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Note

Membrane-Targeted palGFP Predominantly Localizes to the Plasma Membrane but not to Neurosecretory Vesicle Membranes in Rat Oxytocin Neurons

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Recent advances in viral vector technology, specifically using adeno-associated virus (AAV) vectors, have significantly expanded possibilities in neuronal tracing. We have utilized the Cre/loxP system in combination with AAV techniques in rats to explore the subcellular localization of palmitoylation signal-tagged GFP (palGFP) in oxytocin-producing neurosecretory neurons. A distinctive branching pattern of single axons was observed at the level of the terminals in the posterior pituitary. Despite challenges in detecting palGFP signals by fluorescent microscopy, immunoelectron microscopy demonstrated predominant localization on the plasma membrane, with a minor presence on the neurosecretory vesicle membrane. These findings suggest that membrane-anchored palGFP may undergo exocytosis, translocating from the plasma membrane to the neurosecretory vesicle membrane. In this study, we observed characteristic axon terminal structures in the posterior pituitary of oxytocin neurons. This study indicates the importance of understanding the plasma membrane-specific sorting system in neuronal membrane migration and encourages future studies on the underlying mechanisms.

Key words: GFP with a palmitoylation signal (palGFP), plasmalemma localization, neurosecretory vesicle, immunoelectron microscopy, oxytocin

I. Introduction

The viral vector techniques have recently undergone rapid technological advances, enabling tasks that were previously challenging to be accomplished more easily. One notable application is the use of adeno-associated virus (AAV) vectors in neural tracing. By employing the Cre/loxP system, it is possible to express neuron-specific membrane-translocating GFP with a palmitoylation signal (palGFP) [2] using Cre mice. The palGFP that translocates to the membrane can vividly outline the terminals of axons, making it a valuable anterograde neural tracer [2].

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The neurohypophysial neuropeptide hormones, oxytocin and vasopressin, regulate a range of behaviors including territorial, courtship, pair bonding, reproductive, and nurturing behaviors in addition to their peripheral functions in reproduction and antidiuresis [1]. Most of these hormones are synthesized by magnocellular neurosecretory neurons in specific regions of the hypothalamus, namely the paraventricular nucleus (PVN) and supraoptic nucleus (SON) and are released into the systemic circulation from the posterior pituitary. Oxytocin plays a crucial role in facilitating childbirth and lactation, requiring the release of substantial quantities systemically. The magnocellular oxytocin neurons contain numerous neurosecretory vesicles which are transported into the terminal axonal nerve endings and swellings [6]. These dense-cored neurosecretory vesicles each containing about 60,000 molecules of oxytocin have a spherical shape with a diameter of approxi-

mately 160 nm [6]. In addition, the endings of oxytocin (and vasopressin) neurons are characterized by electron-lucent microvesicles with a diameter of approximately 50 nm, containing the excitatory neurotransmitter glutamate. In this study, we employed AAV techniques to express palGFP specifically in oxytocin-producing neuroendocrine neurons to determine at the ultrastructural level whether palGFP is localized not only in the plasma membrane but also in the membrane of intracellular secretory vesicles.

II. Materials and Methods

Animals

Adult male rats of the Wistar strain (10–12-week-old) (Charles River, Yokohama, Japan) used in this study were maintained according to established experimental procedures authorized by The Committee for Animal Research, Okayama University, Japan.

AAV production and purification

All AAV vectors were produced using the AAV Helper-Free System (Agilent Technologies, Inc., Santa Clara, CA, USA) and purified by established methods [3]. Briefly, HEK293 cells were transfected with a pAAV vector plasmid that included the gene of interest, pHelper and pAAV-RC (serotype 9; purchased from Penn Vector Core, Philadelphia, PA, USA, serotype DJ; purchased from Agilent Technologies). The oxytocin mini-promoter (pOT) sequence was kindly gifted by Dr. Valery Grinevich [5] (University of Heidelberg, Germany). A vector coding palGFP was a kind gift from Dr. Takahiro Furuta [2] (Osaka University, Japan).

Stereotaxic AAV injection

Surgery for AAV injections was performed with a stereotaxic instrument. A cocktail consisting of AAV (DJ)-pOT-Cre-WPRE and AAV (Sr9)-CAG-FLEX-palGFP-WPRE, each at a final concentration of 7×10^{11} copies/ml, was prepared. The viral cocktail (0.5 µl) was injected bilaterally into the SON (±1.8 mm lateral, −1.4 mm posterior to the bregma, and +8.8 mm below the skull surface).

Immunofluorescence and immunoelectron microscopy for GFP

Three weeks after AAV injection, rats were deeply anesthetized using intraperitoneal injections of sodium pentobarbital (50 $mg \cdot kg^{-1}$ body weight) and perfused via the left ventricle with physiological saline, followed by 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains and posterior pituitaries were immediately removed, frozen, and 30-µm-thick cryosections were prepared. Immunofluorescence for GFP was performed according to our established methods [4]. After blocking with 1% normal goat serum and 1% BSA, sections were incubated with a 1:2,000 dilution of chicken polyclonal antibody against GFP (Cat# 600-901-215; Rockland, Gilbertsville, PA, USA; RRID: AB_1537402) overnight at 4°C as described previously [7]. The sections were then incubated for 1 hr at room temperature with Alexa Fluor 488-linked goat anti-chicken IgY (Molecular Probes, Eugene, OR, USA). Immunoreacted sections were imaged by using a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan).

Immunoelectron microscopy was performed as previously described [8]. Briefly, after the post-fixation with 0.1% glutaraldehyde for 2 hr at room temperature, the posterior pituitary tissue sample was dehydrated through increasing concentrations of methanol, embedded in LR Gold resin (Electron Microscopy Sciences, Hatfield, PA, USA), and polymerized under UV lamps. Ultrathin sections (70 nm in thickness) were collected on nickel grids, and then incubated with 2% normal goat serum and 2% BSA in 50 mM Tris(hydroxymethyl)-aminomethane-buffered saline (pH 8.2) for 20 min to block non-specific binding. The sections were then incubated with a 1:20 dilution of rabbit monoclonal antibody against GFP (Cat# 2956; Cell Signaling Technology Japan, Tokyo, Japan; RRID: AB_1196615) in Can Get Signal Solution 1 (TOYOBO, Tokyo, Japan) for 1 hr at room temperature, and biotinylated goat anti-rabbit IgG antibody followed by avidin-biotin-HRP complex (a streptavidin-biotin intensification kit, Nichirei, Tokyo, Japan) followed by goat antibody against HRP conjugated to 12 nm gold particles (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) and viewed using an H-7650 (Hitachi, Tokyo, Japan) electron microscope. This rabbit monoclonal antibody for GFP has previously been shown to be specific for GFP [9].

III. Results and Discussion

In this study, we used an AAV vector capable of inducing Cre expression in an oxytocin promoter-dependent manner (AAV-pOT-Cre-WPRE), employing a technique combining the Cre/loxP system (AAV-CAG-FLEX-palGFP-WPRE) to selectively express palGFP in magnocellular oxytocin neurons in the SON. Thus, we administered a cocktail comprising these two AAV vectors (AAV-pOT-Cre-WPRE + AAV-CAG-FLEX-palGFP-WPRE) locally into the rat SON.

Magnocellular neurons expressing the palGFP signal were present mostly in the dorsal part of the SON, suggesting that they are oxytocin neurons. As expected, in the posterior pituitary, numerous axonal varicosities positive for palGFP signaling were observed (Fig. 1A). In many places in the posterior pituitary, we were able to observe the trajectories of individual labeled axons (Fig. 1B). Interestingly, individual axons appeared to terminate in a radial array of terminal dilatations (Fig. 1C). At the light microscopic level, it was challenging to discern whether the palGFP signal originated from the plasma membrane or the neurosecretory vesicle membrane. Therefore, we used immunoelectron microscopy for GFP to study the ultra-

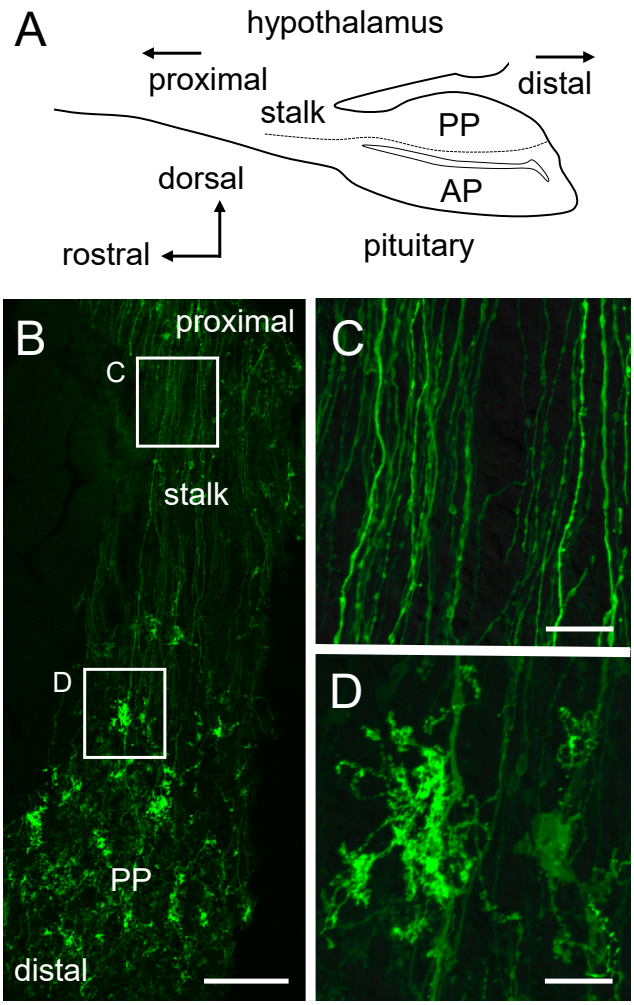

Fig. 1. (**A**) Schematic drawing of the pituitary anatomy and orientation observed in this study. (**B**) In the posterior pituitary, a substantial number of palGFP-positive fibers were present. (**C**) Numerous axons positive for palGFP were observed in the proximal part of the pituitary stalk. In the posterior pituitary, numerous axonal varicosities positive for palGFP signaling were observed. (**D**) A branching pattern radiating from a single axon was observed. (**C**) and (**D**) are at the same magnification. AP, anterior pituitary; PP, posterior pituitary. Bars = 100 µm in (**B**), 20 µm in (**C**, **D**).

structural location of the signal. To enhance the immunoreactivity for GFP, we utilized the streptavidin-biotin intensification method at the electron microscopic level. This demonstrated that palGFP predominantly localizes to the plasma membrane in magnocellular oxytocin neurons in rats. A much smaller amount of label was also present on the membrane of the dense-cored vesicles (Fig. 2A) and the small microvesicles (Fig. 2B). We could interpret this as the result of translocation of plasma membrane-anchored GFP to the neurosecretory vesicle- or microvesicle-membrane during the membrane fusion that occurs in exocytosis [6]. Significantly, the GFP label appeared to be situated on the internal surface of the plasma membrane, but on the external surface of the neurosecretory vesicles (Fig. 2). From

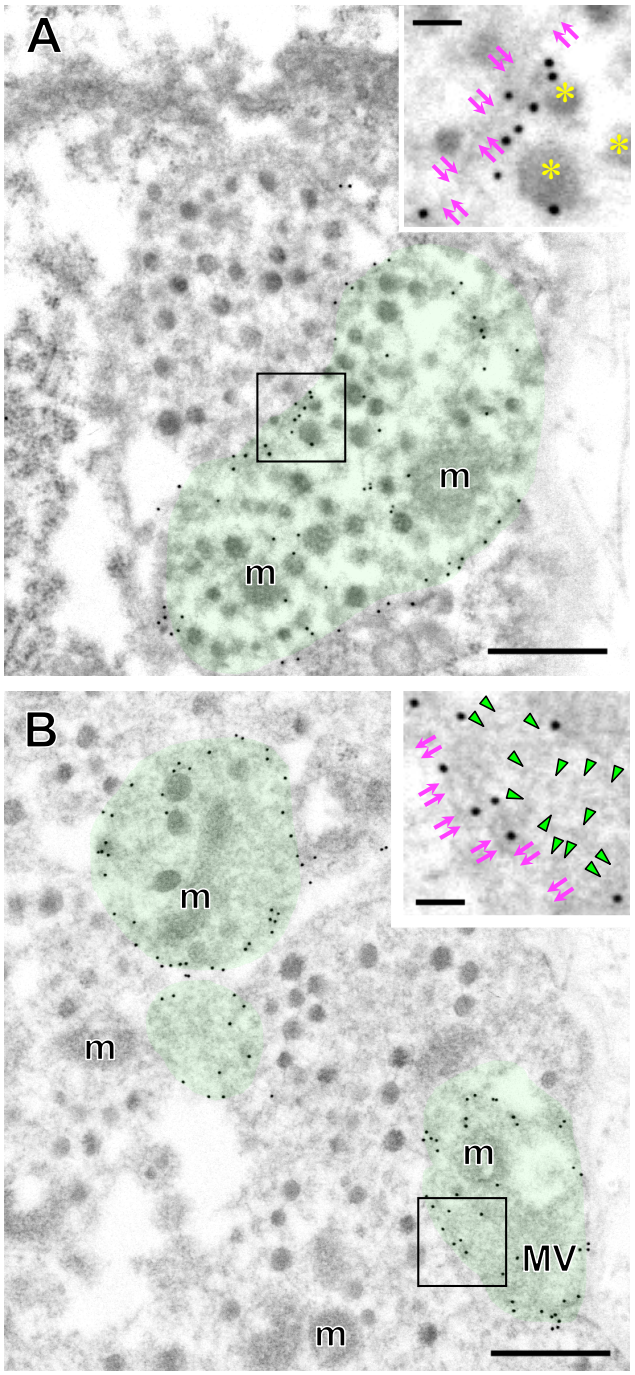

Fig. 2. Immunoelectron microscopy for GFP reveals that palGFP predominantly localizes to the plasma membrane of magnocellular oxytocin neurons in rats; there was also a minor localization to the membranes of the dense-cored vesicles (**A**) and small electron-lucent microvesicles (MV) (**B**). The palGFP-positive axonal varicosities were highlighted in green. Each square represents an enlarged area displayed as a non-highlighted image. Double arrows indicate the plasma membrane. Asterisks mark neurosecretory vesicles. Arrowheads denote clustered microvesicles. m, mitochondrion. Bars = 500 nm for lower magnification and 100 nm for higher magnification.

these results, we suggest that palGFP selectively translocates to the plasma membrane and is anchored on the cytoplasmic side of the plasma membrane. In addition, we recognize that the utilization of avidin-biotin-HRP complexes and anti-HRP antibodies may impact the proximity of gold particles to target molecules. This selection could introduce potential variations in labeling proximity, emphasizing the importance of understanding these nuances for accurately interpreting findings related to the membrane in future studies. While our analysis focused on neuroendocrine oxytocin neurons, these findings could extend to the electron-lucent microvesicles. In other words, palmitoylation serves as a neuron-specific system for protein translocation to the plasma membrane [2] and does not actively promote translocation to neurosecretory vesicles or microvesicles. The mechanism underlying this plasma membrane-specific sorting system in membrane translocation in neurons warrants further investigation.

IV. Conflicts of Interest

The authors declare no conflict of interest.

V. Author Contributions

H.S. and A.I. performed experiments. H.S. wrote the paper. H.S. conceived and supervised the whole study. All authors had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

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