CrossMark

The Neuropeptide Orexin-A Inhibits the GABA$_A$ Receptor by PKC and Ca^{2+}/CaMKII-Dependent Phosphorylation of Its β$_1$ Subunit

Divya Sachidanandan[1] · Haritha P. Reddy[2] · Anitha Mani[3] · Geoffrey J. Hyde[4] ·
Amal Kanti Bera[1]

Abstract Orexin-A and orexin-B (Ox-A, Ox-B) are neuropeptides produced by a small number of neurons that originate in the hypothalamus and project widely in the brain. Only discovered in 1998, the orexins are already known to regulate several behaviours. Most prominently, they help to stabilise the waking state, a role with demonstrated significance in the clinical management of narcolepsy and insomnia. Orexins bind to G-protein-coupled receptors (predominantly postsynaptic) of two subtypes, OX$_1$R and OX$_2$R. The primary effect of Ox-OXR binding is a direct depolarising influence mediated by cell membrane cation channels, but a wide variety of secondary effects, both pre- and postsynaptic, are also emerging. Given that inhibitory GABAergic neurons also influence orexin-regulated behaviours, crosstalk between the two systems is expected, but at the cellular level, little is known and possible mechanisms remain unidentified. Here, we have used an expression system approach to examine the feasibility, and nature, of possible postsynaptic crosstalk between Ox-A and the GABA$_A$ receptor (GABA$_A$R), the brain's main inhibitory neuroreceptor. When HEK293 cells transfected with OX$_1$R and the α$_1$, β$_1$, and γ$_{2S}$ subunits of GABA$_A$R were exposed to Ox-A, GABA-induced currents were inhibited, in a calcium-dependent manner. This inhibition was associated with increased phosphorylation of the β$_1$ subunit of GABA$_A$R, and the inhibition could itself be attenuated by (1) kinase inhibitors (of protein kinase C and CaM kinase II) and (2) the mutation, to alanine, of serine 409 of the β$_1$ subunit, a site previously identified in phosphorylation-dependent regulation in other pathways. These results are the first to directly support the feasibility of postsynaptic crosstalk between Ox-A and GABA$_A$R, indicating a process in which Ox-A could promote phosphorylation of the β$_1$ subunit, reducing the GABA-induced, hyperpolarising current. In this model, Ox-A/GABA$_A$R crosstalk would cause the depolarising influence of Ox-A to be boosted, a type of positive feedback that could, for example, facilitate the ability to abruptly awake.

Keywords GABA · Orexin · Phosphorylation · Sleep-wake cycle · Protein kinase

✉ Amal Kanti Bera
    amal@iitm.ac.in

[1] Present address: Department of Neurophysiology, Institute of Physiology, University of Würzburg, Röntgenring 9, 97070 Würzburg, Germany

[2] Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Sardar Patel Road, Chennai, Tamil Nadu 600036, India

[3] Present address: Life Science Division, AU-KBC Research Centre, MIT Campus, Anna University, Chromepet, Chennai, Tamil Nadu 600044, India

[4] Write About Research, 14 Randwick Street, Randwick, Sydney 2031, Australia

Introduction

Since being independently co-discovered in 1998, the orexins (or hypocretins) have been shown to have important roles in the regulation of metabolism, stress, and behaviours such as feeding, reward seeking, and arousal (Sakurai et al. 1998; de Lecea et al. 1998; Leonard and Kukkonen 2014; Kukkonen and Leonard 2014; Sakurai et al. 2015). Orexins are peptides (orexin-A (Ox-A) and orexin-B (Ox-B)) produced by a relatively small number of neurons that originate in the hypothalamus and project into large areas of the cerebral cortex and many regions of the brainstem and basal forebrain (Peyron et al. 1998; Nambu et al. 1999). Most notably, orexins bind

to class A, G-protein-coupled receptors (predominantly post-synaptic) of two subtypes and distinct (but overlapping) distributions in the brain: OX_1R is highly selective for Ox-A while OX_2R binds both Ox-A and Ox-B equally well (Sakurai et al. 1998; Marcus et al. 2001; Sakurai 2007; Scammell and Winrow 2011; Kukkonen 2013). The most researched function of the orexinergic neurons has been their contribution to the regulation of arousal and the sleep/wake cycle (Sakurai et al. 2015); evidence strongly indicates that their input into various nuclei of the ascending arousal system lowers an animal's arousal threshold and stabilises the waking state, findings of considerable clinical importance (Peyron et al. 2000; Thannickal et al. 2000; Adamantidis et al. 2007; Adamantidis et al. 2010; Saper et al. 2010; Scammell and Winrow 2011; Sakurai et al. 2015; Uslaner et al. 2015).

Given their biological and clinical significance, it is not surprising that the cellular and molecular responses to orexins have been much studied (Kukkonen and Leonard 2014; Leonard and Kukkonen 2014; Sakurai et al. 2015). Upon ligand binding, OX_1R and OX_2R activate one or more heterotrimeric G-proteins, initiating a number of primary and secondary responses. Most significantly, activation at postsynaptic sites results in the altered activity of cation channels, leading to a slow, persistent depolarisation that is sufficient to trigger or augment neuronal firing (de Lecea et al. 1998; Leonard and Ishibashi 2015). In addition to their primary, postsynaptic, depolarising effects, a wide range of secondary responses to orexins, both pre- and postsynaptic, continue to be found (Belle et al. 2014; Leonard and Ishibashi 2015; Palus et al. 2015; Ishibashi et al. 2016). One theme that has emerged from the study of the secondary effects has been that they can oppose the primary postsynaptic effect, suppressing neuronal firing, e.g. by presynaptically boosting release of the inhibitory neurotransmitter GABA (Belle et al. 2014; Palus et al. 2015; Ishibashi et al. 2016). These results, and those that show that some orexinergic neurons directly excite GABAergic neurons (Liu et al. 2002; Burdakov et al. 2003), have led to the proposal that the collective excitation and inhibition mechanisms of the orexins could serve as a negative feedback loop (Liu et al. 2002; Palus et al. 2015). At the organismal level, the potential for one or more forms of crosstalk between the orexinergic and GABAergic systems would be advantageous: their principal neurons co-project into several regions of the brain, e.g. the tuberomammillary nucleus, and the locus coeruleus (Peyron et al. 1998; Sherin et al. 1998); also, any brain region with orexinergic projections will be rich in interneurons, which are primarily GABAergic (McBain and Fisahn 2001; Markram et al. 2004).

Clinically, the complementary symptoms of diseases in which orexin, or GABA signalling, is disturbed and the overlapping effects of orexin antagonists and GABA agonists on insomnia (Scammell and Winrow 2011; Betschart et al. 2013) also indicate that the two systems converge on some of the same regulatory systems, in physiologically significant ways. However, while it is clear that the orexins' modulatory effects on other neuronal players, and particularly GABA, are substantial, the evidence for orexin-GABA crosstalk involving a single cell has been restricted to presynaptic mechanisms.

Here, we have used an expression system approach to examine the feasibility, and nature, of postsynaptic crosstalk between Ox-A and the $GABA_A$ receptor ($GABA_AR$), the brain's main inhibitory neuroreceptor (Sieghart and Sperk 2002). In electrophysiological experiments on single cells, we observed that application of Ox-A to HEK293 cells transfected with OX_1R and subunits of $GABA_AR$ resulted in pronounced calcium-dependent inhibition of the inward Cl^- current typically associated with activation of $GABA_AR$ (Taleb et al. 1986). This inhibition was then investigated using pharmacological agents, and mutants in which the β_1 subunit of $GABA_AR$ had been altered, to dissect the signalling pathway downstream of OX_1R. Protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) were found to have prominent roles in orexin-mediated inhibition of $GABA_AR$. This study has indicated, for the first time, that orexins are capable, in principle, of modulating the activity level of $GABA_AR$, by promoting phosphorylation of its β_1 subunit. If this form of crosstalk also occurs in vivo, it would differ in kind from the previously reported examples of orexin/GABA (system-level) crosstalk, in that the secondary effect of the orexin signal would be to reinforce, not reduce, its primary, depolarising effect.

Materials and Methods

Expression of $GABA_AR$ and OX_1R in HEK293 Cells

Human orexin receptor (hOX_1R) cDNA in pCRII plasmid was a kind gift from Dr. Takeshi Sakurai, Department of Molecular Neuroscience and Integrative Physiology, Kanazawa University, Japan. Human $GABA_A$ receptor subunit clones of α_1 and β_1 in pCIS2 vector and γ_{2S} in pcDNA 3.1(+) vector, respectively, were kindly provided by Dr. Neil Harrison, Department of Anesthesiology and Pharmacology, Columbia University Medical Center, NY, USA. $GABA_AR$ myc-tagged β_1 subunit clone, used for immunoprecipitation experiments, was purchased from OriGene Technologies (Rockville, MD, USA). HEK293 cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X Antibiotic-Antimycotic mixture (Gibco, USA), in a sterile incubator at 37 °C with 5% CO_2. Plasmids encoding the respective proteins were transfected using Lipofectamine 2000 (Invitrogen, USA). Twenty-four-hour posttransfection, the cells were further plated on glass coverslips for electrophysiological experiments.

Calcium Imaging

Ox-A-mediated Ca^{2+} rise was monitored using Fura-2 dye. Cells were loaded with 4 μM Fura-2AM in HEPES-buffered medium (HBM) (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, 1 mM probenecid (dissolved in 1 N NaOH), 20 mM HEPES, 1.2 mM $MgCl_2$, pH 7.4) for 30 min at room temperature. The cells were then washed with fresh HBM and further incubated for 30 min. The coverslip containing Fura-loaded cells was placed in a recording chamber mounted on an Olympus IX-71 inverted microscope, fitted with an Andor CCD camera (Andor Technology, UK). The cells were continuously perfused with HBM, and images were captured by illumination with dual excitation wavelengths of 340 and 380 nm, while capturing the emission signal at 510 nm with the help of appropriate excitation and emission filters (Chroma Technology, USA) at intervals of 5 s. Fast switching between the excitation filters was achieved using a Lambda DG-4 system (Sutter Instrument Company, USA). Ratiometric analysis was done offline, using background-subtracted images using Andor IQ software (Andor Technology, UK).

Electrophysiology

Whole-cell patch-clamp experiments were performed as described earlier (Sachidanandan and Bera 2015). The external bath solution contained the following (in mM): 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4, adjusted with NaOH), and 10 D-glucose. GABA currents were elicited by using 30 μM of GABA dissolved in the abovementioned external solution. Ox-A stock at 50 μM was made in water and stored at −20 °C; the working concentration of Ox-A used was 100 nM. Whole-cell patch-clamp recordings were performed by clamping the cells at −60 mV. All experiments were performed at room temperature. The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunoprecipitation and Western Blotting

HEK293 cells expressing $GABA_AR$ with myc-tagged $β_1$ subunit, in the presence or absence of OX_1R, were treated with 100 nM Ox-A peptide for 10 min and then lysed using RIPA buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 50 mM Tris HCl (pH 8.0)). The cell lysate was further used for immunoprecipitation using pre-washed protein A/G agarose beads and c-myc antibody (Santa Cruz Biotechnology, USA). The samples were separated on a 12% SDS-PAGE gel, transferred to a PVDF membrane and probed with anti-phosphoserine antibody (Santa Cruz Biotechnology, USA) at 1:1000 concentration. A loading control was provided by stripping the blot and probing with anti-c-myc antibody.

Site-Directed Mutagenesis

The mutants of $β_1$ subunit, S384A and S409A, were created using appropriate primers and PCR reactions. The primers used were as follows:

S384A: Forward: 5′-GCCTCGCGGCTGGCCAGGGG CTTGCG-3′
Reverse: 5′-CGCAAGCCCCTGGCCAGCCG CGAGGC-3′
S409A: Forward: 5′ ATCCGCAGGCGTGCCGCCCA GCTCAAAGTCAAGAT 3′
Reverse: 5′ ATCTTGACTTTGAGCTGGGC GGCACGCCTGCGGAT 3′

The mutations were confirmed by sequencing.

Statistics

All data points are represented as mean ± SEM of 3–10 independent experiments, as indicated in the figure legends. Student's t test was performed for analysing statistical significance. Statistical significance of the results is represented as "*" for $P ≤ 0.05$, "**" for $P ≤ 0.01$, and "***" for $P ≤ 0.001$.

Results

Functional Expression of OX_1R in HEK293 Cells

The activity of OX_1R in HEK293 cells was checked by measuring its ability to elevate intracellular calcium upon activation with Ox-A. Cells were co-transfected with the human orexin receptor 1 (hOX_1R) and EGFP, as a reporter. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) were monitored using Fura-2. As shown in Fig. 1a, the application of 100 nM Ox-A increased the $[Ca^{2+}]_i$ immediately in the GFP-positive cells, with the F_{340}/F_{380} ratio increasing almost threefold. In the same samples, non-transfected control cells did not show such a rise, confirming the absence of endogenous OX_1R in HEK293 cells. The efficacy of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) in chelating the $[Ca^{2+}]_i$ was tested. When pre-incubated with 10 μM BAPTA-AM, the $[Ca^{2+}]_i$ of OX_1R-expressing cells did not show any change in response to Ox-A. These results have been summarised in Fig. 1b.

Inhibition of the GABA-Induced Current by Ox-A

Using HEK293 cells transfected with human $GABA_AR$ subunits $α_1$, $β_1$, and $γ_{2S}$ and OX_1R, $GABA_AR$ was activated with 30 μM GABA. When OX_1R was activated with its ligand Ox-A, the GABA-induced current was reduced significantly. One hundred nanomolar of Ox-A inhibited the current by almost
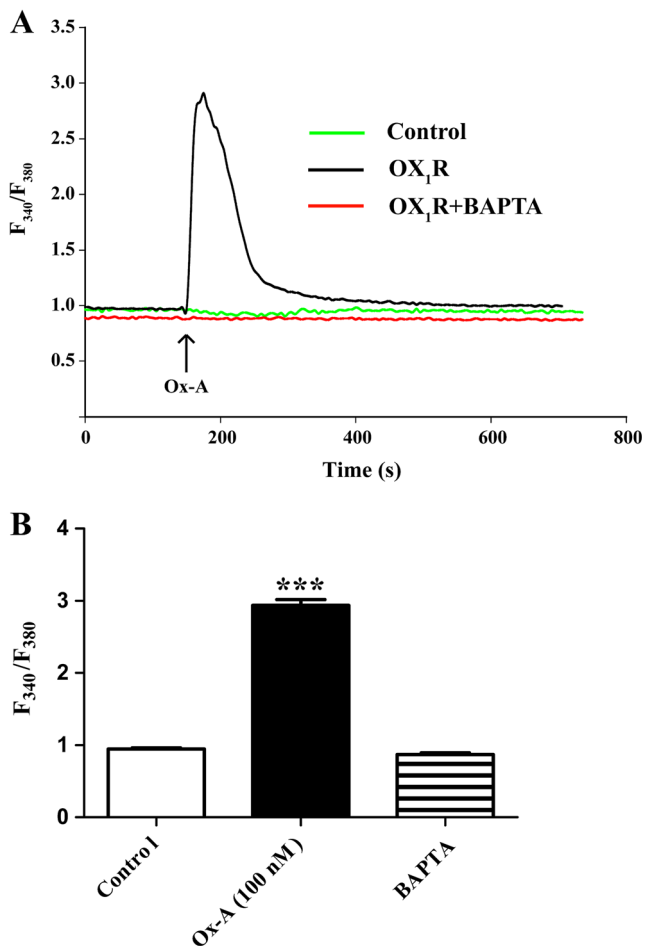
Fig. 1 Functional expression of OX_1R in HEK293 cells. **a** 100 nM Ox-A increased $[Ca^{2+}]_i$ in HEK293 cells expressing OX_1R (*black trace*). Non-transfected control cells showed no change in the basal calcium level (*green trace*). The calcium elevation was not observed in OX_1R-transfected cells, upon incubation with 10 μM BAPTA-AM (*red trace*). **b** *Bar graph* depicts the mean ± SEM of the data obtained from randomly chosen cells ($n = 30$–40). Application of Ox-A resulted in a threefold rise of F_{340}/F_{380} in OX_1R-transfected cells. ****P < 0.001

40% (Fig. 2a). This dosage, which yielded reproducible effects, was used in all subsequent experiments. Also, as the activation of OX_1R was followed by the rise in $[Ca^{2+}]_i$, the latter was used as a proxy for OX_1R activation. When $[Ca^{2+}]_i$ was clamped by including BAPTA (2 mM) in the pipette solution, the inhibitory effect of Ox-A on the GABA-induced current was not seen (Fig. 2b). Likewise, in control cells lacking OX_1R, Ox-A did not inhibit the GABA-induced current, suggesting that it does not interact with $GABA_AR$ directly (Fig. 2c).

Downstream Signalling of OX_1R

Since previous work has shown that activation of the orexin receptors and subsequent rise of $[Ca^{2+}]_i$ are often followed by the activation of several kinases (Nakamura et al. 2015), the effects of various pharmacological agents upon the GABA-

induced current were used to help identify the kinases involved in our system. The control current was first measured by applying 30 μM GABA, followed by incubating the cells with 100 nM Ox-A and one of the inhibitors, after which the GABA current was measured again. The inhibitors used were bisindolylmaleimide-based BIM1 (for protein kinase C, PKC), and the isoquinolinesulfonamide derivatives, KN-62 and PKA-I (for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cAMP-dependent protein kinase A (PKA), respectively). The results are summarised in Fig. 3. Ox-A inhibited the GABA-elicited control current by $42 \pm 4\%$ ($n = 6$). BIM1 and KN-62 attenuated the Ox-A-mediated inhibition significantly, whereas PKA-I had no effect. In the presence of BIM1 (2 μM) and KN-62 (10 μM), Ox-A-mediated inhibition was only $17 \pm 6\%$ ($n = 5$) and $7 \pm 4\%$ ($n = 5$), respectively. PKA-I at a concentration of 4 μM had no effect, as the PKA-I-treated cells exhibited an inhibition of $47 \pm 3\%$ ($n = 5$). Interestingly, phorbol 12-myristate 13-acetate (PMA; 1 μM), a potent activator of PKC, inhibited the GABA-induced current in the absence of Ox-A by $58 \pm 12\%$ ($n = 5$), mimicking the effect of Ox-A. It suggests that Ox-A-mediated inhibition of the GABA-induced current involves the activation of PKC and CaMKII.

Ox-A-mediated inhibition did not require the presence of the γ_{2S} subunit in the transfected cells. When cells containing only the α_1 and β_1 subunits of $GABA_AR$ were treated with Ox-A, the inhibition was as high as when the γ subunit was present, $38 \pm 8\%$ ($n = 10$). Given that the $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1$ forms of $GABA_AR$ responded similarly, the role of the γ_{2S} subunit was not probed further.

Activation of OX_1R Increased Serine Phosphorylation on the $GABA_AR$ β Subunit

There is extensive evidence that the β_1 subunit of $GABA_AR$ is phosphorylated by PKC at a conserved serine, S409, and by CaMKII at S409 and S384 (Krishek et al. 1994; McDonald and Moss 1994). To investigate the effect of Ox-A on the phosphorylation of $GABA_AR$, the myc-tagged β_1 subunit was co-expressed with α_1 and γ_{2S} subunits, along with OX_1R, and these cells were treated with 100 nM Ox-A for 10 min. The β_1 subunit was pulled down using an anti-c-myc antibody and probed using an anti-phosphoserine antibody in a western blot. In the Ox-A-treated cells expressing both $GABA_AR$ and OX_1R, the phosphorylation levels of the β_1 subunit were about three times higher than those in control cells that expressed only $GABA_AR$ and were otherwise treated in the same manner as the test cells (Fig. 4a). The results suggest that Ox-A modulates $GABA_AR$ activity, at least partly, by increasing the phosphorylation level of the β_1 subunit.

Since S409 and S384 are known to be phosphorylation target sites on the β_1 subunit, these sites were mutated to alanine. The effects of Ox-A on GABA current inhibition

Fig. 2 Ox-A attenuated the GABA-induced current. **a** Representative trace of the GABA-induced current, recorded from HEK293 cells transfected with GABA$_A$R (α_1, β_1, γ_{2S}) and OX$_1$R, before and after Ox-A treatment. When incubated with 100 nM Ox-A for 10 min, GABA-induced current was reduced by almost 40%. **b** Inclusion of 2 mM BAPTA in the patch pipette solution blocked this effect, suggesting the role of Ca^{2+} in the process. **c** In HEK293 cells expressing GABA$_A$R, but not OX$_1$R, Ox-A had no effect on the GABA-induced current
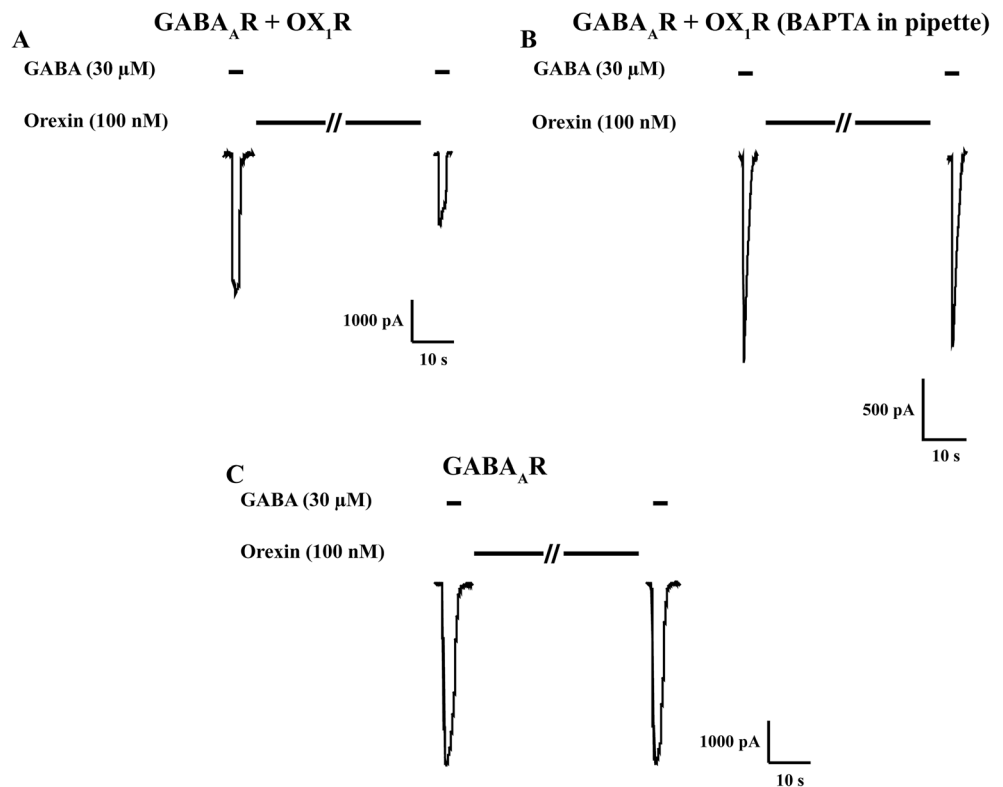
were then tested in cells transfected with OX$_1$R and GABA$_A$R, and containing one of the mutations (S409A or S384A). Interestingly, while cells with the S384A mutation continued to manifest Ox-A-mediated inhibition, similar to the wild type (41 ± 10%, n = 6) (Fig. 4b), cells with the S409A mutation showed a negligible response (10 ± 3%,

Fig. 3 The effect of protein kinase inhibitors on Ox-A-mediated inhibition of the GABA-induced current. Specific inhibitors were used to identify the kinases involved in downstream signalling of Ox-A in HEK293 cells. BIMI, KN-62, and PKA-I are the inhibitors of PKC, CaMKII, and PKA, respectively. **a** The representative traces of GABA-induced current before (*black*) and after (*red*) treatment with the different compounds. **b** BIMI (2 μM) and KN-62 (10 μM), but not PKA-I (4 μM), attenuated Ox-A-mediated inhibition of the GABA-induced current significantly. PMA (1 μM), a PKC activator, mimicked the inhibitory effect of Ox-A. GABA$_A$R, with or without a γ_{2S} subunit, was inhibited to the same extent by Ox-A. Values are the mean ± SEM of 5–10 independent experiments. **$P \leq 0.01$ and ***$P \leq 0.001$
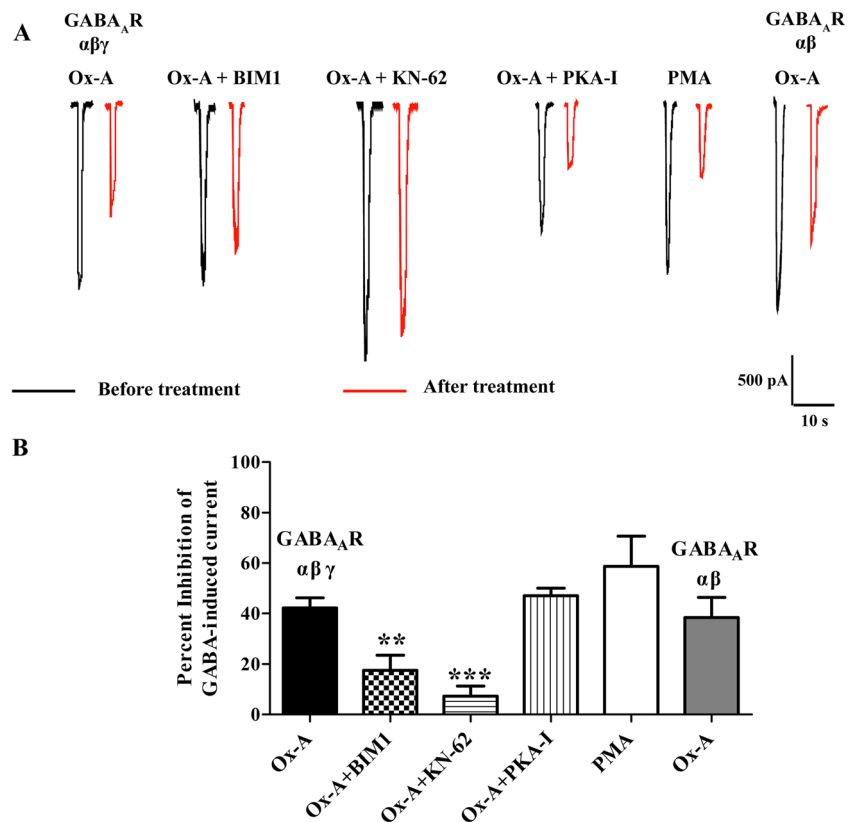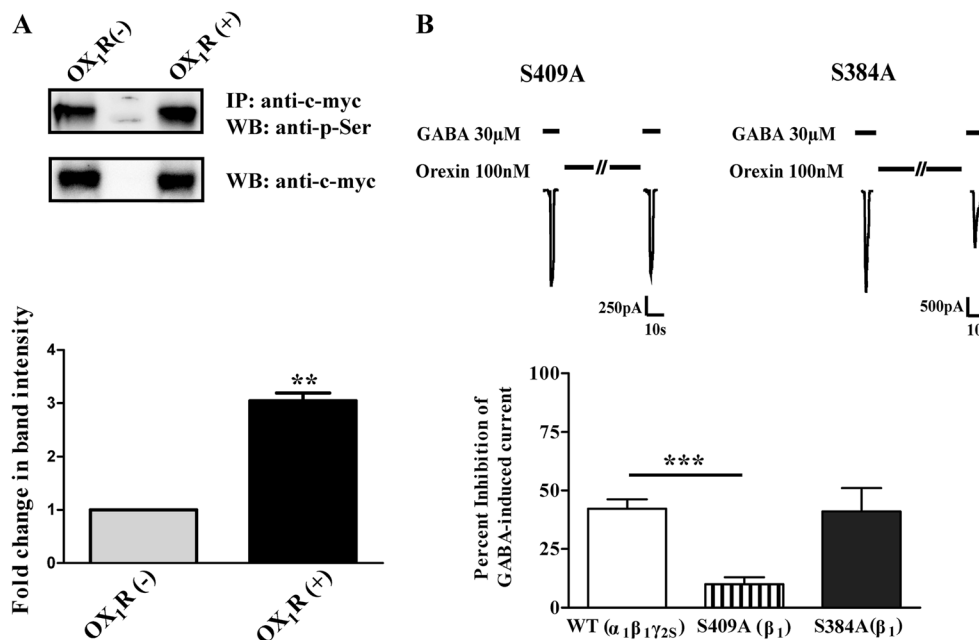
Fig. 4 Ox-A enhanced serine phosphorylation in the β_1 subunit of GABA$_A$R. **a** A representative Western blot depicting the level of phosphorylation in the β_1 subunit. HEK293 cells were transfected with GABA$_A$R, with or without OX$_1$R. Post-Ox-A treatment, immunoprecipitation was performed using anti-c-myc antibody to pull down the myc-tagged β_1 subunit. These subunits were probed with anti-phosphoserine antibody. The level of β_1 phosphorylation is significantly higher in OX$_1$R expressing cells. The blot was stripped and probed with c-myc antibody. Both lanes show equal band intensity, confirming equal loading. The centre lane indicates the marker with two bands. The upper band is 72 kDa and lower band is 55 kDa. *Bar graph* shows the summarised data of three independent experiments. The band intensity of the phosphorylated β_1 subunit was quantified using ImageJ software and normalised with respect to the c-myc-tagged protein band. Bar graph below depicts the fold change, almost three times, in the phosphorylation in the β_1 subunit after orexin treatment. **b** Representative current traces showing the effect of Ox-A on the GABA-induced current when the β_1 subunit of the GABA$_A$ receptor in the transfected cells contained a mutation of serine 409 or serine 384 to alanine. *Bar graph* represents a summary of 6–8 independent experiments. In cells with the S384A mutation, Ox-A inhibited the GABA-induced current, at $41 \pm 10\%$ ($n = 6$) (i.e. as in the wild type) whereas in cells mutated at S409A, the inhibition was significantly less, at $10 \pm 3\%$ ($n = 8$). Values are the mean $\pm$ SEM. $**P < 0.01$, $***P < 0.001$

$n = 8$). This suggests that phosphorylation of the β_1 subunit at S409 is primarily responsible for orexin-mediated inhibition of the GABA-induced current.

Discussion

While multiple lines of evidence (anatomical, physiological, pathological, and pharmacological) indicate that the orexinergic system can modulate the activity of elements of the GABAergic system, these have mainly invoked cell-cell mechanisms, and there has been no report to date of any direct crosstalk between the two systems at the level of the postsynaptic membrane. Below we argue that the results of this in vitro study indicate a range of cellular and molecular responses that could feasibly mediate a physiologically significant postsynaptic interaction between Ox-A and the main inhibitory neuroreceptor, GABA$_A$R.

The first line of evidence for direct crosstalk between Ox-A and GABA$_A$R function was that exposure to Ox-A reduced the amplitude of the GABA$_A$R-mediated, inward Cl$^-$ current in transfected HEK293 cells by 40%. The transfected HEK293 cells also contained the orexin receptor, OX$_1$R, and

their activation by Ox-A is indicated by the immediate rise in intracellular Ca^{2+} levels that followed exposure to Ox-A; such rises are a consistent feature of orexin receptor activation in tissues, and in vitro (van den Pol et al. 1998; Leonard and Ishibashi 2015). When the HEK293 cells contained nearly all the elements of the above system (i.e. GABA$_A$R subunits, GABA, Ox-A) but lacked OX$_1$R, no inhibition was seen, indicating that the inhibition we observed was due to the activation of OX$_1$R by Ox-A, rather than some indirect effect of the peptide. Also consistent with this idea was that, when we used BAPTA to clamp Ca^{2+} levels in the pipette solution, Ox-A had no effect on the GABA current.

Orexin-GABA$_A$R crosstalk was also evidenced, in the transfected HEK cells, by the significantly higher phosphorylation levels of the GABA$_A$R β_1 subunit following exposure to Ox-A. A causal chain that links OX$_1$R activation *to* β_1 subunit phosphorylation *to* GABA current inhibition was supported by our observation that the GABA current inhibition due to Ox-A exposure was attenuated if the preparation also contained an inhibitor of one of the serine threonine/kinases, PKC or CaMKII. Furthermore, when the S409 site of the β_1 subunit, one of the main sites for GABA$_A$R phosphorylation (Nakamura et al. 2015), was mutated to S409A (thus

presumably blocking phosphorylation at this site), the orexin effect was almost abolished.

While this is the first study to provide evidence of this linking pathway (i.e. OX_1R activation *to* β_1 subunit phosphorylation *to* GABA current inhibition), an abundance of other studies support parts of the pathway, or pathways analogous to it. In the most similar example, dopamine has been proposed to cause inhibition of a $GABA_AR$-mediated tonic current via the activation of a G-protein-coupled receptor (D1 or D2), which leads to the phosphorylation of one or more subunits of $GABA_AR$ (Brunig et al. 1999; Hoerbelt et al. 2015). In addition to dopamine, many other factors modulate $GABA_AR$ function (up or down) by boosting phosphorylation levels, including insulin, voltage-gated Ca^{2+} channels, brain-derived neurotrophic factor, glutamate receptors, and $5\text{-}HT_2$ receptors (Nakamura et al. 2015).

Interestingly, in the two other studies that have, as here, induced phosphorylation at the S409 site of the β_1 subunit of $GABA_AR$ in HEK293 cells (by exposure to PKC or PKA, which are both serine/threonine kinases), the amplitude of the GABA current was also decreased (Moss et al. 1992; Krishek et al. 1994). There is one report of an increase in amplitude due to phosphorylation of the $GABA_AR$ β_1 subunit, when mouse L929 fibroblasts were treated with PKC (Lin et al. 1996). That HEK293 and L929 cells, expressing the same β_1 subunit, should respond differently to PKC is not unexpected (nor are the different responses of our cells to PKC/CaMKII and PKA, the latter also being a serine threonine/kinase). Across a wide range of tissues, cell lines, and $GABA_AR$ subunits tested with various phosphorylation modulators, it is clear that phosphorylation of $GABA_AR$ can lead to increased or decreased channel function in a highly context-dependent manner (see review of 25 phosphorylation studies in Nakamura et al. 2015). The natural variations that have been proposed as relevant include the following: the subunit composition of $GABA_AR$; the type or state of the cell; the location of the receptor in the cell; the specific site of phosphorylation, within the same subunit; and the particular isoform of the kinase involved (Lin et al. 1996; Poisbeau et al. 1999; Nakamura et al. 2015). Given that $GABA/GABA_AR$ mediate inhibition throughout the brain, it is neither surprising that so many other systems converge on this system nor that the responses of $GABA_AR$ to modulatory factors, and especially phosphorylation, are so context dependent.

As to how the altered $GABA_AR$ function could result from increased phosphorylation, this was not addressed in this study, but many possibilities exist. If we restrict ourselves to those mechanisms that could be expected to enable changes as rapid as those seen in this study (e.g. excluding the wholesale redistribution of $GABA_AR$ in the cell (Chou et al. 2010)), several reported possibilities remain. Increased phosphorylation could affect the frequency of GABA channel openings, mirroring the way that picrotoxins inhibit the GABA current

(Newland and Cull-Candy 1992). Alternatively, increased phosphorylation has been reported to alter the affinity of $GABA_AR$ for extracellular GABA, thus modulating the GABA current (Brunig et al. 1999; Feigenspan and Bormann 1994).

Conclusions

Expression cell studies have been a pillar of neurobiology, enabling the feasibility of potential mechanisms to be examined in highly tractable systems. In this study, we have demonstrated, for the first time, the feasibility of direct, Ca^{2+}- and phosphorylation-dependent, crosstalk between orexins and $GABA_AR$ in a single cell. Transfected with various $GABA_AR$ subunits, and the receptor for Ox-A, the HEK293 cell is sufficient as a model to test the plausibility of some of the interactions that might occur, in the brain, at the postsynaptic membrane. Previous studies have shown that orexins boost GABA release at the presynaptic membrane in brain tissues, but have not identified the mechanism (Belle et al. 2014; Palus et al. 2015; Leonard and Ishibashi 2015; Ishibashi et al. 2016). In the systems studied in those reports, and in all other systems for which interactions between the orexinergic and GABAergic systems have been reported, the fundamentally hyperpolarising GABAergic signal would be expected to counter the fundamentally depolarising orexinergic signal (Palus et al. 2015). In our study, the suppression of the GABA current by Ox-A represents the first example of an orexin enhancing its depolarising influence via its effect on a component of the GABAergic system. One potential implementation of a positive feedback mechanism such as this, by the brain itself, could be in the regulation of the sleep-wake cycle: orexins could more effectively, and advantageously, accelerate the transition to wakefulness.

Author Contributions D.S. and A.K.B. conceived the idea, analysed the data, and wrote the manuscript. D.S., H.P.R., and A.M. performed the experiments. G.J.H. analysed the data and wrote the manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

References

Adamantidis A, Carter MC, de Lecea L (2010) Optogenetic deconstruction of sleep-wake circuitry in the brain. Front Mol Neurosci 2:31

Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L (2007) Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature 450(7168):420–424

Belle MD, Hughes AT, Bechtold DA, Cunningham P, Pierucci M, Burdakov D, Piggins HD (2014) Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock. J Neurosci 34(10):3607–3621

Betschart C, Hintermann S, Behnke D, Cotesta S, Fendt M, Gee CE, Jacobson LH, Laue G, Ofner S, Chaudhari V, Badiger S, Pandit C, Wagner J, Hoyer D (2013) Identification of a novel series of orexin receptor antagonists with a distinct effect on sleep architecture for the treatment of insomnia. J Med Chem 56(19):7590–7607

Brunig I, Sommer M, Hatt H, Bormann J (1999) Dopamine receptor subtypes modulate olfactory bulb gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 96(5):2456–2460

Burdakov D, Liss B, Ashcroft FM (2003) Orexin excites GABAergic neurons of the arcuate nucleus by activating the sodium–calcium exchanger. J Neurosci 23(12):4951–4957

Chou WH, Wang D, McMahon T, Qi ZH, Song M, Zhang C, Shokat KM, Messing RO (2010) GABAA receptor trafficking is regulated by protein kinase C(epsilon) and the N-ethylmaleimide-sensitive factor. J Neurosci 30(42):13955–13965

de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FSI, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95(1):322–327

Feigenspan A, Bormann J (1994) Facilitation of GABAergic signaling in the retina by receptors stimulating adenylate cyclase. Proc Natl Acad Sci U S A 91:10893–10897

Hoerbelt P, Lindsley TA, Fleck MW (2015) Dopamine directly modulates GABAA receptors. J Neurosci 35(8):3525–3536

Ishibashi M, Gumenchuk I, Miyazaki K, Inoue T, Ross WN, Leonard CS (2016) Hypocretin/orexin peptides alter spike encoding by serotonergic dorsal raphe neurons through two distinct mechanisms that increase the late after hyperpolarization. J Neurosci 36(39):10097–10115

Krishek BJ, Xie X, Blackstone C, Huganir RL, Moss SJ, Smart TG (1994) Regulation of GABA$_A$ receptor function by protein kinase C phosphorylation. Neuron 12(5):1081–1095

Kukkonen JP (2013) Physiology of the orexinergic/hypocretinergic system: a revisit in 2012. Am J Physiol Cell Physiol 3(5):C2–C32

Kukkonen JP, Leonard CS (2014) Orexin/hypocretin receptor signalling cascades. Br J Pharmacol 171(2):314–331

Leonard CS, Ishibashi M (2015) Orexin receptor functions in the ascending arousal system. In: Sakurai T, Pandi-Perumal SR, Monti JM (eds) Orexin and sleep. Springer International Publishing, Switzerland, pp 67–80

Leonard CS, Kukkonen JP (2014) Orexin/hypocretin receptor signalling: a functional perspective. Br J Pharmacol 171(2):294–313

Lin YF, Angelotti TP, Dudek EM, Browning MD, Macdonald RL (1996) Enhancement of recombinant alpha 1 beta 1 gamma 2L gamma-aminobutyric acid A receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both beta 1 and gamma 2L subunits. Mol Pharmacol 50(1):185–195

Liu RJ, van den Pol AN, Aghajanian GK (2002) Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. J Neurosci 22(21):9453–9464

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol 435(1):6–25

Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C (2004) Interneurons of the neocortical inhibitory system. Nat Rev Neurosci 5(10):793–807

McBain CJ, Fisahn A (2001) Interneurons unbound. Nat Rev Neurosci 2(1):11–23

McDonald BJ, Moss SJ (1994) Differential phosphorylation of intracellular domains of gamma-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. J Biol Chem 269 (27):18111-18117

Moss SJ, Smart TG, Blackstone CD, Huganir RL (1992) Functional modulation of GABAA receptors by cAMP-dependent protein phosphorylation. Science 257:661–665

Nakamura Y, Darnieder LM, Deeb TZ, Moss SJ (2015) Regulation of GABAARs by phosphorylation. Adv Pharmacol 72:97–146

Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K (1999) Distribution of orexin neurons in the adult rat brain. Brain Res 827(1–2):243–260

Newland CF, Cull-Candy SG (1992) On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol 447:191–213

Palus K, Chrobok L, Lewandowski MH (2015) Orexins/hypocretins modulate the activity of NPY-positive and -negative neurons in the rat intergeniculate leaflet via OX1 and OX2 receptors. Neuroscience 300:370–380

Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E (2000) A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med 6(9):991–997

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18(23):9996–10015

Poisbeau P, Cheney MC, Browning MD, Mody I (1999) Modulation of synaptic GABA$_A$ receptor function by PKA and PKC in adult hippocampal neurons. J Neurosci 19(2):674–683

Sachidanandan D, Bera AK (2015) Inhibition of the GABA$_A$ receptor by sulfated neurosteroids: a mechanistic comparison study between pregnenolone sulfate and dehydroepiandrosterone sulfate. J Mol Neurosci 56(4):868–877

Sakurai T (2007) Orexins and orexin receptors. In: Nishino S, Sakurai T (eds) The orexin/hypocretin system: physiology and pathophysiology. Humana Press, Totowa, New Jersey, pp 13–23

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92(4):573–585

Sakurai T, Pandi-Perumal SR, Monti JM (eds) (2015) Orexin and sleep. Springer International Publishing, Switzerland

Saper CB, Fuller PM, Pedersen NP, Lu J, Scammell TE (2010) Sleep state switching. Neuron 68(6):1023–1042

Scammell TE, Winrow CJ (2011) Orexin receptors: pharmacology and therapeutic opportunities. Annu Rev Pharmacol Toxicol 51:243–266

Sherin JE, Elmquist JK, Torrealba F, Saper CB (1998) Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. J Neurosci 18(12):4705–4721

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr Top Med Chem 2(8):795–816

Taleb O, Loeffler JP, Trouslard J, Demeneix BA, Kley N, Hollt V, Feltz P (1986) Ionic conductances related to GABA action on secretory and biosynthetic activity of pars intermedia cells. Brain Res Bull 17(5):725–730

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. Neuron 27(3):469–474

Uslaner JM, Renger JJ, Coleman PJ, Winrow CJ (2015) A new class of hypnotic compounds for the treatment of insomnia: the dual orexin receptor antagonists. In: Sakurai T, Pandi-Perumal SR, Monti JM (eds) Orexin and sleep. Springer International Publishing, Switzerland, pp 323–338

van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB (1998) Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. J Neurosci 18(19):7962–7971