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3 **Activation of Bombesin Receptor Subtype-3 Influences Activity of Orexin Neurons**  
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7 **by Both Direct and Indirect Pathways**  
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21 *This manuscript is related to the Yakushima 2009 symposium.*  
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24 a) Running title:  
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28 BRS3 and orexin neurons  
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56 **Number of Figures: 3**  
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**Number of Pages:** 20

**Keywords:** orexin, BRS3, patch clamp, calcium imaging, food intake, sleep

**Acknowledgements**

This study was supported by Grants-in-aid for Scientific Research (B), and for Young Scientists (B). Authors thank Dr. Wendy Gray for critical reading.

1  
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3 **Abstract**  
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7 The neuropeptides orexin A and orexin B (also known as hypocretin 1 and hypocretin 2),  
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10 produced in lateral hypothalamic neurons, are critical regulators of feeding behavior, the  
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12 reward system and sleep/wake states. Orexin-producing neurons (orexin neurons) are  
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14 regulated by various factors involved in regulation of energy homeostasis and  
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16 sleep/wakefulness states. Bombesin receptor subtype 3 (BRS3) is an orphan receptor that  
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18 might be implicated in energy homeostasis, and is highly expressed in the hypothalamus.  
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21 However, the neural pathway by which BRS3 regulates energy homeostasis is largely  
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23 unknown. We examined whether BRS3 is involved in the regulation of orexin neurons.  
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25 Using a calcium imaging method, we found that a selective BRS3 agonist  
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27 (Ac-Phe-Trp-Ala-His-( $\tau$ Bzl)-Nip-Gly-Arg-NH<sub>2</sub>) increased the intracellular calcium  
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29 concentration of orexin neurons. However, intracellular recordings from slice  
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31 preparations revealed that the BRS3 agonist hyperpolarized orexin neurons. The BRS3  
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33 agonist depolarized orexin neuron in the presence of tetrodotoxin. Moreover, in the  
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35 presence of GABA receptor blockers, picrotoxin and CGP55845, the BRS3 agonist  
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37 induced depolarization and increased firing frequency. Additionally, double-label in situ  
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3 hybridization study revealed that *Brs3* mRNA was expressed in almost all orexin neurons  
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7 and many cells around these neurons. These findings suggest that the BRS3 agonist  
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10 indirectly inhibited orexin neurons through GABAergic input and directly activated  
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13 orexin neurons. Inhibition of activity of orexin neurons through BRS3 might be an  
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16 important pathway for regulation of feeding and sleep/wake states. This pathway might  
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21 serve as a novel target for the treatment of obesity.  
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3 **Introduction**  
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7 The neuropeptides orexin A and orexin B were identified as endogenous ligands for two  
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9 orphan G-protein-coupled receptors (GPCRs) (Sakurai, 1998). Orexin-producing  
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11 neurons (orexin neurons) are localized exclusively in the lateral hypothalamic area  
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13 (LHA), which is known as the feeding center. Various evidence has suggested that this  
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15 neuropeptide is involved in the regulation of feeding behavior (Sakurai, 1998; Edwards,  
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17 1999; Haynes, 2000; Haynes, 2002). Subsequently, the finding that orexin deficiency  
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19 causes narcolepsy in humans and animals suggested that these hypothalamic  
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21 neuropeptides also play a critical role in regulating sleep and wakefulness (Chemelli,  
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23 1999; Lin, 1999; Peyron, 2000; Thannickal, 2000; Hara, 2001).  
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39         Recent studies of the efferent and afferent systems of orexin-producing neurons,  
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41 as well as phenotypic characterization of mice with genetic alterations in the orexin  
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43 system, have suggested further roles of orexin in the coordination of emotion, energy  
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45 homeostasis, reward, drug addiction, and arousal. Orexin neurons receive abundant input  
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47 from the limbic system, preoptic sleep-promoting neurons, and hypothalamic neurons  
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49 implicated in energy homeostasis. Orexin neurons are also regulated by peripheral  
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3 metabolic cues, including ghrelin, leptin and glucose, suggesting that orexin neurons  
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7 might provide a link between energy homeostasis and sleep/wake states (Yamanaka,  
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10 2003b).

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14 Bombesin receptor subtype 3 (BRS3) is a member of the bombesin receptor  
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17 subfamily of GPCRs, which also includes the neuromedin B- and gastrin-releasing  
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20 peptide receptors (NMBR and GRPR) (Gorbulev, 1992). BRS3 couples to endogenous  
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23 Gq family proteins and increases intracellular calcium concentration. BRS3 has a unique  
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26 pharmacological profile and does not bind with high affinity with any known  
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29 bombesin-related peptides. Although a number of molecules that can activate human  
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32 BRS3 have been developed (Mantey, 1997; Liu, 2002; Weber, 2003; Boyle, 2005;  
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35 Gonzalez, 2008; Zhang, 2009), its natural ligands are still unknown (Jensen, 2008).  
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42 BRS3 is primarily expressed in the CNS, and is present in the highest amounts  
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45 in the hypothalamic area where orexin neurons are localized (Ohki-Hamazaki, 1997a;  
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48 Yamada, 1999; Liu, 2002; Jennings, 2003; Sano, 2004). Although the normal  
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51 physiological function of BRS3 is largely unknown, mice lacking the BRS3 gene develop  
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56 late onset obesity, accompanied by a reduced metabolic rate and increased food intake  
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3 (Ohki-Hamazaki, 1997b). In good agreement with the *Brs3*<sup>-/-</sup> mouse study, a BRS3  
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7 agonist, Bag-1, reduced food intake and increased metabolic rate (Guan, 2010). These  
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10 studies suggested that BRS3 might be an important regulator of feeding behavior and  
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13 energy homeostasis. A recent study showed that the anorectic effects of the BRS3 agonist  
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16 remained intact in *neuropeptide Y*<sup>-/-</sup>, *agouti-related peptide*<sup>-/-</sup>, *melanocortin 4 receptor*<sup>-/-</sup>,  
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19 *cannabinoid receptor 1*<sup>-/-</sup> and *leptin receptor*<sup>db/db</sup> mice (Guan, 2010). These results  
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22 suggest that the effect of the BRS3 agonist might be mediated through other feeding  
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25 mechanisms. However, the neural pathways of feeding behavior mediated by BRS3 are  
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28 largely undefined. The orexin pathway is one of the possible pathways by which the  
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31 BRS3 agonist evokes its effects, because orexin neurons are regulated by several factors  
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34 involved in energy metabolism, and expression of BRS3 were found in the LHA. To  
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37 investigate this possibility, we evaluated the effect of a BRS3 agonist on orexin neurons.  
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42 In this study, we found that a BRS3 agonist activated orexin neurons directly, while it  
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49 also inhibited these neurons through activation of local GABAergic interneurons.  
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## 56 **Materials and Methods**

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3 **Drugs**  
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7 The BRS3 agonist, neuromedin B (Peptide Institute, Osaka, Japan), tetrodotoxin (TTX)  
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10 (Wako, Osaka, Japan), picrotoxin (Sigma, St. Louis, MO), and CGP55845 (Wako) were  
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13 dissolved in extracellular solution. We also used a synthesized a selective BRS3 agonist  
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17 (Ac-Phe-Trp-Ala-His ( $\tau$ Bzl)-Nip-Gly-Arg-NH<sub>2</sub>) (Boyle, 2005).  
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21 **Animals**  
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24 All experimental procedures involving animals were approved by the Kanazawa  
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28 University Animal Care and Use Committee and were in accordance with NIH guidelines.  
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31 All efforts were made to minimize animal suffering and discomfort and to reduce the  
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35 number of animals used. Genetically-modified mice used in this study were  
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39 *Orexin/YC2.1* mice (Tsuji, 2005) and *Orexin/EGFP* mice (Yamanaka, 2003a).  
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42 ***In situ hybridization***  
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45 Double in situ hybridization was performed according to procedures previously described  
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49 (Mieda, 2006). Digoxigenin (DIG)-labeled riboprobes for *mBrs3* were synthesized from  
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53 a 1196 bp fragment of murine *Brs3* cDNA (nucleotides -172 - +1367 from the initiation  
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57 codon) containing 1196 bp of the whole coding region cloned into the pCRII vector  
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3 (Invitrogen). Fluorescein (FITC)-labeled riboprobes for *prepro-Orexin* were synthesized  
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7 by in vitro transcription. pBluescript II SK (+) containing a 0.5 kb fragment encoding  
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10 Gln33-Val130 of *prepro-Orexin* was used as a template for in vitro transcription (Sakurai,  
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13 1998). The DIG and FITC-labeled probes were detected by means of anti-DIG (1/2000)  
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15  
16 and anti-FITC (1/1000) antibodies conjugated with alkaline phosphatase (Roche  
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18 Diagnostics, Basel, Switzerland). Alkaline phosphatase activity was detected with  
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21 NBT/BCIP and INT/BCIP (Roche Diagnostics).  
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### 27 ***Brain slice preparation***

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30 *Orexin/YC2.1* mice (3-8 weeks old) and *Orexin/EGFP* mice (3-6 weeks old) were  
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32 anesthetized with forane (Abbott, Osaka, Japan). The mice were decapitated under deep  
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35 anesthesia. Brains were isolated in ice-cold cutting solution consisting of (mM): 280  
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38 sucrose, 2 KCl, 10 HEPES, 0.5 CaCl<sub>2</sub>, 10 MgCl<sub>2</sub>, 10 glucose, pH 7.4, bubbled with 100%  
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41 O<sub>2</sub>. Brains were cut coronally into 300- $\mu$ m slices with a vibratome (VTA-1000S, Leica,  
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44 Germany). Slices containing the LHA were transferred for 1 hr to an incubation chamber  
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53 at room temperature filled with physiological solution containing (mM): 125 NaCl, 2.5  
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56 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2.0 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 11 glucose, pH 7.4, bubbled  
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3 with 95% O<sub>2</sub>/5% CO<sub>2</sub>.  
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### 6 7 ***Calcium imaging of orexin neurons*** 8 9

10 Optical recordings were performed on a fluorescence microscope (BX51WI, Olympus,  
11 Tokyo, Japan) equipped with a cooled charge-coupled device (CCD) camera (CoolSNAP  
12 HQ2, Roper Scientific, Tucson, AZ) controlled by MetaFluor 5.0.7 software (Universal  
13 Imaging, West Chester, PA). YC2.1 was excited through a 440DF20 filter, and its  
14 fluorescent image was subjected to dual emission ratio imaging through two emission  
15 filters (480DF30 for ECFP, 535DF26 for EYFP) controlled by a filter changer (ProscanII,  
16 Prior Scientific Instruments, Cambridge, UK).  
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### 34 35 ***Electrophysiological recording*** 36 37

38 *Orexin/EGFP* mice were used for whole cell recordings. The slices were transferred to a  
39 recording chamber (RC-27L, Warner Instrument Corp., CT, USA) at room temperature  
40 on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). Neurons that  
41 showed GFP fluorescence were used for patch-clamp recordings. The fluorescence  
42 microscope was equipped with an infrared camera (C-3077, Hamamatsu Photonics,  
43 Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a  
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4 CCD camera (JK-TU53H, Olympus) for fluorescent imaging. Each image was displayed  
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7 separately on a monitor. Recordings were carried out with an Axopatch 700B amplifier  
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10 (Axon Instruments, Foster City, CA) using a borosilicate pipette (GC150-10, Harvard  
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13 Apparatus, Holliston, MA) prepared by a micropipette puller (P-97, Sutter Instruments,  
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16 Pangbourne, UK) and filled with intracellular solution (4-10 M $\Omega$ ), consisting of (mM):  
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18 125 K-gluconate, 5 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 1.1 EGTA-Na<sub>3</sub>, 5 MgATP, 0.5 Na<sub>2</sub>GTP,  
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21 pH7.3 with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer  
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24 (model 5520, Wescor, Logan, UT). The osmolarity of the internal and external solutions  
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27 was 280-290 and 320-330 mOsm/l, respectively. The liquid junction potential of the  
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30 patch pipette and perfused extracellular solution was estimated to be -16.2 mV and was  
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33 applied to the data. The recording pipette was under positive pressure while it was  
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36 advanced toward an individual cell in the slice. A tight seal of 0.5-1.0 G $\Omega$  was made by  
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39 applying negative pressure. The membrane patch was then ruptured by suction. The  
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42 series resistance during recording was 10-25 M $\Omega$  and was compensated. The reference  
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45 electrode was an Ag-AgCl pellet immersed in the bath solution. During recordings, cells  
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48 were superfused with extracellular solution at a rate of 1.0-2.0 ml/min using a peristaltic  
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3 pump (K.T. Lab, Japan) at RT.  
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## 10 **RESULTS**

### 11 ***Selective BRS3 agonist influences activity of orexin neurons by direct and indirect*** 12 13 **pathways**

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21 Recent studies have suggested that the activity of orexin neurons is influenced by various  
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24 factors involved in sleep/wake states or feeding behavior. It is also possible that BRS3  
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27 may regulate the activity of orexin neurons, because *Brs3*<sup>-/-</sup> mice developed obesity and  
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30 impairment of glucose metabolism (Ohki-Hamazaki, 1997b). To examine the possibility  
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33 that BRS3 is involved in regulation of the activity of orexin neurons, we applied a  
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36 selective BRS3 agonist (Ac-Phe-Trp-Ala-His (τBzl)-Nip-Gly-Arg-NH<sub>2</sub>) to orexin  
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39 neurons using brain slice preparations of *Orexin/YC2.1* transgenic mice, in which orexin  
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42 neurons specifically express the calcium-indicator protein, yellow cameleon (YC) 2.1  
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45 (Tsuji, 2005), and observed the change in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>)  
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49 of orexin neurons. In the presence of tetrodotoxin (TTX), the BRS3 agonist increased the  
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56 YFP/CFP ratio, showing that [Ca<sup>2+</sup>]<sub>i</sub> was increased (Fig. 1A). The BRS3 agonist-induced  
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3 increase in  $[Ca^{2+}]_i$  was concentration-dependent (Fig. 1B) (n=3-6). Of YFP-positive  
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7 neurons tested, 91.8% were activated by the BRS3 agonist (n=49), but they showed no  
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11 response to neuromedin B, an NMBR agonist (n=11).  
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14 We also studied the effect of the BRS3 agonist on orexin neurons in detail by  
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17 slice patch clamp recording. Unexpectedly, BRS3 agonist (1 $\mu$ M) bath application  
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21 potently hyperpolarized and decreased the firing frequency of orexin neurons under the  
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24 whole cell current clamp mode (n=7, Fig. 2A). On the other hand, the BRS3 agonist  
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27 (1 $\mu$ M) induced depolarization of orexin neurons in the presence of TTX (Fig. 2B).  
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31 Furthermore, the BRS3 agonist (1 $\mu$ M) induced depolarization and increased the firing  
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34 frequency of orexin neurons in the presence of both picrotoxin and CGP55845 (GABA<sub>A</sub>  
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36  
37 and GABA<sub>B</sub> receptor blockers, respectively) (Fig. 2C). These observations suggest that  
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42 the BRS3 agonist induced depolarization of orexin neurons directly, but more potently  
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45 hyperpolarized these neurons through activation of GABAergic interneurons.  
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### 48 ***Brs3* and *Orexin* are colocalized in lateral hypothalamic neurons**

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52 To examine the tissue localization of BRS3, we performed double-label in situ  
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56 hybridization. We found that BRS3-expressing neurons were widely distributed in the  
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3 CNS. Relatively large numbers of cells were found in the cortex, hippocampus, amygdala  
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7 and LHA (Fig. 3A and B). We found that almost all neurons expressing *Orexin* mRNA  
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10 expressed *Brs3* mRNA (Fig. 3C and D).  
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## 12 13 14 **Discussion** 15

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17 Although BRS3 still remains an orphan receptor, it is thought to be a possible drug target  
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21 for treating obesity and the metabolic syndrome, because BRS3-deficient mice show late  
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25 onset obesity and impaired glucose metabolism.  
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28 In the present study, we found that a selective BRS3 agonist hyperpolarized all  
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31 orexin neurons under current clamp recording (Fig. 2A) but depolarized them in the  
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35 presence of GABA receptor antagonists, picrotoxin and CGP55845 (Fig. 2C). This result  
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39 suggests that the inhibitory effect on orexin neurons was mediated by activation of  
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43 GABAergic input. Consistently, under inhibition of neural input by TTX, the effect of the  
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47 agonist on orexin neurons was excitatory, and intracellular  $Ca^{2+}$  of orexin neurons was  
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51 increased presumably through activation of the Gq-subfamily of G- proteins (Fig. 2B). In  
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3 Yamada, 1999; Guan, 2010; Jennings, 2003; Sano, 2004). Double-label in situ  
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7 hybridization study showed that almost all orexin neurons also expressed *Brs3* mRNA  
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10 (Fig. 3C and D). Both electrophysiological and histochemical analyses suggested that the  
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14 BRS3 agonist activated orexin neurons directly through activation of BRS3 on orexin  
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17 neurons. However, the direct depolarizing effect of the BRS3 agonist appeared only when  
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21 neural input was suppressed by TTX or a GABA blocker. This suggests that the indirect  
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25 inhibitory effect predominated over the direct excitatory effect. Consistently, we  
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28 observed many BRS3-positive neurons in the LHA by in situ hybridization, which could  
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32 possibly be GABAergic interneurons, although further studies are required to reveal the  
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35 origin of GABAergic input to orexin neurons.  
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39 From a physiological viewpoint, unknown natural ligands of BRS3 also might  
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41  
42 inhibit the activity of orexin neurons and suppress feeding behavior. Although a recent  
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45 report suggested that a BRS3 agonist increased inhibitory synaptic transmission of the  
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48 arcuate nucleus (Guan, 2010), other pathways regulating feeding behavior have not yet  
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52 been clarified. This study revealed a new pathway of a BRS3 agonist. Our results also  
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56 suggest the new possibility that BRS3 might be an important factor in sleep/wake  
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regulation, because the orexin system is considered to be a critical regulator of  
sleep/wake regulation.

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3 **Figure legends**  
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7 Fig. 1  
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10 FRET calcium imaging study showing that the BRS3 agonist influenced orexin neuron  
11 activity, using a hypothalamic slice from *Orexin/YC2.1* mice. A, In the presence of  
12  
13 tetrodotoxin (TTX), bath application of the BRS3 agonist (1 $\mu$ M) increased the YFP/CFP  
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15 ratio, suggesting that the BRS3 agonist (1 $\mu$ M) increased [Ca<sup>2+</sup>]<sub>i</sub> in orexin neurons. B, The  
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17 BRS3 agonist induced a concentration-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> (EC<sub>50</sub>: 45.7  $\pm$  2.4  
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35 Fig. 2  
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38 A, Under whole cell current clamp mode, the BRS3 agonist (1 $\mu$ M) induced  
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41 hyperpolarization of orexin neurons and decreased firing frequency. B, In the presence of  
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44 TTX, orexin neurons were depolarized by the BRS3 agonist (1 $\mu$ M). C, In the presence of  
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48 both picrotoxin (GABA<sub>A</sub> blocker) and CGP55845 (GABA<sub>B</sub> blocker), the BRS3 agonist  
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53 (1 $\mu$ M) depolarized orexin neurons and increased their firing frequency.  
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3 Fig. 3  
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7 Double-label in situ hybridization histochemistry showing distributions of *Orexin*  
8 (brown) and *Brs3* (blue) mRNA. A, *Brs3* mRNA was distributed widely in the CNS,  
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10 especially in the cortex, hippocampus, amygdala and LHA. B, Schematic representation  
11 of distribution of *Brs3* and *Orexin* mRNA (bregma -1.34 mm). C. High power view of  
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13 lateral hypothalamic area. D, High power view of region in rectangle in C. *Orexin* mRNA  
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15 colocalized with *Brs3* mRNA. Yellow arrowheads show colocalization.  
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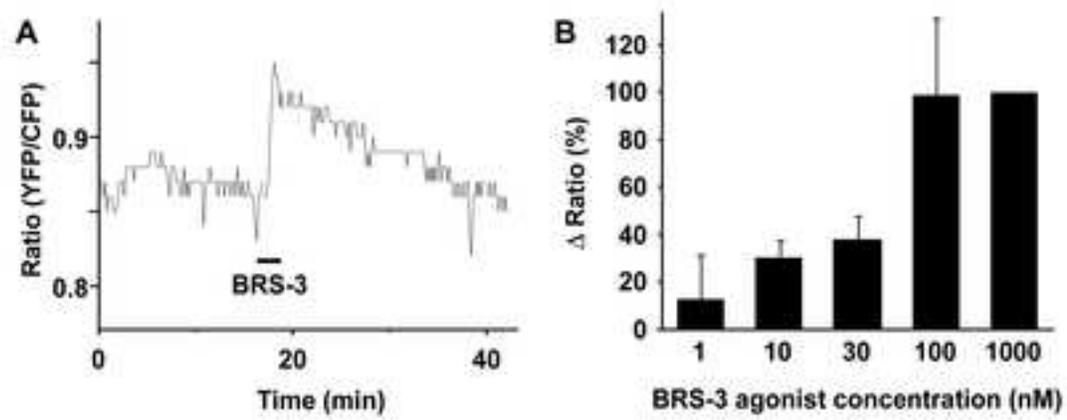
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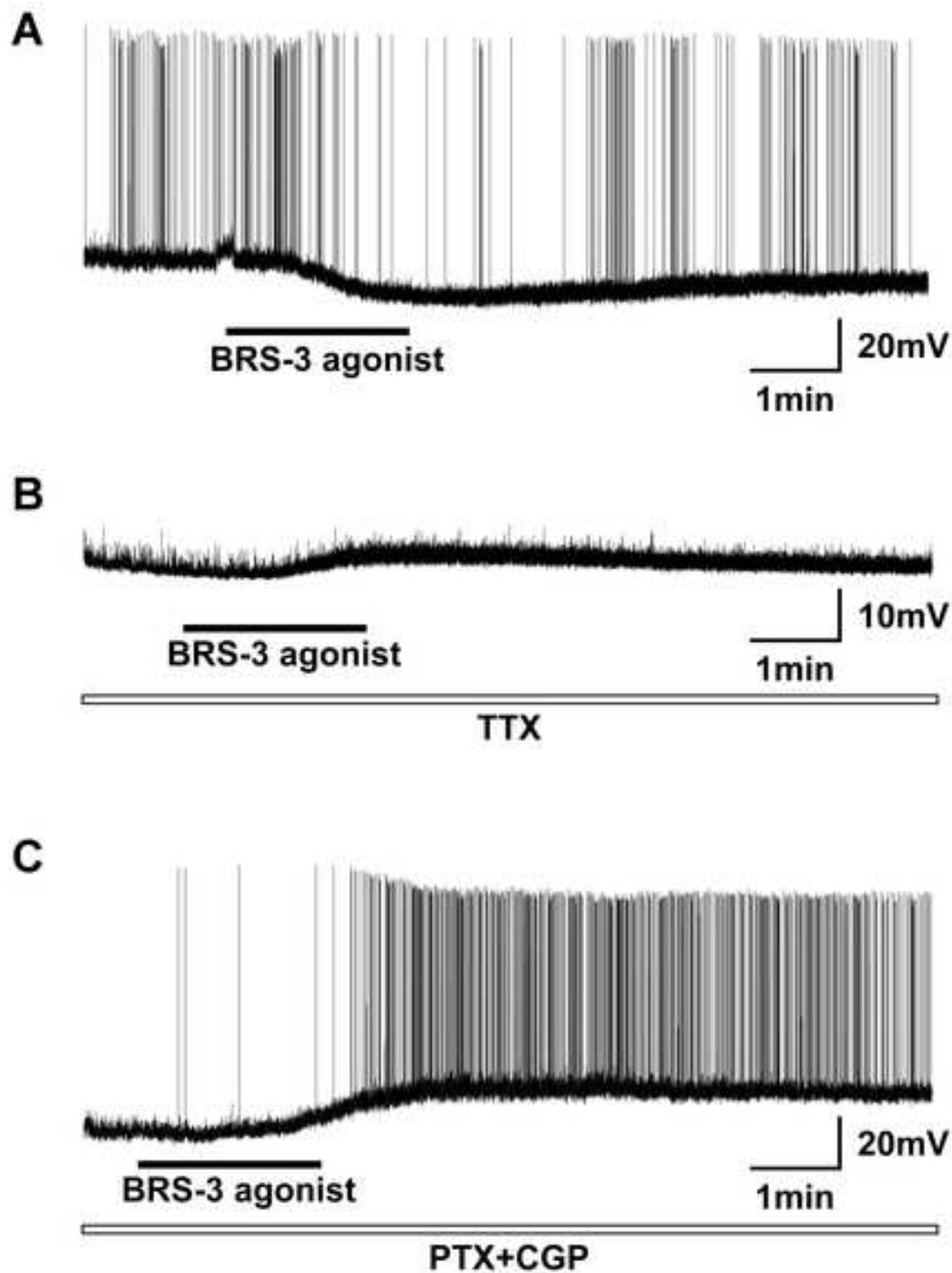
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Figure 1

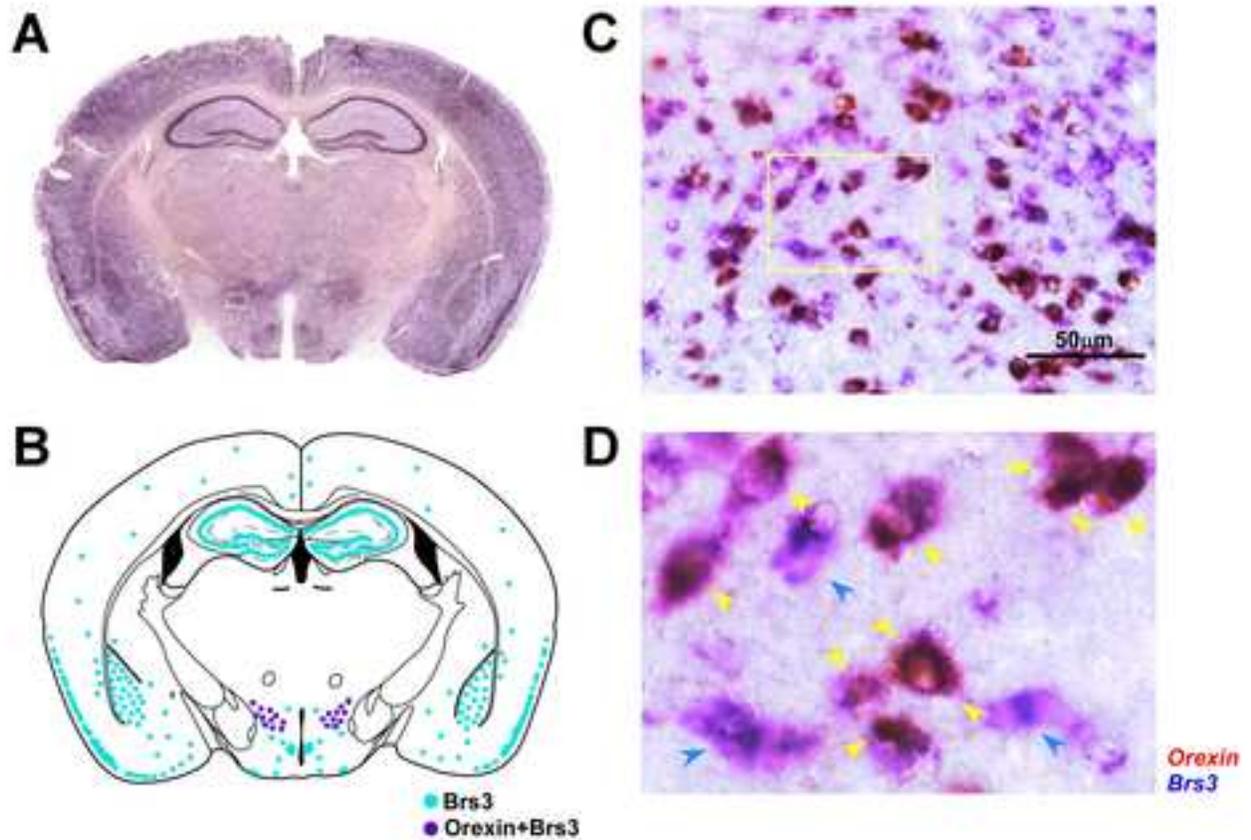
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Furutani et al. Figure 1



Furutani et al. Figure 2



Furutani et al. Figure 3