies, the cytosols of N11 cells or mouse hypothalamus tissues were prepared as follows. N11 cells from eight 100-mm dishes at about 90% confluency or three hypothalami (BALB/C, female, 8-wks) were harvested and resuspended in 1.2 mL PMEE buffer (pH 7.0; 35 mM KOH, 35 mM PIPES, 5 mM MgSO_4 , 1 mM EGTA, 1% BSA, and 0.5 mM EDTA) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and $1 \times$ HaltTM protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Cells were then ruptured by passing them through a 27-gauge needle (Becton Dickinson, Franklin Lakes, NJ) 20 times to break down any vesicular structures. Cell debris and nuclear membranes were removed by centrifugation for 20 min at $14,500 \times g$ to obtain post-nuclear supernatant. 1 mL of the post-nuclear supernatant (5 mg/mL protein) was split into two sets of 500 μL . Each 500 μL of the cytosol was mixed with 100 μL protein A-agarose beads (Sigma) and incubated for 1 h at 4°C on a rotating platform to preclear the cytosol. After removal of the agarose beads, the cytosol was mixed with either 5 μg control IgGs or IgGs against ANXA1 or p150 (dynactin) and incubated for 16 h at 4°C . Then, 60 μL of protein A-agarose beads was added to the tubes and incubated for 8 h. After incubation, the beads were washed four times with PMEE buffer and boiled in 60 μL SDS loading buffer. Co-immunoprecipitation was performed twice per condition.

Differential centrifugation

N11 cells were harvested from three 100-mm culture dishes of about 90% confluency. Hypothalamic tissues were dissected from two mice (BALB/C, female, 8-wks) and used for differential centrifugation. After washing with PBS, cell pellet or hypothalamic tissue was resuspended in 0.3 mL PMEE buffer + proteases/phosphatases inhibitors + 0.25 M sucrose (PMEE-IS) and passed through a 27-gauge needle six times. A postnuclear supernatant (0.5–1 mg/mL) was generated from the lysate after centrifugation at $600 \times g$ for 10 min at 4°C . Post-nucleus supernatant (PNS) was then spun sequentially at 3K, 4.8K, 15K, 100K, and $161K \times g$. After each spin, supernatant was used for next centrifugation and pellet was dissolved in 15 μL of PMEE-IS buffer + 0.5% Igepal CA-630. The wall of centrifuge tube was wiped with cotton Q-tip after each spin to minimize contamination of the pellet with the supernatant. The markers of the plasma membrane, endoplasmic reticulum, Golgi complex, and heavy and light-density vesicles were detected by immunoblotting. Differential centrifugation was performed twice per condition.

Immunoblotting

Proteins in the cytosols extracted from N11 cells and mouse hypothalamus and in the samples of immunoprecipitation and differential centrifugation were boiled with SDS loading buffer, separated in NuPAGE 4–11% Bis-Tris protein gels (Life Technologies), transferred to nitrocellulose membrane (Protran® BA-85, 0.45 μm [Whatman-GE

Healthcare Life Science, Piscataway, NJ]), and detected by immunoblotting using primary antibodies.

Statistical Analysis

All experiments were replicated multiple times with different batches of cell cultures. Microscopic analysis was performed by students blinded to the experimental setup. Statistical significance between two groups was calculated using unpaired student's t-test, with a value of $p < 0.05$ considered statistically significant. Multiple comparisons were done using one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism software, La Jolla, CA).

Acknowledgments

We thank Dr. Andrea Kalinoski (University of Toledo Microscopy Imaging Core) for technical support. We thank to Dr. Y. Peng Loh for 4659 and 4660 antibodies. This research was supported by NICHD K22 (1K22HD056137-01A1) and ARRA funding.

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Fig. 1.

The antibody 4660 recognizes specifically ANXA1 in AtT20 and N11 cells and mouse hypothalamus. Proteins in the cytosols (5 mg/mL) extracted from AtT20 cells (A), untransfected N11 cells (B), and mouse hypothalamus tissues (C) were detected by immunoblotting. Anti-snapin rabbit antibody (α SNAP) and two chicken antibodies (4659 and 4660) were used to detect 15kD snapin (marked with '*') and a 38kD protein (ANXA1) in AtT20 cells (A). The antibodies 4659 and 4660 and mouse anti-ANXA1 antibody (mAb) were used to detect ANXA1 in N11 cells (B) and in mouse hypothalamus (C). (D) The antibody 4660 and mouse anti-ANXA1 antibody (mAb) were used to detect recombinant ANXA1 protein tagged with GST (GST-ANXA1: AN) and GST tag alone (G) that were purified from bacteria.

Fig. 2. ANXA1 colocalizes with oxytocin vesicles (OXT) at the process tips of N11 cells and in the axons of primary hypothalamic neurons. (A) N11 cells grown on poly-L-lysine-coated coverslips were processed for immunocytochemistry with antibodies to oxytocin (green) and ANXA1 (red). Scale bars = 10 μ m. (B–E) Primary mouse hypothalamic neurons (E17) were differentiated on poly-L-lysine-coated coverslips in neurobasal/B27 medium for 14 days (DIV14) and processed for immunocytochemistry with antibody to MAP2 (B), CPE (C), or ANXA1 (D–F) along with anti-oxytocin antibody. (B) Primary oxytocin neurons formed a long axon that was not stained by anti-MAP2 antibody. (C) The image of oxytocin vesicles and CPE at the axon terminals of the neurons was taken by confocal microscope. Scale bars = 5 μ m. The inset of the panel (D) showed the co-localization of oxytocin vesicles with ANXA1 in the axon. (D–F) ANXA1 showed significant co-localization with vesicles

containing oxytocin at the axon terminals. Scale bars = 10 μm . The insets of panel (F) showed the overlapping between the green pixels of oxytocin and the red pixels of ANXA1. The scale bars of the insets = 0.13 μm (= the diameter of one pixel).

Fig. 3. ANXA1 is required for the axon-localization of oxytocin vesicles (OXT) in primary hypothalamic neurons. Primary mouse hypothalamic neurons (E17) differentiated on poly-L-lysine-coated coverslips were transduced with control or anti-ANXA1 shRNA lentiviral particles for 5 days. Transduced neurons were then processed for immunocytochemistry using antibodies to ANXA1 (red) and oxytocin (green). The insets showed that neurons transduced with anti-ANXA1 shRNA (B) had low levels of ANXA1 and oxytocin vesicles in the axons compared to those transduced with control shRNA (A). Scale bars = 10 μ m. (C) Bar graphs show the average intensities $\pm$ SEMs of oxytocin vesicles in the cell bodies and the axons between 0–50, 50–100, 100–150, 150–200, and 200–250 μ m from the cell body of neurons transduced with either control or anti-ANXA1 shRNA (ANXA1 KD). (*: $p < 0.001$)

Fig. 4.

ANXA1, AKAP150, and microtubule motors form a complex in mouse hypothalamus and N11 cells. The cytosols (5 mg/mL) of mouse hypothalamus tissues (A) or N11 cells (B–D) that were treated with DMSO (B), 10 μ M forskolin (C, Forsk), or 10 nM PMA (D) were mixed with the antibody 4660 along with protein A beads (60 μ L) at 4°C for 24 h. Proteins pulled down by anti-ANXA1 antibody (α AN) were probed by immunoblotting. (E) The cytosols (5 mg/mL, post-nuclear supernatant [SN]) of N11 cells were mixed with anti-p150 (dynactin) antibody for co-immunoprecipitation. Proteins bound to dynactin were detected by immunoblotting.

Fig. 5.

Vesicles containing oxytocin (OXT) and ANXA1 co-migrate toward the axon terminal in response to PKA activation. Mouse hypothalamic neurons (E17, DIV14) were treated with DMSO or 10 μ M forskolin for 10 min (A, F10'), 20 min (B, F20'), and 30 min (C, F30') and stained using antibodies to oxytocin and ANXA1. The axons were indicated by arrows. The magnified images in the insets show the co-localization between oxytocin and ANXA1 at the distal axons of neurons treated with forskolin for 10 min (inset i), 20 min (inset ii), and 30 min (inset iii). Scale bar = 50 μ m. (D) To specify the involvement of PKA in forskolin-induced axon-localization of oxytocin vesicles, neurons were pretreated with 10 μ M PKI-ester for 1 h prior to 30-min forskolin treatment (inset iv). (E) The average intensities of oxytocin vesicles in cell bodies and along axons (0–50, 50–100, 100–150, and 150–200 μ m) were quantified using Metamorph software (n=30 per condition). Bar graphs show the average intensities $\pm$ SEMs of oxytocin vesicles along the axons (0–50, 50–100, 100–150,

and 150–200 μm) of neurons treated with forskolin (0, 10, 20, and 30 min). (#: $p < 0.0001$, *: $p < 0.01$ compared to control)

Fig. 6.

Activation of PKA increases the localization of oxytocin-containing vesicles (OXT) to axon terminals while that PKC decreases it. Mouse hypothalamic neurons (E17, DIV14) were treated with DMSO (A), 10 μ M forskolin (B, Forsk), or 10 nM PMA (C) for 30 min and stained using anti-oxytocin antibody. The images of oxytocin vesicles at the axon terminals of the neurons were taken by confocal microscope. Scale bars = 10 μ m. (D) Bar graphs show the average intensities $\pm$ SEMs of oxytocin vesicles at the axon terminals of neurons treated with DMSO, forskolin, or PMA. (*: $p < 0.01$). (E, F) Mouse hypothalamic neurons treated with DMSO (E) or PMA (F) were stained using antibodies to oxytocin and ANXA1. The axons were indicated by arrows. Scale bars = 10 μ m. (G) Bar graphs show the average intensities $\pm$ SEMs of oxytocin vesicles in the axons of neurons treated with DMSO, PMA, and forskolin (Forsk.). (n=60 neurons per condition, *: $p < 0.001$: DMSO vs. forskolin)

Fig. 7.

Activation of PKA increases the association of kinesin-2 with light-density vesicles. (A–D) Post-nucleus supernatant was obtained from either N11 cells (treated with DMSO [A], 10 μ M forskolin [B], or 10 nM PMA [C]) or mouse hypothalamus tissues (D) by spinning cell or tissue extracts at 0.6K $\times$ g to remove nuclei and cell debris. Post-nucleus supernatant was spun sequentially at 3K, 4.8K, 15K, 100K, and 161K $\times$ g. Pellet after each spin was re-suspended in 0.5% Igepal CA-630-containing PMEE to extract proteins from lipid membrane. Proteins in the pellets and cytosol (cyt.) were detected by immunoblotting. Markers for plasma membrane (syntaxin-1), p115 (Golgi complex), calreticulin (ER), CPE (heavy density vesicles), synaptophysin (SYN: light density vesicles), and oxytocin (OXT) in the pellets and cytosol were detected by immunoblotting.

Fig. 8. Schematic illustration of how PKA and PKC affect the interaction of ANXA1 with AKAP150 and kinesin-2, thus changing the direction of movement of oxytocin-containing vesicles. (A) Upon activation of PKA that consists of two catalytic [C] and two regulatory [R] subunits, the regulated subunits withdraw their inhibitory action from the catalytic subunits. Then, the catalytic subunits increase the binding of kinesin-2 and AKAP150 to ANXA1 on light-density oxytocin (OXT) vesicle without affecting the interaction of cytoplasmic dynein-dynactin with ANXA1, resulting in enhancement of anterograde transport of the vesicles to the axon terminal. (B) Activated PKC inhibits the interaction of kinesin-2 with AKAP150-ANXA1 complex without affecting the interaction of cytoplasmic dynein-dynactin with ANXA1 complex, resulting in inhibition of anterograde transport of oxytocin vesicles to the axon terminal. (C) Activation of PKA enhances the anterograde transport of light-density oxytocin vesicles to the axon terminals while activation of PKC inhibits the anterograde transport. Activation of PKC increases somatic exocytosis of oxytocin vesicles. Some heavy density oxytocin vesicles are transported to the axon and dendrites.