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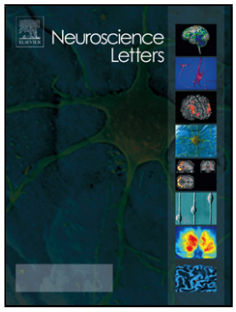

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Orexin A presynaptically decreases inhibitory synaptic transmission in rat locus coeruleus neurons

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Highlights

- Orexin A via OX1 receptors decreased eIPSCs amplitude in LC neurons
- Orexin A decreased eIPSCs amplitude via intracellular Ca^{2+} level and PLC activation
- Orexin A increased PPR and sIPSCs frequency but had no effect on sIPSCs amplitude
- Endocannabinoid CB1 receptors mediate orexin A effects in the LC neurons

Abstract

Locus coeruleus nucleus (LC) is a major noradrenergic nucleus in the brain. It receives dense orexinergic projections from lateral hypothalamus. Whilst it is known that orexin A increases firing rate of LC neurons, its effect on spontaneous and evoked inhibitory postsynaptic currents (sIPSCs and eIPSCs, respectively) has not been yet identified. In this research, we investigated the effect of orexin A on eIPSCs and sIPSCs in LC neurons. Whole-cell recordings revealed that orexin A suppresses eIPSCs amplitude in which this effect was blocked by an orexin type-1 receptors antagonist (SB-334867) and cannabinoid type-1 (CB1) receptors antagonist (AM251). Moreover, exposure of neurons to BAPTA (Ca^{2+} chelator) and U73122 (phospholipase C inhibitor) prevented orexin A-induced eIPSCs depression. On the other hand, orexin A increased pair pulse ratio and sIPSCs frequency but had no effect on sIPSCs amplitude. Our results revealed that eIPSCs suppression in the LC is mediated by CB1 receptor through a presynaptic mechanism.

Keywords: Locus coeruleus nucleus, patch clamp recording, eIPSC, sIPSC, Orexin A, SB-334867, AM251

Introduction

The nucleus locus coeruleus (LC) provides about %50 of brain's norepinephrine content [23]. It is involved in pain modulation [18, 21, 25, 29], attention, consciousness, sleep and awakening cycles [32] as well as drug tolerance and dependence [1, 2, 12, 13, 26, 27]. LC neurons have spontaneous firing and their rate is influenced by inputs from other nuclei [5]. Lateral paragigantocellularis (LPGi) provides the main glutamatergic inputs into the LC and increases its activity [19] while the nucleus prepositus hypoglossi (PrH) sends GABAergic projections into the LC and suppresses its activity [28]. Also, this nucleus receives dense orexinergic inputs from the lateral hypothalamus (LH) [30]. Orexin neuropeptides (orexin A and B) are mainly produced in LH neurons [8, 30] and are spread throughout the brain [35]. Orexins activate two types of G-protein coupled receptors, namely orexin type-1 (OX1) and orexin type-2 (OX2) receptors [30]. These receptors couple with G_q protein and elevate intracellular calcium [22, 37], inhibit potassium channels or activate Na^+/Ca^{2+} exchanger [37]. It was shown that OX1 is more abundantly expressed than OX2 receptors [15]. It appears that activation of postsynaptic Gq-protein-coupled receptors, results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC), producing

diacylglycerol, which is converted by diacylglycerol lipase (DAGL) to 2-arachidonoylglycerol (2-AG), an abundant endocannabinoid in the brain [16]. Endocannabinoids (Anandamide and 2-AG) are lipid-derived transmitters which could activate cannabinoid type-1 (CB1) or type-2 (CB2) receptors [9]. CB1 receptors are also found in the LC nucleus [31]. There is a close interaction between orexin A and endocannabinoid systems. It has been shown that orexin A produces 2-AG in CHO cells [141] and also in the periaqueductal gray [16]. In our previous study, we have reported that microinjection of orexin A into the LC induces nociceptive responses via CB1 receptors [21]. It was demonstrated that CB1 receptors mostly are located in GABAergic terminals [24] and their activation affect neural transmission [6]. Although excitatory effects of orexin A on LC neurons have been shown [17], the effect of orexin A on inhibitory postsynaptic currents in LC neurons remains unknown. Therefore, we hypothesized that activation of postsynaptic OX1 receptors in LC neurons may induce endocannabinoid production and inhibit GABA release by activating CB1 receptors. Here, we examined this hypothesis through electrophysiological recordings in LC neurons.

Materials and methods

Male Wistar rats from 14 to 21 days old were used in this research. This study was performed in accordance with the ethical guidelines of the Faculty of Medical Sciences Ethics Committee, Tarbiat Modares University, which are based on NIH guide for the care and use of laboratory animals. Efforts were made throughout the experiments to minimize animal discomfort and reduce the number of animals used. Rats were housed 4 rats per cage and kept in a temperature-controlled room under a 12 h light–dark cycle with light on at 7 a.m. Food and water were provided ad libitum.

Drugs

Orexin A, 1-(2-methylbenzoxazole-6-yl)-3-[1,5]naphthyridin-4-yl urea (SB-334867, OX1 receptor antagonist), (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN 55,212-2, CB1 receptor agonist), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251, CB1 receptor antagonist) and 1-[6-[[(17)-3-methyl estra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122, phospholipase C inhibitor) were purchased from Tocris Bioscience (Bristol, UK). SB-334867, WIN 55,212-2, AM251, and U73122 were dissolved in dimethylsulfoxide (DMSO) and cyclodextrin 2%. The final concentration of DMSO was <0.1%, which had no effect intrinsically. 6-Cyano-7-nitroquinoxaline-2,3-dione

disodium salt hydrate (AMPA receptor antagonist CNQX disodium salt), 1,2-Bis(2-Aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and DL-2-Amino-5-phosphonopentanoic acid (NMDA receptor antagonist, AP5) were purchased from Sigma Aldrich (USA). These drugs were aliquoted and then were kept at a temperature of -20° C in the freezer. Other salts which are used to make solutions were obtained from Merck Chemicals (Darmstadt, Germany).

Preparation of LC slices and whole-cell patch clamp recording

Male Wistar rats (14 to 21 days old) were anesthetized with ether and then were decapitated. Brains were removed rapidly and trimmed in cold cutting solution. The cutting solution containing (mM): Sucrose 213, KCl 2.6, NaH_2PO_4 1.23, $NaHCO_3$ 26, L-Ascorbic Acid 0.4, D-Glucose 2, $CaCl_2$ 0.1, $MgCl_2$ 2 (pH = 7.4, 290-300 mOsmol/L). The solution was continuously oxygenized with 95% oxygen and carbon dioxide 5%. Two or three 300 μm horizontal slices were obtained using a vibrating microtome (1000 Plus, Vibratome, USA).

Slices were incubated for 30 min at 35° C. Afterwards they were kept at room temperature before being transferred into a recording chamber. Slices were transferred to a submerged recording chamber on the stage of an upright microscope (Axioskop2, Zeiss, Germany) and then perfused with a peristaltic pump at a rate of 1-2 ml/min. The extracellular solution containing (mM): NaCl 125, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 25, D-Glucose 10, $CaCl_2$ 2, $MgCl_2$ 1.3 (pH = 7.4, 290- 300 mOsmol/L) [36].

Recording electrodes (borosilicate glass micropipettes with 3-6 MΩ resistance) were prepared using a programmable microelectrode puller and filled with intracellular solution contained (mM): $CsMeSO_3$ 120, NaCl 5, MgATP 4, NaGTP 0.3, EGTA 1.1, HEPES hemisodium 10, TEA-Cl 10 and QX314 5 (pH=7.3, 282 mOsmol/L) [19]. In one series of experiments, the Ca^{2+} buffering capacity of the internal solution was increased by elevating the BAPTA concentration to 10 mM. In order to keep the osmolarity at 282 mOsmol/L, CsMeSO3 concentration was decreased.

Neurons were visualized with a 40X water immersion objective lens using infrared differential interference contrast (IR-DIC) illumination system. Whole cell current clamp recordings were made from LC neurons using multiclamp 700B amplifiers (Axon Instruments) equipped with Digidata 1440A data acquisition system (Molecular Devices, USA). Electrophysiological responses were filtered at 3 kHz and sampled at 10 kHz and stored on a personal computer for offline analysis.

eIPSCs recording: After fixing the tip of the microelectrode on the surface of the neuron, with a slow suction, the membrane was broken and electrode access was made into the neuron. Neurons that had more than 15% changes in access resistance were not investigated. eIPSCs, were evoked every 20 s by a bipolar electrode placed 100–200 µm away from the recording neuron. eIPSCs were recorded at holding potential of -10 mV in the presence of AP5 (50 µM) and CNQX (10 µM), NMDA and AMPA receptors blocker respectively. When the paired-pulse ratio (PPR) was recorded, paired pulses with 70 ms interval were given every 20 s. The PPR was the ratio of averaged amplitude of the second eIPSC (eIPSC2) to that of the first eIPSC (eIPSC1).

Stable eIPSCs were recorded in each neuron for at least 5 min prior to drug application. To have a paired comparison, an antagonist/agonist was further applied in the same neuron. The amplitudes of 9 eIPSCs were averaged before (the controls) and after antagonist/agonist treatments.

Slices were exposed to orexin A (100 nM), SB-334867 (3 µM), WIN 55,212-2 (3 µM) and U73122 (5 µM) for 20 minutes and eIPSCs amplitude were evaluated between 17 to 20 minutes. When antagonists were used before orexin A, the antagonist was first applied for 15 minutes and then slice was exposed to the orexin A for 20 minutes and eIPSCs amplitude were evaluated between 37 to 40 minutes, and finally slices washout for 15-20 minutes.

sIPSCs recording: Spontaneous IPSCs were recorded at holding potential of -10 mV in the presence of AP5 (50 µM) and CNQX (10 µM), AMPA and NMDA receptor blockers. Spontaneous IPSCs were recorded for 10 min before and at steady state after drug treatments. Kolmogorov–Smirnov test was used to compare the cumulative probability of the frequency or amplitude of sIPSCs between groups. One neuron was recorded from each slice. Data were taken from more than 350 sIPSCs in each group.

Statistical analyses: All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using One-way ANOVA by SPSS software. The Least Significant Difference (LSD) post hoc comparison was subsequently performed if the overall statistic indicated significant differences between groups. Student's t test was used for statistical comparisons between two groups.

Results

Orexin A depressed the amplitude of eIPSCs in LC neurons via affecting OX1 receptors. One-way ANOVA analysis showed that depressive effect of orexin A (11.33, 66 and 100 nM) was concentration dependent ($P < 0.001$) and saturated between 100 and 300 nM (Fig. 1A).

Therefore, 100 nM orexin A was used in the following experiments. The LSD post hoc comparison showed that orexin A (100 nM) significantly depressed ($P<0.001$) eIPSCs amplitude in the LC neurons (Fig. 1A) and this effect was reversible (Fig. 1B).

Pretreatment with SB-334867 (3 µM), an OX1 receptor antagonist, had no effect on eIPSCs amplitude by itself, but it blocked orexin A-induced eIPSC suppression. Moreover, dual comparison showed significant differences ($P<0.05$) between orexin A and SB-334867 plus orexin A groups. These results propose that orexin A decreases eIPSCs amplitude in LC neurons through OX1 receptors (Fig. 1C and 1D).

Orexin A depressed eIPSCs through affecting CB1 receptors. Excitatory effect of the orexin A on LC neurons, suggests that it may inhibits GABA release indirectly. In our previous study, we showed that there is an interaction between OX1 and CB1 receptors in the LC [21]. Furthermore, there are reports that indicate orexin A reduced GABA release through endocannabinoid retrograde signaling in the periaqueductal grey [16] and VTA [34]. Here we examined if this mechanism also exists in the LC.

Pretreatment with AM251 (3 µM), CB1 receptors antagonist, had no effect on eIPSCs amplitude alone but it prevented the effect of orexin A (Fig. 1E and 1F). Dual comparison between orexin A and orexin A plus AM251 showed significant differences ($P<0.01$). WIN 55,212-2 (3 µM), CB1 receptors agonist, also mimicked the orexin A effect and suppressed eIPSCs amplitude ($P<0.001$) significantly (Fig. 2A). This effect was reversed by AM251 (Fig. 2A and 2B). Moreover, dual comparison showed significant differences ($P<0.01$) between WIN 55,212-2 and AM251 plus WIN 55,212-2 groups (Fig. 2A). Notably, application of WIN 55,212-2 after orexin A pretreatment did not enhance suppressive effects of orexin A on eIPSCs amplitude (Fig. 2A). These results suggest that activation of OX1 receptors, reduces GABA release via endocannabinoids, acting at presynaptic CB1 receptors expressed on GABAergic terminals innervating LC neurons. Intracellular increasing of BAPTA, a potent chelator of Ca^{2+}, prevented orexin A-induced eIPSC amplitude depression (Fig. 2C). Dual comparison showed significant differences ($P<0.05$) between orexin A and BAPTA plus orexin A groups (Fig. 2C). The effect of orexin A was also prevented by pretreatment with U73122 (5 µM), a PLC inhibitor (Fig. 2C and 2D). This inhibitor by itself had no effect on eIPSCs amplitude (Fig. 2C). It seems that orexin A inhibits GABAergic transmission, indirectly via 2-AG, an endocannabinoid which is synthesized via a Gq protein-coupled PLC enzymatic cascade.

Orexin A depressed eIPSCs via a presynaptic mechanism. To clarify whether presynaptic or postsynaptic mechanism involves in the orexin A-induced eIPSC depression, we examined the effect of orexin A on the PPR of paired eIPSCs as well as sIPSCs amplitude and frequency.

It was demonstrated that presynaptic modulation result in PPR changes [16, 38]. Orexin A increased significantly (one-way ANOVA, $P<0.05$) the PPR of paired eIPSCs amplitude (Fig. 3G).

Spontaneous IPSCs were recorded for 10 min before and at steady state after drug treatments in the presence of AP5 (50 μM) and CNQX (10 μM). Orexin A significantly reduced the frequency of sIPSCs ($P<0.05$) which was prevented by AM251 and also shifted the cumulative distribution of sIPSCs intervals to the right (Fig. 3E and 3F). Orexin A had no effect on sIPSCs amplitude (Fig. 3C and 3D). These results suggest that orexin A inhibits GABAergic transmission in the LC neurons through the presynaptic mechanism.

Discussion

Immunocytochemical methods have revealed a considerable synaptic innervation of LC neurons by orexinergic terminals in animals. Electrophysiological studies have shown that orexin A and B increase the firing rate of LC neurons in vitro [17]. In addition to glutamatergic signaling, GABAergic transmission is an important modulator for LC neural activity. It was shown that inhibition of LC neurons following PrH stimulation is mediated by $GABA_A$ receptors [11]. Moreover, it was shown that orexin A affects the morphine withdrawal syndrome through decreasing the activity of $GABA_A$ receptors [7].

Our findings showed that orexin A reduces eIPSCs amplitude and this reduction is eliminated by the SB-334867. This finding suggests that orexin A induces the observed effects by affecting the OX1 receptors in the LC neurons.

Application of intracellular BAPTA prevented orexin A-induced the eIPSCs depression in the recording neuron. This result shows that eIPSC depression related to postsynaptic OX1 receptors. On the other hand, AM251 also antagonized orexin A-induced eIPSCs depression, which indicates participation of endocannabinoids CB1 receptor in the LC neurons. The effect of cannabinoids on reducing GABAergic transmission has also been reported [16, 34]. Because orexin A has excitatory effect on neuronal activity, it is unlikely that orexin A directly inhibited GABAergic transmission in LC. Our findings indicate that orexin A increases the pair pulse ratio and decreases the sIPSCs frequency demonstrating that orexin A acts through a presynaptic mechanism. We also showed that WIN 55,212-2 has the same effect on eIPSCs amplitude like orexin A in LC slices. The effectiveness of AM251 in reversing suppressive effect of WIN 55,212-2 suggests that CB1 receptors mediate this effect. It has been shown that WIN 55,212-2 reduces the release of GABA and decrease the eISPC amplitude in the PAG [16] and VTA [34] through CB1 receptors. In presence of orexin A, superfusion of WIN

55,212-2 did not significantly alter the eIPSCs amplitude. It seems that probably OX1 and CB1 receptors use the same signaling pathway. In this regard, there is an evidence indicating that OX1 and CB1 make dimmer on the surface of neurons [10].

It is well established that Gq-protein-coupled receptors such as OX1 receptors, induce endocannabinoid production by phospholipase C (PLC) activation [16] and increasing intracellular calcium [20]. Elevation of intracellular calcium plays an important role in production and release of endocannabinoids [14] which in turn leads to the reduction of neurotransmission in glutamatergic and GABAergic synapses [16]. BAPTA has a large capacity for calcium buffering. Its intracellular application prevented the eIPSCs suppression by orexin A. The ineffectiveness of BAPTA in preventing orexin A-induced eIPSCs depression in the recording neuron may indicate that endocannabinoids were synthesized and diffused from adjacent neurons. The latter case has also been seen in the hippocampus [33] and the cerebellum [4]. Previous studies have shown that PLC activation induces endocannabinoid production [3, 16]. Addition of PLC inhibitor (U73122) into recording chamber prevented eIPSCs amplitude suppression by orexin A. This result is consistent with reports that show the activation of Gq-connected receptors activate the PLC, which ultimately leads to the endocannabinoid formation [16, 34].

In this study, we found that orexin A inhibits GABAergic transmission in LC neurons via a presynaptic mechanism. This effect was antagonized by OX1 receptors, CB1 receptors antagonists, Ca^{2+} chelator as well as PLC inhibitor. These results suggest that orexin A indirectly decreases GABA release possibly by promoting the production of endocannabinoids which activate CB1 receptors on GABAergic terminals. Therefore, it seems that there is an alternative mechanism by which orexin A inhibits the GABAergic transmission, and thereby increases the LC neuronal activity indirectly.

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Figure legends:

Figure 1. **Orexin A suppressed eIPSCs amplitude through OX1 and CB1 receptors in LC slices. A,** orexin A (OXA, 11, 33, 66 and 100 nM) suppressed eIPSCs amplitude in a concentration-dependent manner in LC slices. C, Pretreatment with SB-334867 (3 µM), an OX1 receptors antagonist, had no significant effect on eIPSCs amplitude by itself, but it blocked orexin A-induced eIPSCs suppression. **E,** Pretreatment with AM251 (AM, 3 µM), a CB1 receptors antagonist, reversed orexin A-induced eIPSC depression. AM251 did not altered eIPSCs amplitude by itself. **B, D and F,** The time course effects of the orexin A (100 nM) on eIPSCs amplitude alone (**B**), in the presence of SB-334867 (**D**) or in the presence of AM251 (3 µM) (**F**). Top panels show the averaged eIPSCs trace taken at the time point 1, 2, and 3 (*** P< 0.001 and * P< 0.05 versus control, ## P< 0.01 and # P< 0.05, versus 100 nM orexin A, T student test). Data are mean ± SEM. The number of recording neurons is denoted in the above each bar.

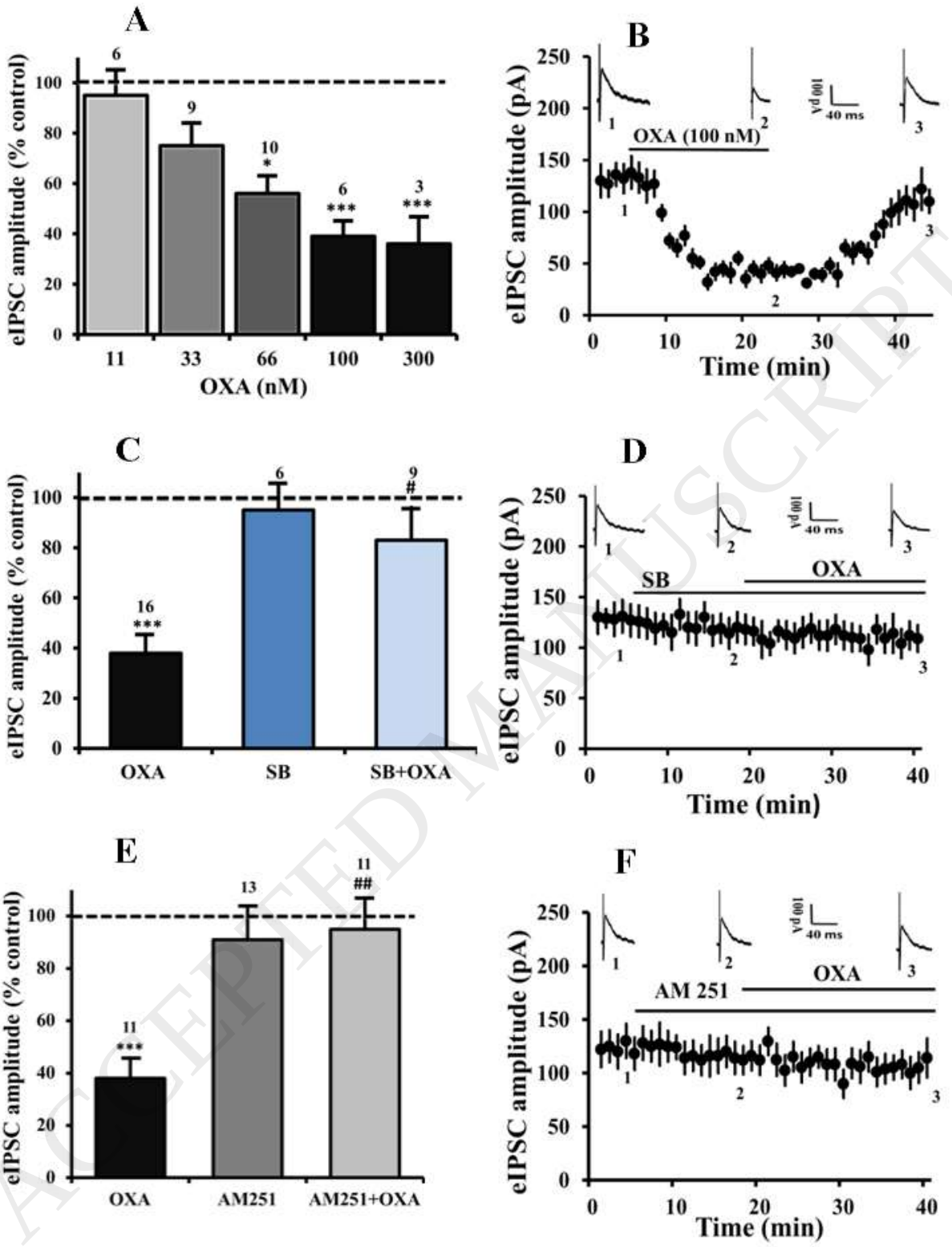

Figure 2. Orexin A-induced eIPSC suppression was mimicked by WIN 55,212-2 while it was prevented by BAPTA and PLC inhibitor. A, WIN 55,212-2 (WIN, 3 µM), CB1receptors agonist, mimicked the effect of the orexin A and depressed eIPSCs amplitude. This effect was reversed by AM251 (3 µM). Notably, pretreatment with orexin A obstructed the effect of

subsequent WIN 55,212-2 (3 µM) application. **B,** Intracellular increase of BAPTA (10 mM), a potent chelator of Ca^{2+}, prevented orexin A-induced eIPSCs amplitude suppression. The effect of orexin A was also prevented by pretreatment with U73122 (5 µM), a PLC inhibitor. This inhibitor by itself had no effect on eIPSCs amplitude. (* P< 0.05, ** P< 0.01 and *** P< 0.001 versus control, ### P< 0.001, and # P< 0.05 versus 100 nM orexin A, ψψ P< 0.01 versus 3 µM WIN 55,212-2, T student test). Data are mean ± SEM. The number of recording neurons is denoted in the above each bar.

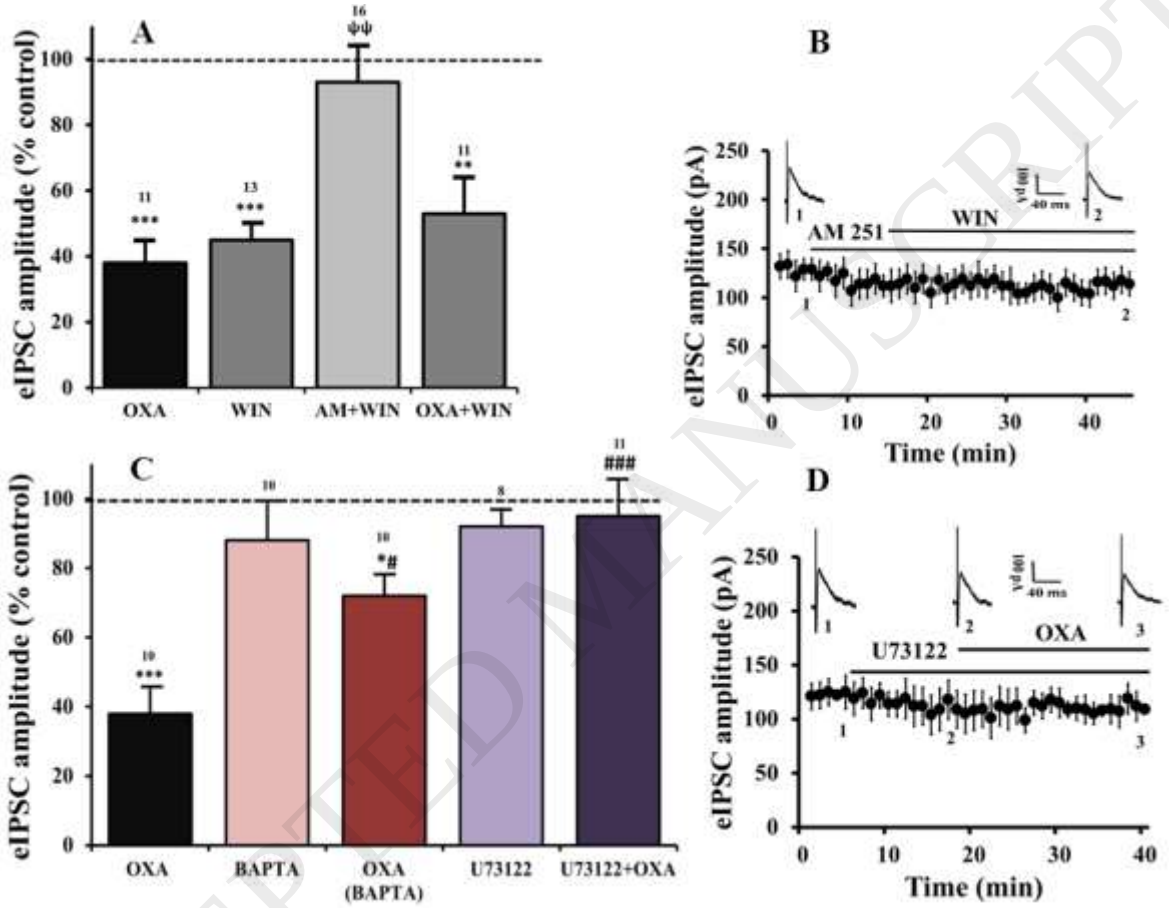

Figure 3. Orexin A decreased the frequency, but not amplitude of sIPSCs and also it increased the PPR of paired eIPSCs in the LC slices. Spontaneous IPSCs were recorded at holding potential of -10 mV for 10 min before and at steady state after drug treatments in the presence of AP5 (50 µM) and CNQX (10 µM). **A, B**, Sample sIPSC traces recorded before (control, left panel) and after treatment with 100 nM orexin A (OXA, right panel) in the LC slices was shown. Orexin A decreased the frequency (**E, F**), but not amplitude (**C, D**), of sIPSCs in a manner reversed by AM251. Kolmogorov–Smirnov test was used to compare the cumulative probability of the frequency or amplitude of sIPSCs. **G**, Paired eIPSCs were evoked by separate pair pulses every 20 s. Orexin A increased the PPR of paired eIPSCs in the LC neuron. This effect reversed by AM251 (3 µM). **H,** The averaged trace of three paired eIPSCs

in a neuron before (control) and 20 min after treatment with 100 nM orexin A (OXA) was shown. (* P< 0.05 versus control, n=6, Data are shown as mean ± SEM, one-way ANOVA).