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Keywords: Moist tobacco snus (snuff); nicotine; endothelin ETB receptor, serotonin 5-HT1B receptor; thromboxane A2 TP receptors, cerebral arteries.

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Abstract: The cardiovascular risk for users of use of Swedish snus/American snuff (moist tobacco) has been debated for a long time. The present study was designed to examine the effects of water- or lipid-soluble (DMSO-soluble) snus and nicotine, the most important substance in tobacco, on the expression of vasocontractile G-protein coupled receptors (GPCR), such as endothelin ETB, serotonin 5-HT1B, and thromboxane A2 TP receptors, in rat cerebral arteries. Studies show that these vasocontractile GPCR show alterations by lipid-soluble cigarette smoke particles via activation of mitogen-activated protein kinases (MAPK). However, the effects of moist tobacco on the expression of GPCR are less studied.
Rat middle cerebral arteries were isolated and organ cultured in serum free medium for 24 h in the presence of water-soluble snus (WSS), DMSO-soluble snus (DSS), or nicotine. The dose of snus and nicotine was kept at plasma level of snus users (25 ng nicotine/ml). A high dose (250 ng nicotine/ml) was also included due to the previous results showing alteration in the GPCR expression by nicotine at this concentration. Contractile responses to the ETB receptor agonist sarafotoxin 6c, 5-HT1B receptor agonist 5-carboxamidotryptamine, and TP receptor agonist U46619 were investigated by a sensitive myograph. The expression of ETB, 5-HT1B, and TP receptors were studied at mRNA and protein levels using quantitative real time PCR and immunohistochemistry, respectively.
Organ culture with WSS or DSS (25 ng nicotine/ml) lowered the 5-HT1B receptor mediated contraction. Furthermore, DSS shifted the TP receptor mediated contraction curve left-wards with a stronger contraction. High dose of nicotine (250 ng nicotine/ml) increased the ETB receptor mediated contraction. The combined 5-HT1B and 5-HT2A receptor mediated contraction was increased, and both the 5-CT and TxA2 induced contractions were left-ward shifted by WSS, DSS, or nicotine (250 ng nicotine/ml). Only the DSS group showed that the increase of 5-HT1B receptor mediated contraction occurred at the transcriptional level, demonstrated by an increased mRNA expression for the receptor.
Snus and nicotine alter the GPCR expression in the cerebral arteries, suggesting that both snus and nicotine may have a potential impact on cerebral vasculature and on the development of cerebrovascular disease like stroke.

Date 2010-11-18

Dear Sirs,

Please find the enclosed manuscript “Alteration in contractile G-protein coupled receptor expression by moist snus and nicotine in rat cerebral arteries” by H. Sandhu, C.B. Xu, and L. Edvinsson. The paper has not been submitted before or published in whole or in part. The authors have no conflict of interest in the results published. . Conflict of interest declaration is attached as a separate document. All the authors have read and approved the submission.

The manuscript text is written in Microsoft word and the fonts are Times New Roman. The contents are as follows:

- Title
- Authors
- Affiliations
- Corresponding author
- Abstract
- Keywords
- Main text
    - Introduction
    - Materials and Methods
    - Results
    - Discussion
    - Conclusion
- Aknowledgement
- References

Figure legends (The text is written in Microsoft word and the fonts are Times New Roman).

Tables (The text is written in Microsoft word and the fonts are Times New Roman).

- Table 1

Figures (Figures have been made in Adobe Illustartor, the fonts in illustrations are Times New Roman, and the figures have been saved in 300 dpi TIFF format):

- Figure 1A-I
- Figure 2A-I
- Figure 3A-J

We are looking forward to hear the views of you and your reviewers.

Sincerely,
Hardip Sandhu
Cang Bao Xu
Lars Edvinsson

Alteration in contractile G-protein coupled receptor expression by moist snus and nicotine in rat cerebral arteries

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Abstract

The cardiovascular risk for users of use of Swedish snus/American snuff (moist tobacco) has been debated for a long time. The present study was designed to examine the effects of water- or lipid-soluble (DMSO-soluble) snus and nicotine, the most important substance in tobacco, on the expression of vasocontractile G-protein coupled receptors (GPCR), such as endothelin ET_B, serotonin 5-HT_{1B}, and thromboxane A_2 TP receptors, in rat cerebral arteries. Studies show that these vasocontractile GPCR show alterations by lipid-soluble cigarette smoke particles via activation of mitogen-activated protein kinases (MAPK). However, the effects of moist tobacco on the expression of GPCR are less studied.

Rat middle cerebral arteries were isolated and organ cultured in serum free medium for 24 h in the presence of water-soluble snus (WSS), DMSO-soluble snus (DSS), or nicotine. The dose of snus and nicotine was kept at plasma level of snus users (25 ng nicotine/ml). A high dose (250 ng nicotine/ml) was also included due to the previous results showing alteration in the GPCR expression by nicotine at this concentration. Contractile responses to the ET_B receptor agonist sarafotoxin 6c, 5-HT_{1B} receptor agonist 5-carboxamidotryptamine, and TP receptor agonist U46619 were investigated by a sensitive myograph. The expression of ET_B, 5-HT_{1B}, and TP receptors were studied at mRNA and protein levels using quantitative real time PCR and immunohistochemistry, respectively.

Organ culture with WSS or DSS (25 ng nicotine/ml) lowered the 5-HT_{1B} receptor mediated contraction. Furthermore, DSS shifted the TP receptor mediated contraction curve left-wards with a stronger contraction. High dose of nicotine (250 ng nicotine/ml) increased the ET_B receptor mediated contraction. The combined 5-HT_{1B} and 5-HT_{2A} receptor mediated contraction was increased, and both the 5-CT and TxA2 induced contractions were left-ward shifted by WSS, DSS, or nicotine (250 ng nicotine/ml). Only the DSS group showed that the increase of 5-HT_{1B} receptor mediated contraction occurred at the transcriptional level, demonstrated by an increased mRNA expression for the receptor.

Snus and nicotine alter the GPCR expression in the cerebral arteries, suggesting that both snus and nicotine may have a potential impact on cerebral vasculature and on the development of cerebrovascular disease like stroke.

Keywords: Moist tobacco snus (snuff); nicotine; endothelin ET_B receptor, serotonin 5-HT_{1B} receptor; thromboxane A_2 TP receptors, cerebral arteries.

1 Introduction

The use of snus (moist tobacco) is an effective way for Swedes to quit/reduce smoking, and thus reduce health risks of burnt tobacco use. However, this occurs at a cost of local oral exposure and higher nicotine levels in the circulation. The toxic effects of using Swedish moist snus on cerebral and cardiovascular diseases have been debated for a long time. Clinical studies show that snus has; (i) mild side-effects on health like few and occasional oral lesions (Andersson *et al.*, 1994), (ii) moderate side-effects such as elevated blood pressure (Bolinder *et al.*, 1992), (iii) rarely, modification of cerebral and cardiovascular diseases, and (iv) seldom larynx/oesophagus cancers (US Department of Health and Human Services, 1986). However, specific and detailed pharmacological studies on snus effects on cerebral arteries are absent.

Nicotine is the addictive substance in snus. In order to reveal possible molecular mechanisms of snus, which could lead to development of cerebrovascular disease, we examined if snus extracts or nicotine could alter GPCR expression and function of contraction in rat cerebral arteries (Hansen-Schwartz *et al.*, 2002;Hoel *et al.*, 2001).

GPCR-mediated vascular smooth muscle contraction, proliferation and apoptosis are important events in the pathogenesis of cerebral and cardiovascular disease (Hansen-Schwartz *et al.*, 2003b). Organ culture of cerebral vessels has been demonstrated as an *in vitro* method for exploring the molecular mechanisms that lead to changes in GPCR (endothelin type B receptor (ET_B), 5-hydroxytryptamine type 1B receptor (5-HT_{1B}), and thromboxane A_2 prostanoid receptor (TP)) expression in a way resembling that observed in cerebral ischemia *in vivo* (Hoel *et al.*, 2001;Rosamond *et al.*, 2008;Hansen-Schwartz *et al.*, 2002;Henriksson *et al.*, 2003). All these three receptors belong to the GPCR family and mediate cerebral vascular contraction and smooth muscle

cell proliferation (Coleman *et al.*, 1994;Masaki *et al.*, 1994;Nilsson *et al.*, 1999) and have been observed to be upregulated after a stroke and after organ culture.

The present study has examined the effects of snus and nicotine at plasma level which resemble that seen in snus users (25 ng nicotine/ml) (Benowitz *et al.*, 1994;Foulds *et al.*, 2003). A high dose was also included (250 ng nicotine/ml) due to previous studies showing that higher doses of nicotine may influences GPCR expression (Zhang *et al.*, 2009). "Normal" plasma level of nicotine of WSS, DSS, or nicotine does not affect the receptor expression of endothelin ET_B. Serotonin 5-HT_{1B} and 5-HT_{2A} mediated contraction curve was lowered by WSS and DSS (normal plasma level), while DSS also left-ward shifted the TP mediated contraction curve. The high dose of nicotine of WSS, DSS, or nicotine enhanced the contraction by 5-HT_{1B} and 5-HT_{2A} and TP. High nicotine level increased the endothelin ET_B receptor mediated contraction curve. The alteration of GPCR expression in the cerebral arteries by snus and nicotine most likely is a molecular mechanism involving cerebral vascular damage. Thus, this suggests that snus and nicotine may have a potential impact on development of cerebrovascular disease.

2 Materials and Methods

2.1 Removal of cerebral vessels and organ culture

Studies were approved by the Danish Animal Experiments Committee guidance (no. 2006/561-1139). Male Sprague-Dawley rats (n = number of rats = 78; 300-350 g) (Taconic, Denmark) were sedated with 70 % CO_2 in O_2 and decapitated while unconscious. The brains were removed and immediately chilled in ice-cold Na^+-Krebs buffer solution (for composition see below). The right

and left middle cerebral arteries were isolated and dissected free of adhering tissue in ice-cold Na^+-Krebs buffer.

Middle cerebral artery segments (1.5 – 3.0 mm long), rings with intact endothelium, were incubated for 24 hrs at 37 °C in humidified 5 % CO_2 and 95 % air in serum free Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4mM L-glutamine, 0.11 mg/ml sodium pyruvate) supplemented with an antibiotics mix (10.000 units/ml of penicillin, 10 mg/ml of streptomycin, and 25 ng/ml of amphotericin B). Each middle cerebral artery was bluntly cut into 4-5 segments, each 2.0 – 3.0 mm long (all in all 8 – 10 segments per rat) for the myograph bath studies. For real time PCR and immunohistochemistry studies the middle cerebral artery was dived into 2 segments, each 6.0 – 7.0 mm long (all in all 4 segments per rat). Middle cerebral artery segments from the rat were used in myograph bath studies for different groups. For real time PCR and immunohistochemistry experiments middle cerebral artery segments were used for both methods. The anatomical portion of middle cerebral artery segments for myograph bath, real time PCR and immunohistochemistry studies were interchanged (n values for each group: myograph bath n = 6-10, real time PCR n = 6-7, and immunohistochemistry n = 4-5). Fresh or organ cultured cerebral artery ring segments were either mounted in myographs for in vitro pharmacology or snap-frozen with dry ice and kept at -80 °C for real time PCR or immunohistochemistry.

WSS or DSS was added to the DMEM medium at the initiation of the incubation. Vehicle (water or DMSO) was added as control. All organ culture experiments were performed for the duration of 24 h. Water, DMSO, WSS, DSS, or nicotine were added at 0 h, giving a total duration of 24 h. The final volume of DMSO added to the 1 ml DMEM was always kept at 1 µl.

2.2 Drugs

Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4 mM L-glutamine, 0.11 mg/ml sodium pyruvate), antibiotics mix (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin), and Trizol were from Invitrogen, USA. 1-Bromo-3-chloropropane, dimethyl sulfoxide (DMSO), carbachol, 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), U46619, absolute ethanol, nicotine and other high grade chemicals were purchased from Sigma-Aldrich, USA. Sarafotoxin 6c was from NeoMPS S.A., France. RNase free water was from Qiagen, USA.

2.3 Extraction of water- or DMSO-soluble snus solution (WSS or DSS)

Bags of snus (General brand, Swedish Match # 0200-113114, batch # 52030-462-1) were dissolved for 1 hr at 37 °C in either water or DMSO (tubes were vortexed every 15 min). The Swedish Match company informed that the pH was 8.5 and the nicotine content was 8 mg/gram snus. Snus bags were weighted before dissolving in water or DMSO, and the final concentrations were 250 ng nicotine / µl (this was diluted ten-times to give a 25 ng nicotine / µl solution also). One µl of these solutions were added to 1 ml DMEM media for organ culture, giving a final concentration of 25 or 250 ng nicotine pr. ml of DMEM. Keeping in mind that nicotine is water soluble, snus was dissolved in DMSO (DSS) in the same way as it was in water.

A previous study has revealed that high doses of nicotine (480 ng/ml and 960 ng/ml) may affect contractions mediated by endothelin ET_B and 5-HT_{1B} receptor in rat mesenteric arteries (Zhang *et al.*, 2009). The E_{max} of the endothelin ET_B receptor mediated contraction induced by the specific agonist S6c is increased dramatically by 2-fold when 480 ng/ml nicotine was added to the organ

culture, whereas 960 ng/ml nicotine gave a 3-fold increase in E_{max}. The 5-HT curve was right-ward shifted (increase in pEC_{50}) by 60 ng/ml and 960 ng/ml nicotine. This shows that a higher dose of 5-HT is needed to promote the same 5-HT receptor mediated contraction, which could be due to fewer 5-HT receptors or lower receptor affinity.

The high doses of nicotine (480 ng/ml and 960 ng/ml) are about 20-40 times higher than that observed in plasma of snus users and does not have any clinical importance (only a toxic dose); hence, we chose to examine more reasonable doses of 250 ng/ml.

2.4 In vitro pharmacology

A sensitive myograph was used to record the isometric tension in isolated cerebral vessel segments (Hogestatt *et al.*, 1983;Mulvany *et al.*, 1977). The vessel segments were threaded on two 40 µm-diameter stainless steel wires and mounted on a Mulvany-Halpern myograph (Danish Myo Technology A/S, Denmark). One of the wires was connected to a force displacement transducer attached to an analog-digital converter unit (PowerLab from ADInstruments, New Zealand), while the other wire was attached to a movable displacement device allowing fine adjustments of vascular tension by varying the distance between the two wires. The measurements were recorded on a computer using the software Chart 5.4.2 (ADInstruments, U.K.).

The segments were immersed into a temperature-controlled (37 °C) Na^+-Krebs buffer solution (composition in mM/ml; NaCl = 119, $NaHCO_3$ = 15, KCl = 4.6, $MgCl_2$ = 1.2, NaH_2PO_4 = 1.2, $CaCl_2$ = 1.5, and glucose = 5.5). The buffer was continuously gassed with 5 % CO_2 in O_2 resulting in a physiological pH at 7.4. The vessels were given an initial pre-tension of 2 mN/mm and were

adjusted to this tension for 1 hour. Contractile capacity was determined by exposed to rich potassium (60 mM K^+-Krebs) buffer solution with the same composition as the Na^+-Krebs buffer solution except that NaCl was exchanged for KCl. The threshold for minimum 60 mM K^+-contraction response was set to 1.0 mN (if the K^+-contraction was lower, the data from the vessel was discarded).

The dilatation induced by carbachol after 5-HT mediated contraction was used as measurement of endothelium functionality. Carbachol mediates an endothelium dependent dilation of vessels via release of nitric oxide. Middle cerebral artery segments were pre-contracted with 300 nM 5-HT and subsequently exposed to 10 μM carbachol. A strong dilation by carbachol indicated intact functional endothelium, whereas a weak or absent dilation indicates damaged or unfunctional endothelium (i.e. due to mechanical damage of the endothelium during mounting on wires). Concentration-response curves were subsequently obtained by cumulative application of the agonist: endothelin ET_B receptor specific agonist S6c (10^{-14} M to 10^{-7} M), serotonin $5\text{-}HT_{1B}$ agonist 5-CT (10^{-12} M to 10^{-4} M), or prostanoid TP receptor specific agonist U46619 (10^{-12} M to 10^{-5} M). Specificity of detailed receptor charactering has been performed before (Henriksson *et al.*, 2003;Sandhu *et al.*, 2010a).

2.5 Molecular Biology

The middle cerebral artery segments were removed and snap-frozen with dry ice in green-cap tubes containing Lysing Matrix D provided in the FastRNA Pro Green Kit (Q Biogene, USA). Total cellular RNA was extracted using the FastRNA Pro Green Kit. The vessels were homogenized in 1 ml of Trizol Solution by using a FastPrep FP120 instrument (Q Biogene, USA). The samples were

centrifuged for 15 min at 4 °C at 12,500 g, and the liquid phase was transferred to 300 µl of 1-bromo-3-chloropropane. After centrifugation the upper phase was transferred to 500 µl of 100 % isopropanol and stored at -20 °C overnight. Next day the samples were centrifuged for 20 min at 4 °C at 15,000 g and the supernatant was removed. The pellet was washed with 500 µl 75 % ethanol, air dried and re-dissolved with RNase free water. Total RNA amount and purity was determined using NanoDrop 2000c (Thermo Science, USA).

Reverse transcription of total RNA to cDNA was performed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, USA) in a GeneAmp PCR System 2400 (Perkin-Elmer, USA). First-strand cDNA was synthesized from 250 ng total RNA in a 20 µl reaction volume with random hexamers used as primers. The reverse transcription reaction was performed with the following setup: 42 °C for 90 min followed by 72 °C for 10 min. The cDNA was diluted with 30 µl of RNase free water when used in the real time PCR reaction.

Real time PCR was performed in a 7500 Fast Real Time PCR sequence detection system (Applied Biosystems, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, USA) with 1 µl cDNA synthesized above as template in a 25-µl reaction volume. A none-template control was included in all experiments. Primer (1 µl of 10 mM) was added to the 25 µl real time PCR reaction. Elongation factor-1 (EF-1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as references genes because they are the product of housekeeping genes and are therefore continuously expressed to a constant amount in cells. Specific primers for the rat endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, prostanoid TP receptor, EF-1, and GAPDH receptors were designed (ET_B receptor primers (NM_017333): forward 5'-CCCGAGCGAACTGCTGAGGATC-3' and reverse 5'-TGCCTTAGGGGGCTTTCGGC-3'; 5-HT_{1B} receptor primers (NM_022225):

forward 5'-TCCGGGTCTCCTGTGTACGT-3' and reverse 5'-GGCGTCTGAGACTCGCACTT-3'; TP receptor primers (NM_017054): forward 5'-ATCTCCCATCTTGCCATAGTCC–3' and reverse 5'-CGATGATCCTTGGAGCCTAAAG-3'; EF-1 primers (NM_175838): forward 5'-CGCAACGGGTTTGCCGTCAG-3' and reverse 5'-AGTCAGCCTGAGATGTGCCCGT-3'; GAPDH primers (NM_017008): forward 5′-GGGCTCTCTGCTCCTCCCTGTT-3′ and reverse 5′-CACACCGACCTTCACCATCTTGTC-3′).

The real time PCR was performed with the following profile: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles with 95°C for 15 seconds and 60 °C for 1 minute.

The 7500 Fast Real Time PCR sequence detection software SDS version 1.4 (Applied Biosystems, USA) monitors the growth of DNA in real time by optics and imaging system via the binding of a fluorescent dye to double-stranded DNA.

2.6 Immunohistochemistry

The middle cerebral artery was dissected out and then placed into Tissue TEK (Sakura Finetek Europe B.V., Netherlands) and frozen. Segments were sectioned into 10-µm-thick slices and mounted on SuperFrost Plus slides (Menzel GMBH & COKG, Germany).

The primary antibodies used were sheep anti-rat endothelin ET_B receptor (ALX-210-506A, Alexis, USA), diluted 1:250; rabbit anti-rat serotonin 5-HT_{1B} receptor (ab13896, Abcam, UK), diluted 1:500; rabbit anti-rat prostanoid TP receptor (ab65150, Abcam, UK), diluted 1:200; and mouse anti-

rat actin (ab11003, Abcam, UK), diluted 1:500. All the dilutions were done in phosphate buffered saline with 2 % donkey serum (017-000-121, Jackson ImmunoResearch laboratories, Inc., USA).

The secondary antibodies used were donkey anti-sheep DL488 conjugated (713-485-003, Jackson ImmunoResearch laboratories, Inc., USA), diluted 1:200; donkey anti-rabbit DL488 conjugated (711-485-152, Jackson ImmunoResearch laboratories, Inc., USA), diluted 1:200; and donkey anti-mouse DL549 conjugated (715-505-150, Jackson ImmunoResearch laboratories, Inc., USA), diluted 1:200. As control, only secondary antibodies were used.

The antibodies were detected at the appropriate wavelength on a confocal microscopy (Nikon, C1plus, Nikon Instruments Inc., USA).

2.7 Calculation and statistical analyses

Data are expressed as mean values ± standard error of the mean (S.E.M.), and n refers to the number of rats. Statistical analyses of myograph data were performed on E_{max}- or pEC_{50}-values with one way ANOVA with Bonferroni's test, and selected groups were compared. When analyzing real time PCR data mRNA levels relative to the reference gene was used, and fluorescence mean intensities values were analyzed for the immunohistochemistry data.

Groups compared in ANOVA test: 24 h organ culture with vehicle (water or DMSO) vs. fresh, or 24 h organ culture with vehicle vs. 24 h organ culture with 25 or 250 ng/ml WSS / DSS / Nicotine.

Statistical information is given in Table 2. P< 0.05 was considered statistically significant (P< 0.05=*, P< 0.01=**, P< 0.001=***).

2.7.1 In vitro Pharmacology

Contractile responses in each segment are expressed as percentage of the 60 mM K^+ induced contraction. E_{max} value represents the maximum contractile response elicited by an agonist and the pEC_{50} the negative logarithm of the drug concentration that elicited half the maximum response. For biphasic responses, $E_{max}(1)$ and $pEC_{50}(1)$ describes the low affinity phase and $E_{max}(2)$ and $pEC_{50}(2)$ describes the high affinity phase.

2.7.2 Real time PCR

Data were analysed with the comparative cycle threshold (CT) method (Hansen-Schwartz *et al.*, 2002). The CT values of EF-1 or GAPDH mRNA were used as a reference to quantify the relative amount of endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, and prostanoid TP receptor mRNA. The relative amount of mRNA was calculated with the CT values of endothelin ET_B receptor, serotonin 5-HT_{1B} receptor, and prostanoid TP receptor mRNA in relation to the CT values of EF-1 or GAPDH mRNA in the sample by the formula $X_0/R_0=2^{CtR-CtX}$, where X_0 is the original amount of target mRNA, R_0 is the original amount of EF-1 or GAPDH mRNA, *CtR* is the C_T value for EF-1 or GAPDH, and C_TX is the C_T value for the target. Data with EF-1 as the reference gene is shown in results section.

2.7.3 Immunohistochemistry

Images were analyzed by Nikon EZ-C1 software version 3.70. Fluorescence intensity was measured on the whole smooth muscle cell area in 3 sections using the entire circumfence; the mean of this

was used. Actin staining was used as reference of smooth muscle cell localization. Analysis was done by having the red 549 nm channel (actin conjugated) overlay.

3 Results

The effects of WSS, DSS, and nicotine were studied in organ culture at the initiation of incubation for 24 h. WSS is the solution containing both snus ingredients and nicotine, while DSS is the solution only containing the lipid-soluble snus ingredients. Together both two solutions along with pure nicotine provides an overall representation of what snus contains, and experimental results show what effect the snus ingredients + nicotine, snus ingredients, or nicotine have on the GPCR. The acute administration of WSS, DSS, and nicotine on resting fresh artery segments produced no vasomotor response (data not shown).

3.1 Endothelial functions

Carbachol (10 µM) elicited dilatation of the 5-HT (300 nM) pre-contracted middle cerebral artery segments by 52 ± 10 % of pre-contraction in fresh middle cerebral vessels. After 24 h of organ culture the dilatation induced by carbachol was 28 ± 8 % (with water), and 27 ± 2 % (with DMSO) ($p>0.05$ for both organ culture types compared to fresh vessels); organ culture nominally reduced the endothelial function. Co-incubation with WSS, DSS, or nicotine (25 ng/ml or 250 ng/ml) did not alter the carbachol-induced dilatation compared to organ culture with vehicle (see Table 2 for all carbachol mediated dilatation values).

3.2 GPCR expression

Addition of 60 mM K^+-induced stable and reproducible contraction of the smooth muscle cells and was used as internal reference (set as 100 %). Incubation for 24 h with vehicle (water or DMSO) did not alter the 60 mM K^+ responses (60 mM K^+ response in mN: fresh = 4.39 ± 1.45, organ culture with water = 4.18 ± 1.75, and organ culture with DMSO = 3.84 ± 1.64).

3.2.1 Endothelin ET_B receptors

The endothelin ET_B receptor mediated contraction was strongly increased by the organ culture (E_{max} in % of 60 mM K^+: fresh = 5 ± 1, organ culture with water = 111 ± 6, and organ culture with DMSO = 80 ± 12; $p<0.001$) (Figure 1A, 1D, 1G, Table 2). Real time PCR and immunohistochemistry showed significant upregulation of endothelin ET_B receptors at mRNA and protein levels (Figure 2A, 2D, 2G, 3A, 3D, and 3G), thus, the increase in the endothelin ET_B receptor mediated contraction occurred at the transcriptional level. The endothelin ET_B receptor mediated contraction in the DMSO vehicle group is slightly lower than that seen in the water vehicle group. The S6c pEC_{50} values did not differ between the two vehicle groups.

3.2.2 5-HT receptors

Addition of 5-CT resulted in a biphasic sigmoid curve. This concentration-effect curve was only slightly increased by organ culture ($E_{max}(1)$ and $E_{max}(2)$ in % of 60 mM K^+: fresh = 5 ± 2 and 52 ± 14, organ culture with water = 4 ± 1 and 60 ± 6, and organ culture with DMSO = 8 ± 4 and 64 ± 7) (Figure 1B, 1E, 1H, Table 2). The mRNA expression of the 5-HT_{1B} receptor was however upregulated significantly by organ culture (Figure 2B, 2E, 2H, $p<0.001$), and so was the 5-HT_{1B} receptor protein level (Figure 3B, 3E, 3H, $p<0.01$ or $p<0.05$). This suggests that the slight increase

in contraction is mediated by production of 5-HT_{1B} receptors. The 5-CT $E_{max}(1)$ and $E_{max}(2)$ or the 5-CT $pEC_{50}(2)$ values did not change in the two vehicle groups.

3.2.3 TP receptors

The U46619 induced contraction was not altered significantly by organ culture (E_{max} in % of 60 mM K^+: fresh = 152 ± 13, organ culture with water = 161 ± 12, and organ culture with DMSO = 137 ± 9) (Figure 1C, 1F, 1I, Table 2). In accord, neither mRNA nor protein levels of TP receptors were changed by organ culture (Figure 2C, 2F, 2I, 3C, 3F, 3I). The U46619 E_{max} or the U46619 pEC_{50} values did not differ between the two vehicle groups.

3.3 Effects of snus or nicotine on the receptor expression

3.3.1 Water soluble snus (WSS)

The WSS incubation experiments did not result in altered endothelin ET_B receptor mediated contractions (E_{max} in % of 60 mM K^+: organ culture with water = 111 ± 6, organ culture with 25 ng WSS = 81 ± 12, and organ culture with 250 ng WSS = 101 ± 28) (Figure 1A, Table 2). There was a tendency towards a weaker contraction by 25 ng WSS after organ culture and a slightly lower ET_B receptor mRNA (Figure 2A), however, without significance. The ET_B receptor protein level remained stable (Figure 3A). There was no alteration in the S6c pEC_{50} values.

The 5-HT_{1B} and 5-HT_{2A} receptor mediated contraction were altered by WSS. Whereas 25 ng WSS reduced the 5-CT contraction of the vessels, 250 ng WSS increase the contraction ($E_{max}(2)$ in % of 60 mM K^+: organ culture with water = 60 ± 6, organ culture with 25 ng WSS = 28 ± 3, and organ

culture with 250 ng WSS = 114 ± 8) (Figure 1B, Table 2) ($p<0.05$ for both doses). The 5-HT_{1B} receptor mRNA and protein levels in the 25 ng WSS group and the 250 ng WSS group remained unaffected (Figure 2B and 3B). The 5-CT $pEC_{50(2)}$ values did change in the WSS incubations ($pEC_{50(2)}$ values of 5-CT: organ culture with water = 5.9 ± 0.1, organ culture with 25 ng WSS = 6.9 ± 0.1, and organ culture with 250 ng WSS = 7.8 ± 0.1)(Table 2) ($p<0.01$ for the high dose).

The TP receptor mediated contraction was not affected by WSS (E_{max} in % of 60 mM K^+: organ culture with water = 161 ± 12, organ culture with 25 ng WSS = 142 ± 6, and organ culture with 250 ng WSS = 164 ± 16)(Figure 1C, Table 2). In addition, mRNA and protein levels of TP receptors did not change by adding WSS (Figure 2C, 3C). The U46619 pEC_{50} values in the high dose increased by addition of WSS to the organ culture (pEC_{50} values of U46619: organ culture with water = 7.6 ± 0.2, organ culture with 25 ng WSS = 7.9 ± 0.1, and organ culture with 250 ng WSS = 8.3 ± 0.1) (Table 2) ($p<0.01$ for high dose).

3.3.2 Nicotine

Addition of nicotine in the high dose increased the ET_B receptor mediated contraction significantly (E_{max} in % of 60 mM K^+: organ culture with water = 111 ± 6, organ culture with 25 ng nicotine = 99 ± 9, and organ culture with 250 ng nicotine = 132 ± 9) (Figure 1D, Table 2) ($p<0.05$). This increased contraction induced by S6c could not be explained by real time PCR or immunohistochemistry (Figure 2D, 3D). Real time PCR showed a significant drop in the ET_B receptor mRNA level at the “normal” nicotine dose (25 ng) ($p<0.001$) in stead. This drop did however not affect the contraction induced by S6c. The S6c pEC_{50} values did not change by addition of nicotine in the organ culture.

The 5-CT induced concentration-effect curve was significantly increased by the high dose of nicotine (E_{max} in % of 60 mM K^+: organ culture with water = 60 ± 6, organ culture with 25 ng nicotine = 83 ± 6, and organ culture with 250 ng nicotine = 97 ± 14) (Figure 1E, Table 2) ($p<0.05$ for high dose). Real time PCR showed a significant drop in 5-HT_{1B} receptor mRNA level in the 25 ng nicotine group (Figure 2E) ($p<0.001$), whereas the protein levels remained unaffected at either dose of nicotine (Figure 3E). The 5-CT $pEC_{50(2)}$ value increased by the higher dose of nicotine ($pEC_{50(2)}$ values of 5-CT: organ culture with water = 5.9 ± 0.1, organ culture with 25 ng nicotine = 6.0 ± 0.1, and organ culture with 250 ng nicotine = 7.7 ± 0.2) (Table 2) ($p<0.01$ for high dose). The increased $pEC_{50(2)}$ value at the high nicotine dose shows that less 5-CT is needed to induce the same contraction when exposed to 250 ng nicotine.

The TP receptor mediated contraction was stable and not affected by nicotine (E_{max} in % of 60 mM K^+: organ culture with water = 161 ± 12, organ culture with 25 ng nicotine = 161 ± 14, and organ culture with 250 ng nicotine = 161 ± 8) (Figure 1F, Table 2). Real time PCR showed a significant drop in the TP receptor mRNA level (Figure 2F) ($P<0.001$), but this drop did not affect the U46619 contraction. Protein levels remained unaffected (Figure 3F). The U46619 pEC_{50} values did change by addition of nicotine to the organ culture (pEC_{50} values of U46619: organ culture with water = 7.6 ± 0.2, organ culture with 25 ng nicotine = 7.6 ± 0.1, and organ culture with 250 ng nicotine = 8.0 ± 0.1) (Table 2) ($p<0.01$ for high dose).

3.3.3 DMSO (lipid) - soluble snus (DSS)

The ET_B receptor mediated contraction was not affected by DSS (E_{max} in % of 60 mM K^+: organ culture with DMSO = 80 ± 12, organ culture with 25 ng DSS = 86 ± 9, and organ culture with 250 ng DSS = 86 ± 9) (Figure 1G, Table 2). Real time PCR showed a significant increase in the ET_B receptor mRNA level (Figure 2G) ($P<0.001$). The S6c induced contraction and the receptor protein level remained unaffected (Figure 3G).

The 5-CT curve was significantly altered in both DSS dose groups ($E_{max(2)}$ in % of 60 mM K^+: organ culture with DMSO = 64 ± 7, organ culture with 25 ng DSS = 35 ± 14, and organ culture with 250 ng DSS = 98 ± 11) (Figure 1H, Table 2) ($p<0.05$). This correlates well with the real time PCR results with a significant rise in the $5\text{-}HT_{1B}$ receptor mRNA level in the 250 ng DSS group (Figure 2H) ($p<0.001$), while the 25 ng DSS group remained. The protein levels remained unaffected (Figure 3H). The 5-CT $pEC_{50(2)}$ values were affected by DSS ($pEC_{50(2)}$ values of 5-CT: organ culture with DMSO = 5.9 ± 0.1, organ culture with 25 ng DSS = 5.9 ± 0.1, and organ culture with 250 ng DSS = 6.5 ± 0.1) (Table 2) ($p<0.01$ for high dose).

The TP receptor mediated contractions (E_{max} in % of 60 mM K^+: organ culture with DMSO = 137 ± 9, organ culture with 25 ng DSS = 156 ± 11, and organ culture with 250 ng DSS = 156 ± 12) (Figure 1I, Table 2), were not statistically modified. This agrees with no effect TP receptor mRNA (Figure 2I) (but not significant) and immunohistochemical TP receptor protein level (Figure 3I). There was a significant increase in U46619 pEC_{50} values by DSS (pEC_{50} values of U46619: OC DMSO = 7.6 ± 0.1, OC DSS 25 ng = 8.1 ± 0.1, and OC DSS 250 ng = 8.1 ± 0.1) (Table 2) ($p<0.01$ for both doses).

4 Discussion

Smokeless tobacco (Swedish moist 'snus') users are often strongly addicted to nicotine (verified by several of smoking-cessation studies), because the amount of nicotine measured in the blood is at much higher level, as compared to that seen in cigarette smokers. The present study demonstrates, for the first time, that water- or DMSO-soluble snus alter the GPCR expression in rat cerebral arteries for endothelin ET_B, serotonin 5-HT_{1B}, and thromboxane TP. This may suggest a potential impact of nicotine and snus on the development of cerebrovascular disease.

There is an increased expression of ET_B receptors in stroke, which is associated with reduced cerebral flow and cerebral ischemia (Ansar *et al.*, 2007;Hansen-Schwartz *et al.*, 2003b). In the present study, we have shown that the expression of the ET_B receptor did not change when exposed to the normal plasma nicotine level (25 ng nicotine/ml seen in snus users), except for reduced ET_B receptor mRNA when nicotine was added to organ culture, and increased ET_B receptor mRNA when DSS was added. On the other hand, a high dose of nicotine (250 ng nicotine /ml) increased the ET_B receptor mediated contraction of cerebral arteries. This gives a caution for using high dose of nicotine.

In previous studies, we demonstrated that in experimental stroke models, 5-HT receptors were involved in the pathogenesis, with increased 5-HT receptor-mediated contractions (Hansen-Schwartz *et al.*, 2003a). 5-HT receptors mediate strong cerebral vessel contractions and the alteration of the serotonin receptor expression is seen after cerebral ischemia (Hansen-Schwartz *et al.*, 2003a) or when vessels are exposed to lipid-soluble cigarette smoke extract (Zhang *et al.*,

2009). Here, snus and nicotine also induced alteration of the 5-HT_{1B} and 5-HT_{2A} receptor-mediated. In the normal dose of plasma level of nicotine WSS and DSS decreases the $E_{max(2)}$ of the 5-CT curve, while high nicotine dose of WSS, DSS, and nicotine increased the $E_{max(2)}$ of the 5-CT curve even though the 5-HT_{1B} receptor mRNA was reduced in the DSS group. This is in line with the present findings that the high dose of WSS, DSS, or nicotine also shifted the 5-CT curve left-wards to the high affinity part.

TP receptor density is enhanced in cardiovascular diseases (Katugampola *et al.*, 2001) and in hypertension (Geoffroy *et al.*, 1989). This increase is likely to be associated with an increase in risk for cardiovascular disease. In addition to being a potent vasoconstrictor TxA_2 also stimulates platelet aggregation, migration, proliferation, and smooth muscle cell differentiation by activating the TP receptor, with molecular mechanisms might be mediated through the ERK and p38 MAPK (Huang *et al.*, 2004;Morinelli *et al.*, 1994;Yun *et al.*, 2009). In addition to this, we found in the present study, that the normal dose of DSS and high dose of WSS, DSS, and nicotine shifted the TP receptor mediated curve left-wards, while the normal plasma dose of nicotine lowered the TP receptor mRNA.

Cigarette smoke is a independent risk factor for stroke. Sweden has much less smokers, and only 17% of Swedish men smoke, because Swedes use another form of moist tobacco called “Snus” instead (Foulds *et al.*, 2003;Haglund *et al.*, 2007). Epidemiological studies have failed to find evidence that moist snus causes cancers, while the adverse effects of snus on the cardiovascular system are debated. Previously, we have demonstrated that there is upregulation of GPCR expression in cardiovascular disease, which leads to an abnormal smooth muscle cell contraction and proliferation, key events in cardiovascular pathology. The change in receptor phenotype can

experimentally be mimicked by organ culture of the arteries in serum-free medium (Hansen-Schwartz *et al.*, 2002;Hoel *et al.*, 2001). Following organ culture of the arteries, there is a time-dependent increase in ET_B receptor mRNA, which is followed by increased levels of ET_B receptor protein and function (Hansen-Schwartz *et al.*, 2002;Sandhu *et al.*, 2010a). Recently, we have reported that organ culture of rat cerebral arteries induced upregulation of ET_B and TP receptors and this receptor upregulation was further increased by DMSO-soluble cigarette smoke extract, but not water-soluble cigarette smoke extract (Sandhu *et al.*, 2010b;Sandhu *et al.*, 2010c). The molecular mechanisms involved are associated with activation of MAPK-mediated intracellular signal pathways. However, using the same model, we have failed to demonstrate this phenomenon of the ET_B and TP receptor upregulation by DMSO-soluble snus extract, suggesting snus may have less effect than cigarette smoke or it may act through different molecular mechanisms. Snus does however increase blood pressure (Hergens *et al.*, 2008) and the outcome of fatal diseases (Hergens *et al.*, 2007). Therefore snus may influence the severity of vascular disease indirectly.

5 Conclusion

In conclusion, we found when cerebral arteries are exposed to extracts from snus or nicotine there was an alteration of the GPCR's (5-HT_{1B} and 5-HT_{2A}, ET_B, and TP) receptor-mediated contractions. Normal nicotine plasma level of WSS and DSS decreased the 5-HT_{1B} and 5-HT_{2A} contraction and left-wards shifted the TP contraction curve, however, this increase was not explained by real time PCR or protein immunohistochemistry, which failed to show an increased mRNA and protein expressions for the GPCR. High nicotine level of WSS, DSS, or nicotine increased the 5-CT mediated contraction curve and left-wards shifted the TP contraction curve. Only the DSS group showed that the increase of the 5-CT curve was at the mRNA level. This

suggests that both transcriptional and post-translational mechanisms are responsible for the 5-HT receptor alteration by WSS, DSS, or nicotine. High nicotine level increased the ET_B mediated contraction curve. The alteration of GPCR expression by snus and nicotine in the cerebral arteries is most likely a molecular mechanism, which is responsible for inducing cerebral vascular damage. Thus, this suggests that snus and nicotine may have potential impact on cerebral vasculature and on the development of cardiovascular diseases.

6 Conflict of interest

Authors declare not having any financial or personal interest, nor having an association with any individuals or organizations that could have influenced inappropriately the submitted work.

7 Acknowledgements

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8 References

1. Andersson, G., Bjornberg, G., and Curvall, M. (1994). Oral mucosal changes and nicotine disposition in users of Swedish smokeless tobacco products: a comparative study. J Oral Pathol.Med. **23**, 161-167.

2. Ansar, S., Vikman, P., Nielsen, M., and Edvinsson, L. (2007). Cerebrovascular ETB, 5-HT1B, and AT1 receptor upregulation correlates with reduction in regional CBF after subarachnoid hemorrhage. Am.J.Physiol Heart Circ.Physiol **293**, H3750-H3758.

3. Benowitz, N. L., Jacob, P., III, Fong, I., and Gupta, S. (1994). Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. J.Pharmacol Exp.Ther. **268**, 296-303.

4. Bolinder, G. M., Ahlborg, B. O., and Lindell, J. H. (1992). Use of smokeless tobacco: blood pressure elevation and other health hazards found in a large-scale population survey. J Intern.Med. **232**, 327-334.

5. Coleman, R. A., Smith, W. L., and Narumiya, S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev **46**, 205-229.

6. Foulds, J., Ramstrom, L., Burke, M., and Fagerstrom, K. (2003). Effect of smokeless tobacco (snus) on smoking and public health in Sweden. Tob.Control **12**, 349-359.

7. Geoffroy, J., Benzoni, D., and Sassard, J. (1989). Antihypertensive effect of thromboxane A2 receptor blockade in genetically hypertensive rats of the Lyon strain. J Hypertens.Suppl **7**, S272-S273.

8. Haglund, B., Eliasson, M., Stenbeck, M., and Rosen, M. (2007). Is moist snuff use associated with excess risk of IHD or stroke? A longitudinal follow-up of snuff users in Sweden. Scand.J Public Health **35**, 618-622.

9. Hansen-Schwartz, J., Hoel, N. L., Xu, C. B., Svendgaard, N. A., and Edvinsson, L. (2003a). Subarachnoid hemorrhage-induced upregulation of the 5-HT1B receptor in cerebral arteries in rats. J Neurosurg **99**, 115-120.

10. Hansen-Schwartz, J., Hoel, N. L., Zhou, M., Xu, C. B., Svendgaard, N. A., and Edvinsson, L. (2003b). Subarachnoid hemorrhage enhances endothelin receptor expression and function in rat cerebral arteries. Neurosurgery **52**, 1188-1194.

11. Hansen-Schwartz, J., Svensson, C. L., Xu, C. B., and Edvinsson, L. (2002). Protein kinase mediated upregulation of endothelin A, endothelin B and 5-hydroxytryptamine 1B/1D receptors during organ culture in rat basilar artery. Br.J.Pharmacol **137**, 118-126.

12. Henriksson, M., Stenman, E., and Edvinsson, L. (2003). Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. Stroke **34**, 1479-1483.

13. Hergens, M. P., Alfredsson, L., Bolinder, G., Lambe, M., Pershagen, G., and Ye, W. (2007). Long-term use of Swedish moist snuff and the risk of myocardial infarction amongst men. J Intern.Med. **262**, 351-359.

14. Hergens, M. P., Lambe, M., Pershagen, G., and Ye, W. (2008). Risk of hypertension amongst Swedish male snuff users: a prospective study. J Intern.Med. **264**, 187-194.

15. Hoel, N. L., Hansen-Schwartz, J., and Edvinsson, L. (2001). Selective up-regulation of 5-HT(1B/1D) receptors during organ culture of cerebral arteries. Neuroreport **12**, 1605-1608.

16. Hogestatt, E. D., Andersson, K. E., and Edvinsson, L. (1983). Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. Acta Physiol Scand **117**, 49-61.

17. Huang, J. S., Ramamurthy, S. K., Lin, X., and Le Breton, G. C. (2004). Cell signalling through thromboxane A2 receptors. Cell Signal. **16**, 521-533.

18. Katugampola, S. D., and Davenport, A. P. (2001). Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT(1) receptor antagonist losartan. Br.J Pharmacol **134**, 1385-1392.

19. Masaki, T., Vane, J. R., and Vanhoutte, P. M. (1994). International Union of Pharmacology nomenclature of endothelin receptors. Pharmacol Rev **46**, 137-142.

20. Morinelli, T. A., Zhang, L. M., Newman, W. H., and Meier, K. E. (1994). Thromboxane A2/prostaglandin H2-stimulated mitogenesis of coronary artery smooth muscle cells involves activation of mitogen-activated protein kinase and S6 kinase. J Biol.Chem. **269**, 5693-5698.

21. Mulvany, M. J., and Halpern, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res **41**, 19-26.

22. Nilsson, T., Longmore, J., Shaw, D., Olesen, I. J., and Edvinsson, L. (1999). Contractile 5-HT1B receptors in human cerebral arteries: pharmacological characterization and localization with immunocytochemistry. Br.J Pharmacol **128**, 1133-1140.

23. Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S. M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M., and Hong, Y. (2008). Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation **117**, e25-146.

24. Sandhu, H. K., Ansar, S., and Edvinsson, L. (2010a). Comparison of MEK/ERK pathway inhibitors on the upregulation of vascular G protein coupled receptors in rat cerebral arteries. Eur J Pharmacol **644**, 128-137.

25. Sandhu, H. K., Xu, C. B., and Edvinsson, L. (2010b). Upregulation of contractile endothelin type B2 receptors by lipid-soluble cigarette smoking particles in rat cerebral arteries via activation of MAPK. Toxicology and Applied Pharmacology **In press**.

26. Sandhu, H. K., Xu, C. B., and Edvinsson, L. (2010c). Upregulation of contractile prostanoid TP receptors by lipid-soluble cigarette smoking particles in rat cerebral arteries via posttranscriptional mechanisms. Basic & Clinical Pharmacology & Toxicology **In press**.

27. US Department of Health and Human Services (1986). The health consequences of using smokeless tobacco. A report of the advisory committee to the Surgeon General. Bethesda, Maryland: Public Health Service, National Institutes of Health **(NIH publication No 86-2874**.

28. Yun, D. H., Song, H. Y., Lee, M. J., Kim, M. R., Kim, M. Y., Lee, J. S., and Kim, J. H. (2009). Thromboxane A(2) modulates migration, proliferation, and differentiation of adipose tissue-derived mesenchymal stem cells. Exp.Mol.Med. **41**, 17-24.

29. Zhang, J. Y., Cao, L., Zheng, X. H., Xu, C. B., and Cao, Y. X. (2009). Dimethylsulfoxide-soluble smoking particles and nicotine affect vascular contractibility. Arch.Pharm.Res. **32**, 1475-1481.

Figure 1

Contractile response to the cumulative application of S6c, 5-CT, or U46619 to fresh or incubated middle cerebral arteries with vehicle (Water or DMSO) or WSS (A-C), Nicotine (D-F), or DSS (G-I). Values given represent means ± S.E.M. (n = 6-10). Statistical values for myograph experiments are given in Table 2.

Figure 2

Real time PCR of fresh or incubated middle cerebral arteries with vehicle (Water or DMSO) or WSS (A-C), Nicotine (D-F), or DSS (G-I). Data are expressed as mean ± S.E.M. values relative to EF-1 mRNA levels (n= 6-7). Comparison of values were done fresh versus organ culture 24 h with vehicle (water or DMSO), 24 h organ culture with water vehicle versus WSS (25 ng/ml or 250 ng/ml) or nicotine (25 ng/ml or 250 ng/ml), and 24 h organ culture with DMSO vehicle versus DSS (25 ng/ml or 250 ng/ml). Statistical analysis: * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$.

Figure 3

Immunohistochemistry staining against endothelin ET_B , serotonin 5-HT_{1B}, or prostanoid TP receptor and actin of fresh or incubated middle cerebral arteries with vehicle (Water or DMSO) or WSS (A-C), Nicotine (D-F), or DSS (G-I). Green channel (488 nm, receptor staining) and green-red channel merge (488 nm and 549 nm, receptor and actin staining) images are shown in J) as an example for the endothelin ET_B receptor staining in the WSS group (100x lense). Data are expressed as mean ± s.e.m. (n= 4-5). Comparison of values were done fresh versus organ culture 24 h with vehicle (water or DMSO), 24 h organ culture with water vehicle versus WSS (25 ng/ml or 250 ng/ml) or nicotine (25 ng/ml or 250 ng/ml), and 24 h organ culture with DMSO vehicle versus DSS (25 ng/ml or 250 ng/ml). Statistical analysis: * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$.

Table 1

		Fresh	OC Water	OC DMSO
60 mM K+ in mN		4.39±1.45	4.18±1.75	3.84±1.64
Endothelium in % of CA dilatation		52 ± 10	28 ± 8	27 ± 2
S6c	**Emax in % of K+**	5 ± 1 c	111 ± 6	80 ± 12
	pEC50	-	10.8 ± 0.2	10.6 ± 0.3
5-CT (low affinity)	**Emax in % of K+**	5 ± 2	4 ± 1	8 ± 4
	pEC50	-	-	-
5-CT (high affinity)	**Emax in % of K+**	52 ± 14	60 ± 6	64 ± 7
	pEC50	5.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
U46619	**Emax in % of K+**	152 ± 13	161 ± 12	137 ± 9
	pEC50	7.2 ± 0.1	7.6 ± 0.2	7.6 ± 0.1
		OC WSS 25 ng	**OC WSS 250 ng**	
60 mM K+ in mN		3.44 ±1.37	3.41±1.67	
Endothelium in % of CA dilatation		35 ± 4	21 ± 3	
S6c	**Emax in % of K+**	81 ± 12	101 ± 28	
	pEC50	10.5 ± 0.3	10.5 ± 0.4	
5-CT (low affinity)	**Emax in % of K+**	6 ± 2	37 ± 12	
	pEC50	-	7.8 ± 0.6	
5-CT (high affinity)	**Emax in % of K+**	28 ± 3 a	114 ± 8 a	
	pEC50	6.9 ± 0.1	7.8 ± 0.1 b	
U46619	**Emax in % of K+**	142 ± 6	164 ± 16	
	pEC50	7.9 ± 0.1	8.3 ± 0.1 b	
		OC Nicotine 25 ng	**OC Nicotine 250 ng**	
60 mM K+ in mN		3.44±1.37	2.99±1.02	
Endothelium in % of CA dilatation		22 ± 3	22 ± 3	
S6c	**Emax in % of K+**	99 ± 9	132 ± 9 a	
	pEC50	10.8 ± 0.2	10.4 ± 0.2	
5-CT (low affinity)	**Emax in % of K+**	40 ± 14	48 ± 19	
	pEC50	6.1 ± 1	7.7 ± 0.4	
5-CT (high affinity)	**Emax in % of K+**	83 ± 6	97 ± 14 a	
	pEC50	6.0 ± 0.1	7.7 ± 0.2 b	
U46619	**Emax in % of K+**	161 ± 14	161 ± 8	
	pEC50	7.6 ± 0.1	8.0 ± 0.1 b	
		OC DSS 25 ng	**OC DSS 250 ng**	
60 mM K+ in mN		3.77±1.34	3.11±1.19	
Endothelium in % of CA dilatation		23 ± 4	25 ± 5	
S6c	**Emax in % of K+**	86 ± 9	86 ± 9	
	pEC50	10.7 ± 0.2	10.7 ± 0.2	
5-CT (low affinity)	**Emax in % of K+**	12 ± 7	19 ± 12	
	pEC50	-	7.1 ± 1.7	
5-CT (high affinity)	**Emax in % of K+**	35 ± 14 a	98 ± 11 a	
	pEC50	5.9 ± 0.1	6.5 ± 0.1 b	
U46619	**Emax in % of K+**	156 ± 11	165 ± 7	
	pEC50	8.1 ± 0.1 b	8.2 ± 0.1 b	

Table 1 – E_{max} and pEC_{50} values of fresh or 24 h incubated (OC = organ culture) middle cerebral artery with vehicle (Water or DMSO) or WSS, Nicotine, or DSS. Endothelium in % of CA dilatation is the value of 10 µM carbachol (CA) dilatation of 300 nM 5-HT pre-contracted vessels. Values are given for all agonist experiments as mean ± S.E.M. Low and high affinity E_{max} and pEC_{50} values are given for 24 h organ culture of middle cerebral artery 5-CT biphasic sigmoid curve. Comparison of values were done fresh versus organ culture 24 h with vehicle (water or DMSO), 24 h organ culture with water vehicle versus WSS (25 ng/ml or 250 ng/ml) or nicotine (25 ng/ml or 250 ng/ml), and 24 h organ culture with DMSO vehicle versus DSS (25 ng/ml or 250 ng/ml). Statistical analysis: a $p<0.05$ and b $p<0.01$, and c $p<0.001$. S6c = sarafotoxin 6c, and 5-CT = 5-carboxamidotryptamine.

Figure 1A

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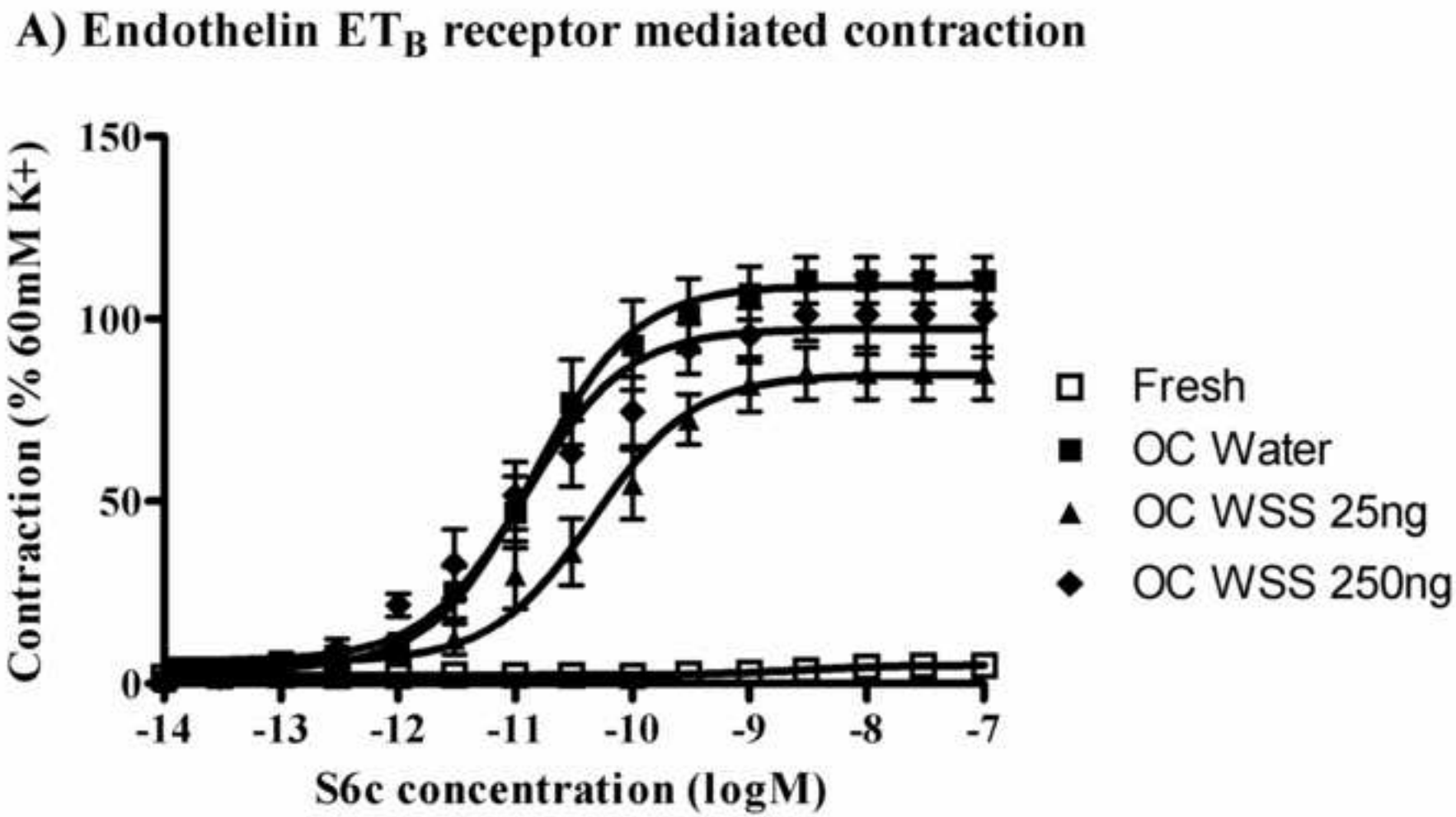

Figure 1B

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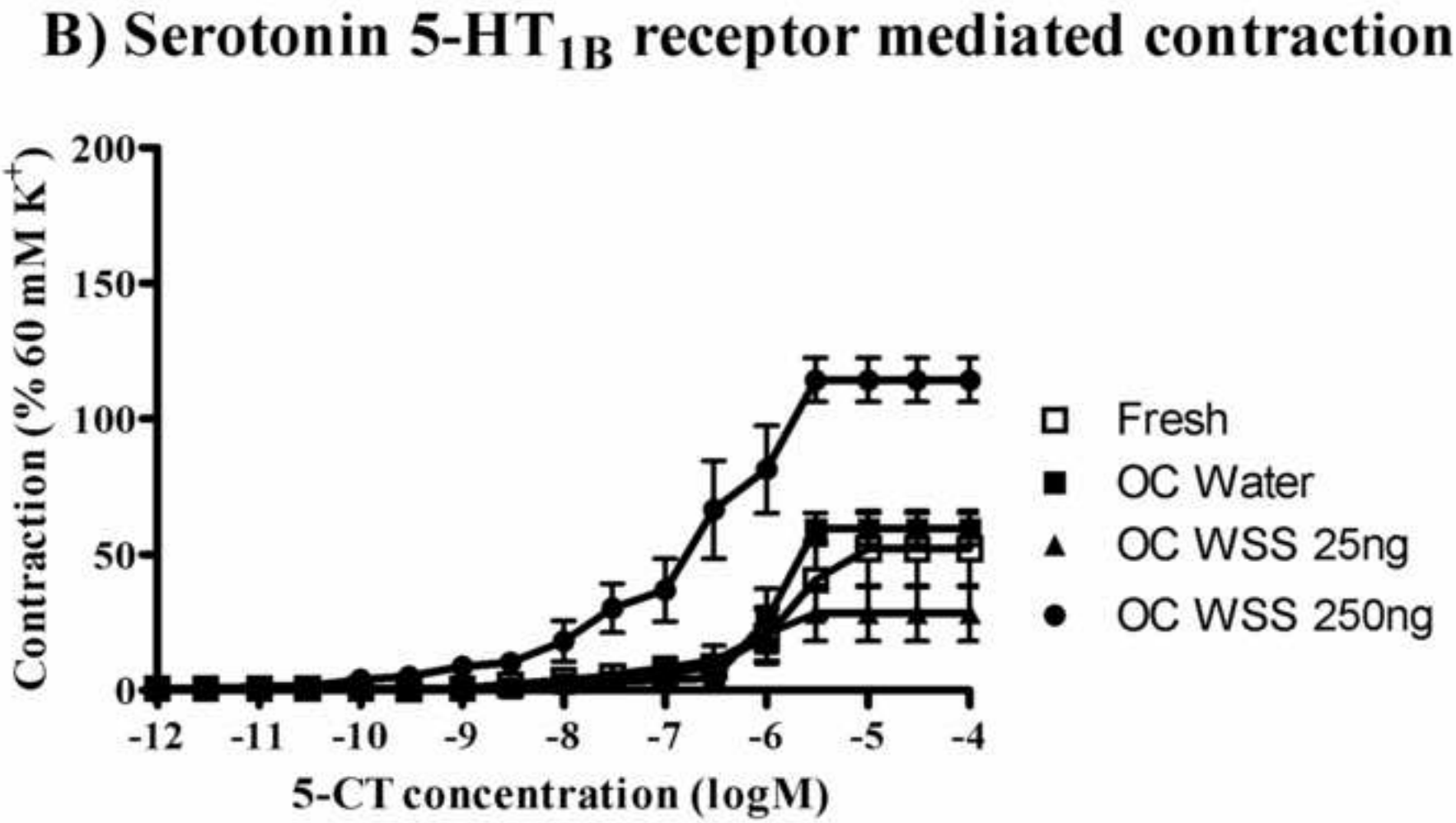

Figure 1C
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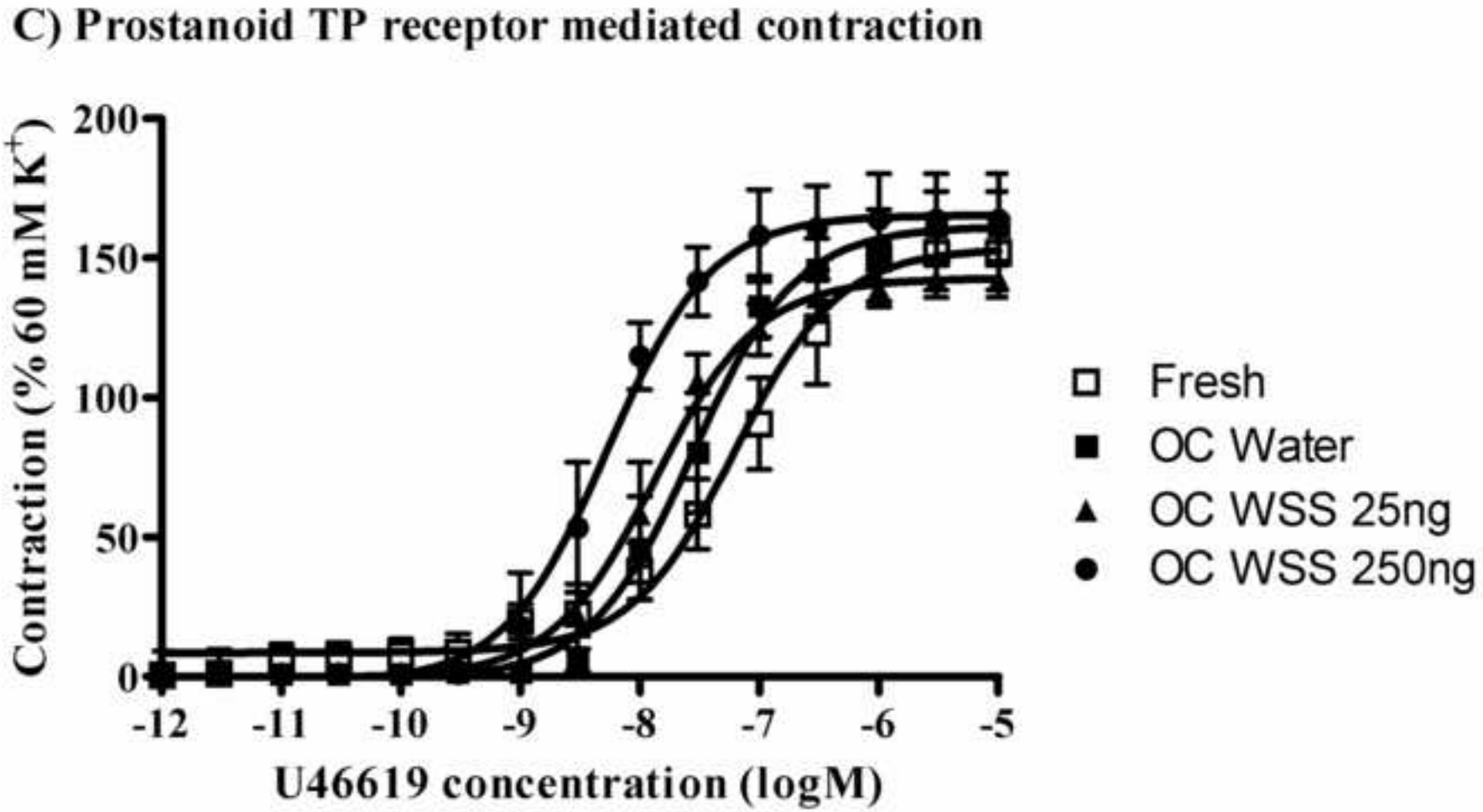

Figure 1D

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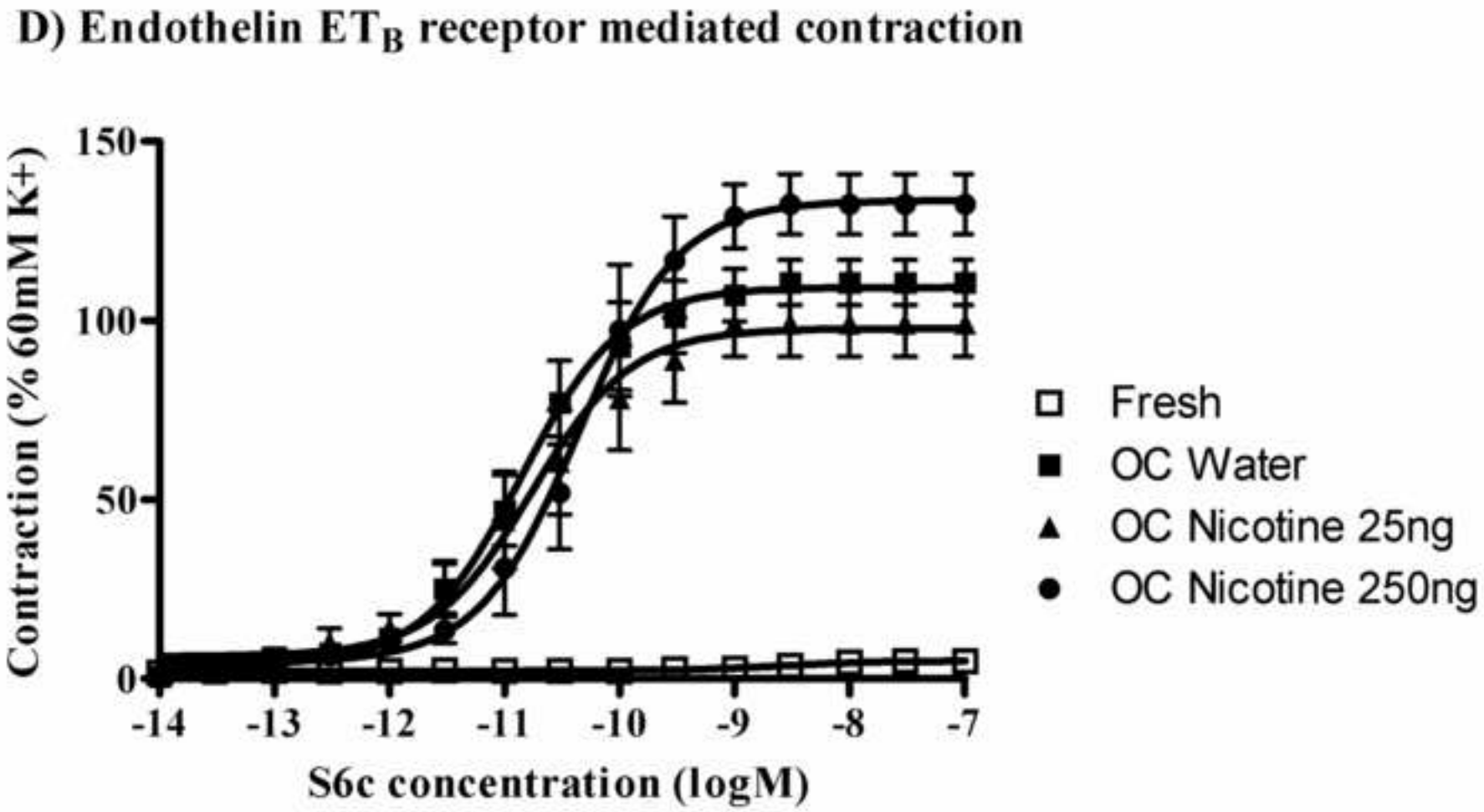

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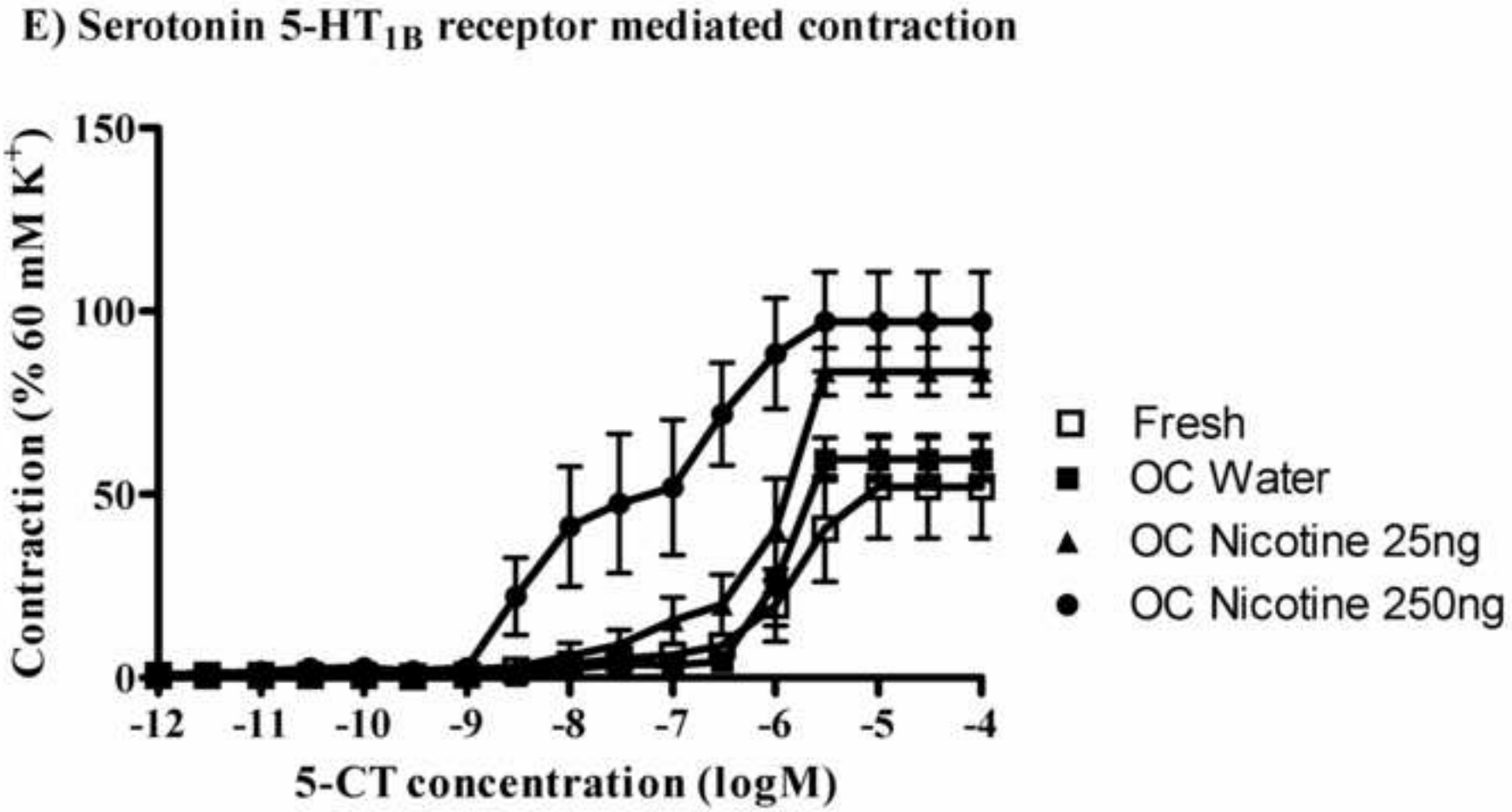

Figure 1F

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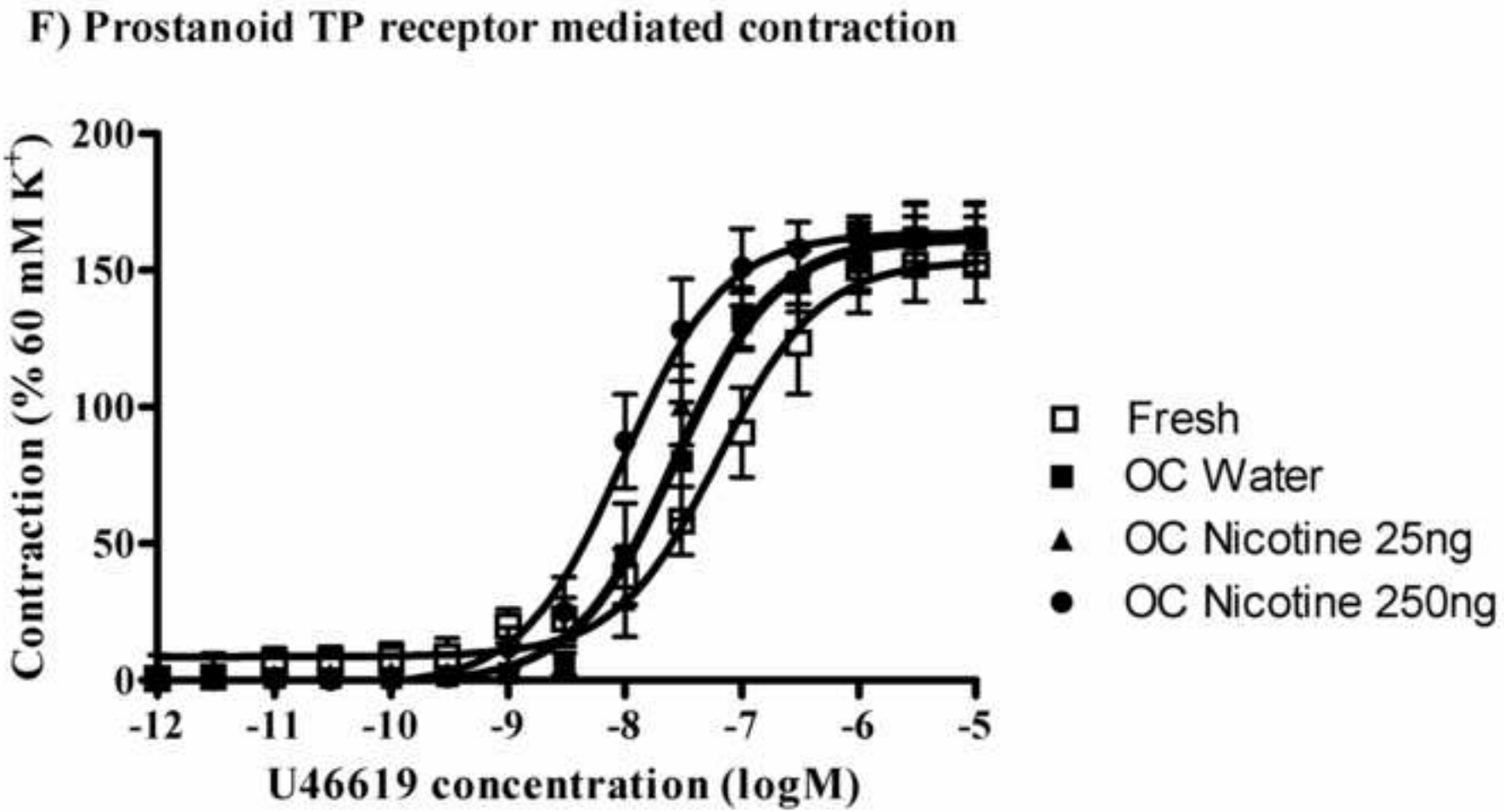

Figure 1G

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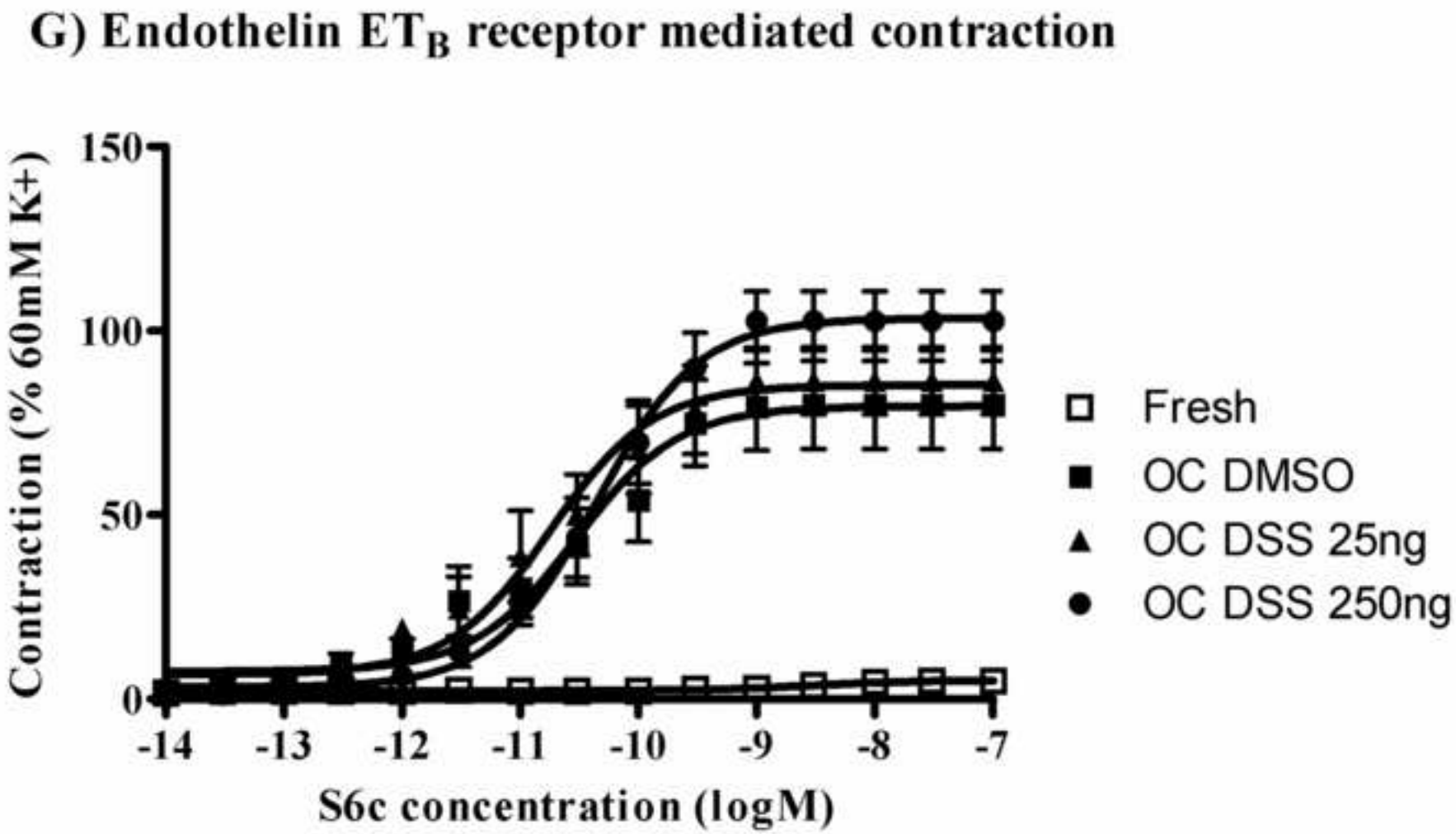

Figure 1H
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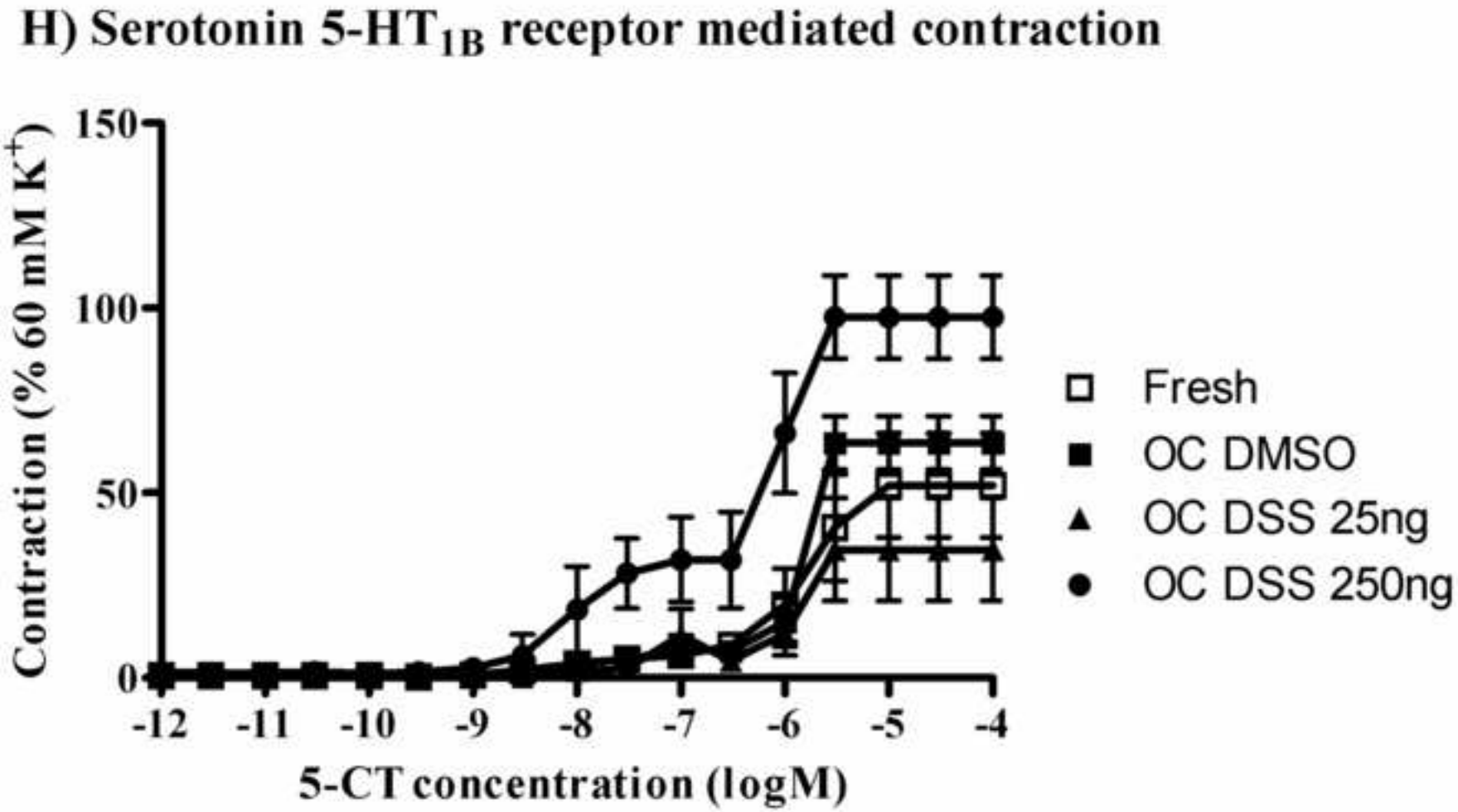

Figure 1I

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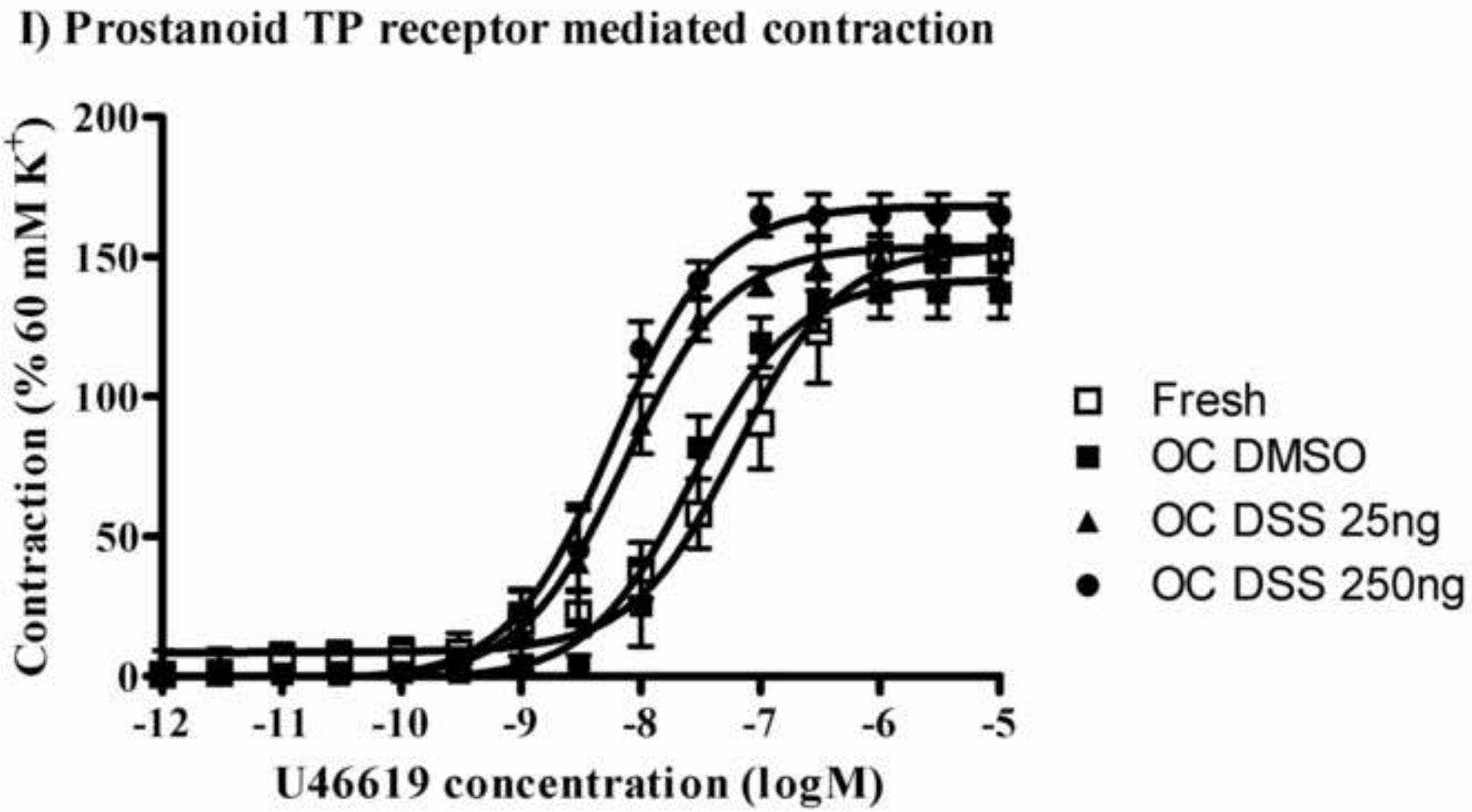

Figure 2A

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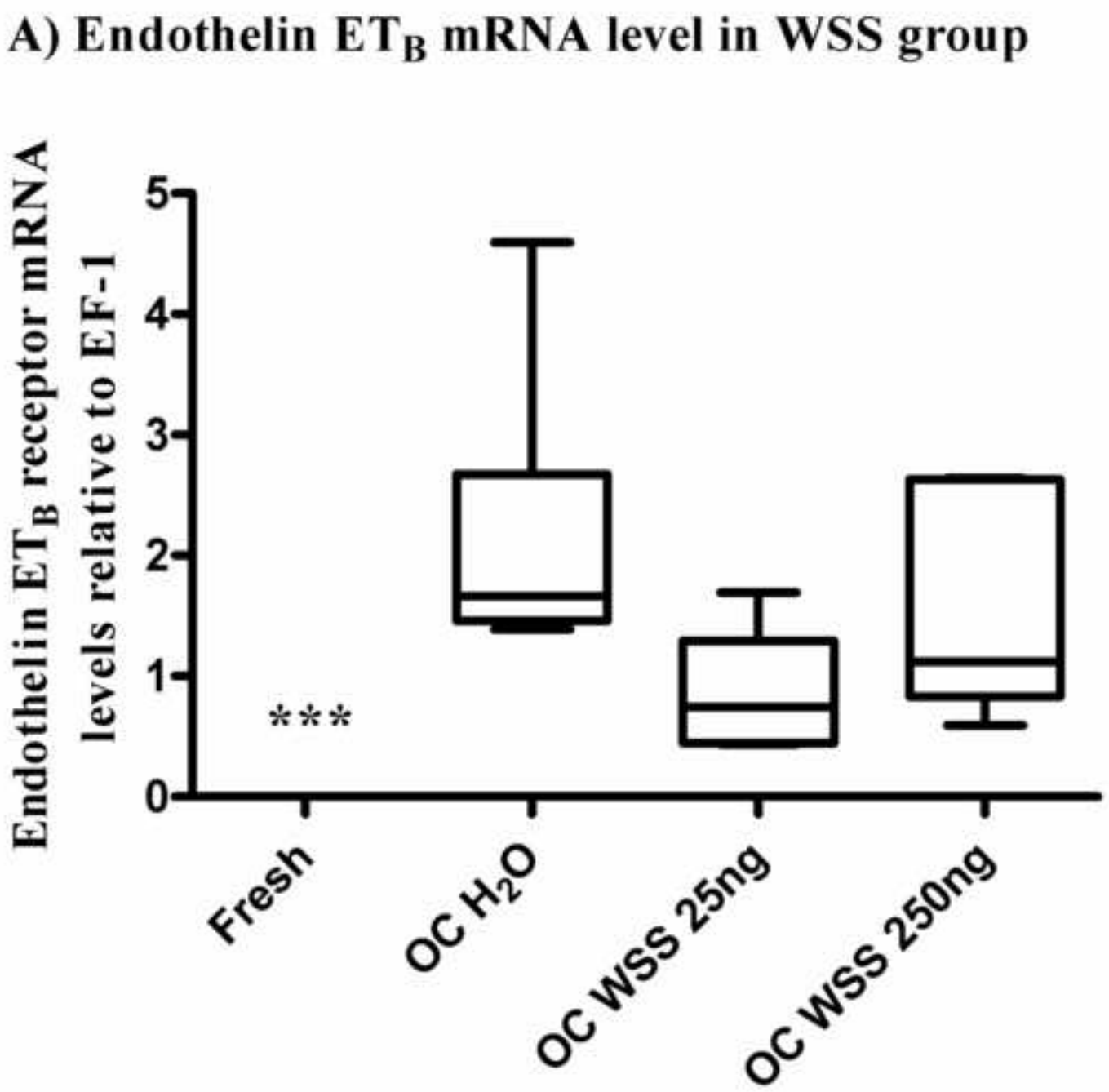

Figure 2B

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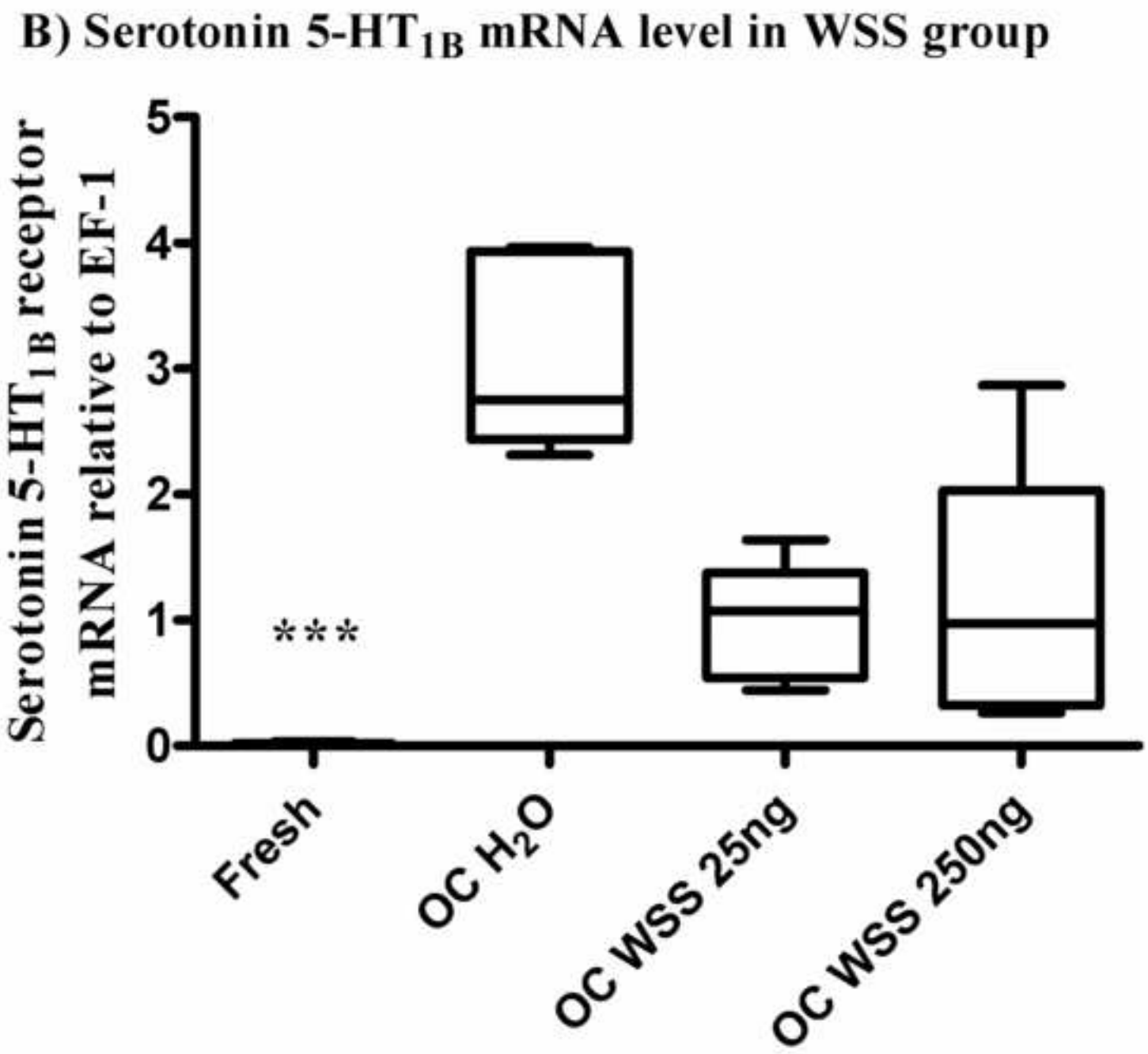

Figure 2C
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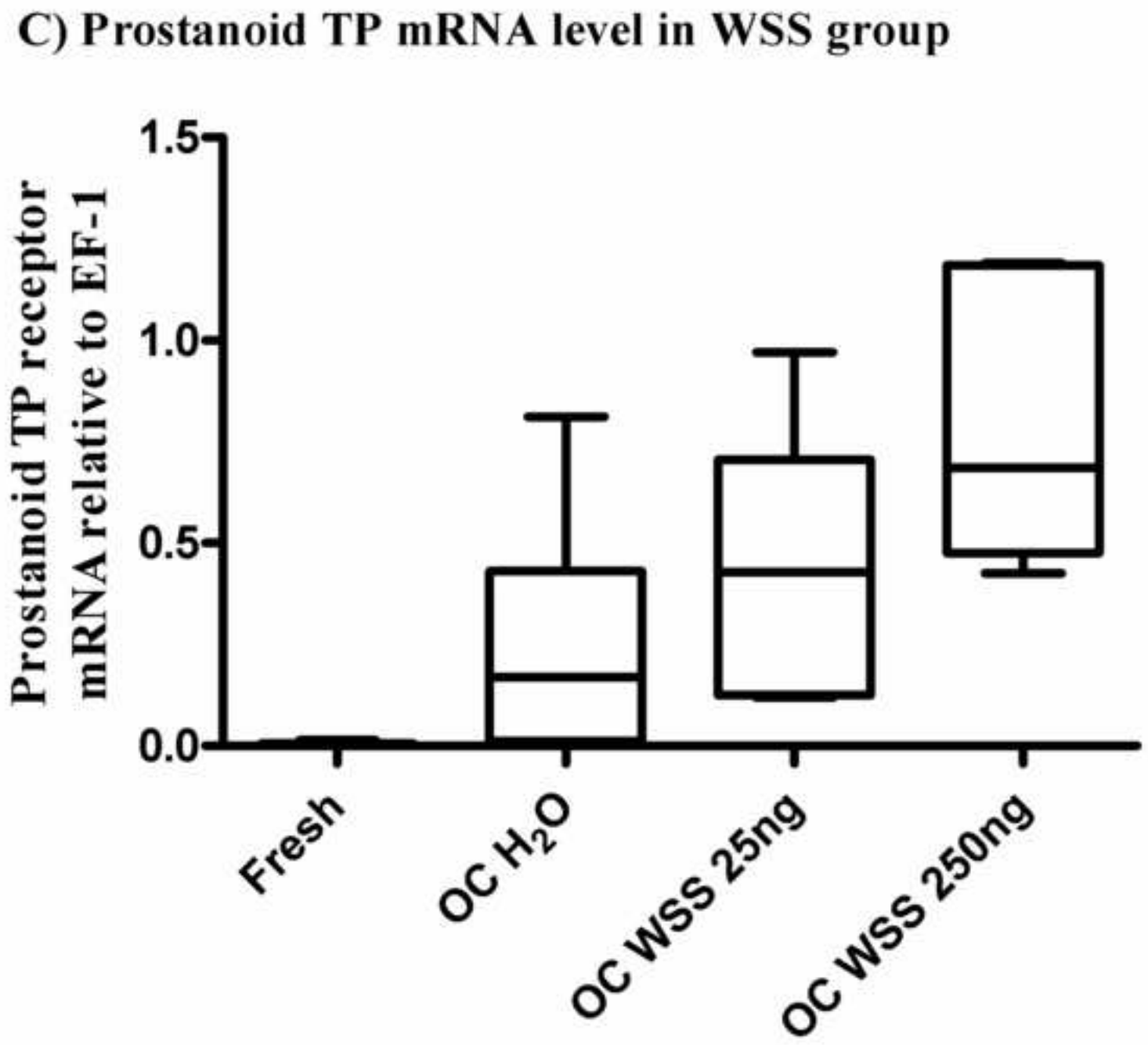

Figure 2D

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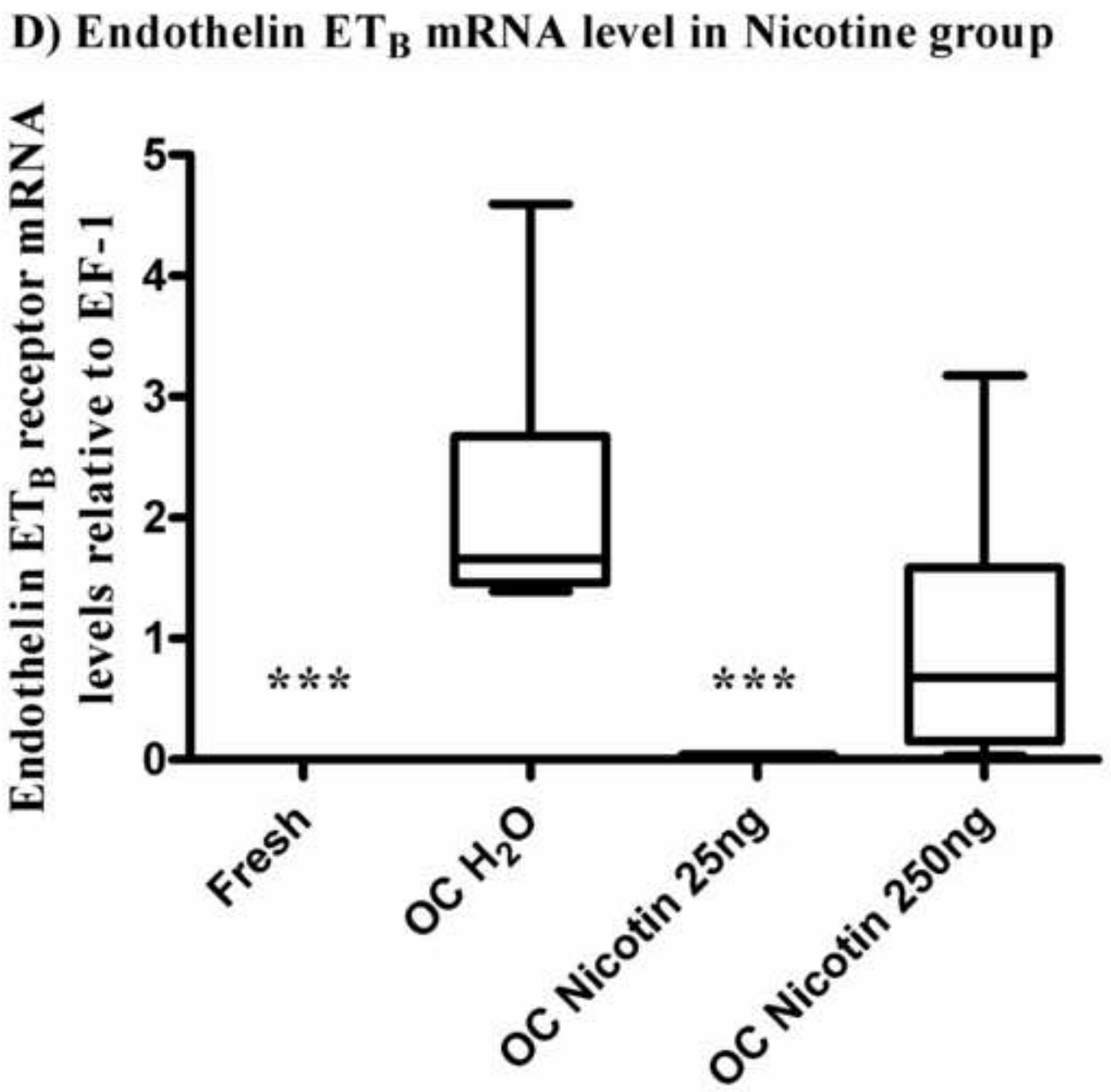

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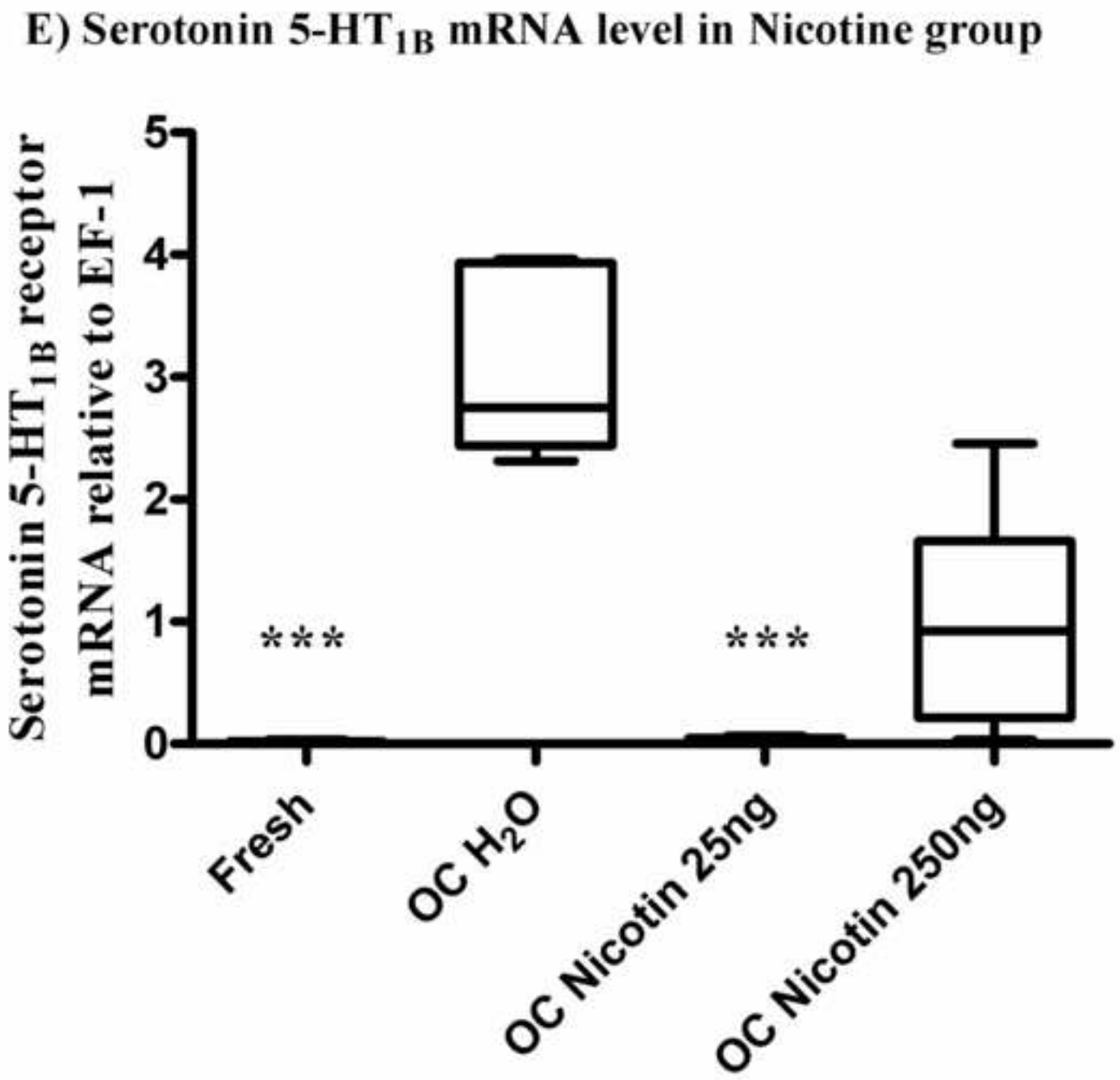

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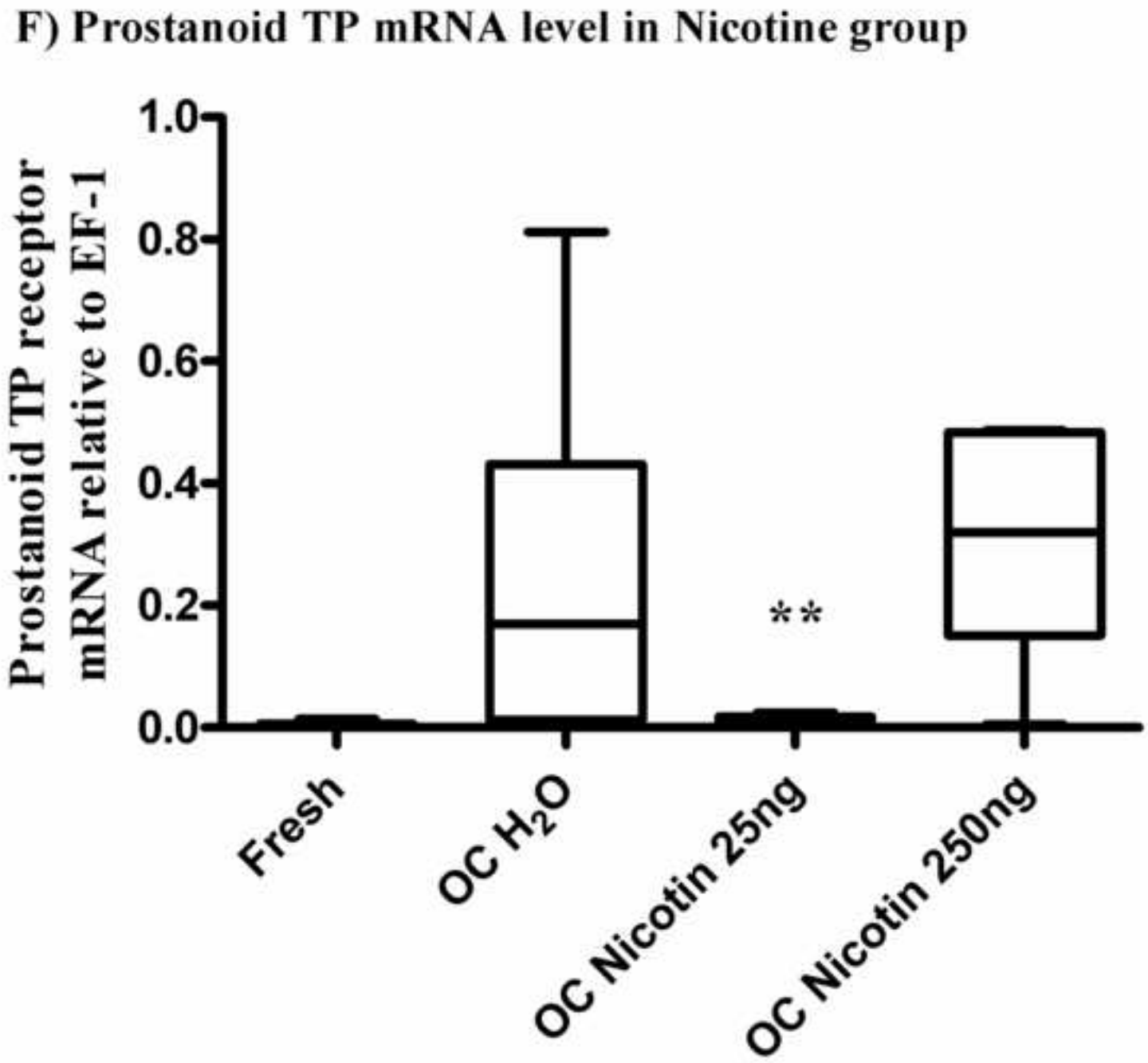

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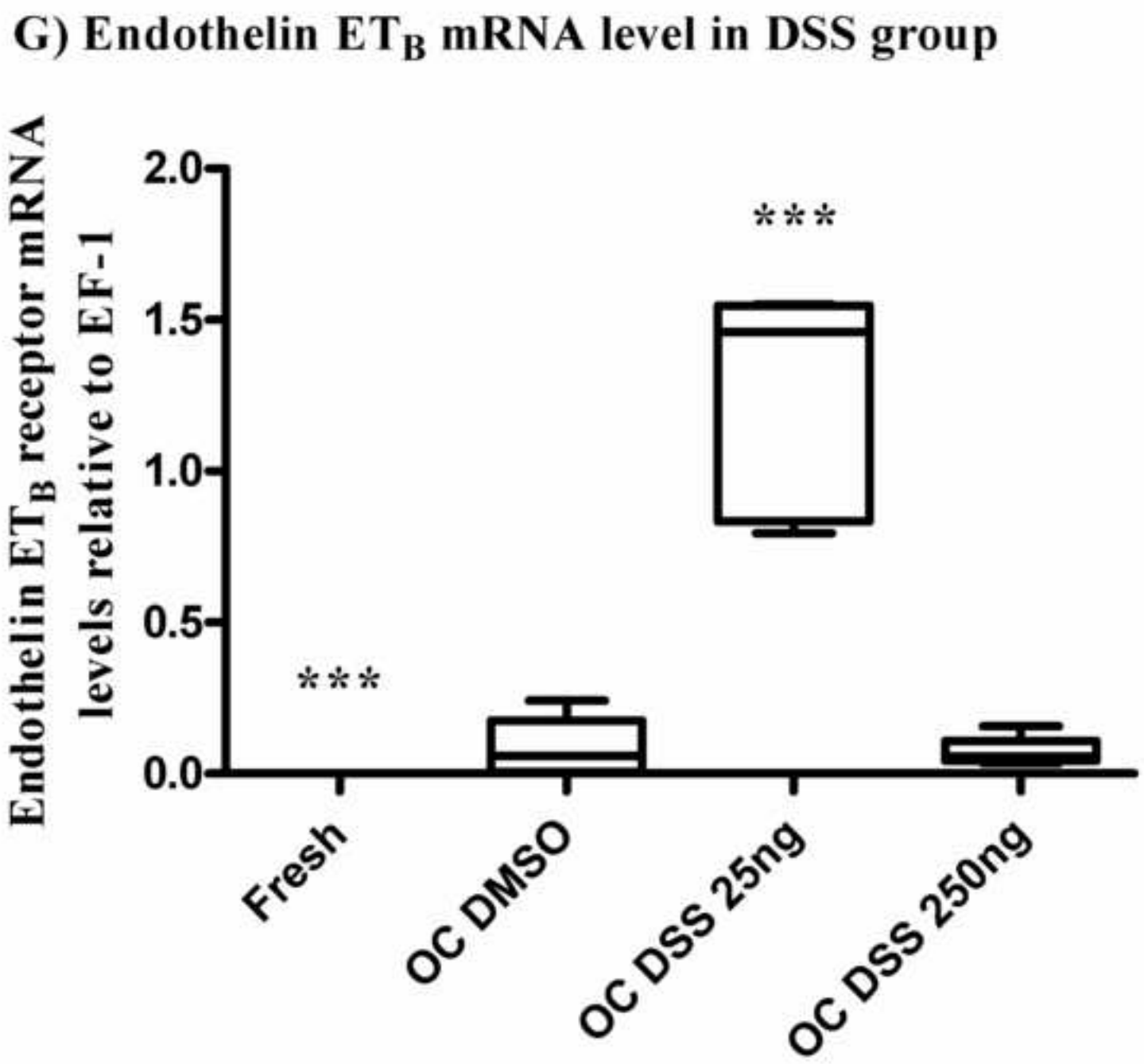

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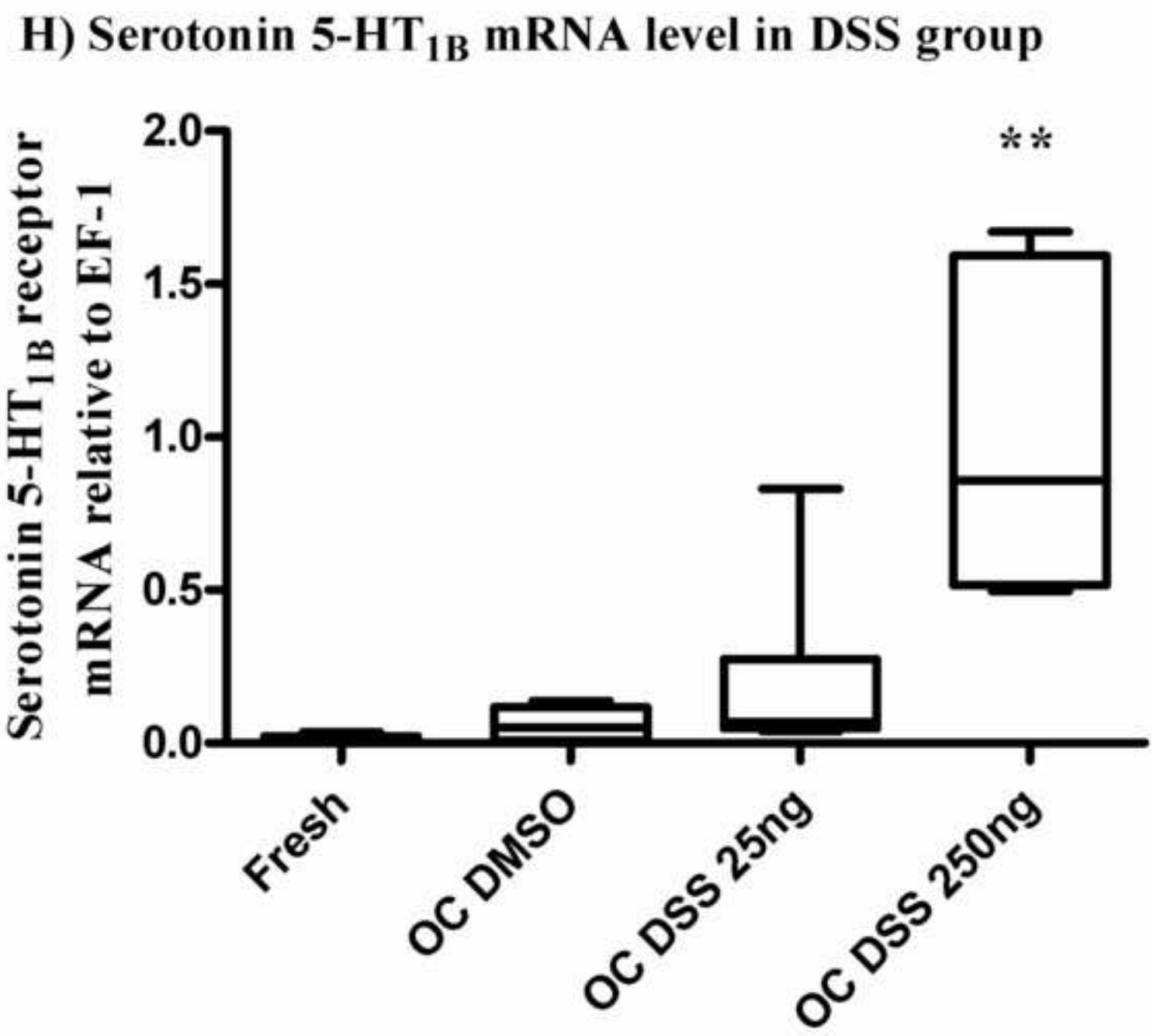

Figure 2I

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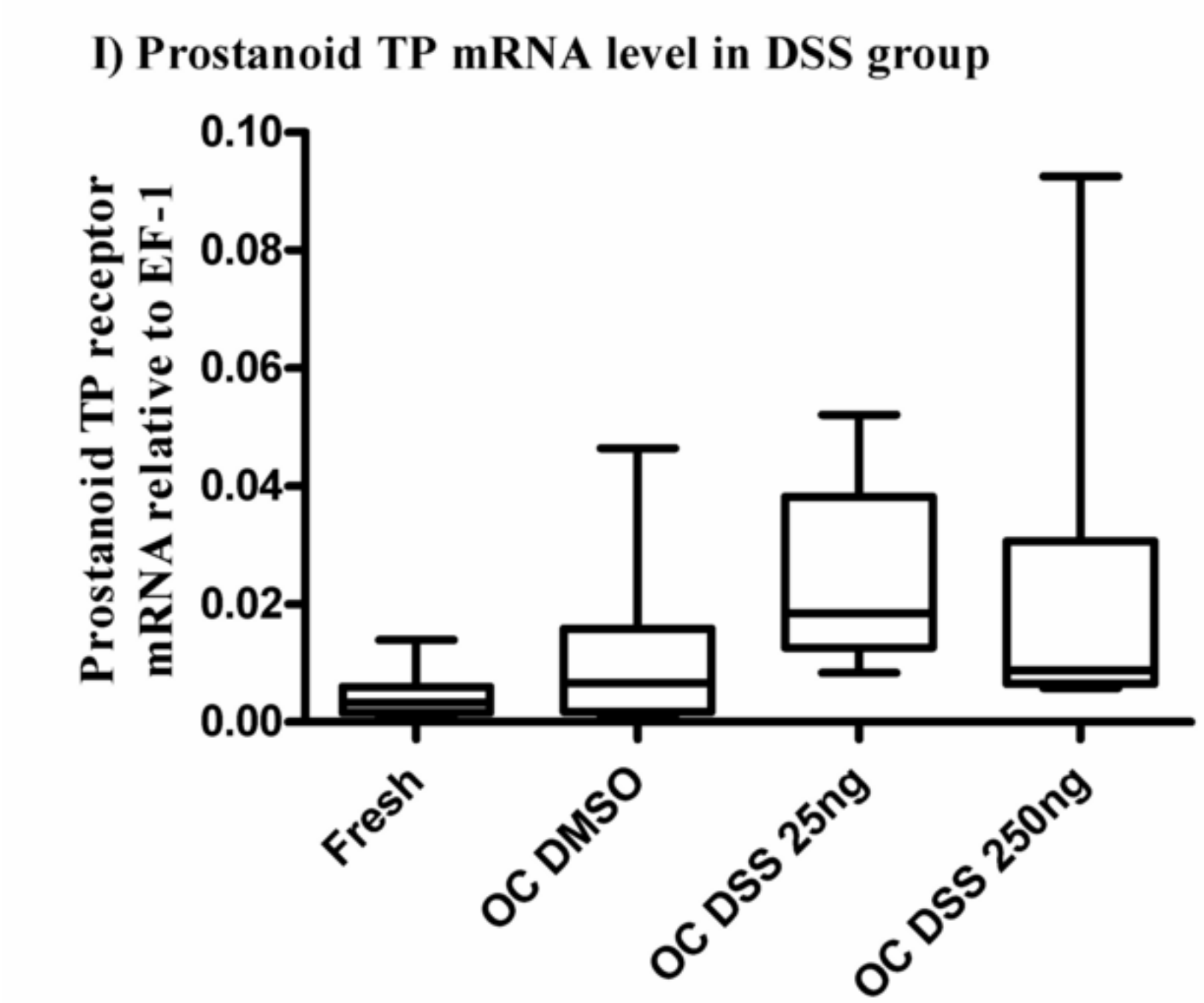

Figure 3A

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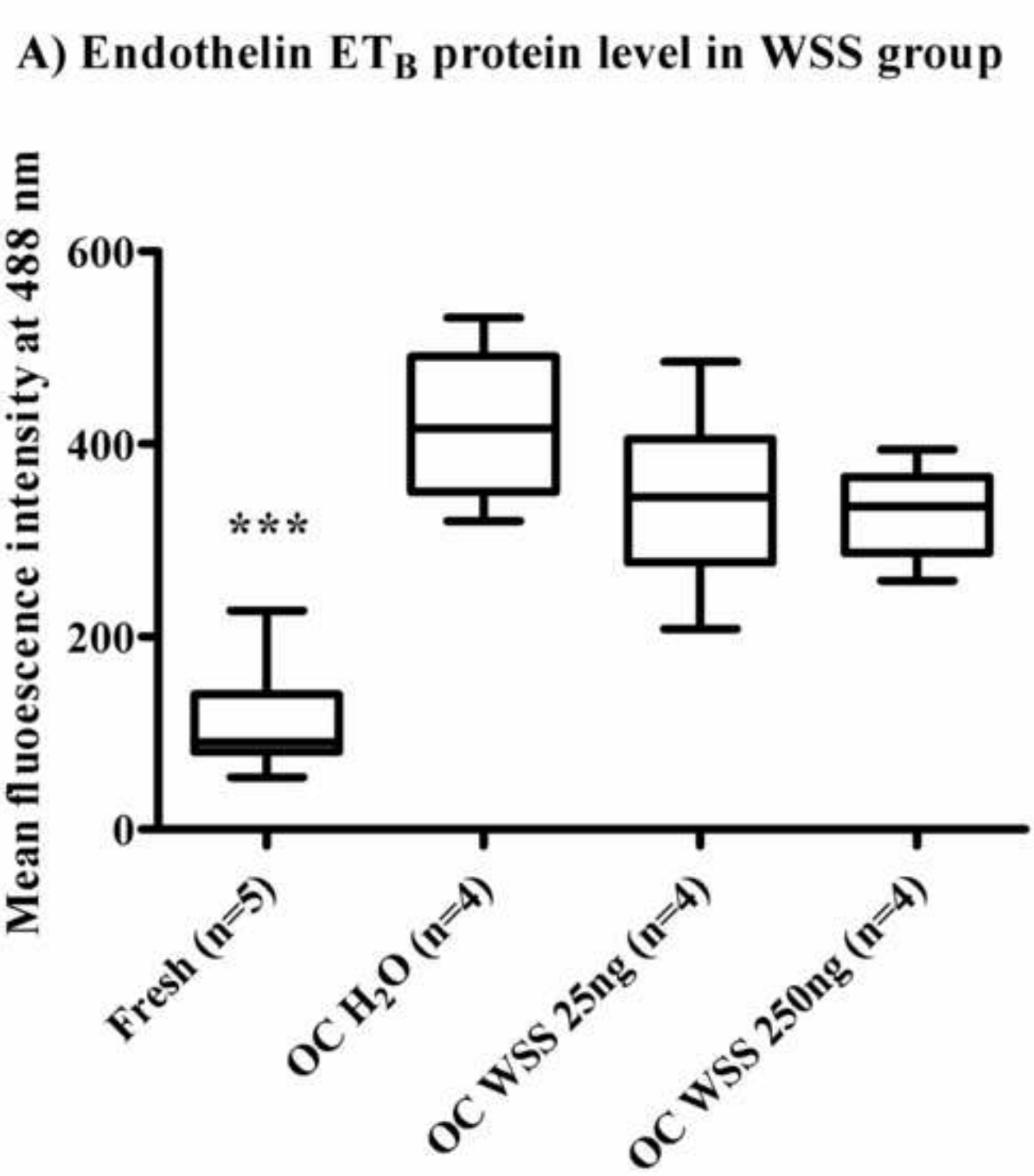

Figure 3B

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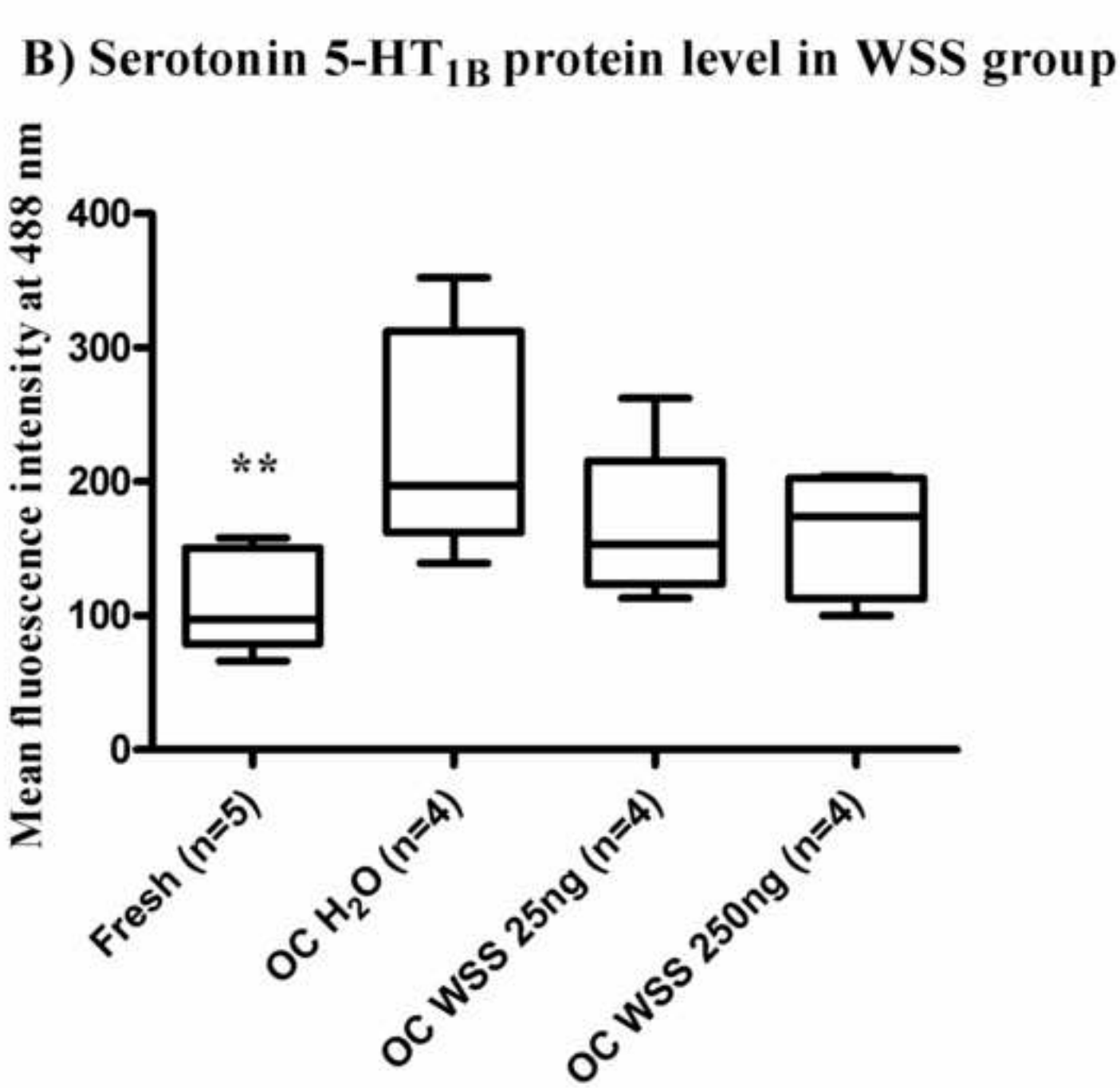

Figure 3C
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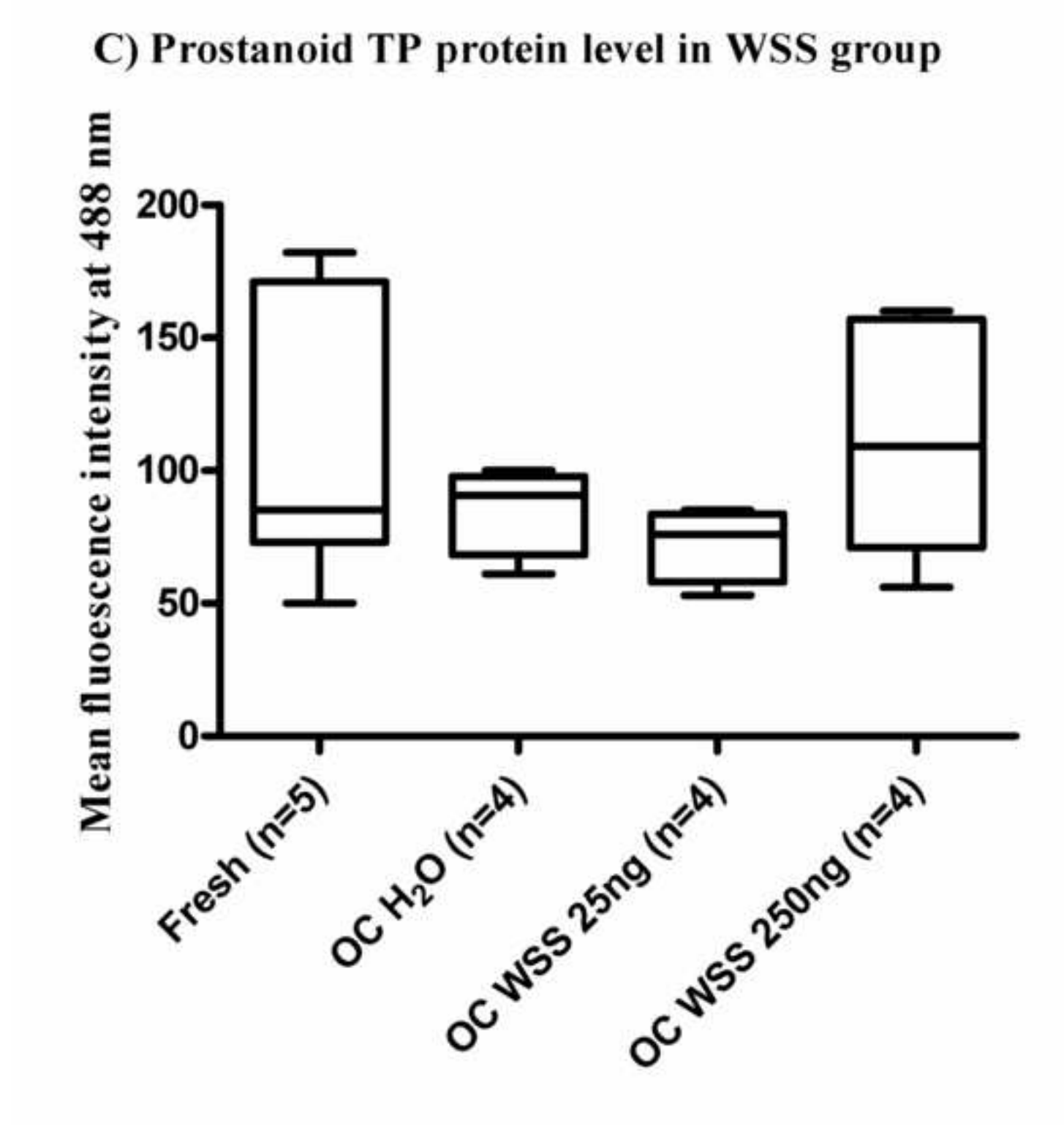

Figure 3D

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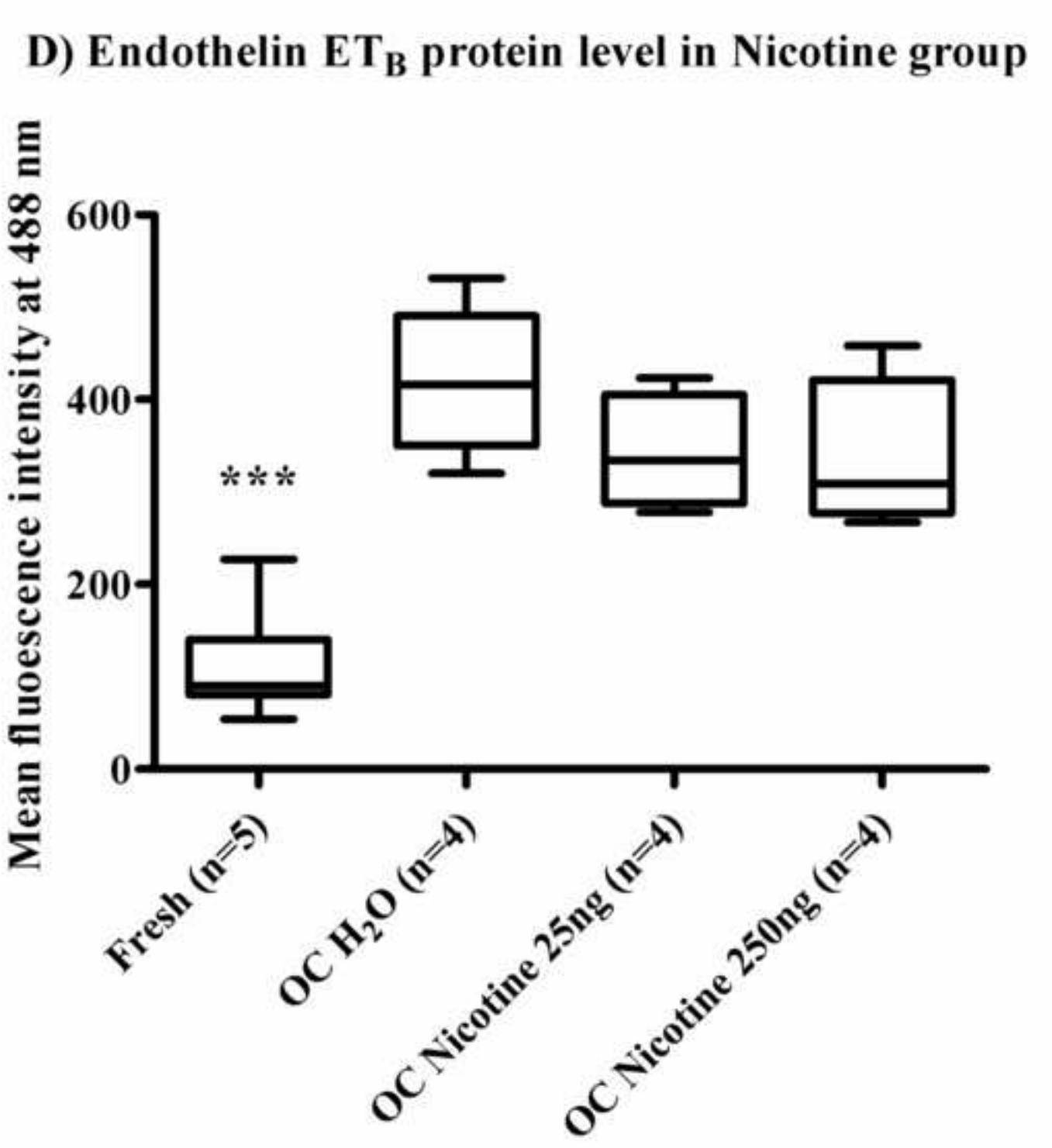

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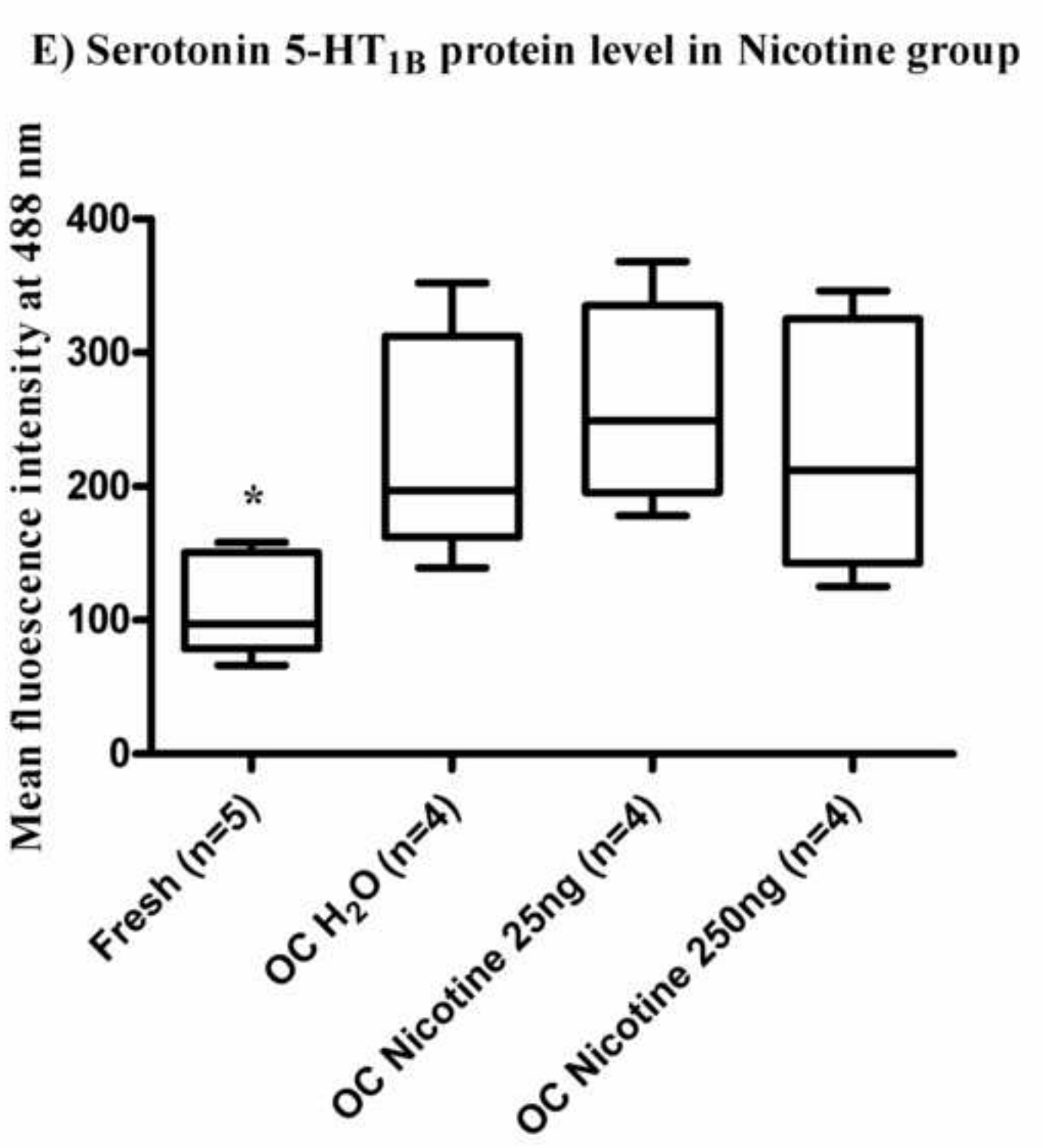

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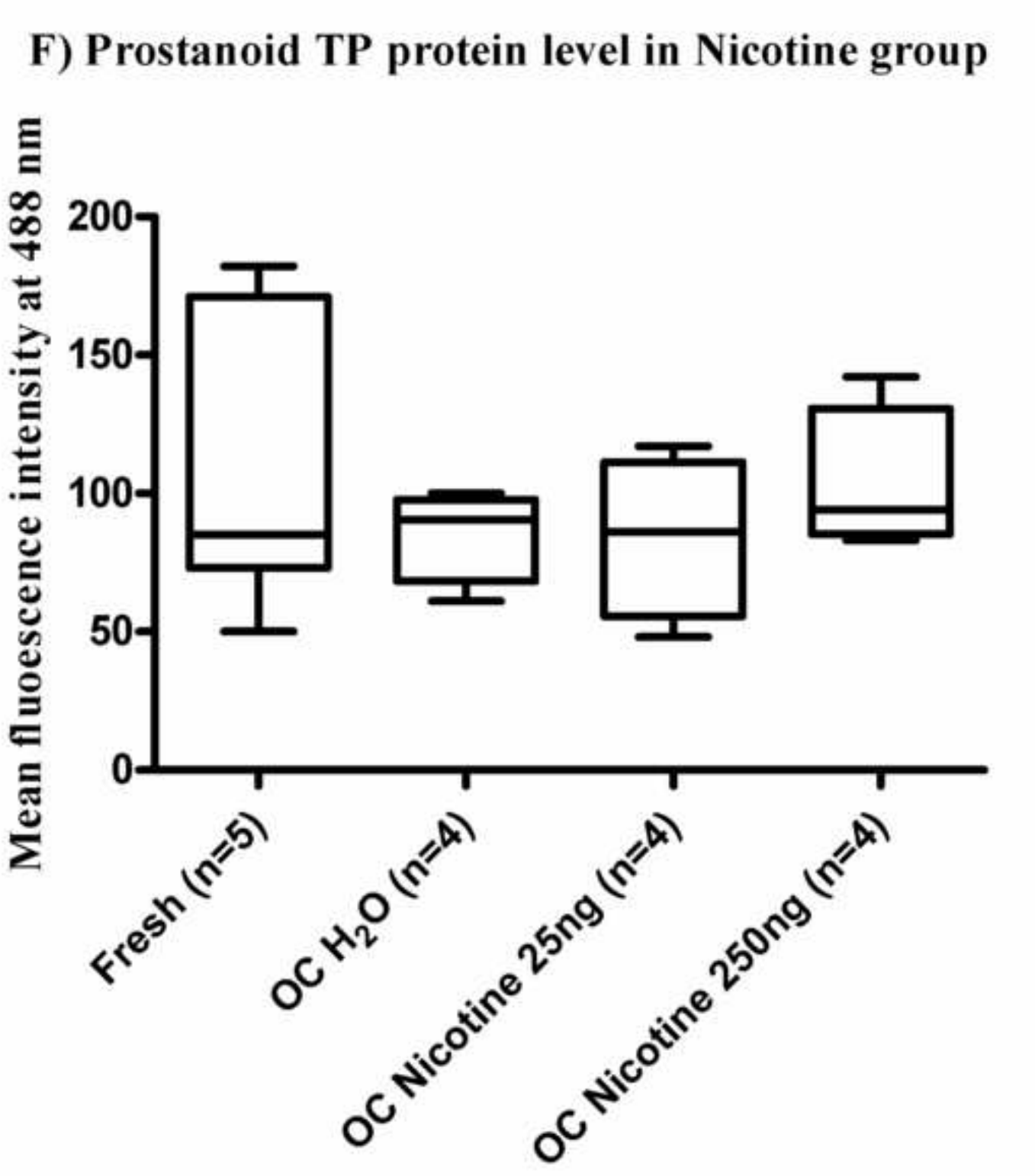

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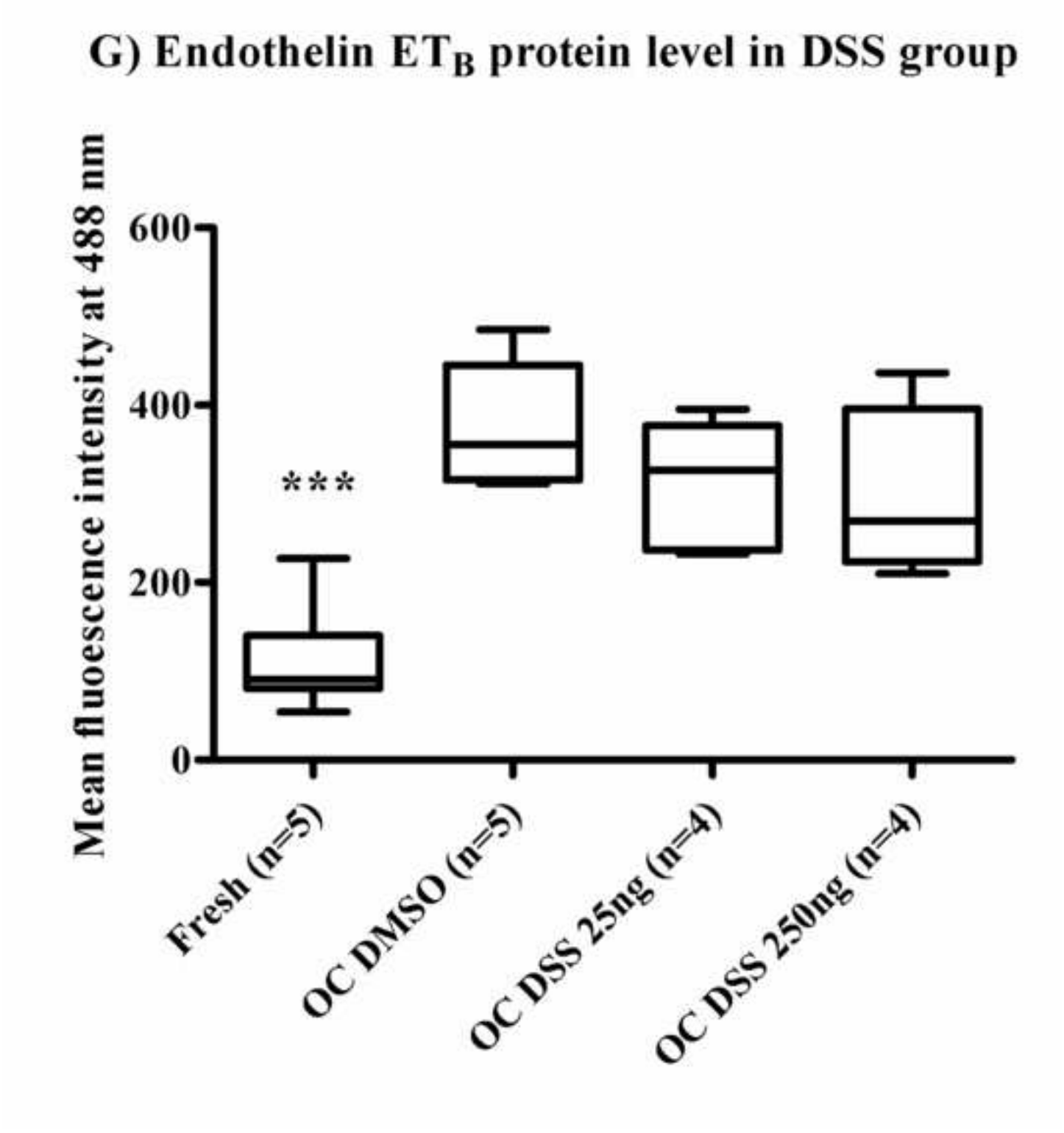

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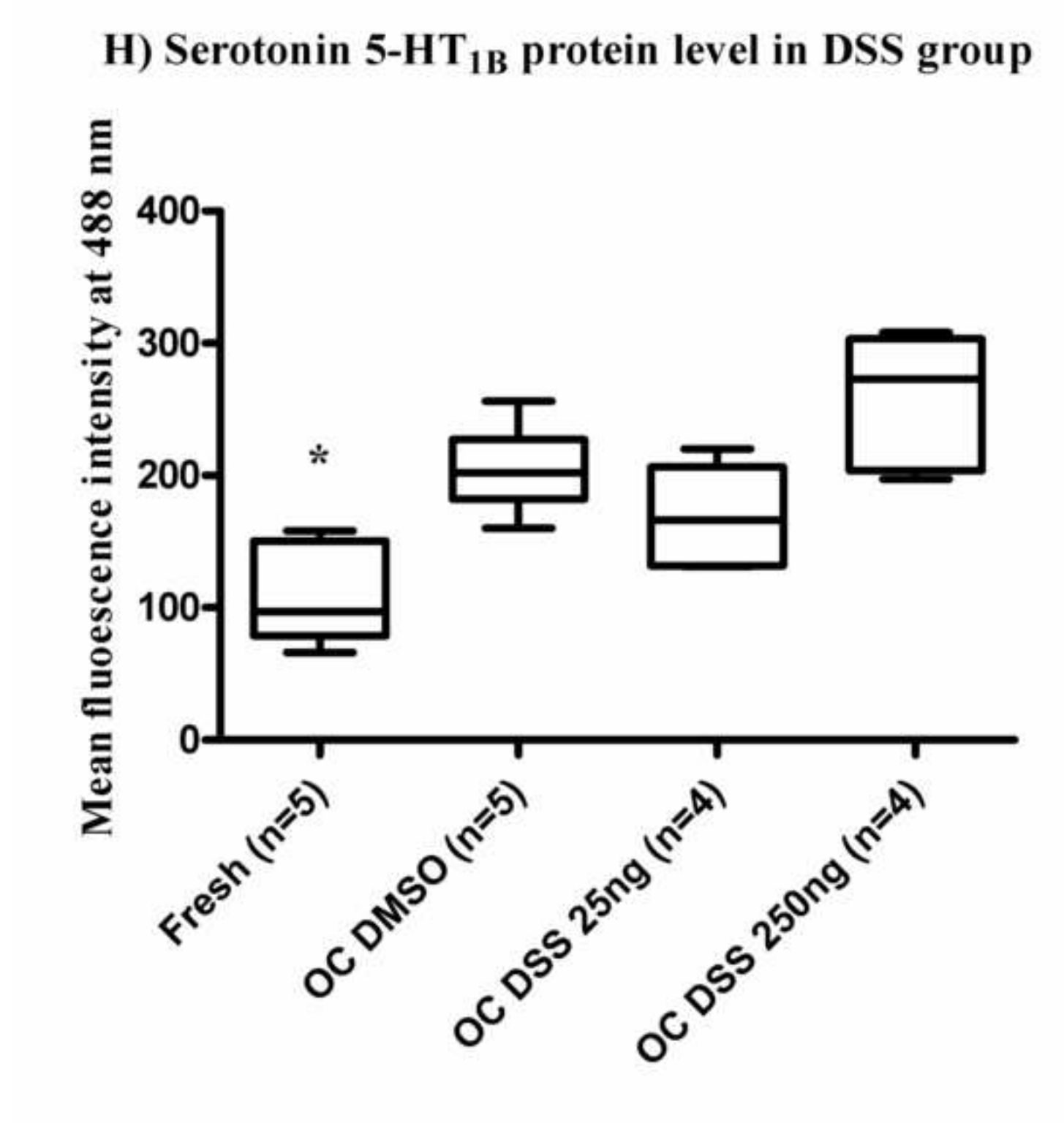

Figure 3I

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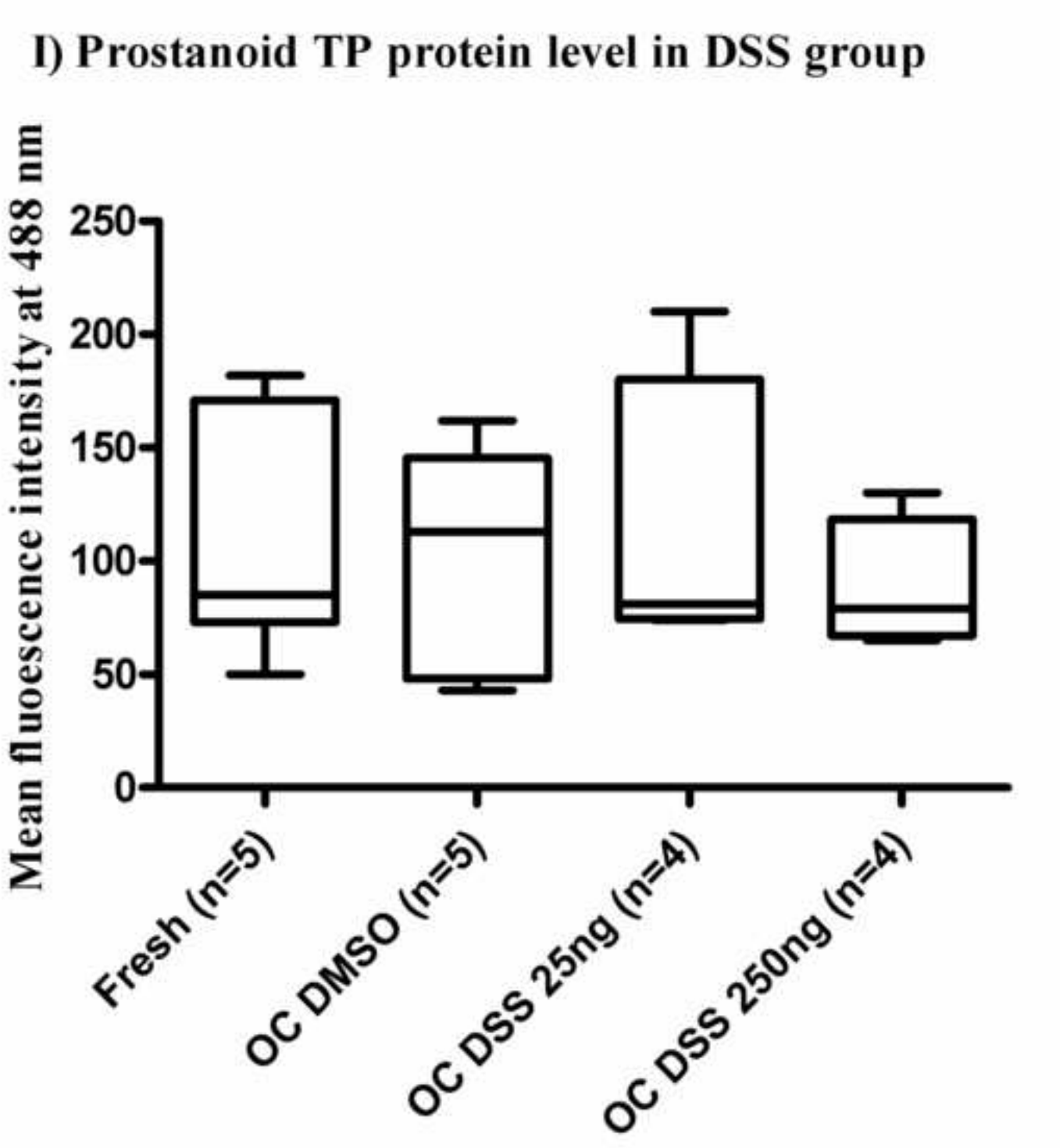

Figure 3J

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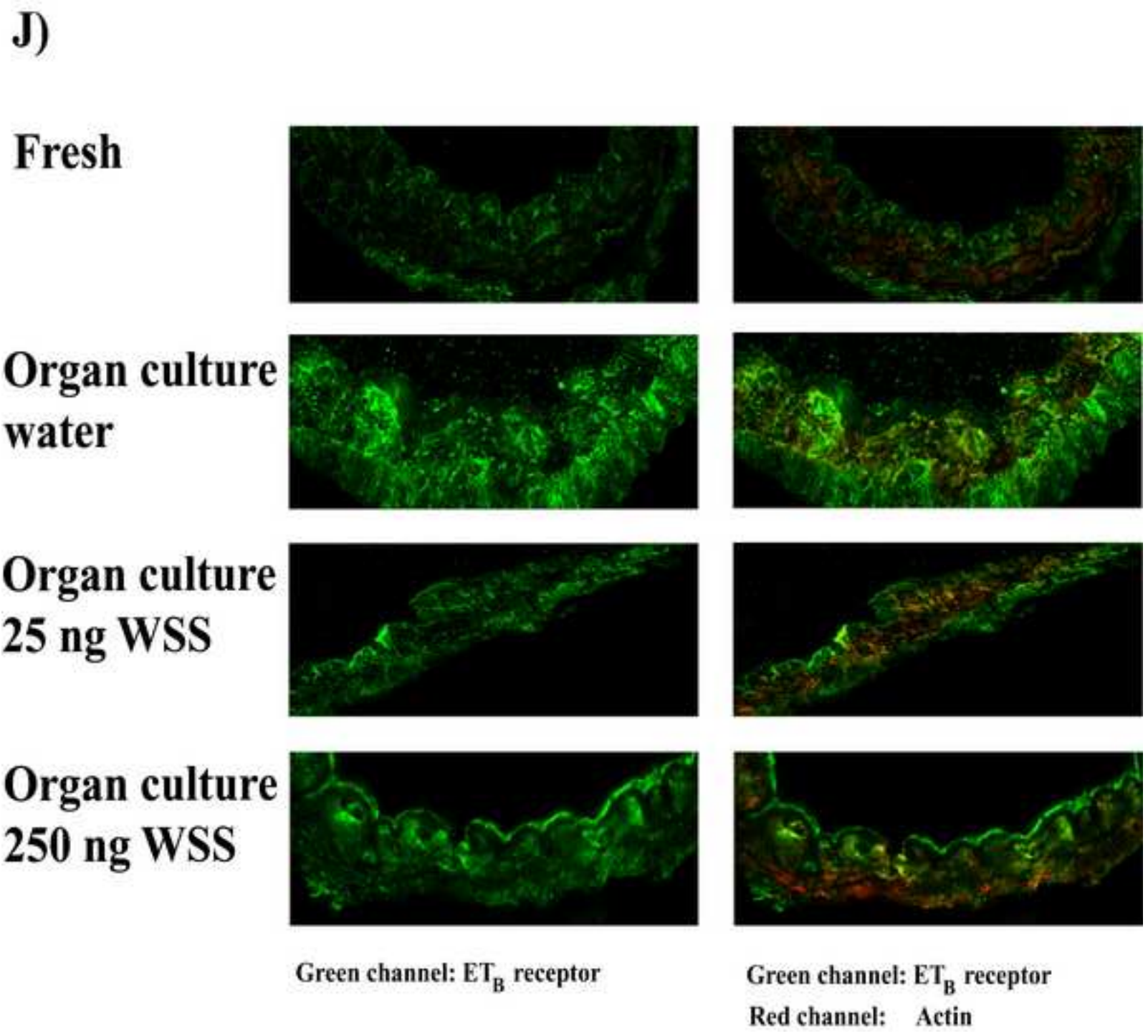

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Conflict of Interest Policy

Title: Alteration in contractile G-protein coupled receptor expression by moist snus and nicotine in rat cerebral arteries

Author name: Hardip Sandhu

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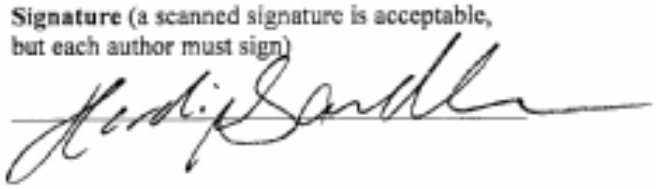

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Title: Alteration in contractile G-protein coupled receptor expression by moist snus and nicotine in rat cerebral arteries

Author name: Cang-Bao Xu

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Author name: Lars Edvinsson

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