Activation of Bombesin Receptor Subtype-3 Influences Activity of Orexin Neurons by Both Direct and Indirect Pathways

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BRS3 and orexin neurons

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Abstract

The neuropeptides orexin A and orexin B (also known as hypocretin 1 and hypocretin 2), produced in lateral hypothalamic neurons, are critical regulators of feeding behavior, the reward system and sleep/wake states. Orexin-producing neurons (orexin neurons) are regulated by various factors involved in regulation of energy homeostasis and sleep/wakefulness states. Bombesin receptor subtype 3 (BRS3) is an orphan receptor that might be implicated in energy homeostasis, and is highly expressed in the hypothalamus. However, the neural pathway by which BRS3 regulates energy homeostasis is largely unknown. We examined whether BRS3 is involved in the regulation of orexin neurons. Using a calcium imaging method, we found that a selective BRS3 agonist (Ac-Phe-Trp-Ala-His-(τBzl)-Nip-Gly-Arg-NH2) increased the intracellular calcium concentration of orexin neurons. However, intracellular recordings from slice preparations revealed that the BRS3 agonist hyperpolarized orexin neurons. The BRS3 agonist depolarized orexin neuron in the presence of tetrodotoxin. Moreover, in the presence of GABA receptor blockers, picrotoxin and CGP55845, the BRS3 agonist induced depolarization and increased firing frequency. Additionally, double-label in situ

hybridization study revealed that *Brs3* mRNA was expressed in almost all orexin neurons and many cells around these neurons. These findings suggest that the BRS3 agonist indirectly inhibited orexin neurons through GABAergic input and directly activated orexin neurons. Inhibition of activity of orexin neurons through BRS3 might be an important pathway for regulation of feeding and sleep/wake states. This pathway might serve as a novel target for the treatment of obesity.

Introduction

The neuropeptides orexin A and orexin B were identified as endogenous ligands for two orphan G-protein-coupled receptors (GPCRs) (Sakurai, 1998). Orexin-producing neurons (orexin neurons) are localized exclusively in the lateral hypothalamic area (LHA), which is known as the feeding center. Various evidence has suggested that this neuropeptide is involved in the regulation of feeding behavior (Sakurai, 1998; Edwards, 1999; Haynes, 2000; Haynes, 2002). Subsequently, the finding that orexin deficiency causes narcolepsy in humans and animals suggested that these hypothalamic neuropeptides also play a critical role in regulating sleep and wakefulness (Chemelli, 1999; Lin, 1999; Peyron, 2000; Thannickal, 2000; Hara, 2001).

Recent studies of the efferent and afferent systems of orexin-producing neurons, as well as phenotypic characterization of mice with genetic alterations in the orexin system, have suggested further roles of orexin in the coordination of emotion, energy homeostasis, reward, drug addiction, and arousal. Orexin neurons receive abundant input from the limbic system, preoptic sleep-promoting neurons, and hypothalamic neurons implicated in energy homeostasis. Orexin neurons are also regulated by peripheral

metabolic cues, including ghrelin, leptin and glucose, suggesting that orexin neurons might provide a link between energy homeostasis and sleep/wake states (Yamanaka, 2003b).

Bombesin receptor subtype 3 (BRS3) is a member of the bombesin receptor subfamily of GPCRs, which also includes the neuromedin B- and gastrin-releasing peptide receptors (NMBR and GRPR) (Gorbulev, 1992). BRS3 couples to endogenous Gq family proteins and increases intracellular calcium concentration. BRS3 has a unique pharmacological profile and does not bind with high affinity with any known bombesin-related peptides. Although a number of molecules that can activate human BRS3 have been developed (Mantey, 1997; Liu, 2002; Weber, 2003; Boyle, 2005; Gonzalez, 2008; Zhang, 2009), its natural ligands are still unknown (Jensen, 2008).

BRS3 is primarily expressed in the CNS, and is present in the highest amounts in the hypothalamic area where orexin neurons are localized (Ohki-Hamazaki, 1997a; Yamada, 1999; Liu, 2002; Jennings, 2003; Sano, 2004). Although the normal physiological function of BRS3 is largely unknown, mice lacking the BRS3 gene develop late onset obesity, accompanied by a reduced metabolic rate and increased food intake

(Ohki-Hamazaki, 1997b). In good agreement with the *Brs3*$^{-/-}$ mouse study, a BRS3 agonist, Bag-1, reduced food intake and increased metabolic rate (Guan, 2010). These studies suggested that BRS3 might be an important regulator of feeding behavior and energy homeostasis. A recent study showed that the anorectic effects of the BRS3 agonist remained intact in *neuropeptide Y*$^{-/-}$, *agouti-related peptide*$^{-/-}$, *melanocortin 4 receptor*$^{-/-}$, *cannabinoid receptor 1*$^{-/-}$ and *leptin receptor*$^{db/db}$ mice (Guan, 2010). These results suggest that the effect of the BRS3 agonist might be mediated through other feeding mechanisms. However, the neural pathways of feeding behavior mediated by BRS3 are largely undefined. The orexin pathway is one of the possible pathways by which the BRS3 agonist evokes its effects, because orexin neurons are regulated by several factors involved in energy metabolism, and expression of BRS3 were found in the LHA. To investigate this possibility, we evaluated the effect of a BRS3 agonist on orexin neurons. In this study, we found that a BRS3 agonist activated orexin neurons directly, while it also inhibited these neurons through activation of local GABAergic internerons.

Materials and Methods

Drugs

The BRS3 agonist, neuromedin B (Peptide Institute, Osaka, Japan), tetrodotoxin (TTX) (Wako, Osaka, Japan), picrotoxin (Sigma, St. Louis, MO), and CGP55845 (Wako) were dissolved in extracellular solution. We also used a synthesized a selective BRS3 agonist (Ac-Phe-Trp-Ala-His (τBzl)-Nip-Gly-Arg-NH_2) (Boyle, 2005).

Animals

All experimental procedures involving animals were approved by the Kanazawa University Animal Care and Use Committee and were in accordance with NIH guidelines. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used. Genetically-modified mice used in this study were *Orexin/YC2.1* mice (Tsujino, 2005) and *Orexin/EGFP* mice (Yamanaka, 2003a).

In situ hybridization

Double in situ hybridization was performed according to procedures previously described (Mieda, 2006). Digoxigenin (DIG)-labeled riboprobes for *mBrs3* were synthesized from a 1196 bp fragment of murine *Brs3* cDNA (nucleotides –172 - +1367 from the initiation codon) containing 1196 bp of the whole coding region cloned into the pCRII vector

(Invitrogen). Fluorescein (FITC)-labeled riboprobes for *prepro-Orexin* were synthesized by in vitro transcription. pBluescript II SK (+) containing a 0.5 kb fragment encoding Gln33-Val130 of *prepro-Orexin* was used as a template for in vitro transcription (Sakurai, 1998). The DIG and FITC-labeled probes were detected by means of anti-DIG (1/2000) and anti-FITC (1/1000) antibodies conjugated with alkaline phosphatase (Roche Diagnostics, Basel, Switzerland). Alkaline phosphatase activity was detected with NBT/BCIP and INT/BCIP (Roche Diagnostics).

Brain slice preparation

Orexin/YC2.1 mice (3-8 weeks old) and *Orexin/EGFP* mice (3-6 weeks old) were anesthetized with forane (Abbott, Osaka, Japan). The mice were decapitated under deep anesthesia. Brains were isolated in ice-cold cutting solution consisting of (mM): 280 sucrose, 2 KCl, 10 HEPES, 0.5 $CaCl_2$, 10 $MgCl_2$, 10 glucose, pH 7.4, bubbled with 100% O_2. Brains were cut coronally into 300-µm slices with a vibratome (VTA-1000S, Leica, Germany). Slices containing the LHA were transferred for 1 hr to an incubation chamber at room temperature filled with physiological solution containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgSO_4$, 26 $NaHCO_3$, 11 glucose, pH 7.4, bubbled

with 95% O_2/5% CO_2.

Calcium imaging of orexin neurons

Optical recordings were performed on a fluorescence microscope (BX51WI, Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device (CCD) camera (CoolSNAP HQ2, Roper Scientific, Tucson, AZ) controlled by MetaFluor 5.0.7 software (Universal Imaging, West Chester, PA). YC2.1 was excited through a 440DF20 filter, and its fluorescent image was subjected to dual emission ratio imaging through two emission filters (480DF30 for ECFP, 535DF26 for EYFP) controlled by a filter changer (ProscanII, Prior Scientific Instruments, Cambridge, UK).

Electrophysiological recording

Orexin/EGFP mice were used for whole cell recordings. The slices were transferred to a recording chamber (RC-27L, Warner Instrument Corp., CT, USA) at room temperature on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). Neurons that showed GFP fluorescence were used for patch-clamp recordings. The fluorescence microscope was equipped with an infrared camera (C-3077, Hamamatsu Photonics, Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a

CCD camera (JK-TU53H, Olympus) for fluorescent imaging. Each image was displayed separately on a monitor. Recordings were carried out with an Axopatch 700B amplifier (Axon Instruments, Foster City, CA) using a borosilicate pipette (GC150-10, Harvard Apparatus, Holliston, MA) prepared by a micropipette puller (P-97, Sutter Instruments, Pangbourne, UK) and filled with intracellular solution (4-10 MΩ), consisting of (mM): 125 K-gluconate, 5 KCl, 1 $MgCl_2$, 10 HEPES, 1.1 EGTA-Na_3, 5 MgATP, 0.5 Na_2GTP, pH7.3 with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer (model 5520, Wescor, Logan, UT). The osmolarity of the internal and external solutions was 280-290 and 320-330 mOsm/l, respectively. The liquid junction potential of the patch pipette and perfused extracellular solution was estimated to be -16.2 mV and was applied to the data. The recording pipette was under positive pressure while it was advanced toward an individual cell in the slice. A tight seal of 0.5-1.0 GΩ was made by applying negative pressure. The membrane patch was then ruptured by suction. The series resistance during recording was 10-25 MΩ and was compensated. The reference electrode was an Ag-AgCl pellet immersed in the bath solution. During recordings, cells were superfused with extracellular solution at a rate of 1.0-2.0 ml/min using a peristaltic

pump (K.T. Lab, Japan) at RT.

RESULTS

Selective BRS3 agonist influences activity of orexin neurons by direct and indirect pathways

Recent studies have suggested that the activity of orexin neurons is influenced by various factors involved in sleep/wake states or feeding behavior. It is also possible that BRS3 may regulate the activity of orexin neurons, because *Brs3*$^{-/-}$ mice developed obesity and impairment of glucose metabolism (Ohki-Hamazaki, 1997b). To examine the possibility that BRS3 is involved in regulation of the activity of orexin neurons, we applied a selective BRS3 agonist (Ac-Phe-Trp-Ala-His (τBzl)-Nip-Gly-Arg-NH2) to orexin neurons using brain slice preparations of *Orexin/YC2.1* transgenic mice, in which orexin neurons specifically express the calcium-indicator protein, yellow cameleon (YC) 2.1 (Tsujino, 2005), and observed the change in intracellular calcium concentration ($[Ca^{2+}]_i$) of orexin neurons. In the presence of tetrodotoxin (TTX), the BRS3 agonist increased the YFP/CFP ratio, showing that $[Ca^{2+}]_i$ was increased (Fig. 1A). The BRS3 agonist-induced

increase in $[Ca^{2+}]_i$ was concentration-dependent (Fig. 1B) (n=3-6). Of YFP-positive neurons tested, 91.8% were activated by the BRS3 agonist (n=49), but they showed no response to neuromedin B, an NMBR agonist (n=11).

We also studied the effect of the BRS3 agonist on orexin neurons in detail by slice patch clamp recording. Unexpectedly, BRS3 agonist (1μM) bath application potently hyperpolarized and decreased the firing frequency of orexin neurons under the whole cell current clamp mode (n=7, Fig. 2A). On the other hand, the BRS3 agonist (1μM) induced depolarization of orexin neurons in the presence of TTX (Fig. 2B). Furthermore, the BRS3 agonist (1μM) induced depolarization and increased the firing frequency of orexin neurons in the presence of both picrotoxin and CGP55845 ($GABA_A$ and $GABA_B$ receptor blockers, respectively) (Fig. 2C). These observations suggest that the BRS3 agonist induced depolarization of orexin neurons directly, but more potently hyperpolarized these neurons through activation of GABAergic interneurons.

Brs3 and _Orexin_ are colocalized in lateral hypothalamic neurons

To examine the tissue localization of BRS3, we performed double-label in situ hybridization. We found that BRS3-expressing neurons were widely distributed in the

CNS. Relatively large numbers of cells were found in the cortex, hippocampus, amygdala and LHA (Fig. 3A and B). We found that almost all neurons expressing *Orexin* mRNA expressed *Brs3* mRNA (Fig. 3C and D).

Discussion

Although BRS3 still remains an orphan receptor, it is thought to be a possible drug target for treating obesity and the metabolic syndrome, because BRS3-deficient mice show late onset obesity and impaired glucose metabolism.

In the present study, we found that a selective BRS3 agonist hyperpolarized all orexin neurons under current clamp recording (Fig. 2A) but depolarized them in the presence of GABA receptor antagonists, picrotoxin and CGP55845 (Fig. 2C). This result suggests that the inhibitory effect on orexin neurons was mediated by activation of GABAergic input. Consistently, under inhibition of neural input by TTX, the effect of the agonist on orexin neurons was excitatory, and intracellular Ca^{2+} of orexin neurons was increased presumably through activation of the Gq-subfamily of G- proteins (Fig. 2B). In situ hybridization study revealed that *Brs3* mRNA was expressed widely in hypothalamic areas (Fig. 3A and B), in agreement with previous studies (Ohki-Hamazaki, 1997a;

Yamada, 1999; Guan, 2010; Jennings, 2003; Sano, 2004). Double-label in situ hybridization study showed that almost all orexin neurons also expressed *Brs3* mRNA (Fig. 3C and D). Both electrophysiological and histochemical analyses suggested that the BRS3 agonist activated orexin neurons directly through activation of BRS3 on orexin neurons. However, the direct depolarizing effect of the BRS3 agonist appeared only when neural input was suppressed by TTX or a GABA blocker. This suggests that the indirect inhibitory effect predominated over the direct excitatory effect. Consistently, we observed many BRS3-positive neurons in the LHA by in situ hybridization, which could possibly be GABAergic interneurons, although further studies are required to reveal the origin of GABAergic input to orexin neurons.

From a physiological viewpoint, unknown natural ligands of BRS3 also might inhibit the activity of orexin neurons and suppress feeding behavior. Although a recent report suggested that a BRS3 agonist increased inhibitory synaptic transmission of the arcuate nucleus (Guan, 2010), other pathways regulating feeding behavior have not yet been clarified. This study revealed a new pathway of a BRS3 agonist. Our results also suggest the new possibility that BRS3 might be an important factor in sleep/wake

regulation, because the orexin system is considered to be a critical regulator of sleep/wake regulation.

Figure legends

Fig. 1

FRET calcium imaging study showing that the BRS3 agonist influenced orexin neuron activity, using a hypothalamic slice from *Orexin/YC2.1* mice. A, In the presence of tetrodotoxin (TTX), bath application of the BRS3 agonist (1μM) increased the YFP/CFP ratio, suggesting that the BRS3 agonist (1μM) increased $[Ca^{2+}]_i$ in orexin neurons. B, The BRS3 agonist induced a concentration-dependent increase in $[Ca^{2+}]_i$ (EC_{50}: 45.7 ± 2.4 nM).

Fig. 2

A, Under whole cell current clamp mode, the BRS3 agonist (1μM) induced hyperpolarization of orexin neurons and decreased firing frequency. B, In the presence of TTX, orexin neurons were depolarized by the BRS3 agonist (1μM). C, In the presence of both picrotoxin ($GABA_A$ blocker) and CGP55845 ($GABA_B$ blocker), the BRS3 agonist (1μM) depolarized orexin neurons and increased their firing frequency.

Fig. 3

Double-label in situ hybridization histochemistry showing distributions of *Orexin* (brown) and *Brs3* (blue) mRNA. A, *Brs3* mRNA was distributed widely in the CNS, especially in the cortex, hippocampus, amygdala and LHA. B, Schematic representation of distribution of *Brs3* and *Orexin* mRNA (bregma -1.34 mm). C. High power view of lateral hypothalamic area. D, High power view of region in rectangle in C. *Orexin* mRNA colocalized with *Brs3* mRNA. Yellow arrowheads show colocalization.

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Figure1

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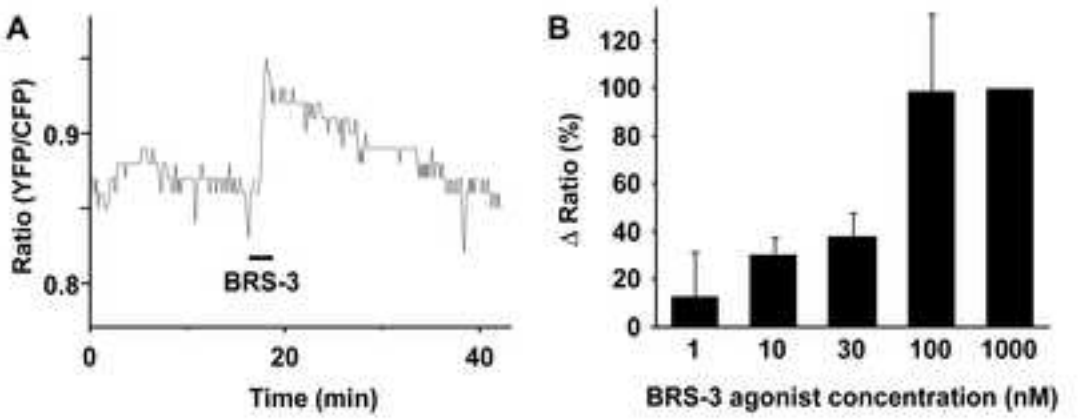

Furutani et al. Figure 1

Figure2

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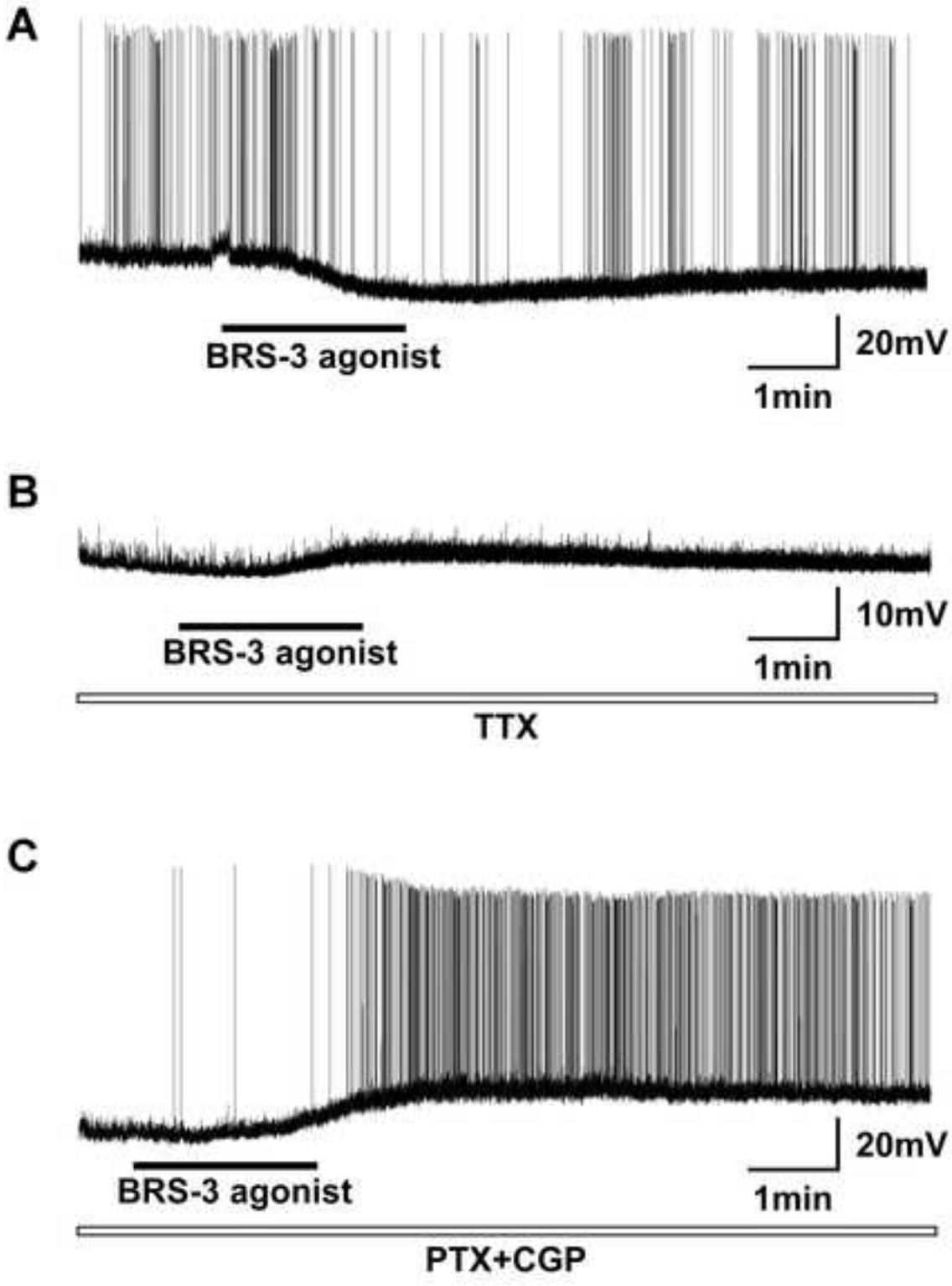

Furutani et al. Figure 2

Figure3

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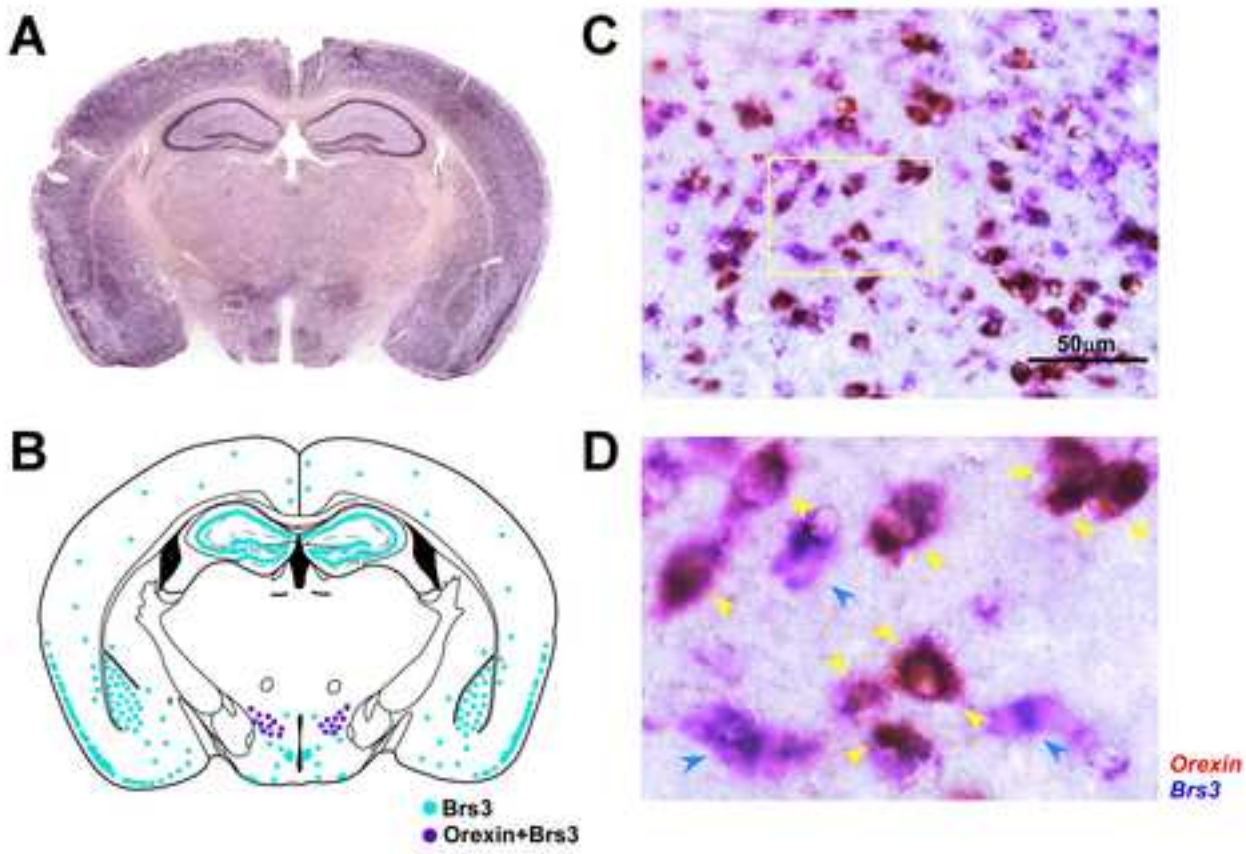

Furutani et al. Figure 3