OREXIN A REVERSES PROPOFOL AND THIOPENTAL INDUCED CYTOSKELETAL REARRANGEMENT IN RAT NEURONS

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Orexin A (OA) is an endogenous peptide regulating awakefulness, known to reduce anaesthesia in animals, but on cellular level its mechanisms to reverse anaesthetics are unknown. Primary cortical cell cultures from newborn rat brains are used and live cell light microscopy is performed to measure 1) neurite retraction after propofol, thiopental, barbituric acid and ketamine exposure and 2) the effect of OA application either before or after anaesthetics. Cytoskeletal reorganization is evaluated with fluorescence microscopy, protein changes are detected with Western blots and mass spectrometry is used to identify proteins after treatment with anaesthetics and/or OA. Adult rats are anaesthesized with propofol, and the cytoskeletal morphology is studied. Orexin A reverses and inhibits neurite retraction and actin ring formation induced by propofol and thiopental. No effect on retraction or actin rings was seen for ketamine (not active on gamma-aminobutiric acid (GABA) receptors), the non-anaesthetic barbituric acid, OA or solvents used. OA increases the tyrosine phosphorylation of a 50 kDa protein, identified as vimentin. Propofol induces an immediate granular appearance of vimentin, which OA reverses to a smooth distribution. Cytoskeletal morphology changes are also induced by propofol in vivo. All OA effects are blocked with an orexin receptor (OX1) antagonist. We conclude that OA reverses the GABA_A receptor mediated cellular effects of both propofol and thiopental in rat brain cells. The morphologic changes of actin and vimentin caused by propofol and thiopental, and the subsequent reversal by OA, deepens our understanding of the mechanisms of anaesthesia.

Key words: orexin A, anaesthesia, orexin receptor 1 antagonist, actin, cytoskeletal morphology, ketamine, vimentin, gamma-aminobutiric acid (GABA) receptor

INTRODUCTION

In anaesthesia, a drug which could quickly reverse the effects of anaesthetic drugs would be a valuable tool: minimizing the time of anaesthesia as well enabling the physician to make more frequent neurological assessment of intensive care patients. Orexin A (OA), also called hypocretin A, is an endogenous peptide produced by neurons in the hypothalamus, and their axons project to the whole brain except the cerebellum. OA interferes with orexin receptor (OX) 1 and 2 (1-3). OA is involved in the regulation of wakefulness but also takes part in the regulation of the autonomic nervous system (4-7), hormone release (8) and stimulates food intake (9). The ability of OA to cause wakefulness makes it interesting as a potential reversing agent of anaesthetics. Previous work has shown that injection of OA into the brain ventriculi reduces the anaesthetic effect of isoflurane (11), ketamine (12) and thiopental (13) in rats. The intravenous anaesthetic propofol interferes in cultured rat cortical neurons with the actin cytoskeleton in a dose- and time-dependent manner (14-16). Propofol also causes retraction of the neurite and reverse the transport of vesicles in rat cortical neurons; these processes are reversible and dependent on the GABA_A receptor and cytoskeletal reorganisation (17, 18). A cytoskeletal protein that is involved cellular adherence and membrane vesicular trafficking (19) is vimentin. This intermediate filament provides spatial-temporal organization of the cell, and its ability to become reorganized is controlled by changes in phosphorylation (20).

The aim of this study is to evaluate if the peptide promoting wakefulness, OA, can interfere with or oppose the cellular effects of anaesthetics. In particular, cytoskeletal dependent processes were studied in cultured brain cells treated with the intravenous anaesthetics propofol, thiopental or ketamine, as well as a non-anaesthetic barbiturate, barbituric acid. We report that OA inhibits and reverses the effects caused by propofol and thiopental on neurite retraction, reorganisation of actin and vimentin and phosphorylation of vimentin via OX1. Neither cytoskeletal effects nor retraction were induced by ketamine or barbituric acid, showing that OA interferes with GABA_A receptor dependent anaesthetics.
MATERIALS AND METHODS

Cell culture

This study was approved by the Linkoping Ethical Committee for Animal Research, Dnr 101/08 and 113/11. Primary cultures of mixed rat neurons/glial cells were prepared as described earlier (14, 21) and used between days 10 to 35.

Cytoskeletal structure identification

Cells growing on cover-slips were rinsed twice in calcium containing buffer (CCM), pH 7.40 at 37°C (22), followed by propofol (23) in the commercial solution Lipuro®, the lipid vehicle Vasolipid® (Braun, Melsungen, Germany), OA, or the OA solvent acetic acid (AE, 0.001%) as described below, at 37°C. For short time experiments, cells were exposed to propofol (20 µM) for 15 s – 5 min. In dose response reversal experiments, OA (10–12–10–7 M) was added after 1 min of propofol (16.8 µM) exposure for another 20 min in the presence of propofol or for inhibition experiments pre-incubated 1 min before propofol exposure for 20 min. To test if the effect of OA vanished over time, OA was given after 1 min of propofol exposure, and experiments terminated after 30 s – 35 min. To evaluate the effects of a brief OA exposure, OA (10–12–10–7 M) was added after 15 min of propofol treatment for the last 5 min. Effects of OA only were tested at 10–12–10–7 M. To block the effects of OA, the OR1 antagonist SB334867 (1 µM) (24) or dimethylsulfoxide (DMSO) at the corresponding volume, was applied to propofol-treated cells (20 µM, 5 min) 1 min before addition of OA (10 nM, 10 min). Solvents were tested for the longest appropriate time at the highest concentration used. In barbiturate experiments, a high dose of thiopental (1 mM) (25, 26) as the commercially available Thiopental Inresa® (Inresa Arzneimittel GmbH, Freiburg, Germany), the non-anaesthetic barbituric acid (BA, 1 mM), or the solvent for thiopental Na2CO3 (150 µM) was added for 2–30 min. Ketamine (30 µM) was added for 2 or 20 min. For reversal experiments, OA (10 nM) was applied after 1 min of thiopental exposure, as described above. Stimulation was stopped by adding 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) for 20 minutes at 37°C, thereafter cells were permeabilised (saponin 0.1%PBS, 5 min at room temperature (rt)). Actin was labelled with Alexa-564-conjugated phalloidine (1:300 in 0.1% saponin/PBS) for 30 minutes in a dark humidified chamber (rt), rinsed 2×5 min in PBS and 1×5 min in distilled water. Vimentin (Novus biologicals, Cambridge, UK) was labelled using goat-anti-vimentin anti-body (1:500) and Alexa-488-conjugated secondary antibody (1:400). Neurons were identified with mouse anti-tubulin-β3 antibody (1:500, Thermo Fisher scientific, Waltham, MA, USA) followed by Alexa-564-conjugated antibody (1:400, Invitrogen, Paisley, UK). All antibodies were diluted in 2% bovine serum albumine (BSA)/0.1% saponin/PBS and incubated for 45 min as described above. Coverslips were mounted using fluorescent mounting medium (DAKO corp., Carpinteria, CA, USA), mixed with DAPI (1.5 µl mL–1) onto glass slides. To ensure consistency in the results, coverslips were viewed (63× oil-fluorescence objective, numeric aperture (NA) 1.4 (Axiovert 200M, Carl Zeiss, Microimaging GmbH, Gottingen, Germany)) in random order by the same person, who was blinded for the treatment. At least 100 cells were counted/cover-slip, and the percentage of cells containing actin rings was calculated. Vimentin and tubulin-β3 marked cells were visually evaluated.

Live cell imaging followed by actin staining in the microscope

Some cells were stimulated with propofol (20 µM) in the Axiovert 200M microscope and the neurite followed for 20 min as described above. After 20 min, the stimulating medium was quickly removed from the cell, PFA/PBS (4%, 20 min) added and actin stained with ALEXA-546-conjugated phalloidine (1:300, 30 min) with the cell still in the viewfield of the microscope. Cells were washed 3 times with 0.1% saponin/PBS, DAPI (1.5 µl mL–1) was added in the 4th washing step, and the cells washed further 2 times. Thereafter fluorescence microscopy with z-stacks (0.25 µm steps) was performed in combination with DIC.

Analysis of cellular proteins

In CCM-25 cm2 culture flask, frozen samples were rinsed twice in CCM, then incubated with propofol (20 µM) or thiopental (1 mM) for 5 minutes before addition of OA (10 nM) for 10 minutes in the presence of the anaesthetics, with their respective solvent used as controls, at 37°C in a waterbath. The OA antagonist SB334867 (1 µM) or DMSO at the corresponding volume was added 1 min before OA (10 µM) for 10 min. OA (10 nM) only, Na2CO3 (150 µM) or BA (1 mM) was used as controls. Ketamine (30 µM) was applied for 10 min after CCM (5 min).

37°C. Differential interference contrast (DIC) images of cells were taken (Zeiss Axiovert 135M (Göttingen, Germany) with a 40×, 1.3 NA oil immersion objective), processed and stored as described earlier (17). We analyzed only superficial cells growing on a glial cell layer. Time-lapse series were obtained with 1 min interval, and neurite length (measured manually by an observer who had not done the experiments) at time –1 was used as reference (100%) (17). Measurement obtained from a single neurite is defined as (n=1) and neurons were obtained from at least 3 different rat litters in each experimental group. Experiments started with CCM (5 min) to establish steady state. Thereafter, propofol (20 µM), or thiopental (0.1–1 mM) was added 10 s before time zero for 5 or 10 min respectively. This was followed by addition of AE (0.001%) or OA (10 nM) and the neurite was followed further for 10 min. To block the effect of OA, SB334867 (1 µM) or DMSO at the corresponding volume was added 1 min before OA (10 µM). In inhibition experiments cells were added with CCM treated with OA (10 nM) 1 min before propofol (20 µM) for 10 min. OA (10 nM) only, Na2CO3 (150 µM) or BA (1 mM) was used as controls. Ketamine (30 µM) was applied for 10 min after CCM (5 min).

Live cell microscopy

The coverslip was rinsed twice in CCM and mounted in a closed bath imaging chamber placed in a heated stage to obtain

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or overnight (4°C). The membrane was washed six times (PBS-Tween 0.05%) and incubated with peroxidase-linked goat anti-mouse antibodies (1:5000–10,000 in 0.05% PBS-Tween, 1 hour, rt). After extensive washing in PBS-Tween, the membrane was incubated with enhanced chemiluminescence (ECL) Western blotting detection reagents and exposed to ECL hyperfilm. To remove bound antibodies some membranes were washed with 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C, blocked and re-probed with a new antibody.

**Mass spectrometry**

Whole cell lysates, IP, or the IP-supernatants were separated as described above, stained with Coomassie blue G-250 for 20 min in room temperature, and destained with 5% AE/5% ethanol in water until the protein bands were clearly visible. Bands with changed phosphorylation were excised from the gel, and in-gel digestion was performed with sequencing grade modified trypsin (Proigma, Madison, WI, USA) (28). The peptides obtained were analyzed on MALDI-MS and data cross-matched using the Mascot database.

**In vivo experiments on rats**

Ethical approval (Dnr 37/11) was given from Linköping Ethical Committee for Animal Research to compare the morphology of CO2 (control) and propofol-treated brains from adult (>4 months old) male Sprague Dawley rats. Control rats were placed in a box, 25 L min−1 CO2 was applied until spontaneous respiratory rate were <3 min−1, thereafter the thorax and abdomen were cut open, the left cardiac ventricle cannulated and the needle inserted into aorta and 4% PFA in 0.1 M PBS, (pH 7.4, 300 mOsm) were infused using an external pump. Experimental rats were first anaesthetized with 3% isoflurane, and the abdomen was cut open, the left cardiac ventricle cannulated and the needle inserted into aorta and 4% PFA in 0.1 M PBS, (pH 6.7) for 30 min at 50°C, blocked and re-probed with a new antibody.

**Oral A reverses propofol induced actin ring formation**

Propofol causes morphological changes of actin by forming a ring structure (Fig. 1A). OA dose-dependently reverses (addition after propofol, n=4) respectively blocks (addition before propofol, n=5) the actin ring formation (pIC50 of −9.02±0.31 M and −9.72±0.31 M respectively, p<0.01), corresponding to IC50 of 0.96 nM and 0.19 nM. OA reverses the propofol effect completely at 10 nM. The lipid vehicle or AE do not cause actin ring formation above the baseline level of approximate 5% in CCM treated cells (Fig 1B and 1C). OA in itself applied at 10−12−7 M do not produce actin ring structures above baseline level (n=4, results not shown). The effect of OA (10 nM) does not vanish when added after one minute of propofol exposure and followed for up to 35 minutes (n=4, p<0.001 compared with the percentage of cells with actin rings caused by propofol (16.8 µM) alone; results not shown). OA added for the last 5 min of a 20 min exposure to propofol (n=5) only partially reverses the percentage of cells with actin ring formation (10.6±1.3% for OA 10−9 M compared with the lipid vehicle (3.4±0.7%, p<0.01)). In this setting, the pIC50 is −10.68±0.38 M, corresponding to an IC50 of 20.9 pM (Fig 1D), p<0.001 compared with the pIC50 of cells treated with OA either one min before or after propofol application. The OR1 antagonist SB334867 (1 µM) blocked the reversal effect of OA on propofol-induced actin ring formation (n=3, p<0.001 compared with propofol/DMSO/OA, Fig 1E).

**Thiopental induces actin rings that are reversed by orexin A**

The barbiturate thiopental (1 mM) causes actin ring formation, with a maximum of 30.0±2.5% cells with actin rings at 20 minutes (n=8, p<0.001 compared with Na2CO3). OA (10 nM) added one minute after thiopental reverses the thiopental effect (11.1±1.3% cells with actin rings, n=6, p<0.001 compared with propofol 20 min, one-way ANOVA). Neither the solvent of thiopental (Na2CO3 (n=7), (Fig 1F), the non-anaesthetic barbiturate butabarbituric acid (n=5, results not shown) or ketamine (30 µM, n=3, results not shown) produce actin rings above baseline.

**Neurite retraction**

Propofol (20 µM)/AE causes time-dependent linear neurite retraction (slope −1.9±0.3, r2=0.44, p<0.0001) to 57.2±9.5% of reference value (n=7), after 15 min stimulation (Fig. 2A and 2B). OA reverses propofol retraction. 92.3±3.9%, n=7, p<0.05 compared with propofol/AE. OA added 1 min before propofol blocks the retraction (91.6±5.9%, n=8, results not shown). SB334867 blocks the effect of OA, the neurite length at 15 min is 63.6±6.6%, n=14, n.s compared with propofol/AE (Fig 2B), and cells receiving DMSO do not differ from propofol/OA-treated cells (results not shown). Thiopental (1 mM) causes a linear retraction (slope −1.6±0.2, r2=0.44, p<0.001), with a
Fig. 1. (A) Cells labelled with ALEXA-546-phalloidin (1:300) after stimulation with propofol 20 μM for 20 minutes. The left arrow points at an actin ring within a cell and above the focal plane another ring structure is seen in the adjacent cell (right arrow). Nuclei are stained blue with DAPI (1.5 µl mL⁻¹).

(B, C, D) The percentage of cells with actin ring structures, in cells treated with calcium medium (Ca), Intralipid 30 µl mL⁻¹ (IL), orexin A (OA) 10⁻⁷ M (O-7), acetic acid (AE), 0.001% or propofol 16.8 µM (P) for 20 min.

(B) OA (10⁻¹²–10⁻⁷ M) is added 1 min after propofol (reversal experiment). OA inhibits the actin ring formation with a pIC₅₀ of –9.02±0.31 M (mean ± S.D.), corresponding to an IC₅₀ of 0.96 nM (n=4).

(C) OA (10⁻¹²–10⁻⁷ M) is added 1 min before propofol (inhibition experiment). OA inhibits the actin ring formation with a pIC₅₀ of –9.72±0.31 M (mean ± S.D.), corresponding to an IC₅₀ of 0.19 nM (n=5).

(D) Orexin A (10⁻¹²–10⁻⁷ M) is added 15 min after propofol for the last 5 min. OA partially reverses actin ring formation (10.6±1.3% for OA 10⁻⁷ M) compared with the lipid vehicle (3.4±0.7%, p<0.01), with an pIC₅₀ of –10.6±0.38 M (mean ± S.D.), corresponding to an IC₅₀ of 20.9 pM (n=5), p<0.01 compared with the pIC₅₀ of cells treated with OA either one min before or after propofol application. Unless otherwise stated, values represent mean ± S.E.M.

(E) The percentage of cells with actin ring structures after treatment with Ca or propofol (P, 20 µM) for 5 min, followed by dimethylsulfoxide (DMSO) or the OR₁ antagonist SB334867 (SB, 1 µM) for 1 min before application of OA (10 nM) or AE for further 10 min, with continuing presence of propofol. SB334867 blocked the effect of OA on propofol-induced actin ring formation (n=3, p<0.001 compared with propofol/DMSO/OA), thereby restoring the amount of rings to propofol-induced levels.

(F) The percentage of cells with actin ring structures, treated with thiopental (1 mM, Pe, n=8), Na₂CO₃ (150 µM, n=7), Ca (n=3) or thiopental (1 mM) with OA (10 nM) addition after 1 min (Pe/OA, n=6) for the time indicated. Thiopental induces actin rings in 30.0±2.5% of cells at 20 minutes, whereas OA reverses this effect to 11.1±1.3% cells with actin rings, p<0.001 (***) compared to propofol 20 min, one-way ANOVA and Bonferroni post hoc test. All other times and treatments were non-significant. Results shown are mean ± S.E.M.; where no error bars are shown, they lie within the symbol.
Orexin A increase tyrosine phosphorylation of vimentin after anaesthetic treatment

Orexin A interferes with phosphorylation upon tyrosine residues on several proteins in both propofol (not shown, n=5) and thiopental-treated cells (Fig. 4A, n=5). After protein enrichment with immunoprecipitation using PTyr antibodies, OA increases the phosphorylation of a protein of about 50 kDa in both propofol and thiopental-treated cells. Mass spectrometry performed on protein bands from the precipitate (Fig. 4B) suggested the 50 kDa band to be the cytoskeletal protein vimentin; this is confirmed by vimentin antibodies (Fig. 4C). The vimentin band is double, with the major tyrosine phosphorylation taking place on the lower band. After OA treatment, the amount of vimentin is increased in the upper band and decreased in the lower band compared to anaesthetic stimulated cells, n=3. The OA antagonist SB334867 blocked the effect of OA (n=4, results not shown).

Fig. 2. (A) Time-lapse imaging reveals dynamics of neurite retraction and outgrowth after addition of propofol and OA. Cortical cell cultures in Ca²⁺-containing medium (CCM) were exposed to 20 µM propofol and observed for 20 min. Images shown are 1, 5, 10 and 15 min after addition of propofol. OA (10 nM) is added 5 min after propofol exposure. Scale bar=10 µm.

(B) Cortical cell cultures in CCM is observed for 5 min and exposed to 20 µM propofol for 15 min. OA (1/10 nM) or AE is added 5 min after propofol exposure. Values are expressed as percentage of neurite length (100%), 1 min before propofol addition (time - 1). Values represent mean ± S.E.M. Data based on n=7 neurites in the propofol/OA group, n=7 neurites in propofol/AE group, n=5 neurites in OA group and n=6 neurites in AE group. Propofol/AE retracts the neurites to 57.2±9.5% after 15 min stimulation. OA reverses propofol retraction (92.3±3.9%, n=7, p<0.05) compared with propofol/AE and not significant compared with baseline. Neither AE (94.2±9.8%, n=6) nor OA (103.6±1.5%, n=5) on their own caused significant changes in neurite length. The OR1 antagonist SB334867 (1 µM) blocked the OA effect, restoring the neurite length to propofol/AE values (63.6±6.6%, n=14, n.s compared with propofol/AE).

(C) Time-lapse imaging reveals dynamics of neurite retraction and outgrowth after addition of thiopental and OA. Cortical cell cultures in CCM were exposed to 1 mM thiopental and observed for 25 min. Images shown are –1, 10, 15 and 20 min following addition of thiopental. OA (10 nM) is added 10 min after thiopental exposure. Scale bar =10 µm.

(D) Cortical cell cultures in CCM were observed for 5 min and exposed to 1 and 0.1 mM thiopental for 20 and 10 min respectively. OA (10 nM) or AE has been added 10 min after thiopental exposure. Values are expressed as percentage of neurite length (100%), 1 min before thiopental addition (time –1) and represent mean ± S.E.M. Data based on n=11 neurites in the thiopental (10⁻³)/OA group, n=11 neurites in BA group, n=9 neurites in the thiopental (10⁻⁴) group, n=10 neurites in thiopental (10⁻⁴)/AE group and n=6 neurites in Na₂CO₃ group (150 µM). Thiopental (1 mM) retracts the neurite to 76±6.3% (n=10) after 10 minutes stimulation, and 0.1 mM retracts the neurite to 90.2±4.3% at 10 min, p<0.001 compared with Na₂CO₃. OA (10 nM) is added 10 min after thiopental exposure whereafter the neurite is 85.1±7.2 % compared with thiopental/AE at 20 min (63.1±7.3%, n=10, p<0.05) and non-significant compared with baseline (Student’s t-test). No retraction is seen by barbituric acid (1 mM, 100.7±3.1%, n=11) Na₂CO₃, (99.5±2.2%, n=6), AE (94.2±9.8%, n=6) or OA (103.6±1.5%, n=5), all non-significant compared with baseline with two-way ANOVA, followed by Bonferroni test.
Vimentin structure is changed by propofol and reversed by orexin A

Vimentin is found in both neurons (cells positive for beta-3 tubulin) and glial cells (Fig. 5A, n=3). The structure of vimentin in propofol treated cells show a more rough, granular appearance in comparison with the smoothly distributed vimentin in cells treated with CCM or propofol for 5 min followed by OA for 10 min. The change to granular appearance of vimentin is visible already after 15 seconds treatment with propofol (n=3, results not shown). In propofol treated cells, application of the OA antagonist SB334867 (1 µM, n=3) before OA, again give vimentin a granular appearance, Fig. 5B. No correlation was seen between vimentin and actin rings at 20 min (results not shown). Ketamine (30 µM) do not change the vimentin structure (n=3, results not shown).

Dorsal membrane ruffles, actin ring formation and rough vimentin are present in propofol-anaesthetised rats

To compare the above described in vitro results with the morphology in vivo, brain-slices from propofol-anaesthetised (20 min) or CO₂ treated animals were evaluated. Propofol-, but not CO₂ treated rats, also show dorsal membrane ruffles, actin rings and granular vimentin in the brain-slices (n=3, Fig. 6).

DISCUSSION

In this study, thiopental induces the same cellular effects (actin ring formation (14, 16, 30) and neurite retraction (17)) as propofol does in cultured neuronal rat brain cells. Orexin A reverses actin ring formation and neurite retraction induced by propofol or thiopental when applied after the anaesthetics and inhibits these effects if given in advance of the anaesthetics. Both propofol and thiopental induce changes in phosphorylation of tyrosine residues on vimentin, and OA reverses these phosphorylation changes. The structural appearance of vimentin in propofol treated cells becomes granular already after 15 s and when OA is applied after propofol, vimentin again becomes smoothly distributed. All these effects of OA are blocked with the OX1 antagonist SB334867. No effects on actin or neurite retraction were
observed by ketamine, the non-anaesthetic barbiturate barbituric acid, the solvents for the drugs or OA alone.

The OA experiments are done in vitro on a mixed neuronal and glial cell culture from rat brain cortex missing their normal input of OA from hypothalamus (1-3). Such deprivation of OA stimulation could make cells more sensitive to OA, however the IC₅₀ of approximate 1 nM for OA to reverse actin ring formation (Fig. 1B) is in the same range as used to reduce anaesthesia in animals (11, 13, 31) suggesting the cellular responsiveness to OA be normal. OA (10 nM) completely reverses both propofol and thiopental stimulated actin ring formation, although the anaesthetics are continuously present at a high concentration (23, 25). The effect of OA is sustained, without relapse to anaesthetic appearance after increasing time of propofol application. Pre-incubation with OA inhibited the propofol effect with an IC₅₀ of 0.2 nM (Fig. 1C), within range of the IC₅₀ of 0.5 nM OA needed to decrease thiopental anaesthesia time in rats (13) and OA pre-treated rats emerge from propofol anaesthesia at 0.3 nM (31). The 5-fold reduction in OA needed to suppress - compared to reverse - the actin effects of propofol suggests the initiation of anaesthesia in vitro to be OA sensitive, whereas inhibition of orexergic signalling do not affect induction but delays emergence from volatile anaesthesia in rodents (32). In humans, plasma OA increases from 20 pM to 30 pM after emergence from propofol-fentanyl anaesthesia (33), proposing OA to be part of awakening mechanisms after anaesthesia. A reduction of anaesthetic effect with 50% at 21 pM OA would hasten the awakening of patients, if the short OA exposure (5 min) data in Fig. 1D can be extrapolated to a clinical setting. OX1 can be inhibited by pentobarbital, ketamine and volatile anaesthetics in Xenopus oocytes(34), whereas in our study the effect of OA on propofol and thiopental induced cellular effects is mediated via the OX1, as they are blocked by the antagonist SB334867. OA has been shown to decrease ketamine anaesthesia time by 20–30% (12), but we

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**Fig. 4.** (A) Immunoblot with anti-phosphotyrosine antibodies (PTyr, 1:500) of cell lysates loaded on a 7.5% polyacrylamide gel. Cells were stimulated with Na₂CO₃ (150 µM) for 15 min, thiopental 1 mM (5 min) followed by addition of either acetic acid (0.001%, 10 min) (Pe/AE) or OA (10 nM, 10 min) (Pe/OA) or OA (10 nM, 15 min). Thiopental decreases the tyrosine phosphorylation of a 50 kDa protein, that is restored to normal phosphorylation by OA. Molecular weight markers to the left (kDa).

(B) Coomassie blue stained 7.5% polyacrylamide gel, showing protein bands after thiopental treatment. The first left hand lane is loaded with two molecular weight (MW) ladders. Lane 2 and 3 show PTyr-immunoprecipitates (IP) of cells treated with thiopental (1 nM) for 5 min followed by either AE (0.001%) or OA (10 nM) for 10 min. Lanes 4 and 5 are loaded with lysate samples collected from the same experiments as were used for IP. In gel mass spectrometry was performed on bands outlined (1–9). Band 3 is identified as myosin, also used in the MW ladders, corresponding to 210 kDa. Band 6 and 7 are vimentin, with the upper more likely to be the c-terminal (70% identity of peptides) and the lower band the n-terminal part (50% identity of peptides). Band 8 is actin. Remaining bands were either not pure enough to retrieve a single protein or the suggested identity not confirmed.

(C) Immunoblots with PTyr (1:500) or anti-vimentin (1:2000) antibodies of lysates, IP supernatant (Sup) or IP with PTyr from cells treated with either thiopental (1 nM), upper row or propofol 20 µM, lower row for 5 min followed by either AE or OA (10 nM) for 10 min, using cells from the same rat litter. The blot membrane was first used to visualize phosphotyrosine (left side), then the same membrane is stripped and reprobed with vimentin antibodies. The blots are shortly exposed to ECL films to avoid saturation of signal from the IPs. Thiopental gels were run for a longer period of time, to increase separation of the two 50 kDa bands, identified as vimentin. The amount of the upper vimentin band is increased by OA, whereas the major phosphorylation takes place on the lower vimentin band.
could not test the effect of OA on ketamine as ketamine did not produce actin rings, retraction or vimentin changes.

The actin ring structure process has been described after initiation with different stimuli that activates tyrosine kinase receptors, such as growth factors (35, 36). After tyrosine kinase receptor activation the cell membrane folds and moves like a wave to the cell interior, where it forms circular DMR. In the live cell experiments we see membrane waves and DMR formation (Fig. 3), simultaneously with neurite retraction after propofol treatment, and actin is shown to be directly underneath the DMR (Fig. 3 and animations), following the shape of the DMR. Membrane folding is initiated by curved activators of actin polymerization (37), and when actin is rearranged to a circle, it folds the membrane into tubes, thereby sequestering tyrosine kinase receptors into the cell (38). The actin tubes are closed, forming vesicles that is either degraded or re-circulate to the membrane. The tiny actin rings visible in the animations and the brain slices might represent such actin/membrane tubes. Propofol causes tyrosine phosphorylation upon the GABA_A receptor (14, 15), and the formation of actin rings/DMR might be involved in removing anaesthetic-activated receptors from the cell surface. The time maximum for DMR formation in other systems is 20 minutes (36), as seen in Fig. 3 and correlates with the propofol (30) and thiopental (Fig. 1E) data; after longer time periods the actin process decline (36). Actin ring structures are an effect of GABA receptor dependent anaesthetics as they are formed by thiopental and propofol but not ketamine or the non-anaesthetic barbiturate BA. Actin rings and DRM are also seen in brain-slices from in vivo experiments on rats (Fig. 6), confirming the in vitro data. Close to the DMR, actin stress fibers are disassembled, leaving a fine actin cortical meshwork (39) used to rearrange cellular shape, such as the propofol induced neurite retraction, a process known to be actin dependent (17).

Fig. 5. (A) Cells treated with calcium-containing medium (CCM) and fixed with 4% PFA-PBS for 20 min, followed by incubation with anti-tubulin-β3 antibody (1:500, upper panel) as a neuronal marker and anti-vimentin anti-body (1:500, middle panel). The two pictures are merged (lower panel) with blue nuclei (DAPI), showing that neurons are positive for vimentin. (B) Cells treated with propofol (20 µM, 20 min; upper panel) and stained with vimentin anti-body (1:500), visualizing a granular, rough appearance of vimentin. Cells treated with propofol (20 µM) for 5 min followed by OA (10 min) for further 10 min, and stained with vimentin anti-bodies (middle panel) reverse vimentin to the same smooth appearance as in CCM treated cells (Fig. 5A, middle panel). Cells treated with propofol (20 µM, 5 min) followed by addition of SB334867 (1 µM) for one minute before OA (10 nM) application for further 10 min in the presence of propofol, show vimentin in a granular appearance (lower panel).
Neurite retraction take place during brain development (irreversible), in hibernation (reversible) (40) and in neurotrauma (partial reversible) (41). Propofol caused neurite retraction is reversible, initiated via the GABA<sub>A</sub> receptor (17, 18) and dependent on actin re-organisation and myosin II. OA (10 nM) could inhibit as well as reverse the retraction caused by propofol and thiopental (Fig. 2). OA had no effect on its own on the neurite length. Neither BA nor ketamine induce retraction,
showing that retraction is an anaesthetic process dependent on the GABA<sub>α</sub> receptor. The retraction is done together with massive rearrangement of the cytoskeleton (Fig. 3) (17).

Vimentin is an intermediate filament that links cytoskeletal proteins, often in close relation to the cell membrane. Vimentin is phosphorylated upon tyrosine (42, 43) and is regulated by the kinases Src and rho-kinase (44). When phosphorylated, it is destabilized (20), allowing it to re-model its architecture. Vimentin is identified as the most phosphorylated protein in IP destabilized (20), allowing it to re-model its architecture.

The morphology of vimentin is changed form granular to smoothly distribution when propofol treated cells receive OA, supporting the immunoblot data that OA interacts with vimentin. Already after 15 s, propofol-treated cells do show granular vimentin, and also in propofol-anaesthesized rats brain cells the vimentin has a granular appearance. OA applied after propofol/thiopental increases the higher molecular weight vimentin combined with a decrease in the lower molecular weight vimentin. Vimentin molecular weight variants might be due to calcium-activated vimentin protease (45). Such shift in the ratio between the higher and lower molecular weight vimentin caused by changed phosphorylation, would influence the remodelling capacity, and the OA effect could be to re-stabilize vimentin. For DRM a close association with the podosome formation (protrusions used by cells to adhere to the substrate and initiate mobility) and the initiation of dorsal ruffles have been seen (36). Vimentin are surrounding actin in podosomes (46) suggesting that changes in vimentin activity are important to help building actin rings. Vimentin organisation is dependent on tyrosine phosphorylation events and actin ring formation is blocked when tyrosine kinases or rhokinase are inhibited (16). OA applied after 15 min of propofol exposure, could only partially reverse the formation of actin rings (Fig. 1D). A possible explanation is that new rings are not formed when vimentin is re-stabilized, whereas actin rings formed before OA application continues their movement to the cell center. For lysophosphatidic acid (LPA) induced neurite retraction, rho-kinase phosphorylates vimentin and locally remodel vimentin architecture, generating a contracting force via myosin. Actin and vimentin are found in the retracting tip of the neurite (44) and neurite retraction caused by propofol is dependent on rho-kinase (47). Vimentin is also involved in membrane vesicle trafficking (19), and propofol causes increased vesicular movement in neurites (18). The cellular effects reported in this study are only shown with anaesthetics. The intermediate cytoskeletal protein vimentin shows immediate changes in its morphology after propofol treatment, both <i>in vitro</i> and <i>in vivo</i>. OA reverses the cellular effects of anaesthetics <i>via</i> OX1 and OA and the GABA<sub>α</sub> receptor dependent anaesthetics interfere upon vimentin; proposing the cytoskeleton as a key regulator of anaesthetic effect. In the future, an OA-agonist could possibly be used to reverse anaesthesia if these <i>in vitro</i> and animal data can be extrapolated to patients. However, more studies on the cellular effects of anaesthetics are needed, where OA could be used as a tool to elucidate key signalling pathways.

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