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Nestorone® (NES) a Novel Progestin for Non-oral Contraception: Structure-activity Relationships and Brain Metabolism Studies

Narender Kumar[1], Jerôme Fagart[2], Philippe Liere[3], Scott J. Mitchell[4], Alanah R Knibb[4], Isabelle Petit-Topin[2], Marion Rame[3] , Martine El-Etr[3], Michael Schumacher[3], Jeremy J Lambert[4], Marie-Edith Rafestin-Oblin[2] and Regine Sitruk-Ware[1]

[1] Population Council, Center for Biomedical Research, New York, NY, USA

[2] U773 INSERM, Paris, France

[3] U1195 INSERM, University Paris Sud, Le Kremlin Bicêtre, France

[4] Division of Neuroscience, Ninewells Hospital & Medical School, Dundee University, Dundee, Scotland, UK.

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Corresponding Author: Dr Regine Sitruk-Ware, MD
The Population Council, Center for Biomedical Research
1230 York Avenue, New York, NY 10065, USA
regine@popcbr.rockefeller.edu

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Abstract (250 words):

Nestorone® (NES) is a highly potent non-androgenic progestin being developed for contraception. NES is a synthetic progestin that may possess neuroprotective and myelin regenerative potential as an added health benefits. In receptor transactivation experiments, NES displayed greater potency than progesterone to transactivate the human progesterone receptor (hPR). This was confirmed by docking experiments which revealed that NES adopts the same docking position within the PR ligand-binding domain (LBD) as progesterone and forms additional stabilizing contacts between 17α-acetoxy and 16-methylene groups and PR LBD supporting its higher potency than progesterone. The analogue 13-ethyl NES also establishes similar contacts as NES with Met909, leading to comparable potency as NES. In contrast, NES is not stabilized within the human androgen receptor (hAR)-LBD leading to negligible AR transactivation. Since progesterone acts in the brain by both PR-binding and indirectly *via* the metabolite allopregnanolone binding to $GABA_A$ receptor ($GABA_AR$), we investigated if NES is metabolized to 3α, 5α-tetrahydronestorone (3α, 5α-THNES) in the brain and if this metabolite could interact with $GABA_AR$. In female mice, low concentrations of reduced NES metabolites were identified by Gas Chromatography-Mass Spectrometry in both plasma and brain. However, electrophysiological studies showed that 3α, 5α-THNES exhibited only limited activity to enhance $GABA_AR$-evoked responses with WSS-1 cells and did not modulate synaptic $GABA_ARs$ of mouse cortical neurons. Thus the inability of reduced metabolite of NES (3α, 5α-THNES) to activate $GABA_AR$ suggests that the neuroprotective and myelin regenerative effects of NES are mediated *via* PR binding and not via its interaction with the $GABA_AR$.

Introduction:

NES (16-methylene-17α-acetoxy-19-nor-pregn-4-ene-3, 20-dione, also known as ST1435), belongs to the group of “19-nor pregnanes” (1, 2). It has been evaluated as a contraceptive agent and is also being considered as a potential progestin for use in HRT. NES has been synthesized with the objective of creating a highly specific progestin, with selective binding to the progesterone receptor (PR). NES exerts potent progestational and antiestrogenic actions on the uterus and does not interact with estrogen, or androgen receptors (1, 3). NES is inactive when given orally to humans or animals (4 - 7) but is very potent when delivered parenterally, with a progestational activity 100 times higher than that of progesterone (1, 3). Low doses of NES have been found to be very effective in preventing ovulation, allowing its development as a

contraceptive in humans in different non-oral delivery systems such as silastic implants, vaginal ring and transdermal gel (8 - 11).

Synthetic steroidal progestins are structurally related to either testosterone and characterized by a 17β-hydroxyl group (*e.g*. norethindrone and levonorgestrel), or to the natural hormone progesterone (P) and harbor a 17β-methyl ketone function (*e.g.* NES). They act by binding to and transactivating the progesterone receptor (PR), a transcription factor belonging to the nuclear receptor superfamily.

Since different progestins differ in their progestational and anti-estrogenic activity, we used different approaches to assess the progestational activity of NES, as well as its lack of androgenic effect as compared to levonorgestrel (LNG). In previous experiments we showed that NES was active in stimulating neuroprotection and neuroregeneration (12, 13) and myelin repair (14 - 16) and these effects were shown to be exerted *via* the PR (13, 17). However, we wanted to determine whether part of this neuro-regenerative activity could be mediated *via* one of the reduced metabolites of NES as exemplified by allopregnanolone [5α-pregnan-3α-ol-20-one; 3α, 5α-tetrahydroprogesterone (3α, 5α-THPROG)], a neuroactive metabolite of progesterone in the brain (17, 18).

Numerous biochemical studies have established a relationship between the chemical structure of steroidal ligands and their ability to transactivate PR and AR. Moreover, X-ray crystal structures of the ligand-binding domain of PR and AR complexed with various ligands have brought a new insight on the ligand binding mode (19 - 22). Our aim was to characterize the activity of NES, a progesterone related progestin, and its potent analogue 13-ethyl-NES derivative (23) on PR and AR and to identify the binding contacts responsible for their increased potency and those involved in their selectivity. 13-ethyl NES was previously synthesized by Gedeon Richter (Hungary) to compare its oral bioactivity compared to NES. Here, we used 13-ethyl NES to compare PR and AR binding with that of LNG, also a 13 ethyl substituted, but 19-nortestosterone derivative.

Further to the transactivation studies we conducted NES metabolite profiling in mice to determine whether NES is metabolized similarly to progesterone and if reduced metabolite(s) of NES are active on the mouse brain tissue. In this regard, some of the reduced metabolites of progesterone are established as potent and selective positive allosteric modulators of the γ-aminobutyric acid type A receptor ($GABA_AR$). Upon administration, these steroids exhibit clear

behavioral effects that include anxiolysis, sedation and analgesia, they are anticonvulsant and at high doses induce a state of general anesthesia (24, 25). The demonstration that certain neurons and glial cells within the central nervous system (CNS) can synthesize these steroids either *de novo*, or from peripherally derived progesterone, has led to the proposal that these steroids (neurosteroids) can additionally function in an autocrine, or paracrine manner, to locally influence GABAergic transmission (26).

Not all progestins exert the same behavioral effects as natural progesterone, possibly due to lack of conversion into 5α-reduced metabolites, or due to the lack of an interaction with the $GABA_AR$. We therefore assessed the potential of NES progestin to be metabolized in mouse brain tissue and using electrophysiology compared the $GABA_AR$ modulatory action of one of these metabolites 3α, 5α-tetrahydronestorone (3α, 5α-THNES) with the progesterone metabolite 3α, 5α-THPROG.

METHODS

A. STRUCTURE ACTIVITY EXPERIMENTS

AR and PR transactivation by NES, 13-ethyl NES and LNG

The expression vectors pchPR code for human PRB. The human AR expression vector pcDNA-hAR was kindly provided by G.A. Coetzee. The plasmid pcβgal, which contains the β-galactosidase sequence, was used to standardize the transfection experiments. The reporter vector GRE2Luc was the kind gift from Drs. A. Biola-Vidamment and M. Pallardy. HEK 293T cells were routinely cultured in a high-glucose DMEM medium (Invitrogene, Cergy Pontoise, France), 20 mM HEPES, 2 mM glutamine, 1X non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere at 37°C and with 5% CO_2. One day before transfection, the cells were seeded at $3x10^6$ cells/80-mm diameter culture Petri dish and cultured overnight in the same medium. Six hours before transfection, the FCS supplemented medium was replaced by the same medium supplemented with 10% dextran-charcoal treated FCS. Transfections were carried out using the calcium phosphate precipitation method. The calcium phosphate precipitate was prepared with one of the steroid receptor expression vectors (0.5 µg pchPRB or 2 µg pchAR,) plus 7 µg of the reporter vector GRE2-Luc and 1 µg of pcβgal, in 1 ml of 140-mM NaCl, 0.75 mM Na_2HPO_4, 25 mM HEPES, 125 mM $CaCl_2$, pH 7.05 and added to the cells 30 minutes later. After incubating

for 16 h, the transfected cells were washed with PBS containing 2.5 mM EDTA, trypsinized and pooled. The transfected cells were replated in 24-well plates (100,000 transfected cells/well). 4h later, ligands (NES, 13-ethyl NES, or LNG at various concentrations) were added to the transfected cells and the incubation continued for a further 24h at 37°C. Cells were lysed in 300 µl PBS 1X, 25-mM glycylglycine, 4 mM EDTA, 15% glycerol, 1% triton X-100, 15 mM $MgSO_4$, pH 7.8 supplemented with 2 mM β-mercaptoethanol. The luciferase activities were quantified using a Mithras LB940 microplate reader (Berthold). To standardize the transfection efficiency, the relative light units (RLU) obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay.

Docking of NES within the ligand-binding pocket of the androgen and progesterone receptors

NES was build using the build fragment function of the Discovery Studio package (version 3.1, Accelrys, San Diego, USA). The C17 side chains of NES were oriented to superimpose those of Ulipristal Acetate (27) After minimization using the charmm27 forcefield, NES was manually docked within the X-ray crystal structure of the AR and PR LBDs (PDB identification numbers 2AMA and 1A28, respectively) by taking as guides the probe accessible volume of each cavity. The orientation of the ligand or protein side chains were fixed during the docking process.

B. METABOLISM STUDIES IN BRAIN

B.1. CHEMICALS

Nestorone (NES) was synthesized under GMP conditions at Crystal Pharma, Spain. In addition, reduced metabolites of NES such as: 5α-dihydronestorone (5α-DHNES), 20α-dihydronestorone (20α-DHNES), 3α, 5α-tetrahydronestorone (3α, 5α-THNES) and 3β, 5α-tetrahydronestorone (3β, 5α-THNES) were custom synthesized by Crystal Pharma Valladolid Spain based on structure designs that we intended to explore corresponding to the known progesterone metabolite(s) potentially interacting with the GABA-A receptor (Fig 1). The structures of synthetic reference reduced metabolites were confirmed and purity determined to 95-98%.

B.2. STEROIDS MEASUREMENTS IN MOUSE BRAIN AND PLASMA BY GC/MS/MS

An acute subcutaneous injection of NES (10 µg/mouse or 200 µg/mouse) was administered to 3-month old C57Bl6 female mice. Mice were sacrificed by decapitation after anesthesia with isoflurane at 0.5h, 1h and 2h post-administration for low dose experiment and 0.5h, 1h, 2h, 4h and 24h post-administration for high dose experiment (n=6 per group). Blood samples were collected, centrifuged at 3000 g for 10 min at 4°C to obtain plasma samples (200-300 µl). The brain (400 - 500 mg) was dissected out on a bed of crushed ice and weighed. The samples were stored at -20°C until gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) analysis.

NES, 5α-DHNES, 20α-DHNES, 3α, 5α-THNES and 3β, 5α-THNES were determined by GC/MS/MS according to the purification protocol described by Meffre et al (28) with minor modifications. Extraction was performed by adding 10 volumes of methanol (MeOH) and internal standards were introduced for steroid quantification: 2 ng [$^{2}H_{6}$]5α-DHPROG (CDN Isotopes, Pointe Claire, Canada) for 5α-DHNES, 2 ng [$^{13}C_{3}$] androstenedione (Isoscience, PA, USA) for NES and 20α-DHNES and 2 ng of epietiocholanolone for 3α, 5α-THNES and 3β, 5α-THNES. Samples were purified and fractionated by solid-phase extraction with the recycling procedure (29). The unconjugated steroids-containing fraction was filtered and further purified and fractionated by HPLC. The HPLC system is composed of a WPS-3000SL analytical autosampler and a LPG-3400SD quaternary pump gradient coupled with a SR-3000 fraction collector (Thermoscientific, USA). The HPLC separation was achieved with a Lichrosorb Diol column (25 cm, 4.6 mm, 5 µm) in a thermostated block at 30°C. The column was equilibrated in a solvent system of 90% hexane and 10% of a mixture composed of hexane/isopropanol (85/15). Elution was performed at a flow-rate of 1 ml/min, first with 90% hexane and 10% of hexane/isopropanol (85/15) for 8 min, then with a linear gradient to 100% of hexane/isopropanol (85/15) in 2 min. This mobile phase was kept constant for 10 min and a linear gradient to 100% MeOH was applied. The column was washed with MeOH for 15 min. The fraction containing 5α-DHNES was collected in the time range 4-15 min. and then derivatized with 50 µl de MSTFA/NH_4I/DTE (1000/2/5) for 1h at 80°C. NES, 20α-DHNES, 3α, 5α-THNES and 3β, 5α-THNES were collected in the time range 15-28 min. and were derivatized with 25 µl heptafluorobutyric anhydride and 25 µl anhydrous acetonitrile for 1h at 80°C.

Brain and plasma extracts were analyzed by GC/MS/MS with an AI 1310 autosampler (Thermoscientific, USA). The Trace 1310 gas chromatograph is coupled with a TSQ 8000 mass

spectrometer (Thermoscientific, USA). The mass spectrometer was used in tandem mode using Argon as collision gas. Injection was performed in the split-less mode at 250°C (1 min of split-less time) and the temperature of the gas chromatograph oven was initially maintained at 50°C for 1 min and ramped between 50 to 200°C at 20°C/min, then ramped up to 300°C at 10°C/min and finally ramped to 350°C at 30°C/min. The helium carrier gas flow was maintained constant at 1 ml/min during the analysis. The transfer line and ionization chamber temperatures were 330°C and 200°C, respectively. Electron impact ionization was used for mass spectrometry with ionization energy of 70 eV. GC/MS/MS signals were evaluated using a computer workstation by means of the software Excalibur®, release 3.0 (Thermoscientific, USA). Identification of steroids was supported by their retention time and according to two or three transitions. Quantification was performed according to the more abundant transition for the calibration solutions and for the biological extracts. The GC/MS/MS parameters for identification and quantification of NES and its metabolites are summarized in Table 1 in the supplemental information.

The evaluation of the analytical procedure included the limit of detection, sensitivity, linearity and intra- and inter-assay precision. The limit of detection was determined as the lowest amount of compounds that can be measured by GC/MS/MS with a signal-to-noise ratio greater than 3. It ranged from 1 pg for NES and 20α-DHNES to 10 pg for 5α-DHNES, 3α, 5α-THNES and 3β, 5α-THNES (see Table 1 in the supplemental information). Method sensitivity was evaluated by analysing extracts of 100 mg of mouse brain and of 200 µl of mouse plasma containing progressively lower concentrations of NES and its metabolites down to 0.01 ng/g or 0.01 ng/ml. The sensitivity was 0.1 ng/g or 0.1 ng/ml for NES and 20α-DHNES and 1 ng/g or 1 ng/ml for 5α-DHNES, 3α, 5α-THNES and 3β, 5α-THNES. Linearity was determined by analysing mice brain samples prepared at concentrations of 0.01, 0.1, 1 and 10 ng/g of NES and its reduced metabolites. The assay was linear in this concentration range with a coefficient of correlation of 0.995. Intra-assay precision was determined by analysing 3 replicates per run of 100 mg of mice brain supplemented with 1, 10 and 100 ng/g of NES and its metabolites. Inter-assay precision was determined by analysing these samples for a 4-day period. The intra-assay coefficients of variation were 9.3 - 12.5%, 5.6 - 8.3% and 4.5 - 5.6% and inter-assay coefficients of variation were 10.1 - 13.2%, 7.2 - 9.4% and 4.8 - 6.5% for 1, 10 and 100 ng/g of NES and its metabolites, respectively.

B.3. PR TRANSACTIVATION BY NES AND ITS REDUCED METABOLITES

Test compounds (NES and its metabolites) were prepared in DMSO. Diluted stock solutions were stored at 4°C. Human PR, in the test system described below, was challenged with eight concentrations of NES starting at 100 nM and continuing with 1:5 serial dilutions, and 5α DHNES, and 3α, 5α THNES, starting at 10,000 nM and continuing with 1:5 serial dilutions.

The nuclear receptor assay system for this transactivation experiment utilized proprietary human cells engineered to provide constitutive, high-level expression of the full-length human Progesterone Receptor (hPR). Reporter Assay System 96-well Format Assays Product # IB05001 (Indigo Biosciences, PA, USA). The reporter vector used in these studies comprise the firefly luciferase gene functionally linked to an upstream PR response element.

A suspension of Reporter Cells was prepared in Cell Recovery Medium (CRM; containing 10% charcoal stripped FBS). For PR agonist activity assay, 100 µl of the Reporter Cell suspension was dispensed into wells of a white, cell culture treated, 96-well assay plate. Immediately prior to assay setup, test compounds were diluted using compound screening medium (CSM; containing 10% charcoal stripped FBS) to generate 2x-concentration treatment media. 100 µl of each treatment medium was dispensed into triplicate assay wells pre-dispensed with Reporter Cells. Assay plates were incubated at 37°C for 24 hr. Following the 24 hr incubation period, treatment media were discarded and 100 µl/well of Luciferase Detection Reagent was added. Relative light units (RLUs) were quantified from each assay well to determine PR agonist activity. The performance of PR agonist assays was validated by performing a Reference Agonist (Progesterone) dose response curve.

B.4. INTERACTION OF REDUCED NES METABOLITES WITH $GABA_A$ RECEPTORS

Chemicals and reagents for electrophysiological studies of WSS-1 cell and mouse cortical neurons.

GABA (γ-aminobutyric acid), tetrodotoxin and strychnine (Sigma/Tocris), kyneurenic acid (Ascent). The neurosteroid 3α, 5α-THPROG was a gift from Dr. Kelvin Gee. The Nestorone metabolite 3α, 5α-THNES was synthesised by Crystal Pharma Valladolid Spain based on structure designs supplied by the Population Council. Stock solutions of 3α, 5α-THPROG and 3α, 5α-THNES, in DMSO, were diluted to achieve a final DMSO concentration of ≤ 0.01%. Note the vehicle had no effect on the GABA-evoked currents recorded from WSS-1 cells, or on the miniature inhibitory postsynaptic currents (mIPSCs) recorded from mouse cortical neurons.

Cell culture and maintenance of WSS-1 cells

WSS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 400 µg/ml geneticin (G-418) used to positively select cells that express $GABA_AR$s. Resistance to the antibiotic is conferred by the vector containing a cDNA encoding the α1 subunit (30 - 31). The cells were incubated at 37°C with 5% CO_2 atmosphere and re-seeded into a T25 flask every 3 days *i.e.* when the cells were 80 - 95% confluent. For experiments, 20 - 50µl of solution containing WSS-1 cells were plated onto a 35mm cell culture dish. Such cells were used 17 - 96 hr. following plating for electrophysiological experiments. All reagents used were obtained from Invitrogen.

Electrophysiological studies on WSS-1 cells

Electrophysiological studies were performed using the whole-cell voltage-clamp technique. WSS-1 cells were voltage-clamped at -60mV using an Axopatch 200B amplifier, low pass filtered with a cut-off frequency of 2 kHz. The data were recorded and digitized using a Digidata 1440A interface (Axon Instruments, Union City, CA). Data were acquired at 10 kHz and saved onto a PC hard drive. Currents were analysed using pCLAMP 10.1.3 software (Axon instruments). Cells were continually superfused (5ml min^{-1}) with an extracellular solution containing (in mM) NaCl, 140; KCl. 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.5; glucose, 10; and HEPES, 10 (pH 7.4 with 1M NaOH). Patch pipettes were pulled from 1.5 mm borosilicate glass capillaries with a micropipette puller (P-87, Sutter Instruments). Each microelectrode was fire polished and had a final resistance of 1 - 3MΩ when filled with an electrode solution containing (in mM): CsCl, 140; $MgCl_2$, 2.0; EGTA, 11; ATP (Mg^{2+} salt) 3; and HEPES, 10 (pH 7.4 with 1M CsOH). With these intracellular and extracellular solutions the chloride equilibrium potential was approximately 0 mV. GABA and steroids were rapidly applied for 5 sec. (solution exchange time of 15 - 17ms) using a three-barrel Perfusion Fast-Step (SF-77B) solution exchange system (Warner Instruments, Hamden, CT, USA). Solution flow was regulated at 0.3 ml min^{-1} by a syringe pump (Cole-Parmer, Vernon Hills, IL, USA). Upon establishment of the whole-cell recording mode, cells were lifted by movement of the recording electrode *via* the micromanipulator from the base of the dish and positioned within the stream of the control extracellular recording solution. To deliver GABA, or GABA + the steroid, the perfusion barrels were stepped rapidly from side to side, exposing the cell to the test drug solution for 5 sec. Solution exchanges for each cell were repeated for 4 trials. Experiments were performed at room temperature (20 - 24°C). All reagents

used were obtained from Sigma-Aldrich Co. All data are expressed as the arithmetic mean ± S.E.M. Statistical significance of mean data was evaluated using a paired Student's *t*-test.

Preparation of murine brain slices for electrophysiology

The methods were essentially as recently described (32). All rodent studies were approved by the University of Dundee Ethical Review Committee (Home Office Project Licenses 60/4005 and 70/8161, Dr. Belelli), and complied with Schedule 1 of the UK Government Animals (Scientific Procedures) Act, 1986. Cortical slices were prepared from postnatal day (P) P20 - 24 C57/Bl6 129 SVJ mice of either sex. Mice were killed by cervical dislocation, the brain dissected and placed in ice-cold oxygenated (95% O_2/5%CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 225 sucrose, 2.95 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 glucose, (pH 7.4; 328-330 mosmol l^{-1}). Coronal brain slices were cut to 300 - 350 µm thickness, using a Vibratome series 1000 PLUS Sectioning System (Intracell, Royston, Hertfordshire, UK) and then immediately transferred to a nylon mesh platform housed within a chamber containing circulating oxygenated extracellular solution (ECS, in mM: 126 NaCl, 26 $NaHCO_3$, 2.95 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose [306-309 mOsm]). Such slices remained at room temperature for a minimum of 1 hr before electrophysiological recording.

Voltage-clamp recording

During recording, cortical slices were perfused (3 - 5 ml min^{-1}) with ECS maintained at 35 °C, which was recycled to a 50 ml oxygenated reservoir. For whole-cell recordings, an intracellular solution (ICS) containing (in mM): 135 CsCl, 10 HEPES, 10 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 2 Mg-ATP and 5 QX-314 (pH 7.2-7.3, 290-300 mOsm) was employed. Patch pipettes were made from thick-walled borosilicate glass (0.95 mm I.D. 1.55 mm E.D. Garner Glass Co. Claremont, CA), using a PC-10 electrode puller (Narashige, Japan). When filled with ICS, such pipettes had an open tip resistance of 2 - 6 MΩ. Neurons were visually identified with an upright Olympus BX50WI microscope (Olympus, Southall, UK) equipped with IR-DIC optics. Pyramidal neurons located within cortical L II/III were identified based on their canonical pyramidal morphology. Neurons were voltage-clamped (Vh = -60 mV) using an Axopatch 1D amplifier (Molecular Devices, CA, USA) and filtered at 2 kHz. The $GABA_AR$-mediated mIPSCs were isolated by supplementing the ECS with kynurenic acid (2 mM), tetrodotoxin (TTX, 500 nM) and strychnine (1 µM). Data was acquired and digitised (10 kHz) using a NIDAQ mx card (National Instruments,

TX, USA) and stored directly to PC using WinEDR software (Strathclyde University, UK). Series resistance compensation was applied up to 80%.

Analysis of mIPSCs (33)

Digitized data was analyzed offline using WinEDR/WinWCP software (Strathclyde University, UK). The mIPSCs were identified by an algorithmic detection protocol, then visually inspected and spurious events omitted. To eliminate distal events, Gaussian distributions of 10 - 90% rise times were generated and mIPSCs falling outside the Gaussian limits were excluded. For each neuron, 50 or more mIPSCs were analyzed to determine their peak amplitude, 10 - 90 % rise time. Accepted mIPSCs recorded from a single neuron were averaged and fitted with either a mono-exponential ($y(t) = Ae^{(-t/\tau)}$), or a bi-exponential ($y(t) = A_1e^{(-t/\tau 1)} + A_2e^{(-t/\tau 2)}$) decay function, where y(t) is the current amplitude at time t, A is the current amplitude and τ is the decay time constant. To compare goodness of fit between a mono- or bi-exponential decay, an F test was applied to the standard deviation of the residuals. The mIPSC decay times were best fit by a bi-exponential function. Therefore, a mean weighted decay constant (τ_w) was calculated to accommodate the relative contribution of each decay component whereby: $\tau_w = \tau_1P_1+\tau_2P_2$. Here, τ_1 and τ_2 are the decay time constants for the first and second exponential functions, and P_1 and P_2 are the proportions of current amplitude described by each component *i.e*.

$$P1 = \frac{A1}{A1+A2} \qquad P2 = \frac{A2}{A1+A2}$$

Data are expressed as mean values ± standard error of the mean (S.E.M.). To determine statistical significance a one way ANOVA followed *post-hoc* by Tukey's HSD test (SPSS software, Chicago, IL, USA) was performed.

RESULTS

NES STRUCTURE ACTIVITY RELATIONSHIPS

Transactivation Studies

Transactivation assays performed in HEK293T cells transiently expressing PR revealed that NES and 13-ethyl NES display the same potency to activate PR (EC_{50}: 8.2 pM; Fig.2A). These two molecules are much more potent than progesterone (EC_{50}: 98 pM; Fig. 2A) and

norethindrone (EC_{50}: 53 pM; Fig. 2A), a testosterone derived progestin characterized by a 13-methyl group. Interestingly, NES and 13-ethyl NES are nearly as potent as LNG, a testosterone derived progestin characterized by a 13-ethyl group substitution (EC_{50}: 5.8 pM; Fig.2A). These results show that the replacement of a methyl group by an ethyl group at the C13 position does not modify the already high potency of NES, whereas it increases that of norethindrone. Transactivation assays performed in HEK293T cells transiently expressing AR revealed that NES and 13-ethyl NES are nearly unable to activate AR (Fig.2B), in contrast to LNG which is a potent AR agonist (EC_{50}: 1.18 ± 0.01 x 10^{-10} M; Fig. 2B). These results show that the ability of progestin to activate AR is greatly ensured by the C17 substituents. The 17β-hydroxyl and 17α-ethynyl groups of LNG are much more efficient than the 17β-methylketone and 17α-acetoxy groups of NES for producing an androgenic response.

NES binding mode to PR and AR LBD

To link the high potency of NES to activate the PR on its chemical structure, we followed a modeling approach based on the X-ray crystal structure of the PR ligand-binding domain (LBD) complexed with progesterone (19). Within the ligand-binding cavity of PR NES can adopt the same positioning as progesterone. The C3 ketone function of NES can be hydrogen bonded to the Gln725 (3.0 Å) and Arg766 (1.7 Å) (Fig. 3A) and its 17β-methylketone can be anchored to Cys891 through a CHO hydrogen bond (2.8 Å) (Fig. 3A) as is observed for progesterone (19). The main difference between NES and progesterone is the presence of the 17α-acetoxy and 16-methylene groups in NES. Both groups can establish additional stabilizing contacts within the ligand-binding pocket of PR, the 17α-acetoxy group forming van der Waals contacts with Leu715, Leu718 and Phe794 (Fig.3B) (3.3, 4.4 and 3.2 Å, respectively) and the 16-methylene group contacting Tyr890 (3.4 Å). These observations may well explain the higher potency of NES as compared to progesterone. Docking studies of the 13-ethyl NES further revealed that this molecule adopts the same anchoring positioning in the LBD as NES and that the 13-ethyl group can be accommodated within the binding pocket at the vicinity of Met909 as the 13-methyl group does (data not shown). This result is in good agreement with the observation that NES and 13-ethyl NES display the same potency in activating PR. A striking difference was observed between LNG and norethindrone, since the accommodation of 13-ethyl group of LNG requires a change in the Met909 orientation permitting additional stabilizing contacts which are responsible for the higher potency of LNG as compared to norethindrone (22).

We also used a modeling approach to compare the anchoring mode of NES and LNG within AR. LNG docking within the X-ray crystal structure of the AR LBD complexed with dihydrotestosterone (DHT) (PDB ID: 2AMA) reveals that it adopts the same positioning as DHT and that its C3-ketone function can be hydrogen bonded to the Gln711 and Arg752 and its C17-hydroxyl anchored to Asn705 and Thr877 (data not shown). Docking of NES within the AR LBD showed that it can also adopt the same orientation as DHT with its C3-ketone function hydrogen bonded to the Gln711 and Arg752 (3.5 and 2.9 Å) (Fig. 3C). However, NES is unable to be anchored to Asn705 (Fig. 3C). Furthermore, the 17β-methylketone and 17α-O-acetyl group of NES make strong unfavorable contacts with Leu701 (2.0 Å), Leu704 (2.7 Å), Asn705 (2.4 Å) and Met780 (2.9 Å) (Fig. 3D). Thus, NES is not stabilized within the AR binding pocket leading to an extremely low capacity of this molecule to activate AR.

METABOLITE PROFILING IN MOUSE BRAIN AND PR TRANSACTIVATION

Metabolite profiling of NES in mice

After acute administration of a high dose of NES (200 µg/mouse) in female mice, maximal level of NES was measured at 1h post-administration both in plasma (4.7 ± 1.1 µg/ml - Fig. 4A) and brain (0.62 ± 0.09 µg/g - Fig. 4C) with a marked decline to 4h post-administration. GC/MS/MS analysis revealed that 5α-reduced metabolites of NES such as 5α-DHNES as well as 3α, 5α-THNES and 3β, 5α-THNES were detected both in plasma and brain, but in limited amounts as compared to NES (Fig 4A, C). 5α-DHNES was the most abundant metabolite in brain at 30 min post-administration (80.5 ± 25.5 ng/g Fig. 5C) whereas 3β, 5α-THNES was the major metabolite in plasma at 1h post-administration (116.6 ± 26.7 ng/ml - Fig 5A). Maximal levels of 5α-DHNES and 3α, 5α-THNES were also found in plasma at 1h post-administration (36.3 ± 5.4 and 58.5 ± 17.1 ng/ml, respectively, Fig.5A). 20α-DHNES was not detected.

In the 2nd experiment, a much lower dose of NES (10 µg/mouse) was administered. Higher levels of NES were found at 30 min post-administration both in plasma and brain (39.1 ± 5.0 ng/ml and 24.4 ± 2.5 ng/g - Fig. 4B, D) and 5α-DHNES was the only detectable NES metabolite found at comparable levels relatively to NES in mice brain (11.0 ± 2.2 ng/g - Fig. 4D). High concentrations of 5α-DHNES were shown in brain (Fig 5D) as compared to the very low levels

found in plasma (0.13 – 0.19 ng/ml - Fig. 5B). Both THNES (3α, 5α and 3β, 5α) were the major NES metabolites measured in plasma (1.0 – 1.5 ng/ml - Fig. 5B).

PR transactivation with NES and its metabolites

PR agonist activity of NES, 5α-DHNES, 3α, 5α-THNES was evaluated in the human PR transactivation assay. Based on these results, the putative synthetic NES metabolites possess significantly lower PR agonist activities as compared to NES, the parent molecule. Progesterone the reference PR agonist had an EC_{50} of 650 pM and NES had EC_{50} value of 24 pM whereas the EC_{50} of 5α-DHNES and 3α, 5α-THNES were 410 pM and 2,850 pM respectively. Compared to NES, 5α-DHNES, 3α, 5α-THNES were approximately 17x, and 120x, respectively less potent in activating PR agonist activity. Thus reduction of NES leads to loss of PR activity which may be due to the lack of strong binding to PR of 5α-DHNES and 3α, 5α-THNES in this assay system.

DOES THE NES METABOLITE 3α, 5α-THNES MODULATE $GABA_A$Rs?

The reduced metabolite of progesterone; allopregnanolone (3α, 5α-THPROG) is an established potent positive allosteric modulator of the $GABA_A$R (34 - 36). Indeed, following *in vivo* metabolism of progesterone, this steroid enhances $GABA_A$R function and contributes to the behavioral effects of the parent steroid, progesterone (34). As described above, in the *in vivo* study in mice, Nestorone® (NES) was partially converted into 5α-DHNES, 20α-DHNES, 3β, 5α-THNES and 3α, 5α-THNES. Structure activity studies have revealed that progesterone and 5α-DHPROG have little, or no direct effect on the $GABA_A$R (26, 35). By contrast, reduction of the 3-ketone group of 5α-DHPROG, to a 3α-hydroxy (*i.e*. 3α, 5α-THPROG), confers potent $GABA_A$R activity (26). Therefore, we investigated whether the equivalent metabolite of Nestorone (3α, 5α-THNES), which crucially incorporates the A ring 3-hydroxy in the α configuration, was similarly active at the $GABA_A$R.

Electrophysiological studies on WSS-1 cells

Preliminary experiments revealed the rapid application of GABA (10 nM – 1 µM) to WSS-1 cells expressing α1βγ2 $GABA_A$Rs, (Vh = -60 mV) produced a concentration-dependent inward current. For all subsequent experiments, a GABA concentration of 3 µM, which gave a response ~ 10% of the maximum response to GABA (EC_{10}) was utilised. The co-application of GABA (3 µM) with 3α, 5α-THPROG (100 nM) produced a large increase of the GABA-evoked response (461 ± 69% of control; n = 4; $p < 0.001$ paired Student's t-test) - Figure 6. Under identical conditions, the Nestorone metabolite 3α, 5α-THNES (100 nM) produced only a modest, albeit significant ($p < 0.01$ Student's paired t-test), enhancement of the GABA-evoked response (131 ± 4% of control; n = 10 cells) - see Figure 6. Furthermore, an increased concentration of 3α, 5α-THNES (1µM) did not produce a further increase of the GABA-evoked current and indeed was now statistically ineffective in this respect (129 ± 13 % of control; n = 5 cells; ($p > 0.05$; student's paired t-test) – see Figure 6.

Electrophysiological studies on mouse cortical neurons

The $GABA_A$Rs engineered to be expressed in a cell line may not accurately represent the properties of native neuronal $GABA_A$Rs. We therefore investigated whether 3α, 5α-THNES may influence the function of synaptic $GABA_A$Rs of mouse cortical pyramidal neurons. We have previously demonstrated that the duration of miniature inhibitory postsynaptic currents (mIPSCs) mediated by GABA activation of synaptic $GABA_A$Rs is prolonged by 3α, 5α-THPROG (32). Due to issues associated with poor penetration of the steroid into the mouse brain slice preparation we incubated the slice for 2 hr in 3α, 5α-THNES (100 nM, or 1µM). However, under these conditions, the steroid had no effect at either concentration on the mIPSC decay time – see Figure 6; Table 1. By contrast, equivalent treatment with 3α, 5α-THPROG (100 nM), produced a large prolongation of the mIPSC decay time (Figure 6) see (32). See also Table 2 in supplemental information)

DISCUSSION

Nestorone is a new chemical entity (NCE) used as a progestin component in female and male contraceptive methods (8 - 11). It was shown to exert additional non-contraceptive effects that may lead to health benefits (12 – 16). Previously we showed that NES binds to PR with much greater affinity than P, but does not bind to AR. These *in vitro* results were also confirmed by

bioassays in *in vivo* animal models (3). Our previous results showed the high selectivity of NES in terms of its high progestational activity without any androgenic or estrogenic activity. In this study we show that NES and 13-ethyl NES, two progesterone related structures, with a 13-methyl and 13-ethyl substituent respectively, display the same high potency to activate the human PR. In contrast, LNG, a 19-nortestosterone-related progestin with a 13-ethyl substituent was shown to be much more potent than norethindrone, the corresponding testosterone-related progestin with a 13-methyl substituent (22). Accordingly, NES and 13-ethyl NES are accommodated in the ligand-binding cavity of PR in a similar mode, creating the same network of stabilizing contacts, as revealed by docking studies. In contrast, the binding mode of LNG and norethindrone to the human PR are quite distinct. Thus, the replacement of a methyl group by an ethyl does not modify the activity of progesterone-related progestins, although it increases the activity of testosterone-related progestin structures.

In addition, in this structure activity relationship study, we showed that the ethylene radical in C16 position for both NES and 13-ethyl NES creates an additional anchor increasing the potent progestational action of NES and 13-ethyl NES. In contrast to LNG, NES did not bind to the ligand binding domain of AR and did not transactivate the AR confirming its lack of androgenic activity previously shown in an *in vivo* bioassay (3). The differential action of NES on PR and AR was also confirmed by Attardi et al (37) using other *in vivo* models.

In several earlier studies, neuroregenerative and neuroprotective effects of NES were observed in a stroke model (13, 14) and remyelination effects of NES were observed in a cuprizone demyelinated mice model (14, 15). These findings were shown to be PR related as the effects were not observed in knock-out mice models (14). In our earlier pharmacology studies, we did not investigate if NES converts to reduced metabolites comparable to metabolites formed with progesterone, specifically 3α, 5α-THPROG, a potent neurosteroid (allopregnanolone) with neuroprotective effects (17, 18). Therefore, we assessed whether NES would convert into reduced metabolites in mouse plasma and brain tissue at different time periods following NES subcutaneous administration of low and high NES dose. The low dose (equivalent to a neuroprotective dose in mice) and high dose (pharmacological) of NES was chosen so as to show the potential for NES conversion to dihydro and tetrahydro-NES (equivalent to allopregnanolone formation with progesterone) metabolites using GC/MS/MS method. The high dose was used in these experiments to make sure that the reduced metabolites are detected by

GC/MS/MS with a sufficient sensitivity. The metabolites were identified and quantified by sensitive and specific GC/MS/MS method using synthetic reference standards (28, 29). We demonstrated the presence of reduced NES metabolites in both brain tissue and plasma when NES is injected in mice subcutaneously. However the concentrations of reduced metabolites detected in brain and plasma appeared to be much lower than the concentration of reduced metabolites obtained with progesterone.

The 5α-DHNES was converted to 3α, 5α-THNES in the brain from mice treated with a high dose of NES which is similar to the formation of 3α, 5α-THPROG (allopregnanolone) from 5α–DHPROG, but at a much lower conversion rate. Indeed, the concentration of 3α, 5α-THNES in the brain tissue samples was very low compared to NES and 5α-DHNES levels in brain tissue samples. In mice injected with a low dose of NES, the 3α, 5α-THNES metabolite was undetectable in brain tissue. Only 5α-DHNES was detectable following injection of the low dose of NES. The high concentration of 5α-DHNES found in the brain as compared to the very low levels found in plasma clearly indicate a high 5α-reductase activity in brain.

Dense expression of the 5α-reductase enzyme in the white matter has been well demonstrated (38). An active 5α-reductase activity has also been shown to be present in peripheral myelinated nerves (39). The localization in myelin membranes may suggest a possible involvement of 5α-reduced metabolites of the different steroids in the process of myelination. However, the major metabolite observed with the low dose of 10 µg/animal (therapeutic dose in demyelination and stroke models) was the 5α-dihydro NES which would not be expected to interact with the $GABA_AR$, given the presence of keto but not the hydroxyl function at the critical 3 position of the steroid A ring (26).

We investigated whether these reduced metabolites may have potent activity on the PR transactivation and if they modulate the function of the $GABA_AR$ *i.e*. in common with progesterone could they act as neuroprotective in the brain. We tested the bioactivity of reduced metabolites compared to NES in the PR transactivation assay. NES was very potent in transactivation of PR, followed by 5α-DHNES, and 3α, 5α-THNES. These findings suggest that 5α-DHNES does bind to PR with a binding affinity sufficient to transactivate PR but was almost 17 times less potent than the parent NES in this transactivation. These observations suggested that NES is metabolized in the brain by reductive pathway but the overall metabolite

concentrations may not be enough for $GABA_A$-R receptor activation. This finding leads to our conclusion that most neurotropic activities of NES relate to the parent molecule and not to its metabolites.

While allopregnanolone interacts with the $GABA_AR$ and *via* this interaction is responsible for certain behaviors produced by progesterone such as sedation, the small conversion of NES into 3α, 5α-THNES and its limited interaction with the $GABA_AR$ supports the clinical findings of a lack of such adverse events with NES at contraceptive doses (10). In rat studies, evaluating the behavioral profiles indicative of positive allosteric modulation of the $GABA_AR$ modulatory activity, another progestin of the same class of 19-norprogesterone structure, trimegestone (TMG) was shown to be less active on this likely undesirable endpoint than progesterone and norethindrone acetate, which may translate into fewer mood-related side effects (40). However the authors did not assess the metabolites of TMG in brain tissue in these studies.

In conclusion, we show here for the first time that NES has strong docking and binding interactions with the ligand binding domain of PR receptor but not AR based on the structure activity relationship studies. The binding and transactivation properties of NES in regard to the PR and AR structures confirm the potent selective progestational activity of NES *in vivo*, as well as its lack of androgenic effect.

In addition, we identified the metabolites of NES produced in the brain tissue and plasma from mice treated with NES and demonstrated that the 3α-hydroxy ring A reduced metabolite of NES exhibit little or no enhancement of $GABA_AR$ activity. These results support the conclusion that the beneficial effects of NES on the brain are not related to its metabolite(s) but mainly *via* its direct high affinity binding to PR.

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Figure legends

Figure 1. Structures of Nestorone and its tentative metabolites identified by in vitro metabolism studies. Reference metabolites were custom synthesized and used for metabolite profiling, PR transactivation and $GABA_A$ receptor binding studies

Figure 2. **PR and AR transactivation activity in response to progestins**. HEK-293T cells transiently expressing the human PR (A) or AR (B) were incubated for 16 h with progesterone (Prog), levonorgestrel (Levo), Nestorone (NES), 13 ethyl Nestorone (13NES) or Norethindrone (Noret).The cell extracts were assayed for luciferase and β-galactosidase activities. The PR and AR transactivation activity was determined by the luciferase activity normalized by the β-galactosidase activity. Values are the means ± SEM of three independent experiments performed in triplicate. The GraphPad Prism software was used for curve fitting and calculation of the EC_{50} values.

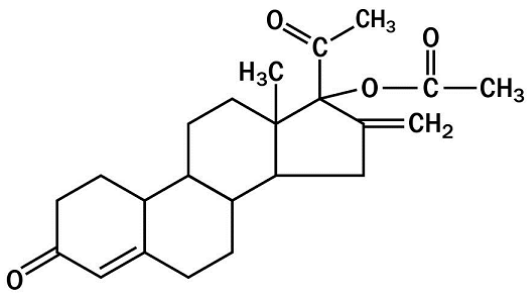
Figure 3. Docking of Nestorone within the PR and AR ligand-binding cavity. The accommodation mode of Nestorone within the PR LBD (PDB ID: 1A28) is shown in 3A and 3B. The α-helices of the PR LBD are depicted as grey ribbons. Nestorone accommodation within the AR LBD (PDB ID: 2AMA) is presented in 3C and 3D. The α-helices of the AR LBD are depicted as blue ribbons. Some residues in the binding pockets are shown with their carbon, oxygen, nitrogen and sulfur atoms colored in gray, red, blue and green respectively. The carbon and oxygen atoms of the ligand Nestorone are colored in gold and red respectively. The figure panels were generated using the Dino package (DINO: Visualizing Structural Biology 2002) (http://www.dino3d.org). The view in the B panel (and D) was obtained by applying 90° rotations (around the x and z axis) on the A view (and C).

Figure 4 – NES metabolism in plasma and brain of female mice after NES administration. NES and its metabolites were measured by GC/MS/MS. NES: nestorone; 5α-DHNES: 5α-dihydronestorone; 20α-DHNES: 20α-dihydronestorone; 3α5α-THNES: 3α5α-tetrahydronestorone; 3β5α-THNES: 3β5α--tetrahydronestorone. Steroid concentrations are measured in plasma after administration of NES at 200 µg (A) and 10 µg (B) and in brain at 200 µg (C) and 10 µg (D) at different post-administration time points. Steroid concentrations are expressed as ng/ml ± SEM or ng/g ± SEM.

Figure 5 – NES metabolism in plasma and brain of female mice after NES administration at 10 and 200µg/mouse. NES metabolites were measured by GC/MS/MS. 5α-DHNES: 5α-

dihydronestorone; 20α-DHNES: 20α-dihydronestorone; 3α5α-THNES: 3α5α-tetrahydronestorone; 3β5α-THNES: 3β5α--tetrahydronestorone. Steroid concentrations are measured in plasma after administration of NES at 200 µg (A) and 10 µg (B) and in brain at 200 µg (C) and 10 µg (D) at different post-administration time points. Steroid concentrations are expressed as ng/ml ± SEM or ng/g ± SEM.

Figure 6: *The nestorone metabolite 3α5α-THNES has little effect on $GABA_AR$s.* **A)** Illustrated are the GABA (3 µM)-induced inward currents recorded from a representative WSS-1 cell in the absence (black trace) and presence (grey trace) of the neurosteroid 3α5α-THPROG (100 nM). Note the large enhancement of the GABA-evoked response produced by this neurosteroid. **B)** The nestorone metabolite 3α5α-THNES (100 nM) produced only a modest increase in the GABA (3µM) – evoked current. **C)** A bar chart summarizing the effect of 3α5α-THPROG (100 nM; n = 4) and 3α5α-THNES (100 nM, n = 10, 1 µM, n = 5) on the peak amplitude of the GABA-evoked response. *** = $p < 0.001$; ** = $p < 0.01$ (Student's t-test). **D, E).** The black traces illustrate averaged mIPSCs recorded from representative mouse cortical pyramidal neurons under control conditions. Superimposed upon these control recordings are representative mIPSCs recorded from cortical neurons obtained from brain slices incubated for ~ 2 hr. in **D)** 3α5α-THPROG (100 nM) and **E)** 3α5α-THNES (100 nM) – grey traces. **F)** A bar chart summarizing the effect of 3α5α-THPROG (100 nM; n = 5 neurons) and 3α5α-THNES (100 nM, n = 4 neurons 1 µM, n = 4 neurons) on the control (n = 25 neurons) mIPSC decay time (quantified as the τw the weighted time constant of decay [ms]). # # # $p < 0.001$ (one way ANOVA). Note the large prolongation produced by 3α5α-THPROG (100 nM), whereas 3α5α-THNES (100 nM) was inert in this respect. The data for the control τw and the τw in the presence of 3α5α-THPROG (100 nM) is reproduced from Brown *et. al*., 2016.



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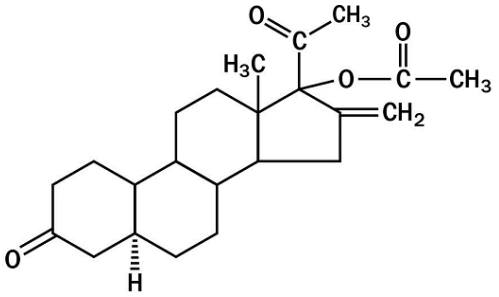

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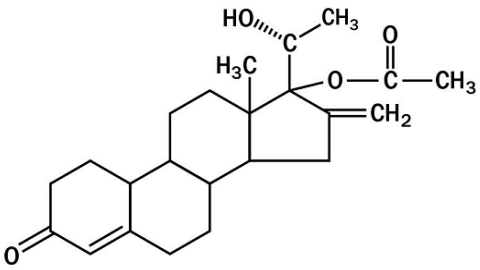

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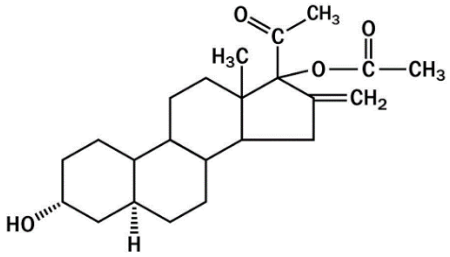
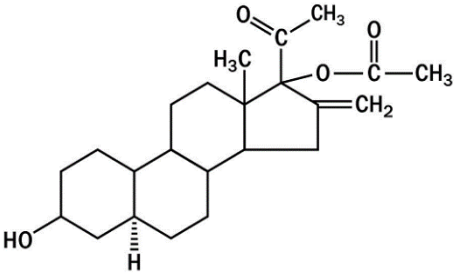


3α, 5α-THNES



3β, 5α-THNES

Figure 2

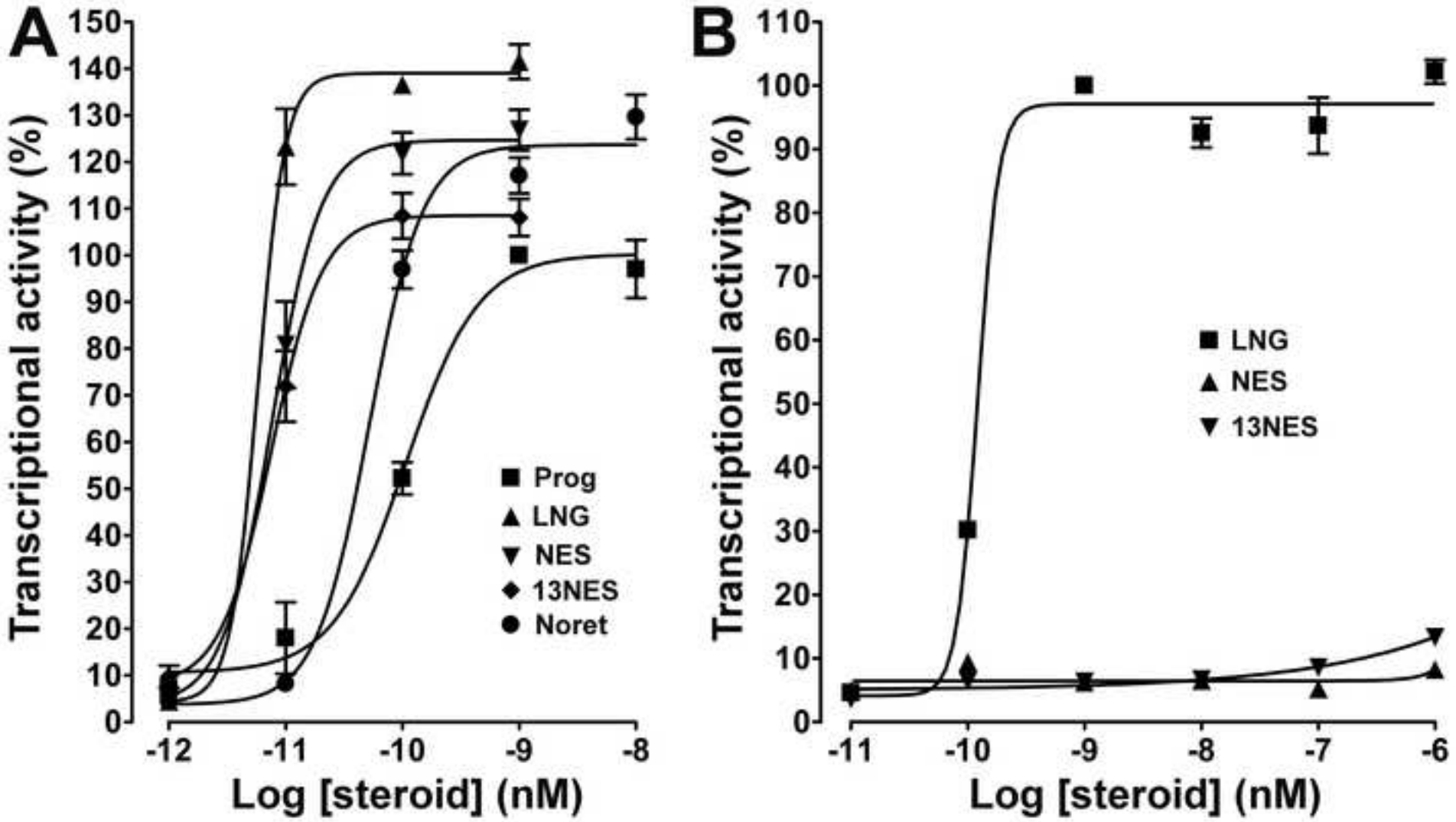


Figure 3

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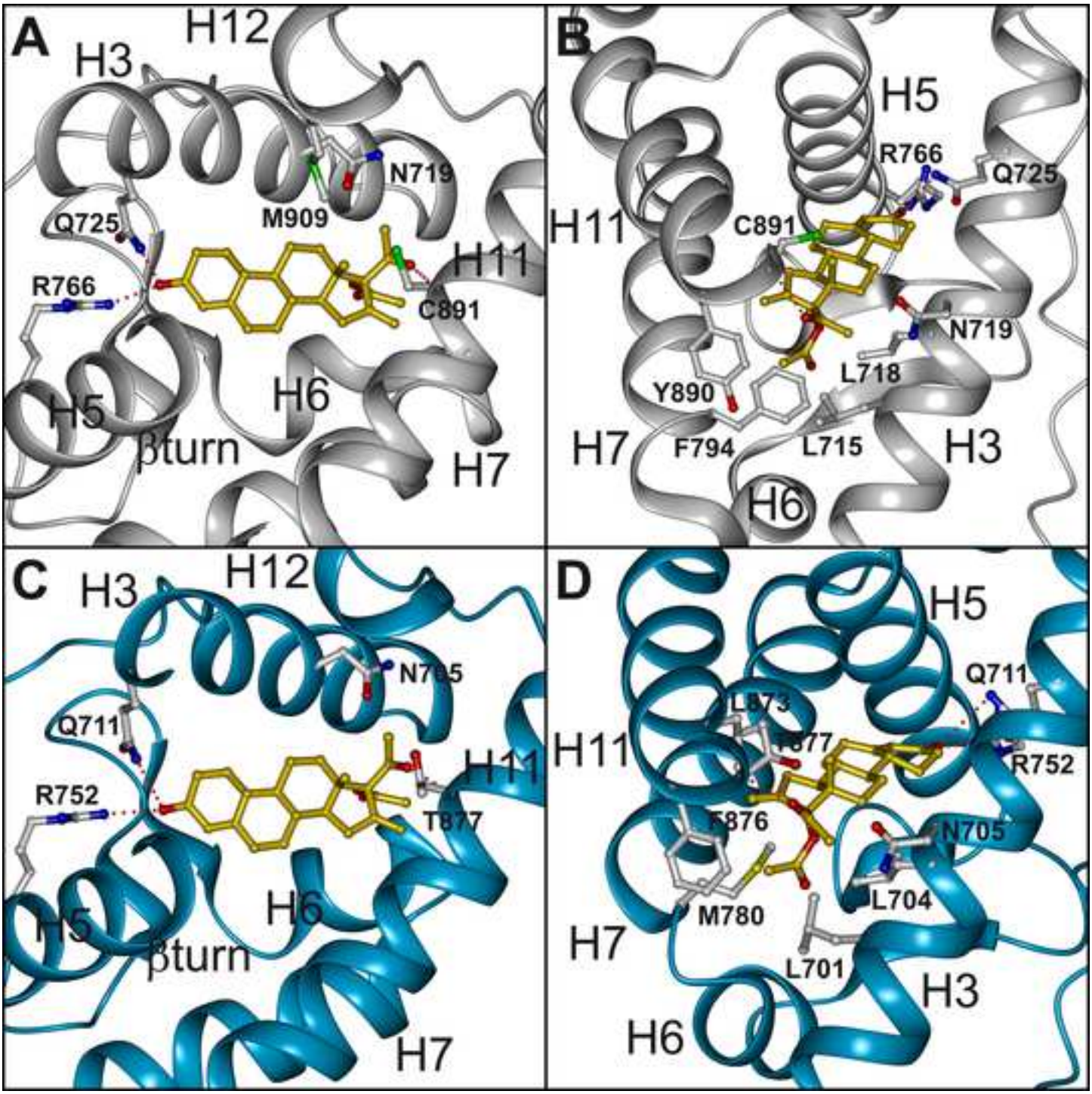

Figure 4

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figure 4

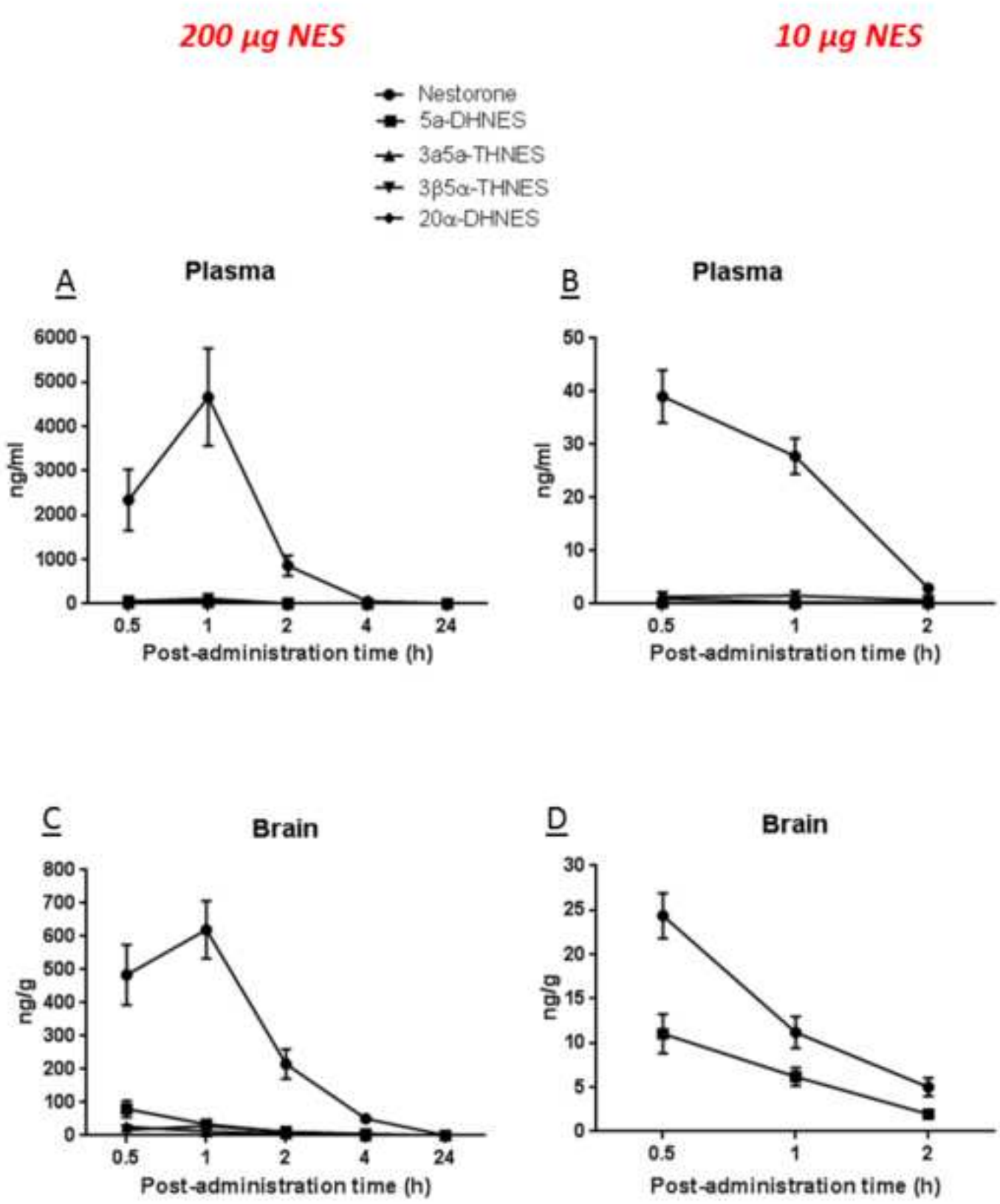

Figure 5

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figure 5

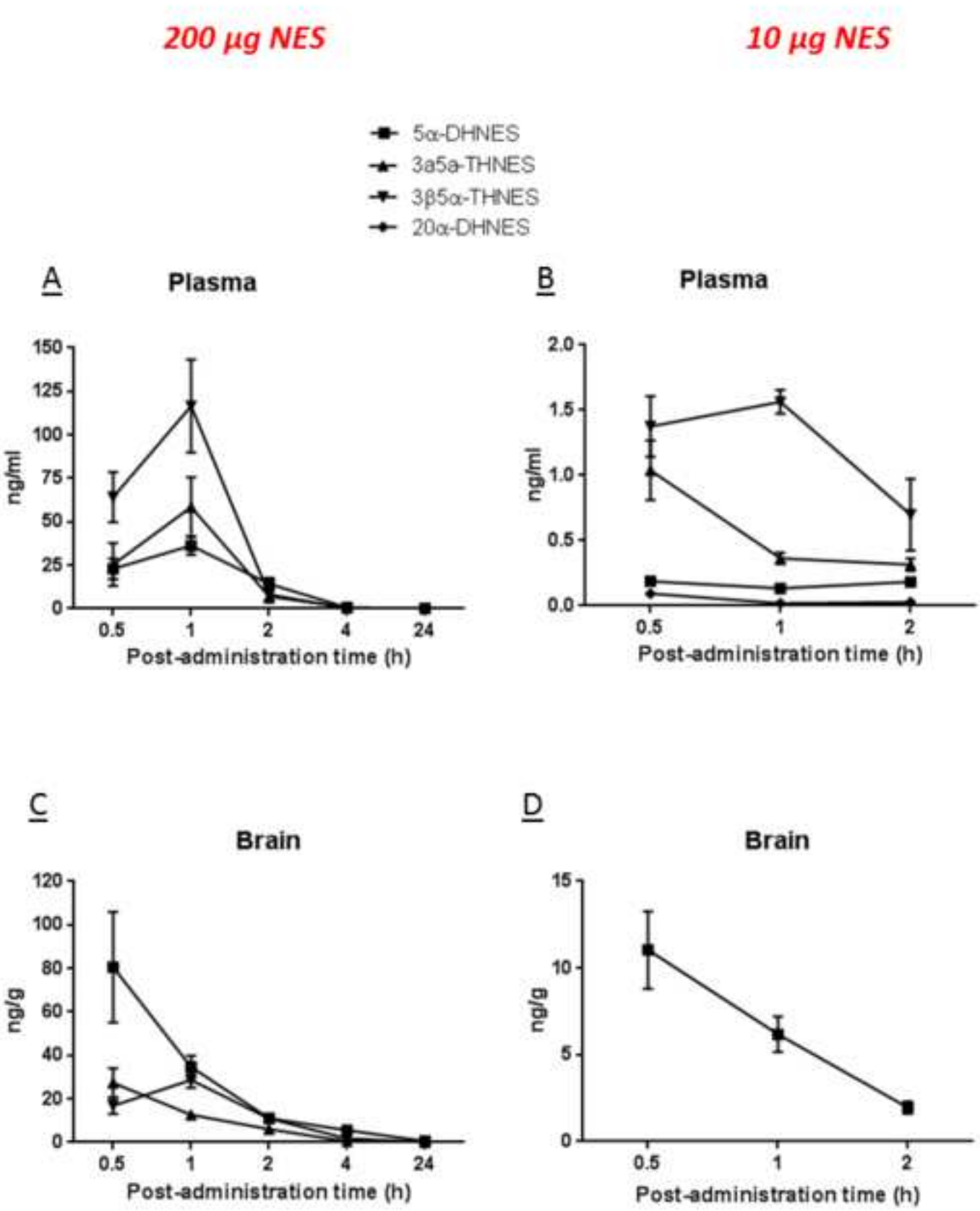
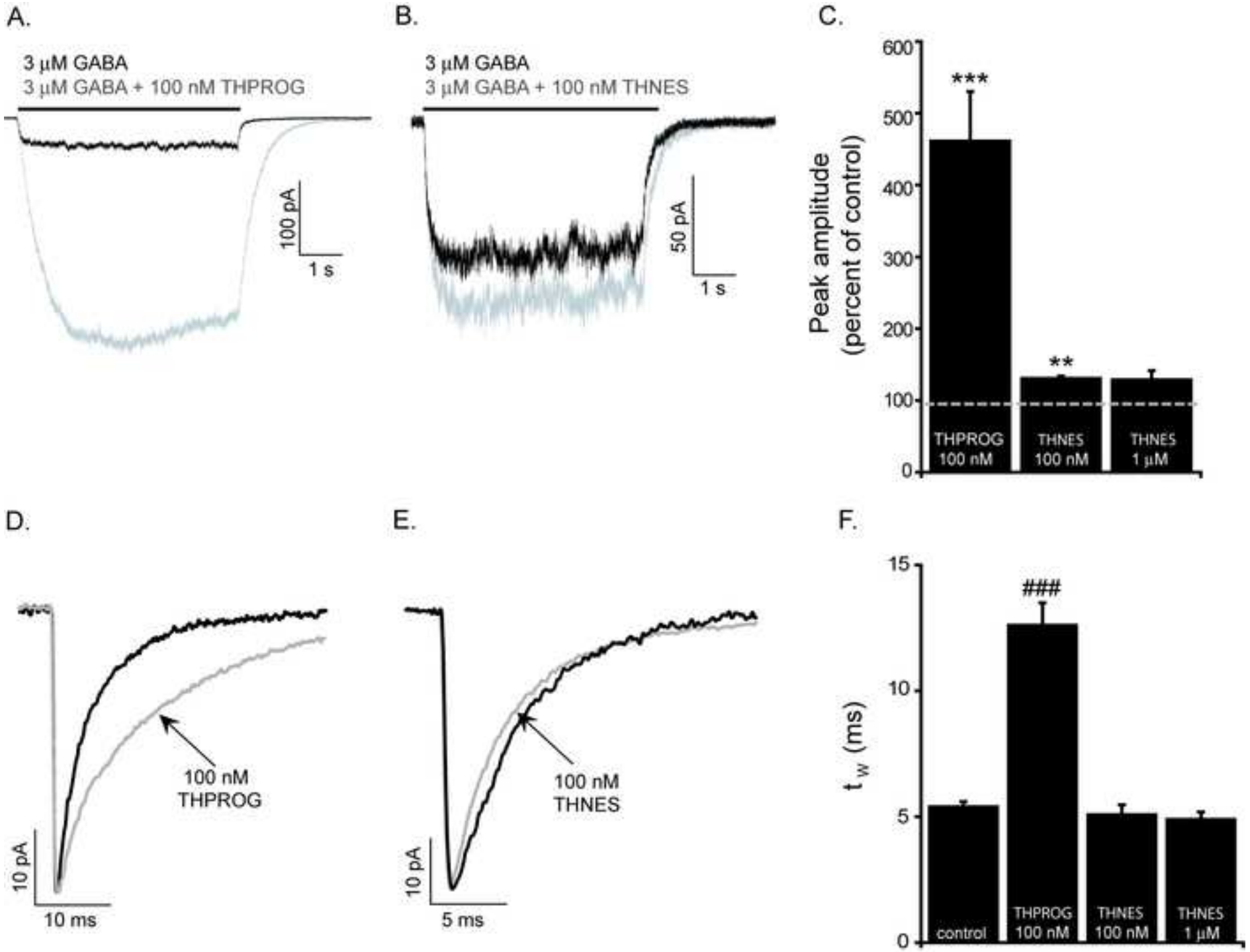


Figure 6



Supplementary table 1

Supplementary table 2