Orexin A affects $GABA_A$ receptor recirculation after stimulation with Propofol

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TUTOR AUTHORIZATION

I, Henrik Andersson, Department of medicin and health, division of drug research, Linköping University, authorize the presentation of the bachelor thesis titled "Orexin A affects GABA A receptor recirculation after stimulation with Propofol" by Sofia León Francí performed under my supervision.

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ABSTRACT

Propofol, the intravenous anaesthetic, involves the $GABA_A$ receptors in its signalling cascade, altering the vesicular transport and inducing neurite retraction. The neuropeptide Orexin A, produced by the lateral and posterior hypothalamus has an essential role regulating the arousal system. It is capable of reducing the anaesthetic effects of various anaesthetic drugs as well as enhancing wakefulness in rats. Recent data from our lab suggests that Orexin A can affect the recirculation of the $GABA_A$ receptors. In order to further understand the anaesthetic mechanism of Propofol and how Orexin A can revert its effect, we wanted to investigate the effect of Orexin A on $GABA_A$ receptors in Propofol-treated SH-SY5Y cells. Samples were treated with medium (control), Propofol, Orexin A or Orexin A + Propofol and immunofluorescent labelling was performed against $GABA_A$ receptor β2 and α1 subunits. We could see the number of surface receptors was reduced in the double-treated cells compared to the others. However, the quantitation of signal intensities did not result in any significant differences between treatments.

INTRODUCTION

In the brain, there are two main kind of neurons; inhibitory and excitatory. Each one has its own neurotransmitters. In the central nervous system (CNS) inhibitory neurons are constituted by GABAergic and glycinergic neurons whereas excitatory neurons are mainly glutamatergic. During development, both type of neurons are born in different brain locations and then migrate reaching its correct cortical region. About one in five neurons is GABAergic in many species, for that reason it is needed a huge regulation with some feedback mechanism to produce the correct balance of neuron numbers since an imbalanced generation of these kind of neurons is the cause of many neurodevelopmental disorders[1, 2].

Sleep regulation

Sleep is a vital daily process which is characterized by a reduction of the voluntary motor activity, a highest external stimuli threshold and a stereotyped position[3]. It is regulated by interactions between wakefulness and sleep regulating neural circuitry[3, 4]. The arousal system is coordinated by two ascending pathways, each one associated with different neurotransmitters and different cerebral regions[4,5]. On one hand, there are cholinergic and monoaminergic neuronal activity discharging to the thalamus; on the other hand, the lateral hypothalamus, basal forebrain and cerebral cortex are innervated by monoaminergic neurons, including noradrenergic, serotoninergic, dopaminergic, histaminergic neurons which all have a specific function but no one is crucially needed since wakefulness is not altered if some of them are not present[3, 4, 5]. Every 24 hours, there is a network located on the ventral lateral preoptic area (VLPO) that switches off the wakefulness system sending descendent inhibitory projections to wakefulness neurons. It contains the inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and galanin[3, 4, 5, 6].

GABA

GABA is the main inhibitory neurotransmitter in the CNS and it is required for the balance between excitation and inhibition[3, 7, 8, 9]. It produces inhibition by interacting with different GABA receptors. There are two types of GABA receptors; ionotropic receptors, such as $GABA_A$ and $GABA_C$ receptors, that belong to the nicotinicoid superfamily and are ligand gated ion channels, or metabotropic receptors, like $GABA_B$ receptor, that are G-protein coupled receptors which act via second messengers. The $GABA_A$ receptor is a heteromeric pentamer made of two α subunits, two β and one γ subunit or δ[10, 11] and every subunit has many subtypes: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ and π[12], which combine giving rise to a functional chloride channel[7]. It belongs to the Cys-loop ligand-gated ion channel superfamily with

four transmembrane (TM) domains and an intracellular loop between the TM3 and TM4 important for being a protein interaction domain since it is a critical phosphorylation site[13,14,15]. Once activated, its β subunit becomes phosphorylated[8,9,10,16] and consequently, the channel is opened, allowing chloride ions to enter into the neuron. This causes a hyperpolarisation of the cell, reducing the transmission of new action potentials and decreasing cell excitability[8, 9, 10, 13]. Moreover, it's function is also very important during brain development since it controls some events such as proliferation, differentiation, migration and dentritic maturation of neurons.[11]

GABA receptor modulation/regulation

As mentioned above, the neurotransmitter GABA and its receptor have a crucial role in the cell inhibition excitability and for that reason there must be a strong cellular mechanism to regulate $GABA_A$ receptor activity.[17] This important mechanism has been suggested to be receptor phosphorylation[13, 18, 19], which has implications in altering the opening, the kinetics and the sensitivity of the channel[13]. Nevertheless, the phosphorylation of some specific residues in the large intracellular loop of the $GABA_A$ receptor subunits have been proposed to, on one hand, modulate the receptor activity[17, 18] and, on the other hand, to be crucial for $GABA_A$ receptor trafficking.[11,13,20]

Several laboratories have demonstrated that depending on the subunit expressed[11,17], the $GABA_A$ receptor can be differentially modulated by distinct kinases such as cAMP-dependent protein kinase (PKA)[13], protein kinase C (PKC)[11, 19], the tyrosine kinase Src[17, 18], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[11, 17] or even by Akt (also known as PKB)[11, 13]. The β subunits contain multiple phosphorylation sites; those receptors with the β1 subunit are phosphorylated by the PKA and PKC at Ser409[21, 22] which negatively modulates its activity. In contrast, in the β3 subunit, PKA phosphorylation of Ser408 and Ser409 increases the activation of the $GABA_A$ receptor[22]. Moreover, the PKC is able to phosphorylate the β2 subunit at the Ser410[18, 21] as well as the γ2 subunit[21, 23] decreasing GABA-stimulated Cl^- currents[17, 20].

Besides modulating the $GABA_A$ receptor activity, these phosphorylation sites are critical for its trafficking and endocytic pathway because they overlap with the interacting domains with some proteins that promote the binding and recruitment of the receptor[12, 24, 25].

$GABA_A$ receptor trafficking

$GABA_A$ receptors can be found in the axons of different kinds of cells and both synaptic and extrasynaptic, depending on its subunits all of them contributing to GABAergic currents.[11] But how does the cell control the number of synaptic

receptors? First of all, the whole process is regulated by the number of assembled receptors. Once the complex is functional, since the receptors are not constitutively expressed in the cell membrane, what defines the number of surface receptors is the maturation and traffic between intracellular compartments as well as cell membrane insertion and endocytosis.[13]

Likewise, what determines the number of $GABA_A$ receptors at the synapses is the exchange between extrasynaptic and synaptic receptors, this being crucial for the strength of the inhibition due to its dependence on the number of synaptic receptors.[18]

In order to facilitate the functioning of these processes, there are many proteins that facilitate receptor's transport from the endoplasmatic reticulum (ER) to the cell membrane passing by Golgi reticulum. First of all, $GABA_A$ receptors assembly involves the binding of the chaperones immunoglobulin protein (Bip) and Calnexin, proteins from the ER. There, the α and β[11] subunits also interact with the protein that links integrin-associated protein with the cytoskeleton-1 (Plic-1), that inhibits the degradation and increases the insertion into the surface membrane.[13] The Golgi-specific DHH zinc finger protein (GODZ, zDHHC3) interacts with the palmitoylation sites from the γ2 subunits and allows the accumulation of the receptor at inhibitory synapses.[11] Another protein located in the trans Golgi is brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2), involved in traffic and maturation due to it creates vesicles from the Golgi apparatus to the membrane.[11, 13] $GABA_A$ receptor associated protein (GABARAP) colocalizes at the Golgi compartment with $GABA_A$ receptors[13] and interacts with its γ2 subunits and the microtubuli. This is important for receptor's membrane trafficking[13] and its translocation to the cell surface[11, 18]. It interacts with N-ethylmaleimide sensitive factor (NSF), an ATPase[11, 26] that is also able to directly interact with the β subunit of the receptor. It regulates GABARAP trafficking of the receptor and decreases the number of surface receptors.[13, 27] GABARAP also interacts with the phospholipase C-related catalytically inactive protein 1 (PRIP-1), a kinase and phosphatase adaptor. By binding to the β subunit, it regulates, in one hand, receptor phosphorylation and dephosphorylation[11, 13] and on the other hand, receptor trafficking[27, 28]. Moreover, PRIP-1 competes with GABARAP for the γ2 subunit.[29] Furthermore, the endocytosis via clathrin and dynamin regulates the number of surface and synaptic $GABA_A$ receptors through the interaction between $GABA_A$ receptor β and γ subunits phosphorylation sites with the clathrin adaptor protein AP2. Intriguingly, its interaction, which is indirectly controlling the strength of the inhibitory synapse, is negatively regulated by phosphorylation, triggering the internalization when the receptor is not phosphorylated. Moreover,

huntingtin-associated protein 1 (HAP1), which binds to the β subunit, promotes the recycling of the receptors back to the cell surface and the function of the inhibitory synapses.[11, 13]

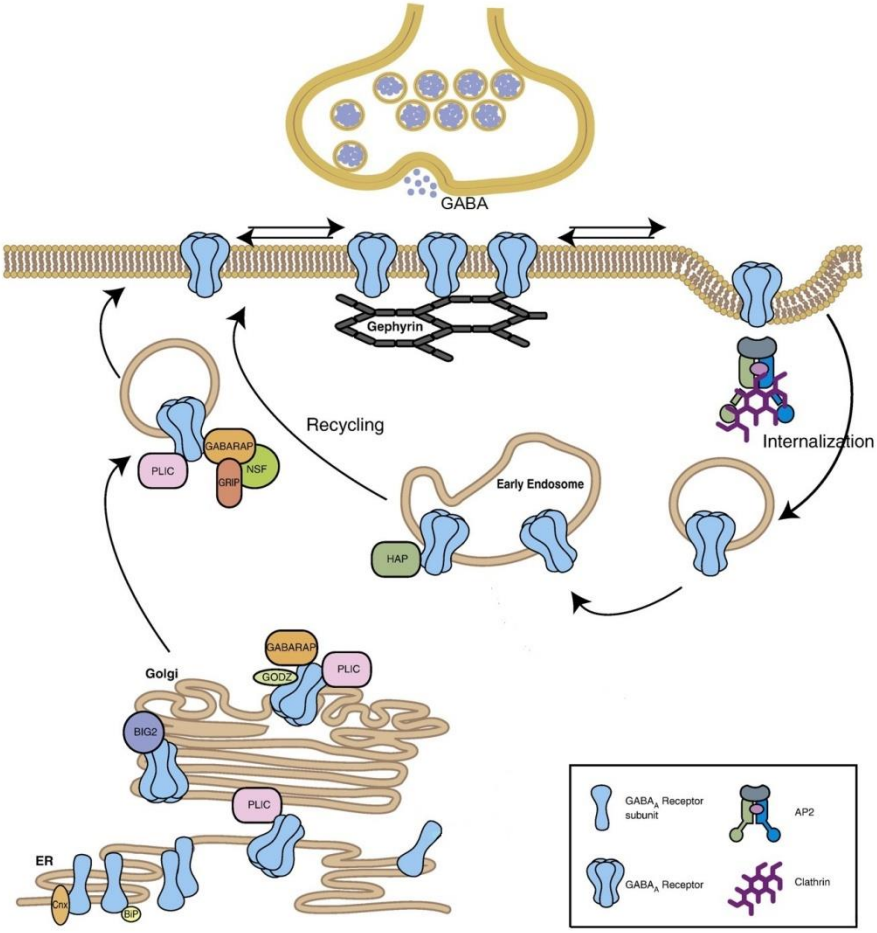
As mentioned previously, the phosphorylation state of the $GABA_A$ receptors β subunits by PKA or PKC may modulate its endocytosis In addition, the internalization is also regulated by the interaction with the adaptor PRIP. When it becomes phosphorylated, the phosphatase PP1α is activated promoting the interaction of AP2 with de β3 subunit since it becomes dephosphorylated by PP1 α[29] triggering receptor endocytosis. Nevertheless, PRIP also acts as an adaptor for the Akt kinase, promoting the insertion of $GABA_A$ receptors to the cell surface[30].

Related to that phosphorylation dependence, PKC activates NSF decreasing the expression of the $GABA_A$ receptors in the surface.[26]

A part from all that regulation that allows the insertion of the receptor to the cell surface, there are some proteins which are essential to target $GABA_A$ receptors to the inhibitory synapses. Gephyrin, which forms clusters at inhibitory synapses, is the most important for the receptor stabilization; it can build a hexagonal lattice making dimers and trimers of itself that is capable of recruiting the inhibitory receptors.[31, 32] Moreover, gephyrin interact with tubulin and GABARAP, suggesting they connect the receptor to microtubes, having an important role in the synaptic transport.[11, 13, 33]

Orexin A

Orexin A (OA) is a neuropeptide produced exclusively by the lateral and posterior hypothalamic neurons although they have efferences throughout the CNS.[34] It causes the depolarization of the neurons enhancing the excitability and firing rate.[35] For that reason it is essential in the regulation of sleep and wake states due to its effect on the activation of monoaminergic and cholinergic neurons of the hypothalamus and brainstem as well as for its neuroendocrine functions[3, 4, 5, 34, 35]. OA can interact with both Orexin receptor 1 (OX1R) and Orexin receptor 2 (OX2R), which are coupled to a seven transmembrane Gq, Gs and Gi proteins even though it has more affinity to OX1R.[3, 35, 36] Both receptors have a critical role; OX1R regulates sleep and wakefulness while OX2R is involved in promoting arousal.[35, 37] However, its activity is regulated by other neuromodulators like noradrenaline, serotonin and GABA, that inhibits their function creating a negative feedback which stabilize the activity of Orexin and monoaminergic neurons, whereas cholecystokinin, neurotensin and oxytocin activates Orexin neurones.[3, 6]



Modified from Kittler et al. 2009 (13)

Figure 1. Receptor's membrane trafficking and associated proteins.
The assembly of $GABA_A$ receptors occurs in the ER by the action of Bip and Calnexin and the subunits bind to Plic-1, which inhibits their degradation and enhances the insertion into the surface membrane. GODZ and BIG2 are two proteins from the Golgi apparatus that are involved in the traffic and maturation of the receptor. GABARAP interacts with the $GABA_A$ receptor β subunit as well as NSF, both promoting the translocation of the receptor to the cell surface. Gephyrin, capable of recruiting receptors at inhibitory synapses, by interacting with GABARAP facilitate the synaptic transport of the $GABA_A$ receptors. The internalization is mediated by clathrin and its adaptor AP2, that interact with the phosphorylation sites of the β and γ subunits. Also binding to the β subunit, HAP1 facilitates the recycling of the receptors and their expression in the membrane surface.

When Orexin A binds to OX1R, which is coupled to a Gq protein, cause a receptor structural change and activates the phospholipase D (PLD) as well as phospholipase C (PLC) and their signalling pathways. PLD then hydrolyses phosphatidylcholine (PC) to choline and phosphatidic acid (PA), which by the action of phosphatidic acid phosphohydrolase (PAP) is converted in diacyl glycerol (DAG).[38, 39, 40] With the activation of PLC, the phosphatydilinositol (PIP2) cascade is initiated and DAG and inositol triphospate (IP3) are generated. On one hand, IP3 increases cytosolic calcium concentration. On the other hand, DAG also activates protein kinase Cε (PKCε)[41], promoting its translocation to the cell membrane[37]. PKCε then

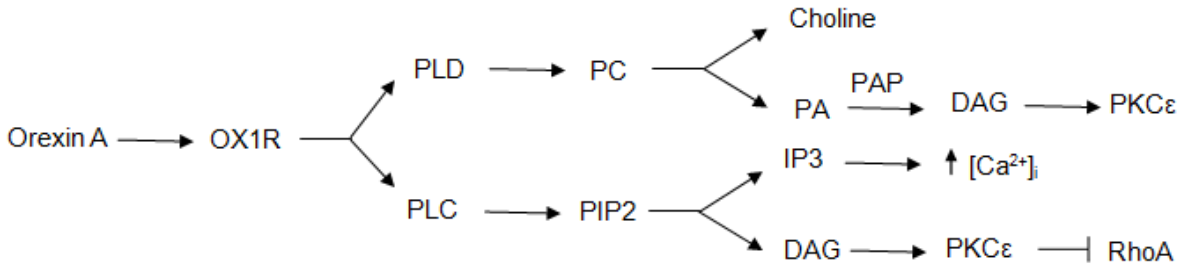


Figure 2. Suggested signalling pathway for Orexin A.
Orexin A interacts with Orexin receptor 1. It activates on one hand the PKC, which increases the intracellular calcium concentration. On the other hand, it stimulates phospholipase C protein with the subsequent triggering of the phosphatydilinositol cascade and diacyl glycerol (DAG) and inositol triphospate (IP3). IP3 increases cytosolic calcium concentration while DAG activates PKCε promoting its translocation to the cell membrane. Then PKCε is able to phosphorylate its substrates such as Rho A.

phosphorylates and inhibits Rho A.[42, 43]

Previous studies have described some models which establish that what regulates the sleep state is a circuit called "Flip-Flop", in which Orexin has an important role. It creates wire neuronal circuits with different neurochemical features that interact regulating the initiation and maintenance of sleep and wakefulness state.[3, 4, 5]

Therefore, there is a connection between arousal and sleep system; they inhibit each other in order to keep the balance among inhibition and excitation. Unbalance between them can cause many symptoms; too little inhibition or too much excitation cause anxiety, uneasiness and insomnia. Too little excitation or too much inhibition can lead to depression, sedation, sleep or even coma.[7] Afferents from the wakefulness system, monoaminergic, cholinergic and serotoninergic neurons, projects to the VLPO. However, during sleep, VLPO discharges on the hypothalamus and brainstem creating a reciprocal inhibitory loop.[4, 5] This circuit is called "flipflop" system, and as said above, if there is a lack of control, some disorders appear.[3, 4, 5]

<u>Induced sleep</u>

Propofol (2.6-di-isopropylphenol) is an intravenous anaesthetic drug extensively used worldwide. It is one of the most used anaesthetic agents due to its numerous advantages such as minimal side effects, quick onset and clearance, controllable anaesthetic effect and a fast recovery of psychomotor and cognitive function of the patient.[44] It interacts with the $GABA_A$ receptor.[8, 10, 16] However, its signalling pathway is not the same as for GABA, with differences in phosphorylation of its subunits.[9, 16] Nevertheless, this interaction generates a longer hyperpolarization of the neuron compared to the physiological caused by GABA due to induction of

spontaneous release of GABA creating a positive feedback.[9, 10]

Once Propofol has activated the $GABA_A$ receptor, on one side, it stimulates a tyrosine kinase protein which phosphorylates phosphatidylinositol 3-kinase (PI3K) and initiates its signalling pathway. It regulates the G-protein Rac, releasing calcium from extracellular and intracellular stores.[8, 9, 10, 16] This increase in calcium amount leads to actin phosphorylation[9] despite its independence from extracellular calcium, but when calcium is available from both extracellular and intracellular sources, actin in the whole cell is reorganized[45] and actin rings are originated[10, 16].

On the other side, Propofol activates RhoA, a GTPase which activates Rho-associated kinase (ROK)[9, 16, 46]. Once activated, ROK phosphorylates and inhibits myosin light chain (MLC) phosphatase and consequently, increases phosphorylation of myosin regulatory light chains by the action of MLC kinase, that regulates the motor protein myosin II. This pathway enhances the interaction between actin filament and myosin, leading to neurite retraction, a reversible process mediated by the force generation between actin and myosin filaments.[8, 42, 46, 47]

Neuronal network functioning

The proper function of the CNS depends on specific connections between neurons wiring a functional network. During the development period, selective retraction of recently created axons happens in order to clean out inappropriate axonal projections and obtain a proper network which will allow a good neuronal development.[48]

Because of the retraction, the synaptic transmission is altered. Many anaesthetic agents, even Propofol, reduce neuronal activity in cortical networks[49] since they decrease synaptic neurosecretion, which has an important role in synaptic transmission. The movement of the neurite cytoplasm toward the cell body causes

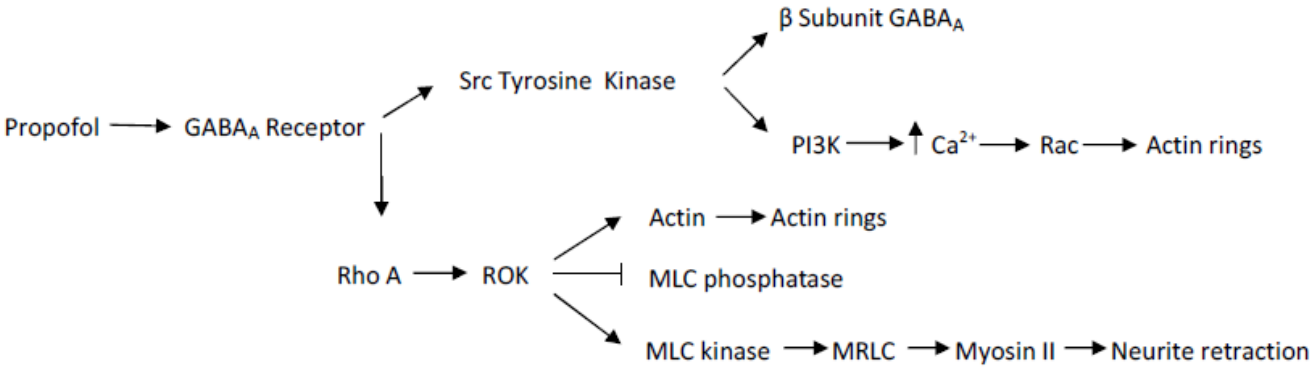


Figure 3. Suggested signalling pathway for Propofol.

Propofol interacts with the $GABA_A$ receptor which becomes tyrosine phosphorylated on the β subunit by the Src tyrosine Kinase. The Src kinase also phosphorylates phosphatidyl inositol 3-kinase (PI3K) initiating its own signalling pathway, increasing cytosolic [Ca2+] from the intracellular and extracellular sources. It activates Rac, a G-protein that regulates actin promoting ring formation. Furthermore, Propofol activates the GTPase RhoA, which stimulates ROK, a protein kinase. It phosphorylates and inhibits myosin light chain (MLC) phosphatase and consequently, increases phosphorylation of myosin regulatory light chains (MRLC) by the action of MLC kinase, that regulates the motor protein myosin II. Taken together, this cause neurite retraction, mediated by force generation between actin and myosin filaments.

retrograde vesicle movement and the number of vesicles secreted and the quantity of neurotransmitter released modulates the postsynaptic response.[50, 51, 52]. So this neurite retraction may explain the anaesthetic mechanism of Propofol as well as actin phosphorylation and rearrangement.

Previous work has shown that the anaesthetic and arousal signalling pathways may be connected; on one hand OA interferes with the Propofol-induced neurite retraction by inhibiting RhoA through PKCε. On the other hand, Propofol decreases the degree of phosphorylation of the PKCε and consequently its activation.[42] Furthermore, the $GABA_A$ receptor phosphorylation caused by PKCε, decreases its sensitivity to Propofol since it diminish the amount of receptors in the cell surface.[20] These results support the idea that PKC modulates $GABA_A$ receptor activity.[42] In this way, it is possible that Orexin A, by activating PKCε inhibit Propofol after-effects on the CNS, reverting its anaesthetic effect.

Moreover, a flow cytometry indicate that there is a reduction of $GABA_A$ receptors on the cell surface when cells are treated with Orexin A before being stimulated with Propofol (unpublished data).

The effect of Orexin A in the modulation $GABA_A$ receptor activity by the action of PKCε has still not been studied and to further elucidate the molecular mechanism of anaesthesia and how to revert its effects, the aim of this study was to investigate the effect of OA on the availability of $GABA_A$ receptors in Propofol-treated SH-SY5Y cells.

We hypothesized that pre-treating SH-SY5Y cells with Orexin A would block the recirculation pathway of the $GABA_A$ receptors to the cell surface and consequently, the global number or receptors in the membrane would be reduced.

In order to prove that theory, we stimulated the cells with medium, Propofol, Orexin A and both Orexin A and Propofol before performing an immunofluorescence.

MATERIALS AND METHODS

Reagents

Reagents were acquired from the following companies: bovine serum albumin (BSA), fetal calf serum (FCS), Trypsin-EDTA 0.25% solution, Dulbecco's modified Eagle's medium (DMEM), nutrient mixture F-12 HAM, streptomycin, penicillin, glutamine, Orexin A and retinoic acid (Sigma-Aldrich, St Louis, MO, USA); paraformaldehyde (PFA) (Histolab, Göteborg, Sweden); 2.6-diisopropylphenol (Propofol) (BRAUN, Melsungen, Germany); anti-$GABA_A$ receptor β2-subunit antibody and anti-$GABA_A$ receptor α1-subunit (Abcam, Cambridge, UK) Alexa Fluor 488 goat anti-rabbit antibody and Alexa Fluor 546 goat anti-mouse antibody (Invitrogen, Eugene, Oregon, USA); fluorescence mounting medium (DAKO, Glostrup, Denmark); 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Paisley, UK). SH-SY5Y cells were also acquired from Sigma-Aldrich, St Louis, MO, USA.

Cell culture

The tree time-cloned SH-SY5Y cell line was used. SH-SY5Y cells were originally from a neuroblastoma of a four year old patient taken from a bone marrow biopsy. They have neuron biochemical, functional and transmitter properties.[53, 54] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 HAM (1:1), penicillin (20 units/ml), streptomycin (20 mg/ml), and 10% (vol/vol) heat-inactivated FCS. Cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO2 and were seeded on poly-L-lysine-coated 25 mm ϕ sterile cover-slips in Petri dishes at an initial density of 2X10^5 cells/well. For the cells to stop dividing and differentiate into a more neuronal phenotype, retinoic acid (RA) was used. RA was added the same day the cells were plated at a final concentration of 10 μM in medium. Every 2-3 days, the cultures received new differentiation medium. Differentiation was carried out for 7 days before cell stimulation.

Cell stimulation

Differentiated SH-SY5Y cells were treated with medium only (untreated), Propofol (2 μM) for 10 min, Orexin A (10nM) for 15 min or Orexin A for 5 min followed by Propofol for 10 min. The medium was removed and then cells were fixated in 900 μL of preheated PBS-PFA (4%) for 20 minutes at 37ºC. After that, all glasses were rinsed once in 2mL of fresh PBS. Cells were stored in PBS at 4ºC until the immunofluorescent labelling was performed.

Immunofluorescence

Immunofluorescence is a method which combines immunological and biochemical

techniques in order to indentify antigens using specific antibodies. A secondary antibody with a conjugated fluorophore is used to visualize the distribution and location of the primary antibody which is bound to the specific cellular component within the cell.[55]

All antibodies were titrated in order to determine the best concentration. All cell cover-slips were incubated for 45 min with primary antibodies, anti-$GABA_A$ receptor β2-subunit and anti-$GABA_A$ receptor α1-subunit diluted as indicated in 45 µL PBS-BSA (3%) on slow shaking at room temperature. They were rinsed 3 times, 5 minutes each, in PBS-BSA solution. This was followed by the secondary antibodies, but performed in the darkness due to its light sensitivity. The secondary antibody, Alexa Fluor 488 and Alexa Fluor 546 were diluted 1:400 in PBS-BSA. Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse react with IgG heavy chains and all classes of immunoglobulin light chains from rabbit or mouse respectively. After the secondary antibody incubation, cell cover-slips were again rinsed 3 times, 5 minutes each, in PBS-BSA (3%) solution and once in PBS solution. The cover slips were mounted on a slide with fluorescence mounting medium containing 1 µL/mL of DAPI in order to see the nuclei and stored over night in the dark.

Microscopy

Microscopy was performed in a Car Zeiss Axiovert 200M inverted microscope, using an alpha Plan-Fluar 63X 1'4 NA oil immersion fluorescence objective (Carl Zeiss Gmbh, Göttingen, Germany). All images were captured using a AxioCam MRm (Carl Zeiss).

Data quantification and statistical analysis

Cell and background intensity were measured using Fiji/Image J software. The background was subtracted from the cell intensity in order to obtain the real cell intensity.

The final number of slides per stimuli was 11 and 5 cells per slide were measured. The mean and the standard error of the mean (SEM) were calculated. Overall significant differences between conditions were determined by two-sided Student's T test. All statistical analyses and graphing were done using Excel and P-value of <0.05 was considered statistically significant.

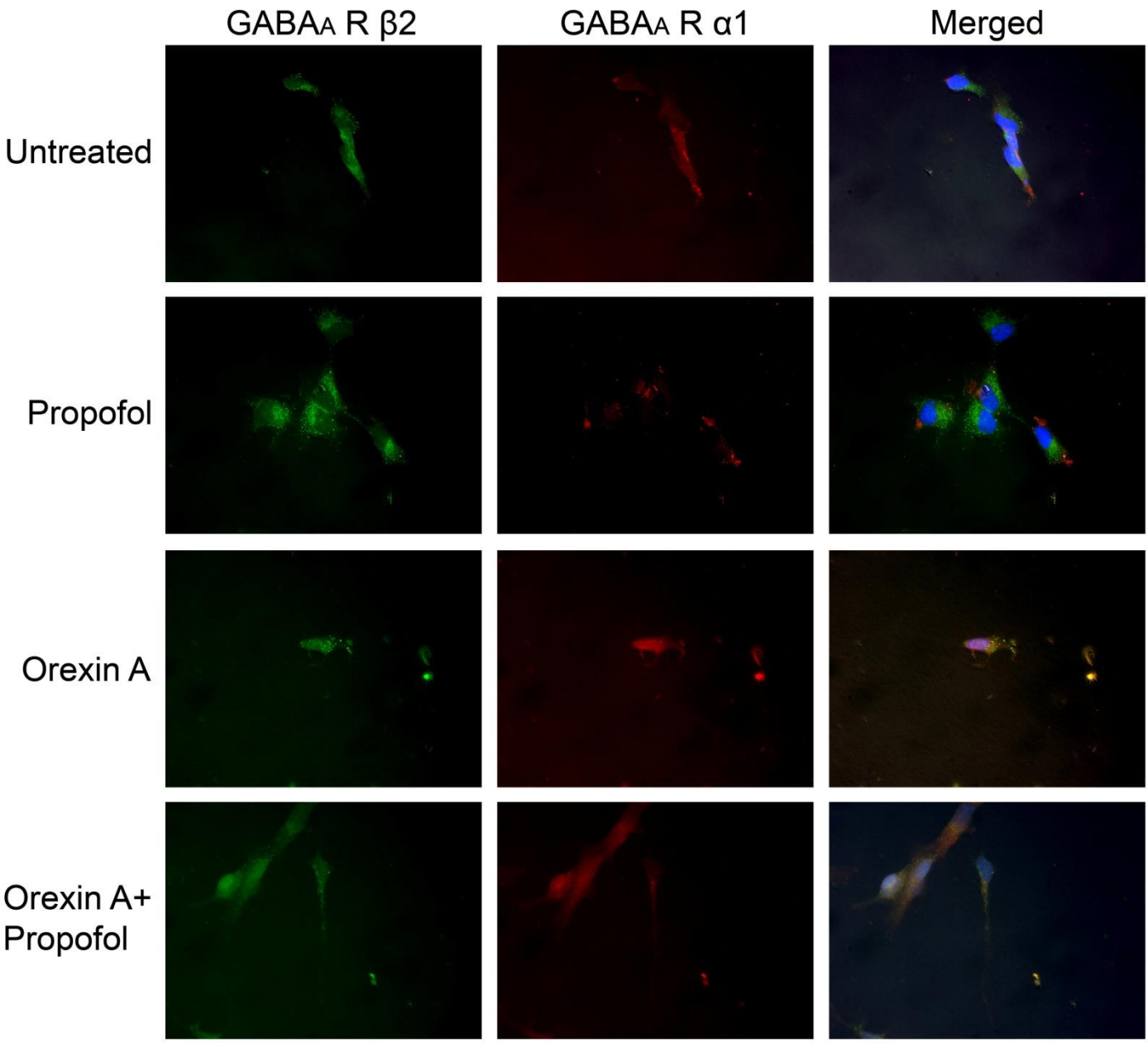
Results

We wanted to study how Orexin A affects the expression of $GABA_A$ receptor on the cell surface. We differentiated SH-SY5Y cells 7 days with RA and then treated the cultures with medium (untreated), Propofol (2 μM), Orexin A (10 nM) and Orexin A + Propofol respectively. We had 3-4 slides in each replicate and the experiment was repeated four times. To see where the $GABA_A$ receptors are localized in the cells and how the treatment with a combination of those three different substances alter their location, a immunofluorescence procedure was performed. The antibodies used, anti-$GABA_A$ receptor α1-subunit and anti-$GABA_A$ receptor β2-subunit, were titrated in order to determine the optimal concentration. In both cases, the best concentration was 1:50 dilution (1:50 and 1:200 were tested) which allowed us to see in a clear and transparent way the receptors. Furthermore, DAPI was used so visualize the nuclei. As we wanted to see if those receptors were only inserted on the cell surface, not in the intracellular compartment, no permeabilization agent was used.

To investigate if Orexin A, the regulator of wakefulness, could interfere with the anaesthetic propofol by causing any change in the location of $GABA_A$ receptor, SH-SY5Y cells were pre-treated 5 min with OA and then Propofol was added and cells were incubated an additional 10 min. Cells were fixated with PFA followed by a immunofluorescent staining.

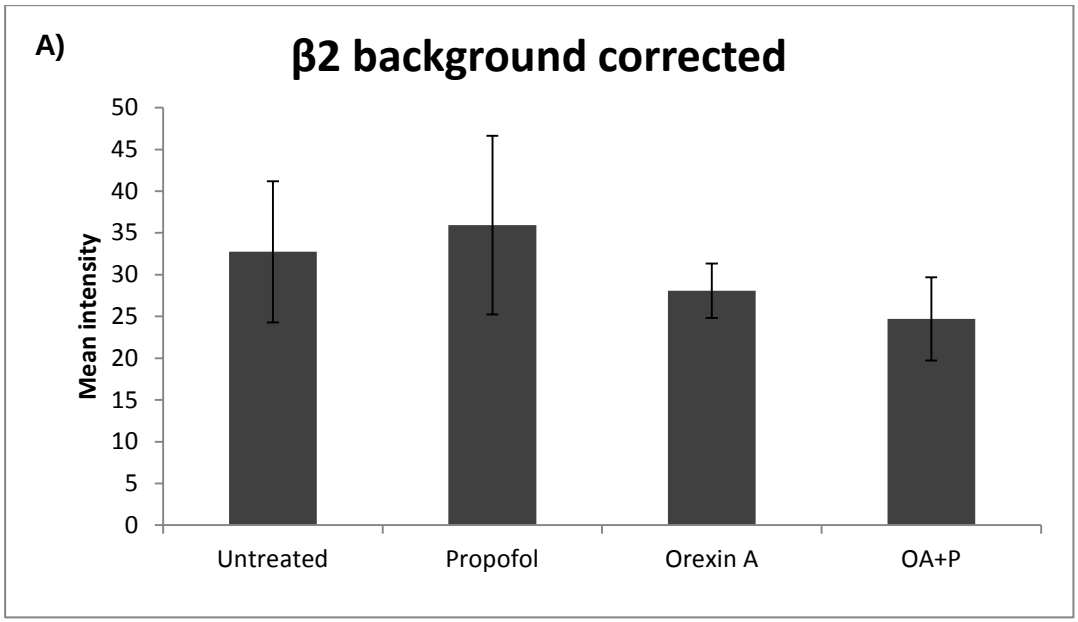
After the microscopy analysis, we could clearly see that the number of surface receptors was reduced when cells were pre-treated with OA prior to Propofol compared those exposed to medium or Propofol or Orexin only (figure 4). The amount of receptors in untreated and Orexin A samples was similar while slightly reduced in the Propofol treated samples.

To quantitatively compare untreated, Propofol, OA and OA + Propofol treated SH-SY5Y cells, we did a quantitation of signal intensities by measuring the mean intensity of the whole cell from both antibodies. However, we did not find any significant differences between treatments (figure 5).



Fiugre 4. An immunofluorescence reveals a reduction of $GABA_A$ receptors in SH-SY5Y cells after the addition of OA prior to Propofol.

Cell cultures were treated with Propofol (2 μM) for 10 min with or without pre-treatment with Orexin A (10 nM) for 5 min. Immunofluorescence staining with antibodies against $GABA_A$ receptor subunits β2 and α1 was performed. The double-treated samples show a reduction of $GABA_A$ receptors. Images shown are from one representative experiment of four.



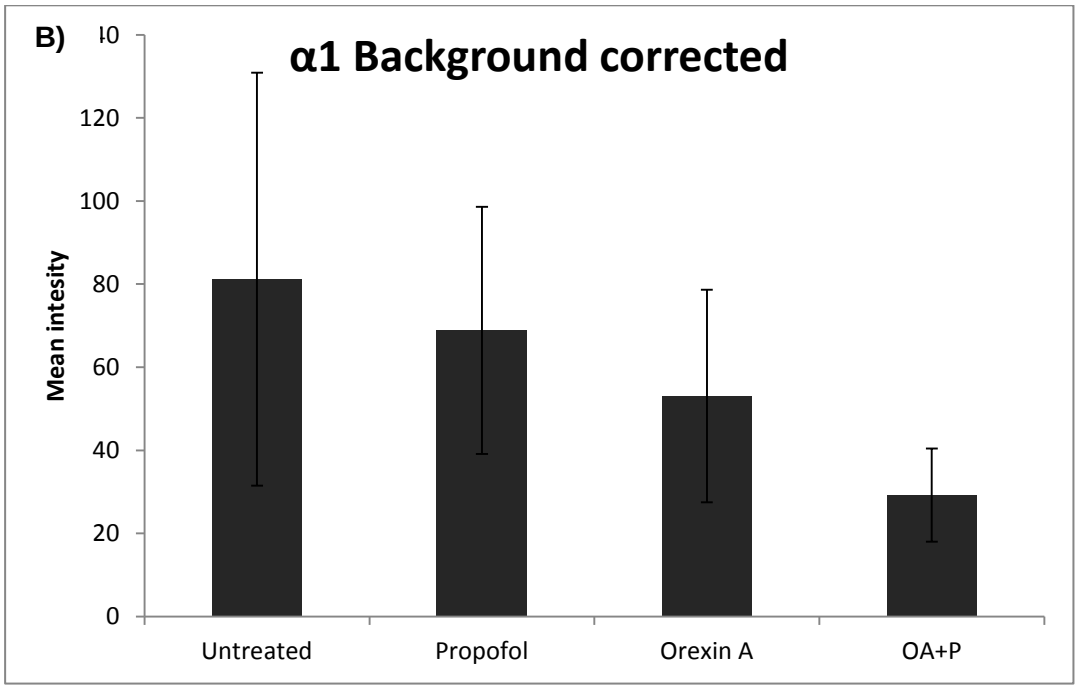


Figure 5. Mean intensity of SH-SY5Y cells after treatment with OA and/or Propofol.
Cell cultures were treated with Propofol (2 μM) for 10 min with or without pre-treatment with Orexin A (10 nM) for 5 min. Immunofluorescence staining with antibodies against $GABA_A$ receptor subunits β2 and α1 was performed. Mean intensities from $GABA_A$ receptor β2-subunit (A) and $GABA_A$ receptor α1-subunit (B) were analysed. No significant differences were found (Student's T test). Bars represent mean +/- SEM (n=4).

DISCUSSION

The aim of this study was to investigate the effect of Orexin A on $GABA_A$ receptors in Propofol-treated SH-SY5Y cells.

In the quantitative analysis we could not see any significant difference between the number of $GABA_A$ receptors in the cell surface in the double treated cells compared to those treated with medium or only Propofol or Orexin A. Nevertheless, in the images we can clearly see there is a reduction in the Orexin A + Propofol samples which indicate that the method followed to calculate overall differences is not the most appropriate.

The technique used to determine the number of receptors on the cell surface was to measure the mean intensity of the cells. However, the number of pixels that correspond to the receptors are only a small proportion in comparison to the whole cell. For that reason, the effect of the $GABA_A$ receptors in the mean intensity cannot be appreciated. If we had used a specific software to calculate only the number of individual receptors, the results would have been different.

Furthermore, during the whole procedure we did not use any saponin or other detergent to permeabilize the cells with the intention to stain only $GABA_A$ receptors on the surface. However, it is well known that with PFA fixation, the cells get somewhat permealized. As a consequence, we had staining of internalized $GABA_A$ receptors as well as those on the cell surface.

Previously, similar experiments were made with cortical rat neurons, but the number of neurons in the primary cultures was 10%-20% compared to 80%-90% that were glial cells. So in order to solve that problem and to further improve the results, the findings of our study were obtained from *in vitro* experiments utilizing the neuronal cell line SH-SY5Y. Moreover, some other benefits we obtained using this kind of cells are, first of all, we could avoid the ethical concerns of performing animal experiments and using human cells increases relevance. Also, we had unlimited access to cells since their care is not as tedious and long as rat cortical neurons, improving the efficiency of the protocol. However, SH-SY5Y cells are an immortalized cell line and not primary cells.

The intravenous drug Propofol, as many other anaesthetic drugs, involves the $GABA_A$ receptors in its signalling cascade, altering the vesicular transport and inducing neurite retraction.[46, 50] It has been shown that signalling events involving of RhoA and ROK, contributes to the contraction of the neurons by phosphorylating myosin light chain.[10, 16]

The neuropeptide Orexin A, produced by the lateral and posterior hypothalamus

which has an essential role regulating the arousal system[34], is capable of reducing the anaesthetic effects of various anaesthetic drugs as well as enhancing wakefulness in rats[56, 57, 58] Although the exact molecular mechanism still has to be elucidated it is known that OA is able of activate PKC through its signalling pathway.[36] Recent data from our lab show that, on one hand, OA can inhibit the retraction of the neurons caused by Propofol[42] through the stimulation of PKCε, on the other hand, OA can also affect to the recirculation of the $GABA_A$ receptors (unpublished data). These findings suggests that understanding how OA works may be a relevant tool to understand the anaesthetic mechanism of Propofol.

It is known that through its activation, PKC acts as a modulator for many ionic channels.[60] It is able to phosphorylate the intracellular loop of the $GABA_A$ receptors[21], and some reports claim this triggers their internalization.[21,24] In fact, the phosphorylated state of the receptor promotes its interaction with several proteins that facilitate the endocytosis, either for recycling it to the cell membrane or to degrade the receptor.[11, 13]

According to the above mentioned reports, it is tempting to speculate that OA, through its signalling cascade and the activation of the PKCε, can promote the activation of those proteins that initiate the endocytocis of the $GABA_A$ receptors decreasing the number of available receptors for Propofol's use. However, our data suggests that Orexin A does not affect the endocytosis directly, rather blocks the recirculation of the $GABA_A$ receptor since OA alone did not reduce cell surface receptors.

CONCLUSIONS

In this study we wanted to investigate if pre-incubation of SH-SY5Y cells with Orexin A before treating them with Propofol , the number of $GABA_A$ receptors available for Propofol were decreased. After performing the experiments, our conclusion is that even though the quantitative analysis showed no significant differences between the treatments, in the images we could clearly see a reduction of the number of $GABA_A$ receptors on the cell surface. So the method used to quantify overall differences was not the most appropriate.

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