Int. J. Morphol.,
42(5):1267-1277, 2024.

Neuroanatomical Relationship Between Nucleus Accumbens $A_{2A}R$ Neurons and Orexin Neurons in the Mouse Brain

Relación Neuroanatómica entre las Neuronas del Núcleo Accumbens $A_{2A}R$ y las Neuronas Orexina en el Cerebro del Ratón

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ZHANG, J. P & LI, R. X. Neuroanatomical relationship between nucleus accumbens $A_{2A}R$ neurons and orexin neurons in the mouse brain. *Int. J. Morphol., 42(5)*:1267-1277, 2024.

SUMMARY: To explore morphological evidence for neuronal circuits between adenosine A_{2A} receptor ($A_{2A}R$) neurons in the nucleus accumbens (NAc) and orexin neurons. The utilized adeno-associated virus (AAV) encoding humanized Renilla green fluorescent protein (hrGFP) as a tracer to visualize the expression of the A2AR neurons in the NAc and their axon distributions in orexin neurons. The Cre-dependent AAV was injected into the NAc in adenosine $A_{2A}R$-Cre mice. Immunohistochemistry was then used to visualize hrGFP and their projections in orexin neurons. The data revealed that NAc $A_{2A}R$ neurons, mainly expressed in γ-aminobutyric acidergic (GABAergic) projection neurons, projected to the hypothalamus, with apparent contacts of hrGFP containing boutons onto orexin-immunoreactive (IR) soma and dendrites. We also observed strong staining of orexin-IR varicose terminals in the NAc, with apparent contacts of orexin-IR terminals onto $A_{2A}R$ neurons. Furthermore, in the NAc, orexin-IR varicosities contained vesicular glutamate transporter 2 ($VGluT_2$) and synaptophysin (Syp). These data provide morphological evidence for exploring the function between NAc $A_{2A}R$ neurons and orexin neuron in the hypothalamus, particularly in regulating sleep processes.

KEY WORDS: Nucleus accumbens; Adenosine A2A receptor; Hypothalamus; Orexin; Mouse; Arousal.

INTRODUCTION

The nucleus accumbens (NAc) pathways regulates sleep-wake behavior and mediates arousal effects of the midbrain dopamine system and stimulant modafinil (Lazarus *et al*., 2012; Qiu *et al*., 2012). Also, it has been reported that adenosine A_{2A} receptors ($A_{2A}Rs$) in the NAc are critical for the arousal effect of caffeine (Lazarus *et al*., 2011) and mediate slow-wave sleep of classic endogenous somnogen adenosine (Zhou *et al*., 2019). The behavior induced by NAc adenosine $A_{2A}Rs$ is the result of the combined action of NAc $A_{2A}R$ neurons and its neuronal pathways. Fortunately, projections of NAc adenosine $A_{2A}R$ neurons were mapped utilizing adeno-associated virus (AAV) encoding humanized Renilla green fluorescent protein (hrGFP) as a tracer to a wide range of targets, including the ventral pallidum (VP), the hypothalamus, the ventral tuberomammillary nucleus (VTM), the ventral tegmental area (VTA), the dorsal raphe nucleus (DR), the ventrolateral periaqueductal gray (VLPAG), and the locus coeruleus (LC), that may contribute to wakefulness (Zhang *et al*., 2013).

Also, neurons in hypothalamic nuclei and their associated neural pathways have been shown to regulate the initiation and maintenance of sleep (Zha & Xu, 2015). Orexin neurons are exclusively situated in the lateral, perifornical, and posterior part of the hypothalamic area in the brain, which project in a diffuse manner to the whole brain and spinal cord (Peyron *et al*., 1998; Nambu *et al*., 1999). At the rostro-caudal level, the lateral hypothalamus (LH) may be divided into the anterior part (LHa), tuberal part (LHt), and mammillary part (LHm) (Sano & Yokoi, 2007). It has been reported that orexin neurons in the LH play an indispensable role in stabilizing and maintaining wakefulness (Adamantidis *et al*., 2007; Sakurai, 2007; De Luca *et al*., 2022).

Furthermore, neuronal circuits between the NAc and the hypothalamus has been hypothesized to play an important role in the feeding behavior (Maldonado-Irizarry *et al*., 1995; Baldo *et al*., 2004; Thorpe & Kotz, 2005) and locomotor activity (Thorpe & Kotz, 2005). Stimulation of NAc

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FUNDING.This work was supported by the National Natural Science Foundation of China (82074146, to Z.J.P.)

Received: 2024-05-22 Accepted: 2024-06-07

significantly inhibited the activity of orexin neurons in the LH (Wei *et al*., 2015). The sleep-promoting process induced by the microdialysis-perfusion of the NAc with $A_{2A}R$ agonist is associated with a decline in the activity of orexin neurons (Satoh *et al*., 2006). Orexin neurons can directly depolarize NAc neurons, and that NAc neurons are involved in the cellular mechanisms through which orexin neurons participate in the regulation of the arousal behavior (Mukai *et al*., 2009). The sleep-promoting action of adenosine can be reversed by orexin A applied to the LH (Cun *et al*., 2014). Neuronal circuits between the NAc adenosine $A_{2A}R$ and orexin neurons in the hypothalamus are hypothesized to regulate sleep-wake (Lazarus *et al*., 2012), but an anatomical basis for this circuit remains unclear. Although the lateral hypothalamic area has been reported to receive axonal projections from NAc projection neurons (Zahm *et al*., 1993; Kirouac & Ganguly, 1995; Usuda *et al*., 1998), it is not known whether orexin neurons in the LH receive direct inputs from the NAc $A_{2A}R$ neurons.

The conditional anterograde tracing based on Cre/loxP technology has been used to trace $A_{2A}R$ neurons, in which an AAV encodes a hrGFP that is transcriptionally silenced by a neo-cassette flanked by LoxH/LoxP sites (AAV-loxH- Neo-loxp-hrGFP) which can be activated as a tracer by Cre recombinase (Gautron *et al*., 2010). The AAV is injected into the brain of adenosine $A_{2A}R$-Cre mice that expresses Cre under the control of the $A_{2A}R$ promoter, causing robust hrGFP expressions in $A_{2A}R$ neurons (Durieux *et al*., 2009). In addition, hrGFP is effectively transported to the projection fields. Here we showed, by unilaterally injecting the AAV into the NAc, that the LHt orexin neurons receive the direct GABAergic inputs from NAc $A_{2A}R$ neurons. We also observed strong staining of orexin-immunoreactive (IR) varicose terminals in the NAc, with apparent contacts of orexin-IR terminals onto $A_{2A}R$ neurons. Furthermore, we found that orexin-IR varicosities in the NAc contained the vesicular glutamate transporter 2 (VGluT2) and synaptophysin (Syp), a presynaptic marker for small synaptic vesicles (SSVs) of most glutamatergic terminals (Navone *et al*., 1986; Valtorta *et al*., 2004; Bragina *et al*., 2007), but not the vesicular transporter for GABA (VGAT). These observations provide neuroanatomical relationship between NAc adenosine $A_{2A}R$ and orexin neurons in the hypothalamus, improving our understanding of the possible roles of the NAc adenosine $A_{2A}R$, especially in the regulation of sleep.

MATERIAL AND METHOD

Animals. The adenosine $A2_{2A}R$-Cre genetic mice have been well characterized in a previous study (Durieux *et al*., 2009). The adenosine $A_{2A}R$-Cre mice (18-20 weeks old, weighing 24-28 g) were kindly provided by the department of Pharmacology (School of Basic Medical Sciences, Fudan University). All mice were individually housed with an automatically controlled light-dark cycle, appropriate ambient temperature and humidity, and ad libitum water and food in the facility.

Ethical consideration. All experimental procedures were approved by the Laboratory Animal Research Centre of Zhejiang Chinese Medical University, Hangzhou, in Zhejiang province (Protocol Number: IACUC-20220627-07).

Intracranial stereotaxic injection of AAV. Under pentobarbital anesthesia (50 mg/kg, i.p), the adenosine $A_{2A}R$-Cre mice were positioned in a stereotaxic apparatus. Under aseptic conditions, a burr hole was made and a fine glass micropipette (10-20 μm tip) containing the AAV-lox-Stop-hrGFP was bilaterally into the NAc (AP: + 1.1 mm; ML: 1.0 mm; DV: 3.8 mm) referring to the Franklin and Paxinos mouse brain atlas (Paxinos & Franklin, 1997). Using an air compression system, the AAV-lox-Stop-hrGFP was delivered by slow pressure injection for at least 15 min. After remaining the pipette in the brain for additional 10 min, the pipette was slowly retracted. The burr hole was filled with gel-film, and scalp incisions were closed with wound clips. After recovering and awakening, mice were repositioned into cages.

Tissue preparation. Four weeks after stereotaxic AAV injection, the adenosine $A_{2A}R$-Cre mice were deeply anesthetized and transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde. Brains were immediately removed, post-fixed in the paraformaldehyde solution at 4 °C for 2 h, and transferred to 30 % sucrose solution until the brains sank in the 30 % sucrose solution. The brains were then frozen and 30 μm serial coronal sections were cut on a Leica freezing microtome.

Immunohistochemistry. To identify NAc adenosine $A_{2A}R$ neurons, one series of tissues was processed for hrGFP immunofluorescent staining. Nonspecific binding was blocked with a solution containing 5 % normal goat serum (NGS) and 0.3 % Triton X-100 in phosphate-buffered saline (PBS). Sections were incubated in rabbit anti-hrGFP antiserum (Table I; Stratagene; cat. no. 240142; 1:5,000) in 5 % NGS with 0.3 % Triton X-100 in PBS over night at 4 °C. Brain sections were subsequently incubated in Alexa Fluor-488-conjugated goat anti-rabbit IgG (Invitrogen, La Jolla, CA; cat. no. A11034; lot no. 1073084; 1:500) for 2 h at room temperature.

For confirmation of the chemical identity of NAc adenosine $A_{2A}R$ neurons, brain sections were subjected to

double-immunofluorescence staining of hrGFP/GABA or hrGFP/dopamine and cAMP-regulated phosphoprotein 32 (DARPP-32) or hrGFP/parvalbumin (PV). Brain sections were incubated in 10 % normal donkey serum (NDS) in PBS containing 0.3 % Triton X-100 for 1 h followed by in a mixture of rabbit anti-hrGFP antiserum (Stratagene; cat. no. 240142; 1:5,000) and mouse anti-GABA antiserum (Sigma; cat. No. A0310; 1: 3,000) or mouse anti-$DARPP_{32}$ antiserum (Santa Cruz Biotechnology, Inc; cat. No. sc-135877; 1: 5000) or mouse anti-PV antiserum (Millipore; cat. No. MAB1572; 1: 7,000) in PBS with 5 % NDS over night at 4 °C. The sections were then rinsed and incubated in a secondary antibody mixture of Alexa Fluor-488-conjugated donkey anti-rabbit IgG (Invitrogen, La Jolla, CA; cat. no. A21206; 1:2,000) and Alexa Fluor-594-conjugated donkey anti-mouse IgG (Invitrogen, La Jolla, CA; cat. no. A21203; 1:2,000) for 2 h at room temperature.

For double immunofluorescence staining of orexin-A/hrGFP (in the hypothalamus or NAC) or orexin-A/DARPP32 (in NAC), brain sections were incubated in a mixture of goat anti-orexin-A antiserum (Santa Cruz Biotechnology, Inc; cat.no. sc-8070; 1:4,000) and rabbit anti-hrGFP antiserum (Stratagene; cat. no. 240142; 1:5,000) or mouse anti-DARPP32 antiserum (Santa Cruz Biotechnology, Inc; cat. No. sc-135877; 1:5,000) in PBS containing 5 % NDS over night at 4 °C. After thorough rinsing in PBS, the sections were incubated in a mixture of Alexa Fluor-594-conjugated donkey anti-goat IgG (Jackson Immunoresearch; cat. no. 705-585-147; 1:2,000) and Alexa Fluor-488-conjugated donkey anti-rabbit IgG (Invitrogen, La Jolla, CA; cat. no. A21206; 1:2,000) or Alexa Fluor-488-conjugated donkey anti-mouse IgG (Jackson Immunoresearch; cat. no. 715-485-151; 1:2,000) for 2 h at room temperature.

For double immunofluorescence staining of orexin-A/vesicular glutamate transporter 2 (VGluT2) or orexin-A/vesicular GABA transporter (VGAT) or orexin-A/synaptophysin (Syp) in NAc, brain sections were incubated in a mixture of goat anti-orexin-A antiserum (Santa Cruz Biotechnology, Inc; cat.no. sc-8070; 1:4,000) and mouse anti-VGluT2 antiserum (Millipore, cat.no. MAB5504; 1:1,000) or rabbit anti-VGAT antiserum (Millipore, cat.no. AB5062P; 1:1,000) or rabbit anti-Syp antiserum (Abcam, cat.no. Ab68851; 1:1,000) in PBS containing 5 % NDS and 0.3 % Triton X-100 over night at 4 °C. The sections were then rinsed and incubated in a mixture of Alexa Fluor-594-conjugated donkey anti-goat (Jackson Immunoresearch; cat. no. 705-585-147; 1:2,000) and Alexa Fluor-488-conjugated donkey anti-mouse IgG (Jackson Immunoresearch; cat. no. 715-485-151; 1:2,000) or Alexa Fluor-488-conjugated donkey anti-rabbit IgG (Invitrogen, La Jolla, CA; cat. no. A21206; 1:2,000) for 2 h at room temperature.

Microscopy. Finally, all sections were rinsed in PBS, mounted on slides using Fluoromount-GTM (Southern Biotech; Catalog No, 0100-01). Non-confocal images were captured by a fluorescence microscope (IX71, Olympus). High-resolution fluorescence images were collected on a confocal microscope (FV-1000, Olympus). Images were analyzed using Adobe Photoshop CS2 software. Images are representative of at least three biological replicates.

RESULTS

Chemical identity of NAc adenosine A_{2A}R neurons. To genetically visualize NAc A_{2A}R neurons, A total of 8 transgenic A2AR-Cre mice unilaterally received the AAV-lox-Stop-hrGFP injections at the NAc, in which 5/8 were restricted within the NAc. Of the five restricted injections, 3 were selected for further analysis. The location of a typical injection was schematically depicted and visualized around the anterior commissure (ac) by hrGFP immunofluorescence staining (Fig. 1A, B).

The NAc lacks glutamatergic neurons but is instead mostly composed of GABAergic projection neurons, the remaining neurons being cholinergic and GABAergic interneurons (Meredith *et al*., 1993), and GABAergic projection neurons can be further sub-characterized into enkephalinergic and dynorphinergic neurons. In the NAc, adenosine A_{2A}Rrs are predominantly localized in the GABAergic enkephalinergic neurons (Ferré *et al*., 2007; Schiffmann *et al*., 2007). Therefore, we performed double immunofluorescence staining against GABA, DARPP32 (a marker for GABAergic projection neurons), and PV (a postsynaptic marker for GABAergic interneurons) on the A_{2A}R-Cre mouse brain sections and revealed that hrGFP systematically co-localized with GABA (Fig. 1C-C2) and DARPP32 (Fig. 1D-D2), but not with PV (Fig. 1E-E2) in the NAc. These data suggest hrGFP was expressed in the NAc GABAergic projection neurons of and hrGFP-IR plexus in the hypothalamus may contain the GABAergic axon terminals in A_{2A}R-Cre mice.

The terminals of NAc A_{2A}R neurons target orexin neurons. We next asked whether the terminals of NAc A_{2A}R neurons directly innervate the orexin neurons. We performed immunostaining using an anti-orexin-A in the hypothalamus of the A_{2A}R-Cre mice sections and showed that orexin-IR neurons (Fig. 2A,B) and hrGFP-IR varicosities (Fig. 2C,F) were sensitively observed in the LHt. Moreover, some hrGFP-IR terminals were found to contact with the soma (Fig. 2E) or the beaded processes (Fig. 2G) of orexin neurons. Our data suggest that the axon terminals of NAc A_{2A}R neurons directly innervate orexin neurons in the LHt.

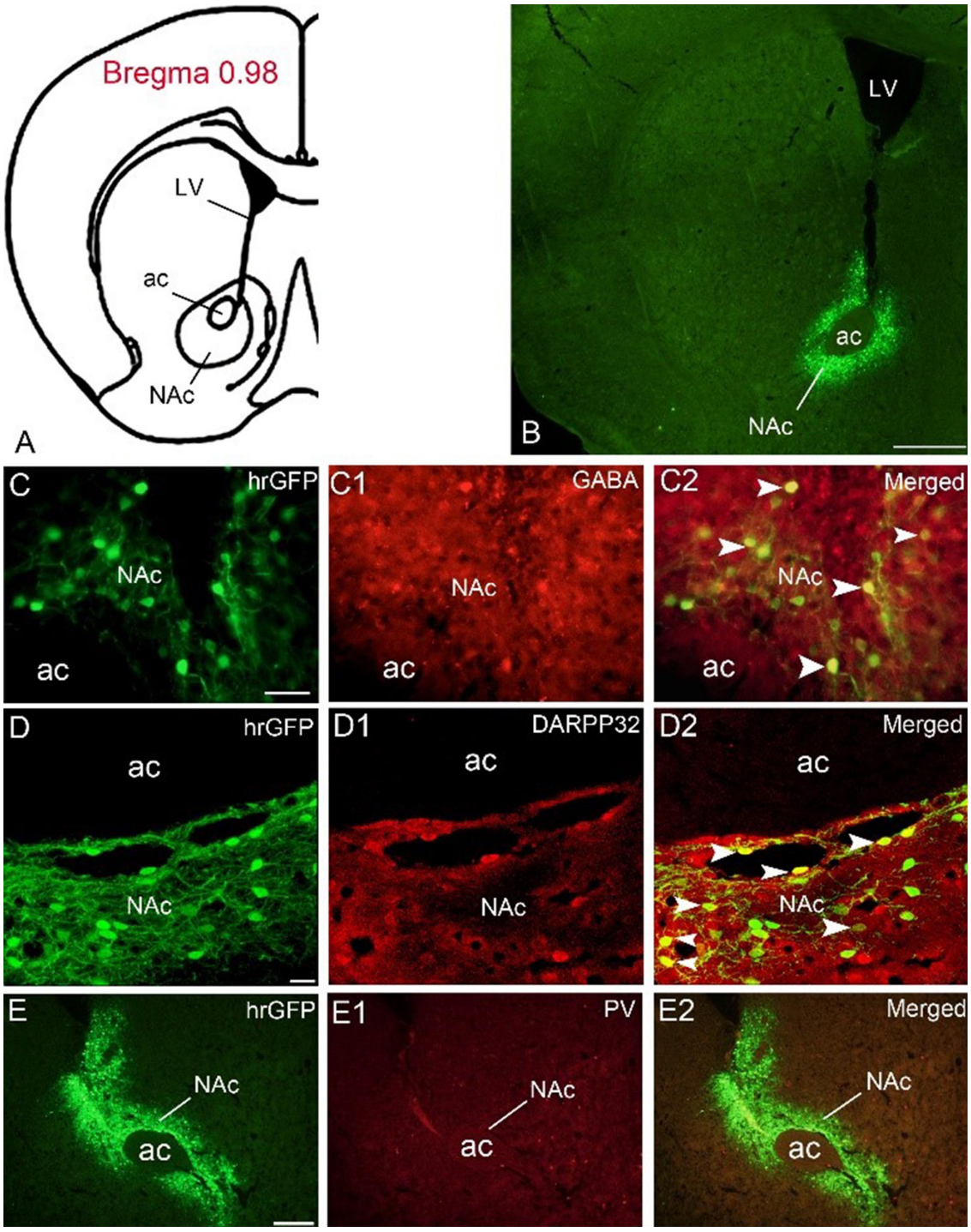

Fig. 1. Representative fluorescence images of hrGFP-IR showing examples of the AAV-lox-Stop-hrGFP injection site in NAc and restricted expression of hrGFP in GABAergic neurons. A: Schematic of the NAc area showing the injection site of AAV-lox-Stop-hrGFP. B: Representative fluorescence photomicrograph through the NAc showing the injection site of AAV-lox-Stop-hrGFP. C-C2: Images showing hrGFP-IR neurons (C), GABA-IR neurons (C1), and merged images of hrGFP with GABA in NAc (C2). D-D2: Images showing hrGFP-IR neurons (D), DARPP-32-IR neurons (D1), and merged images of hrGFP with DARPP-32 in NAC (D2). E-E2: Images showing hrGFP-IR neurons (E), PV-IR neurons (E1), and merged images of hrGFP with PV in NAc (E2). White arrows indicating hrGFP/GABA-positive (C2) or hrGFP/DARPP-32-positive (D2) cells. Abbreviations: ac: anterior commissure; NAc: nucleus accumbens; LV: lateral ventricle. Scale bar=500 μm in B, E-E2; 100 μm in A-C; 10 μm in D-F.

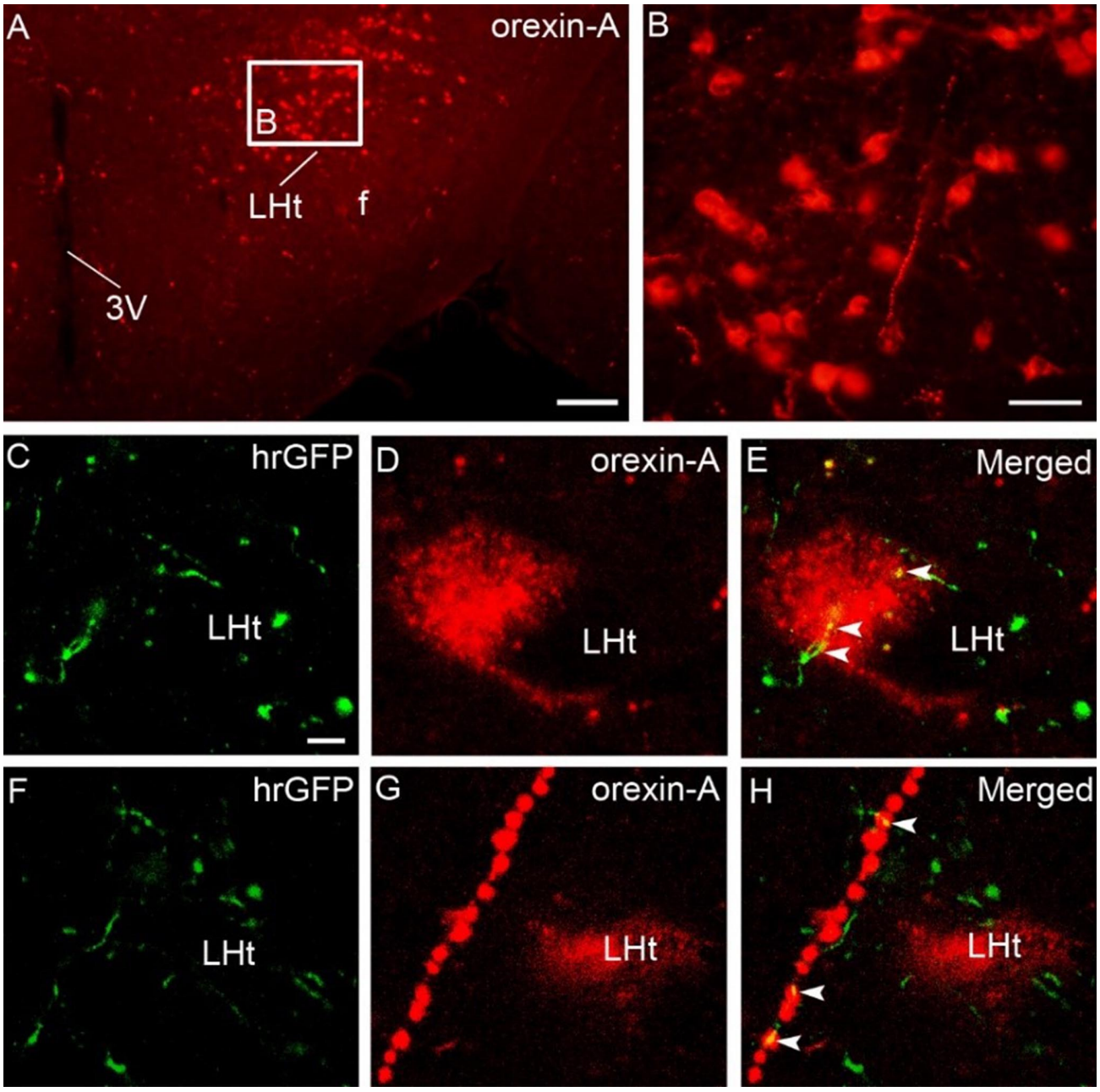

Fig. 2. Representative fluorescence images for showing the terminals of NAc A2AR neurons directly innervating orexin neurons in the LHt. A-B: Images showing orexin-A-IR neurons (A) in the LHt, higher-magnification images of the boxed region showing the morphology of orexin-A-IR neurons. C-H: Confocal images showing hrGFP-IR fibers and terminals in the LHt (C or F), Orexin-IR neurons and processes (D or G), and merged images of hrGFP with orexin-A in LHt (E or H). White arrows indicating hrGFP-IR terminals contacting with the soma (E) or the beaded processes (H). Abbreviations: 3V: 3rd ventricle; f: fornix; LHt: the tuberal part of lateral hypothalamus. Scale bars: 500 μm in A; 50 μm in B; 5 μm in C-H.

Orexin terminals may form excitatory synaptic connections with A2AR neurons in the NAc. Orexin-IR fibers and terminals were found in the NAc (Fig. 3A1-A3), as previously described (Peytron *et al.*, 1998). However, whether these terminals appear to make a contact with the NAc A2AR neurons remains uncertain. We performed dual immunofluorescence for orexin-A/DARPP32 and orexin-A/hrGFP in the A2AR-Cre mice. We clearly detected that orexin-A-IR terminals appeared to contact with DARPP32-IR (Fig. 3B1-B3) or hrGFP-IR (Fig. 3C1-C3) soma and dendrites in the NAc.

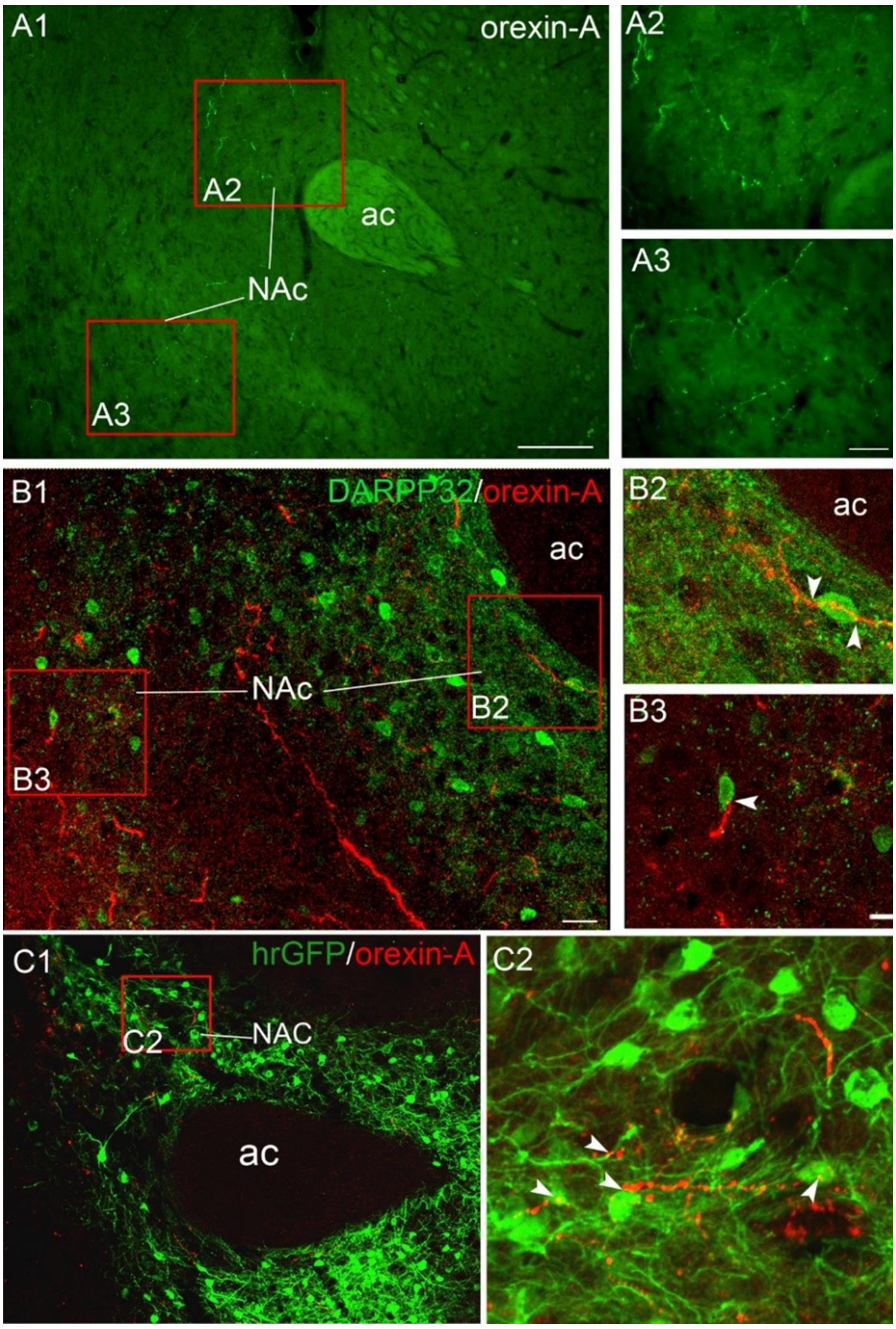

Fig. 3. Representative fluorescence images for showing orexin-A-IR terminals directly innervating the NAc $A_{2A}R$ neurons. A1-A3: Images showing orexin-A-IR terminals in the NAc, higher-magnification images of the boxed region showing the small beaded processes morphology of orexin-A-IR fibers and terminals. B1-B3: Images showing intermingled orexin-A-IR terminals and DARPP32-IR neurons and dendrites were seen in the NAc, higher-magnification images of the boxed region showing apparent contacts of the small beaded processes morphology of orexin-A-IR fibers and terminals onto DARPP32-IR neurons. C1-C2: Images showing intermingled orexin-A-IR terminals and hrGFP-IR neurons and dendrites were seen in the NAc, higher-magnification images of the boxed region showing apparent contacts of the small beaded processes morphology of orexin-A-IR fibers and terminals onto hrGFP-IR neurons. White arrows indicating orexin-A-IR terminals contacting with the soma or the dendrite. Abbreviations: ac: anterior commissure; NAc: nucleus accumbens. Scale bars: 200 μm in A1 and C1, 50 μm in A2 and A3, 20 μm in B1, 5 μm in B2, B3 and C2.

Further, dual-immunostaining for orexin-A and $VGluT_2$ revealed that some orexin-A-IR varicosities also contained $VGluT_2$ (Fig. 4A). No orexin-A-IR varicosities were immunopositive for VGAT (Fig. 4B). In addition, the orexin-A-IR varicosities contained Syp (Fig. 4C). The co-location of orexin-A and $VGluT_2$ or Syp in varicosities was confirmed with confocal microscopy in orthogonal views of image stacks (Fig. 4B,C). Thus, we suggest that the orexin varicosities might utilize glutamate as a neurotransmitter and form glutamatergic synapses upon NAc $A_{2A}R$ neurons.

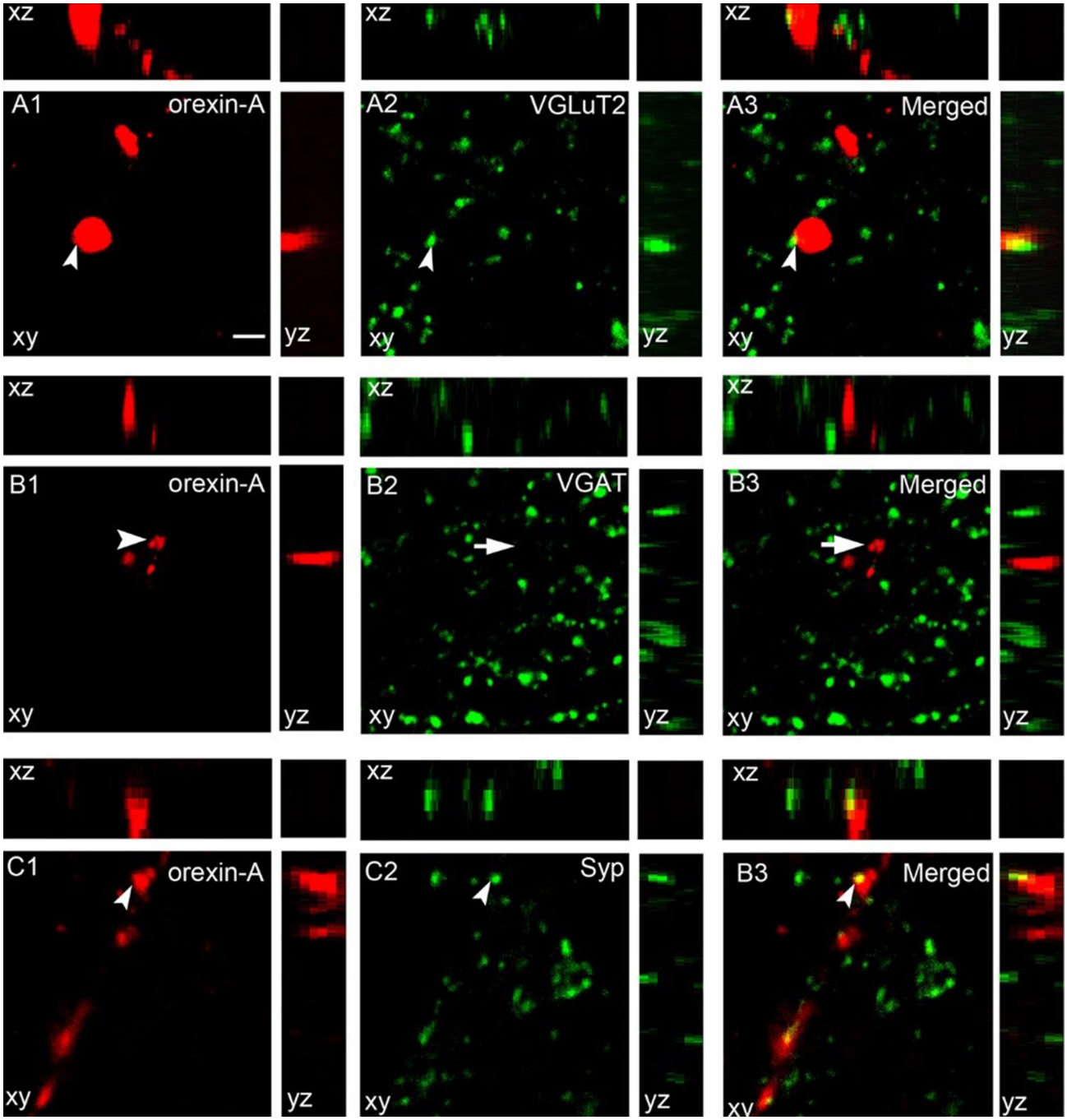

Fig. 4. Representative confocal images of orexin-A/$VGluT_2$, VGAT or Syp dual-immunostaining in the NAc. A1-A3: An orexin-A-IR varicosity (A1, arrowhead) is positively immunostained for $VGLUT_2$ (A2, arrowhead), as evident in the merged image (A3, arrowhead) and the corresponding three orthogonal views (xy, xz and yz sub-panels). B1-B3: Multiple orexin-A-IR varicosities (B1, arrowhead) are not immunostained for VGAT (B2, arrow), as evident in the merged image (B3, arrow) and confirmed in the orthogonal views. C1–C3: An orexin-A-IR varicosity (C1, arrowhead) is positively immunostained for Syp (C2, arrowhead), as evident in the merged image (C3, arrowhead) and confirmed in the orthogonal views. The center images of each panel (xy view) represent single optical sections. The xz and yz views are orthogonal views of the 3D data volume. Scale bar=2 μm (for all panels and sub panels).

DISCUSSION

In the present study, we visualized NAc $A_{2A}R$ neurons by unilaterally injecting a tracer of AAV-lox-Stop-hrGFP into the NAc of adenosine $A_{2A}R$-Cre mice. Then, we detected that the LHt orexin neurons receive the direct GABAergic projections from NAc $A_{2A}R$ neurons. Orexin-IR terminals were also observed in the NAc, with apparent contacts of orexin-IR terminals onto $A_{2A}R$ neurons. Furthermore, we found that orexin-IR varicosities in the NAc contained the VGluT2 and Syp, but not the VGAT. Our results, thus, would suggest a neuroanatomical circuit between NAc $A_{2A}R$ neurons and orexin neurons of the hypothalamus.

Conditional anterograde tracing based on Cre/loxP technology has been used to trace $A_{2A}R$ neurons (Zhang *et al*., 2013). In the NAc, adenosine $A_{2A}Rs$ are predominantly localized in the GABAergic enkephalinergic neurons (Rosin *et al*., 2003a; Ferré *et al*., 2007; Schiffmann *et al*., 2007). Therefore, it has been thought that NAc adenosine $A_{2A}R$ neurons are GABAergic projection neurons, which was also proved in the present experiment by double immunofluorescence staining for hrGFP/GABA-positive and hrGFP/DARPP-32. However, Figure 1 showed some single-labeled hrGFP cells instead of double-labeled cells, which may be due to ectopic Cre expression in the NAc in the $A_{2A}R$-Cre mice (Cre expression in cells that are GABA negative) or due to sufficient Cre expression in neurons only weakly expressing GABA mRNA or protein (Stuber *et al*., 2015). It is also plausible that transient expression during development may be enough to drive Cre-recombinase in cells that do not express $A_{2A}R$ in the adult (Liu *et al*., 2021).

The classic endogenous somnogen adenosine affects behavior arousal via excitatory adenosine $A_{2A}Rs$ on neurons in the NAc (Satoh *et al*., 1999; Lazarus *et al*., 2011). Previous study showed that elevated adenosine levels in the NAc core promote slow-wave sleep (SWS) via adenosine $A_{2A}Rs$, which suggested that adenosine is a plausible candidate molecule for activating NAc core $A_{2A}R$ neurons to induce SWS (Zhou *et al*., 2019). Orexin A induces a robust increase in the firing activity of orexin neurons, while adenosine has an inhibitory effect, and orexin neurons are an important target in endogenous adenosine-regulated sleep homeostasis (Cun *et al*. 2014). Inhibitory responses in the LH are induced by stimulating NAc in the electrophysiological experiments (Mogenson *et al*., 1983). Stimulation of NAc significantly inhibits the activity of orexin neurons in the LH (Wei *et al*., 2015). The sleep-promoting process induced by the microdialysis-perfusion of the NAc with $A_{2A}R$ agonist is associated with a decline in the activity of orexin neurons (Satoh *et al*., 2006). However, the morphological basis of this kind of interaction remains uncertain. Previous tracing studies demonstrated that NAc projections terminate throughout the rostro-caudal extent of the LH (Heimer *et al*., 1991; Usuda, 1998). Consistent with these data, NAc $A_{2A}R$ neurons projection to the LHa, the LHt, and the LHm (Zhang *et al*., 2013). It has been found that NAc medium spiny neurons (MSNs) terminal area in the LHa is distinctly separate from orexin neurons (Sano & Yokoi, 2007). We also did not observe apparent contacts of hrGFP-IR terminals onto soma or dendrite of orexin neurons in the LHa. But the present study showed that NAc $A_{2A}R$ neurons directly innervate the orexin neurons of the LHt.

Furthermore, it has been demonstrated that orexin neurons send efferent projections to NAc (Peyron *et al*., 1998; Nambu *et al*., 1999), but it is not known whether NAc adenosine $A_{2A}R$ neurons also receive direct inputs from orexin neurons. The electrophysiological studies showed that orexin neurons can directly depolarize NAc neurons, and that NAc neurons are involved in the cellular mechanisms through which orexin neurons participate in the regulation of the arousal behavior (Mukai *et al*., 2009). Neuronal circuits between the NAc adenosine $A_{2A}R$ and orexin neurons in the hypothalamus are hypothesized to regulate sleep-wake (Lazarus *et al*., 2012). Our present results also showed that the axons of orexin neurons appeared to contact soma and dendrites of NAc GABAergic and adenosine $A_{2A}R$ neurons.

The present results showed orexin varicosities contain VGluT2 and Syp, which indicates orexin varicosities may have the capacity to store and release Glu in the NAc. Thus, orexin-containing neurons can excite NAc GABAergic neurons and adenosine $A_{2A}R$ neurons through the synaptic release of Glu. Similar results have also been shown that some orexin-positive varicosities contain separate populations of Orexinergic and glutamatergic vesicles in the tuberomammillary nucleus (TMN) (Torrealba *et al*., 2003; Schöne *et al*., 2012), the VLPO (De Luca *et al*., 2022), and the LC (Henny *et al*., 2010). So, glutamate has been thought to be the likely candidate for co-release with orexin (Ziegler *et al*., 2002; Rosin *et al*., 2003b). No orexin terminals are found to be immunostained for the vesicular transporter for VGAT. That orexin terminals might contain an inhibitory amino acid vesicular transporter was thought possible since not all orexin terminals were seen to make asymmetric synapses in electron microscopic studies within the VTA (Balcita-Pedicino & Sesack, 2007). However, GABA was not detected by electron microscopic studies of orexin terminals in the hypothalamus (Torrealba *et al*., 2003), mRNA for the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD), was also not detected by *in situ* hybridization in orexin neurons (Rosin *et al*., 2003b), and these reports confirms our results.

Based on our present findings, we suggest a neuroanatomical circuit model between NAc adenosine $A_{2A}R$ neurons and orexin neurons in the hypothalamus (Fig. 5). In this model, NAc adenosine $A_{2A}R$ neurons send GABAergic inhibitory efferent projections to the orexin neurons, which then send excitatory efferent to NAc adenosine $A_{2A}R$ neurons. Previous pharmaco-behavioral study partly supports this proposed neuronal circuit model. For example, high-frequency stimulation of NAc induced an increase in GABA level in the LHA and significantly inhibited the activity of orexin neurons in the LH (Wei *et al*., 2015). Intra-NAc of orexin was found to induce feeding response and stimulate locomotor activity (Thorpe & Kotz, 2005), and feeding behavior elicited from the NAc was shown to reduce by pharmacological inactivation of the LH (Maldonado-Irizarry *et al*., 1995). Also, infusion of carbachol into the LH was shown to induce antinociception, and the antinociception induce by LH stimulation was shown to be prevented by NAC pre-administration of SB334867 (orexin-A receptor antagonist) (Sadeghi *et al*., 2013). Besides feeding behavior, locomotion, and antinociception, both the LH and NAc have been implicated in sleep-arousal (Lazarus *et al*., 2012). Orexin-A can directly depolarize NAc neurons, and that NAc neurons are involved in the cellular mechanisms through which orexin participates in the regulation of the arousal behavior (Mukai *et al*., 2009). The sleep-promoting process induced by the microdialysis-perfusion of the NAc with $A_{2A}R$ agonist is associated with a decline in the activity of orexin neurons (Satoh *et al*., 2006). It is possible, therefore, that the neuroanatomical circuit between the NAc adenosine $A_{2A}R$ neurons and orexin-containing neurons visualized in the present study might also be involved in regulating these behaviors.

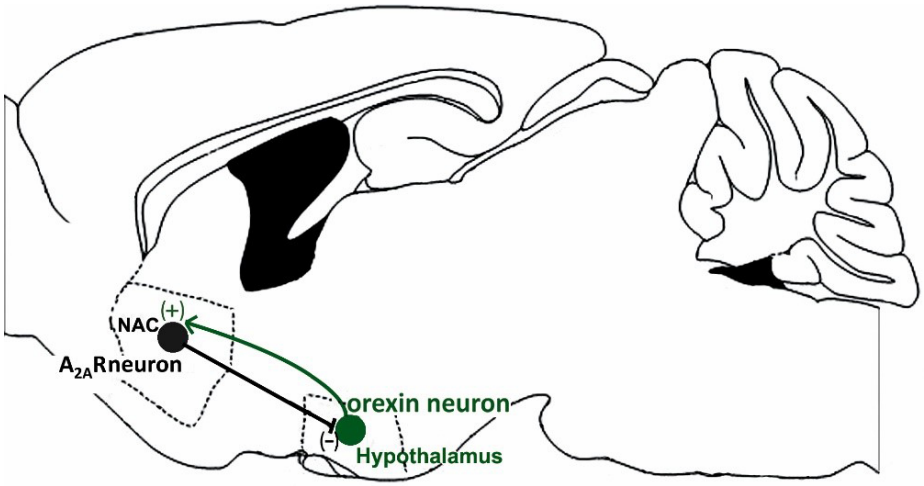

Fig. 5. A possible neuroanatomical circuit model composed of NAc A2AR neurons and the orexin neurons. GABAergic NAc A2AR neurons send inhibitory (-) efferent to the orexin neurons. orexin neurons send excitatory (+) efferent to NAc A2AR neurons.

CONCLUSIONS

Based upon the evidence in this study, the present study proposes a neuroanatomical circuit model between NAc adenosine $A_{2A}R$ neuron and orexin neurons. This improves our understanding of the possible roles of the NAc $A_{2A}R$, especially in the regulation of sleep.

ACKNOWLEDGMENTS. We would like to thank Prof. Zhi-Li Huang for technical assistance and discussions.

ZHANG, J. P & LI, R. X. Relación neuroanatómica entre las neuronas del núcleo accumbens $A_{2A}R$ y las neuronas orexina en el cerebro del ratón. *Int. J. Morphol., 42(5)*:1267-1277, 2024.

RESUMEN: El objetivo de este trabajo fue explorar la evidencia morfológica de los circuitos neuronales entre las neuronas del receptor de adenosina A_{2A} (A2AR) en el núcleo accumbens (NAc) y las neuronas de orexina. El virus adenoasociado (AAV) que codifica la proteína fluorescente verde Renilla humanizada (hrGFP) se utilizó como trazador para visualizar la expresión de las neuronas $A_{2A}R$ en la NAc y sus distribuciones de axones en las neuronas de orexina. El AAV dependiente de Cre se inyectó en la NAc en ratones adenosina $A_{2A}R$-Cre. Luego se utilizó inmunohistoquímica para visualizar hrGFP y sus proyecciones en las neuronas de orexina. Los datos revelaron que las neuronas NAc $A_{2A}R$, expresadas principalmente en neuronas de proyección acidérgicas γ-aminobutíricas (GABAérgicas), se proyectaban hacia el hipotálamo, con contactos aparentes de botones que contenían hrGFP en el soma y las dendritas inmunorreactivas con orexina (IR). También observamos una fuerte tinción de las terminales varicosas de orexina-IR en la NAc, con contactos aparentes de las terminales de orexina-IR en las neuronas $A_{2A}R$. Además, en la NAc, las varicosidades de orexina-IR contenían transportador vesicular de glutamato 2 ($VGluT_2$) y sinaptofisina (Syp). Estos datos proporcionan evidencia morfológica para explorar la función entre las neuronas NAc $A_{2A}R$ y la neurona orexina en el hipotálamo, particularmente en la regulación de los procesos del sueño.

PALABRAS CLAVE: Núcleo accumbens; Receptor de adenosina A_{2A}; Hipotálamo; Orexina; Ratón; Excitación.

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