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The Pheromone Androstenol (5α-Androst-16-en-3α-ol) Is A Neurosteroid Positive Modulator of $GABA_A$ Receptors

Rafal M. Kaminski, Herbert Marini[1], Pavel I. Ortinski, Stefano Vicini, and Michael A. Rogawski

Epilepsy Research Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892-3702 (R.M.K, H.M., M.A.R.); and Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC 20057-1460 (P.I.O., S.V.)

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Corresponding Author:

Michael A. Rogawski, M.D., Ph.D.

Epilepsy Research Section

Porter Neuroscience Research Center

NINDS, NIH

Building 35, Room 1C-1002

35 Convent Drive MSC 3702

Bethesda, MD 20892-3702

Telephone: 301-496-8013

Fax: 775-249-7715

E-mail: michael.rogawski@nih.gov

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Abstract

Androstenol is a steroidal compound belonging to the group of odorous 16-androstenes, first isolated from boar testes and also found in humans. Androstenol has pheromone-like properties in both animals and humans, but the molecular targets of its pheromonal activity are unknown. Androstenol is structurally similar to endogenous A-ring reduced neurosteroids that act as positive modulators of $GABA_A$ receptors. Here we show that androstenol has neurosteroid-like activity as a $GABA_A$ receptor modulator. In whole-cell recordings from cerebellar granule cells, androstenol (but not its 3β-epimer) caused a concentration-dependent enhancement of GABA-activated currents (EC_{50}, 0.4 μM in cultures; 1.4 μM in slices), and prolonged the duration of spontaneous and miniature inhibitory postsynaptic currents. Androstenol (0.1–1 μM) also potentiated the amplitude of GABA-activated currents in HEK 293 cells transfected with recombinant α1β2γ2 and α2β2γ2 $GABA_A$ receptors, and at high concentrations (10–300 μM) directly activated currents in these cells. Systemic administration of androstenol (30–50 mg/kg) caused anxiolytic-like effects in mice in the open-field test and elevated zero-maze, and antidepressant-like effects in the forced swim test (5–10 mg/kg). Androstenol but not its 3β-epimer conferred seizure protection in the 6 Hz electroshock and pentylenetetrazol models (ED_{50} values, 21.9 and 48.9 mg/kg, respectively). The various actions of androstenol in the whole animal models are consistent with its activity as a $GABA_A$ receptor modulator. $GABA_A$ receptors could represent a target for androstenol as a pheromone, for which it is well suited because of high volatility and lipophilicity, or as a conventional hormonal neurosteroid.

Introduction

The C_{19} 16-unsaturated steroid androstenol (5α-androst-16-en-3α-ol; Fig. 1) is a musk-smelling substance first isolated from boar testes (Prelog and Ruzicka, 1944) that is well recognized to act as a pheromone in pigs and possibly other species (Melrose et al., 1971; Gower and Ruparelia, 1993; Grammer et al., 2005). Boars secrete the volatile steroid during sexual activity, which evokes a mating response in the estrus sow. The alteration in sexual receptivity induced by androstenol is believed to be mediated by absorption of the steroid through the nasal mucosa (Stefanczyk-Krzymowska et al., 2000), but the site and mechanism of action is unknown.

Substantial amounts of androstenol are present in human urine (Brooksbank and Haslewood, 1961) and the steroid has also been found in pig and human plasma and saliva, and human axillary sweat (Brooksbank et al., 1974; Bicknell and Gower, 1976; Smals and Weusten, 1991). The best studied site of androstenol synthesis is the testis (Gower and Haslewood, 1961; Hurden et al., 1984; Smals and Weusten, 1991). The crucial synthetic reaction is the conversion of pregnenolone (Brooksbank and Wilson, 1970; Hurden et al., 1984) to androstadienol by the recently described 16-ene-synthetase activity of cytochrome P450c17 (steroid 17α-monooxygenase) (Soucy et al., 2003). Androstadienol can then be sequentially converted to androstenol by 3β-hydroxysteroid dehydrogenase, 5α-reductase and 3α-hydroxysteroid dehydrogenase (Dufort et al., 2001), a series of reduction and oxidation steps analogous to the metabolic pathway for androgens in which dehydroepiandosterone is converted to testosterone and then to androstanediol. In humans, androstenol may be synthesized in the adrenal gland and ovary rather than the testis (Smals and Weusten, 1991), under the control of adrenocorticotrophic hormone and gonadotropins (Cleveland and Savard, 1964; Gower and Stern, 1969).

Several studies have indicated that androstenol is able to modulate behavioral and social responses in humans (Cowley and Brooksbank, 1991; Gower and Ruparelia, 1993; Grammer et al.,

2005), presumably reflecting effects of the steroid on the nervous system. In examining the structure of androstenol, we recognized a similarity to the C_{19} 5α,3α-reduced testosterone metabolites androstanediol (5α-androstan-3α-ol-17β-ol) and androsterone (5α-androstan-3α-ol-17-one) (Fig. 1). Androstandiol is a positive modulator of $GABA_A$ receptors (Frye et al., 1996) with potent anxiolytic and anticonvulsant activity (Frye and Reed, 1998; Reddy, 2004ab); recently, we confirmed that androsterone has similar systemic activities (Kaminski et al., 2005). A structural homology also exists between androstenol and the C_{21} 5α,3α-reduced progesterone and deoxycorticosterone metabolites allopregnanolone (5α-pregnan-3α-ol-20-one) and allotetrahydrodeoxycorticosterone (5α-pregnane-3α,21-diol-20-one). These latter compounds are prototypic neurosteroids, which also act as positive modulators of $GABA_A$ receptors (Puia et al., 1990) and have diverse behavioral properties characteristic of such GABA modulators, including anticonvulsant (Rogawski and Reddy, 2004) and anxiolytic (Rodgers and Johnson, 1998; Bitran et al., 1991; Finn et al., 2003) actions. Androstenol has previously been shown to inhibit [^{35}S]*t*-butylbicyclophosphorothionate binding to rat cortical membranes, indicating that it interacts with $GABA_A$ receptors (Bolger et al., 1996).

In the present study, we sought to demonstrate that androstenol, like the aforementioned 5α,3α-hydroxy A-ring reduced congeners, has activity as a positive modulator of $GABA_A$ receptors. In addition, we wished to determine if the steroid has central nervous system (CNS) actions typical of $GABA_A$ receptor modulators when administered systemically, providing support for the hypothesis that endogenously produced androstenol or environmental exposure to the steroid could influence brain function.

Materials and Methods

In vitro studies

Cell culture and tissue preparation. Primary cultures of mouse cerebellar granule cells were prepared from postnatal day 5–7 mice uncontrolled for sex. Mouse pups were sacrificed by decapitation in accordance with the guidelines of the Georgetown University Animal Care and Use Committee. The cerebella were dissociated with trypsin (0.25 mg/ml; Sigma, St. Louis, MO) and plated in 35 mm Nunc dishes at a density of 1.1×10^6 cells/ml on 12 mm glass coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-L-lysine (10 μg/ml; Sigma). For the first 5 days, the cells were cultured in high (25 mM) K^+ basal Eagle's medium supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamycin (all from Invitrogen, Carlsbad, CA), and maintained at 37 ºC in 5% CO_2. At culture day 5, the medium was replaced with minimal essential medium (MEM; containing 5 mM K^+) supplemented with 5 mg/ml glucose, 0.1 mg/ml transferrin, 0.025 mg/ml insulin, 2 mM glutamine, 20 μg/ml gentamicin (Invitrogen) and 10 μM cytosine arabinofuranoside (Sigma) as previously described (Chen et al., 2000; Losi et al., 2002). The cultures were generally used for recording on the 7th day in vitro (DIV).

Human embryonic kidney 293 (HEK 293) cells (American Type Culture Collection, Rockville, MD; CRL1573) were grown in MEM, supplemented with 10% fetal bovine serum, 100 units /ml penicillin, and 100 units/ml streptomycin (all from Invitrogen) in a 5% CO_2 incubator. Growing cells were dispersed with trypsin and seeded at approximately 2×10^5 cells/35-mm dish in 2 ml of culture medium on 12 mm glass coverslips coated with poly-L-lysine. The cells were transfected with mouse $GABA_A$ receptor subunit cDNAs (kind gifts of Dr. Peter H Seeburg, Max-Planck-Institute for Medical Research, Heidelberg, Germany and Dr. Hartmut Lüddens, University of Mainz) and green fluorescent protein (GFP) using calcium phosphate precipitation. Mixed plasmids

(5 μg total) were added to the dish containing 2 ml culture medium for 8–12 h. The cells were used for electrophysiological recording 2–3 days after transfection.

Sagittal cerebellar slices (200 μm thick) were prepared from postnatal day 11–12 hybrid mice of mixed genetic background (C57BL/6J × 129/Sv/SvJ). Slices were cut with a Vibratome (Technical Products International, St. Louis, MO). The ice cold slicing solution contained (in mM): 85 NaCl, 2.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose and was brought to pH 7.4 by continuous bubbling with carbogen gas (95% O_2 and 5% CO_2).

Recording of $GABA_A$ receptor currents. Cultured granule cells and HEK 293 cells transfected with $GABA_A$ receptors subunits (on 12 mm glass coverslips), and mouse cerebellar slices were placed on the stage of an Nikon E600FN upright microscope equipped with Nomarski optics and an electrically insulated 60× water immersion objective. Cultured granule cells and HEK 293 cells were washed with phosphate buffered saline and then continuously perfused with extracellular solution containing (in mM, except as noted): 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 5 glucose, 15 sucrose, 0.25 mg/l phenol red and 0.01 D-serine adjusted to pH 7.4 with NaOH. For recordings of miniature IPSCs, 0.5 μM tetrodotoxin (Sigma) was added to the extracellular solution. Transfected HEK 293 cells were identified by the fluorescent label. The extracellular solution for cerebellar slices contained (in mM): 120 NaCl, 3.1 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4, 25 $NaHCO_3$, 2.5 glucose, 30 sucrose (pH 7.4 when continuously bubbled with carbogen gas). All recordings were performed at room temperature (24–26 °C). Electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) and filled with recording solution containing (in mM): 145 KCl, 10 HEPES, 5 ATP.Mg, 0.2 GTP.Na, and 10 BAPTA, adjusted to pH 7.2 with KOH. Pipette resistance was 5–8 MΩ. Whole cell voltage-clamp recordings were made at –60 mV with an Axopatch-1D amplifier (Axon Instruments, Union City, CA). Access resistance was

monitored throughout the recordings. Cell capacitance was determined from the transient current response to a 10 mV hyperpolarizing voltage step. Currents were filtered at 1 kHz with an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA), digitized at 5–10 kHz using an IBM-compatible microcomputer equipped with a Digidata 1322A data acquisition board and pCLAMP 9 software (both from Axon Instruments). Off-line data analysis and figure preparation were performed as previously described (Ortinski et al., 2004). In the analysis of sIPSCs and mIPSCs, infrequent AMPA receptor-mediated excitatory postsynaptic currents were easily identified by their rapid decay (<2 ms) and were excluded from the analysis. The decay phase of sIPSCs and mIPSCs was fitted by the least-squares method using a simplex algorithm according to the triple exponential equation $I(t) = I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) + I_3 \exp(-t/\tau_3)$, where $I_{k=1..3}$ is a peak amplitude of a decay component and $\tau_{k=1..3}$ is the corresponding decay time constant. To allow for easier comparison of decay kinetics between experimental conditions, weighted time constants are reported that were calculated from the individual component time constant values according to the formula $\tau_w = \tau_1 [I_1/(I_1 + I_2 + I_3)] + \tau_2 [I_2/(I_1 + I_2 + I_3)] + \tau_3 [I_3/(I_1 + I_2 + I_3)]$. For display, 30 or more traces were averaged.

Test substance application in $GABA_A$ receptor current recordings. GABA (Sigma) was dissolved in water and androstenol (Sigma), its 3β-epimer 5α-androst-16-en-3β-ol (Steraloids, Newport, RI) and allopregnanolone (Sigma) in hydroxypropyl-β-cyclodextrin (Trappsol; CTD, High Springs, FL). Stock solutions were diluted in the extracellular medium to the concentration desired. The concentration of hydroxypropyl-β-cyclodextrin in the extracellular medium never exceeded 0.04%. To investigate the potentiation of whole-cell GABA responses in cerebellar granule cell cultures, slices, and transfected HEK 293 cells, a fixed concentration of GABA was coapplied locally with varying concentrations of androstenol by means of a Y-tube (Murase et al., 1989). The steroid perfusion was begun 15–20 s before the GABA application. The GABA concentration differed among the various

experimental preparations and was taken to be the EC_{20} (concentration eliciting 20% of maximal current amplitude). Under our experimental conditions, the EC_{20} value for cerebellar granule cell cultures (DIV 7) and cerebellar slices (P11–12) was 0.25 μM, and varied from 1–3 μM for α1β2γ2 and α2β2γ2 transfected HEK cells. In experiments examining the direct effect of androstenol where high (1–300 μM) concentrations were used, responses to 5 μM GABA (EC_{30} concentration; Huang et al., 2004) were determined in several α1β2γ2-transfected cells in each culture before the androstenol applications. The average GABA current amplitude obtained in these naive cells was then used to normalize whole-cell responses evoked by application androstenol. Since high concentrations of androstenol tended to accumulate in the cultures, this procedure allowed a reliable baseline GABA response to be determined.

In vivo studies

Animals. Male NIH Swiss mice (25–30 g) and Sprague-Dawley rats (120–180 g) (Taconic Farms, Germantown, NY) were housed two per cage. Animals were kept in a vivarium under controlled laboratory conditions (temperature 22–26 °C; humidity 40–50%) with an artificial 12 h light/dark cycle and free access to food and water. Animals were allowed to acclimate to the vivarium for at least 5 days. The experimental groups consisted of 6–8 animals. The experiments were performed during the light phase of the light/dark cycle (between 9:30 and 15:30 h) after at least a 30 min period of acclimation to the experimental room. Animals were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and experiments were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke (NINDS) in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington. D.C.; http://www.nap.edu/readingroom/books/labrats/).

Test substance and convulsant administration. Solutions of androstenol and its 3β-epimer were made fresh daily in 40% hydroxypropyl-β-cyclodextrin in sterile 0.9% saline. Further dilutions were made using sterile saline. The steroids were injected intraperitoneally (i.p.); control animals received injections of 40% hydroxypropyl-β-cyclodextrin. The convulsant agent pentylenetetrazol (PTZ; Sigma-Aldrich) was dissolved in saline immediately before use. All drug solutions were administered in a volume equaling 0.01 ml/g of the animal's body weight.

Open-field test and spontaneous locomotor activity. Spontaneous exploratory activity was assessed with a VersaMax automated locomotor activity monitoring systems (Accuscan Instruments, Columbus, OH). The test area was a square arena (40 × 40 × 30 cm) with clear Plexiglas walls and floor, evenly illuminated by overhead fluorescent white room lighting. Sixteen sets of infrared beams and photocell detectors spaced 2.5 cm apart were arrayed on each side of the arena 2.2 cm above the floor, at right angles to one another, forming a grid of 256 equally sized squares. To detect vertical movement, a third set of 16 photocell beams was located above the square grid capable of sensing movement at a height of up to 8.4 cm off the floor. Mice were injected with the test compound and placed in the center of the open field and allowed to freely explore for 60 min. The number of horizontal and vertical beam breaks was taken as a measure of horizontal and vertical activity. Time spent in the central square of the open field (2.5 cm away from the walls) was recorded by the VersaMax system as center time. Data were collected in 10-min epochs during the 60 min observation time. Center time is expressed as a percentage of total time of the epoch. Stereotypy counts represent the number of beam breaks detected by one sensor when the same beam was broken 3 or more times in succession without another beam being broken. The test chamber was cleaned with 70% ethanol solution after each subject to prevent subsequent test subjects from being

influenced by deposited odors. For each behavioral measure the values in 10 min epochs were analyzed with a two-way ANOVA with repeated measures. Specific comparisons between the treatments were made by a post-hoc multiple comparisons procedure (Bonferroni's *t*-test). The area-under-the-curve for the total time spent in the center of the arena was determined by the trapezoidal method and the values for various androstenol doses were compared to vehicle by Student's *t*-test.

Horizontal screen test. Androstenol was evaluated for motor toxicity by using a modification of the horizontal screen test as previously described (Kokate et al., 1994). Mice were placed on a horizontally oriented grid (consisting of parallel 1.5-mm diameter rods situated 1 cm apart), and the grid was inverted. Animals that fell from the grid within 10 s were scored as impaired. The dose producing impairment in 50% of mice (TD_{50}) and the 95% confidence limits were estimated by log-probit analysis using the Litchfield and Wilcoxon method (PHARM/PCS Version 4.2, MicroComputer Specialists, Philadelphia, PA).

Elevated zero-maze. Testing was performed as originally described by Shepherd et al. (1994). The elevated zero-maze test is a behavioral test of anxiety based on the naturalistic tendency of rodents to avoid open and elevated areas. It is similar to the more widely used elevated plus maze, except that the open and closed arms are arranged circularly, thus eliminating the center area which removes ambiguity in interpretation of time spent in the central square of the traditional design. The elevated zero-maze (Hamilton-Kinder, Poway, CA) was positioned in the center of a room under dim lighting. Each mouse was individually removed from its home cage and placed just inside a closed arm. Five-min test sessions were video recorded with a tripod-mounted camcorder. Several anxiety related behaviors were subsequently scored from the taped records using Observer 3.0 software (Noldus, Wageningen, The Netherlands). The measures included: (a) latency to first open area entry, (b) number of open area entries, and (c) percent of time spent in open areas. An animal was considered

to have entered the open arm if all four paws had left the closed arm. Open-arm time was considered terminated once a single paw was placed back into the closed arm.

Forced swim test. This test was performed as originally described by Porsolt et al. (1977). Briefly, mice were pretreated with either androstenol or vehicle and forced to swim in a glass cylinder (21 × 12 cm) containing fresh water (25 ± 2 °C) at a depth of 9 cm for a period of 6 min. The last 4 min of the test session was video recorded and scored for immobility time by two observers blind to the treatment. Mice were judged to be immobile if they ceased struggling and remained motionless making only those movements necessary to keep their head above water. Reduction in the duration of immobility by a drug was considered an antidepressant-like effect. Mean immobility time values for androstenol-treated animals were compared to vehicle values by Student's *t*-test.

Pentylenetetrazol (PTZ) seizure test. Testing was carried out as previously described (Kokate et al., 1994). In brief, mice were injected subcutaneously with PTZ (80 mg/kg) and were observed for a 30-min period. Mice failing to show clonic seizures lasting longer than 5 s were scored as protected. To construct dose-response curves, steroids were tested at several doses spanning the dose producing 50% protection (ED_{50}), or if no protection was obtained at a dose of 100 mg/kg. At least 6–8 mice were tested at each dose. ED_{50} values and corresponding 95% confidence limit were determined by log-probit analysis as for the horizontal screen test.

6 Hz Seizure Test. Testing was carried out as previously described (Kaminski et al., 2004). In brief, 3-s corneal stimulation (200 μs-duration, 32-mA monopolar rectangular pulses at 6 Hz) was delivered by a constant current device (ECT Unit 5780, Ugo Basile, Comerio, Italy). Ocular anesthetic (0.5% tetracaine) was applied to the corneas 15 min before stimulation. Immediately

before stimulation, the corneal electrodes were wetted with saline to provide good electrical contact. Following the stimulation, the animals exhibited a “stunned” posture associated with rearing and automatic movements that lasted from 60 to 120 s in untreated animals. Animals resumed their normal exploratory behavior after the seizure. The experimental endpoint was protection against the seizure: an animal was considered to be protected if it resumed its normal exploratory behavior within 10 s of stimulation. Statistical comparisons were as for the PTZ test.

Physiochemical properties of steroids

Vapor pressure values were estimated using the Clausius-Clapeyron equation from boiling point and enthalpy of vaporization values calculated using Advanced Chemistry Development (ACD/Labs, Toronto, ON) software version 4.67. Octanol-water partition coefficient values (log *P*) were also calculated using the ACD/Labs software.

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Results

Androstenol potentiates GABA responses in cerebellar granule cells

To investigate the physiological actions of androstenol on $GABA_A$ receptor responses, we first characterized the effects of the steroid on currents induced by exogenously applied GABA in mouse cultured cerebellar granule cells. Application of 0.25 μM GABA for ~2–5 s evoked brief inward current responses that were enhanced in a concentration-dependent fashion by increasing concentrations of androstenol, preapplied for 15– 20 s and then coapplied during the GABA application (Fig. 2 and 3A). The effect of androstenol was fully reversible, but required long wash times at higher concentrations (800 s following 1000 nM androstenol; Fig. 2). At a concentration of 100 nM, the mean percent of control value for androstenol was 109.9 ± 12.7 and the mean value in a separate series of experiments for allopregnanolone was 150.1 ± 44.6 (*n*=7). Androstenol produced a similar concentration-dependent enhancement of GABA-evoked currents in recordings from granule cells in cerebellar slices obtained from 11–12 day-old mice (Fig. 3B). To determine if the potentiating effect is structurally specific, we compared the response to androstenol with that of its 3β-epimer in cultured cerebellar granule cells. As shown in Fig. 4, only the natural 3α-isomer was active at concentrations of 1000 and 3000 nM.

Androstenol potentiates GABA currents in HEK 293 cells transfected with α1β2γ2or α2β2γ2$GABA_A$ receptor subunit combinations

We next assessed the ability of androstenol to potentiate currents generated by recombinant $GABA_A$ receptors. HEK 293 cells were transfected with α1β2γ2 or α2β2γ2 $GABA_A$ receptor subunit combinations (along with GFP as a marker to identify transfected cells). These two subunit combinations are representative of the most abundant $GABA_A$ receptor subunit combinations in brain (McKernan and Whiting, 1996). Androstenol caused a concentration-dependent increase in peak

current responses evoked by 1 and 3 μM GABA, and at high concentrations seemed to enhance desensitization (Fig. 4). α2β2γ2 transfected cells showed slightly smaller responses than cells transfected with α1β2γ2 but this effect did not reach statistical significance. Overall, the magnitude of the androstenol potentiation of recombinant GABA receptors was comparable to that obtained with native receptors in cultured granule cells, even though lower GABA concentrations were used in the experiments with native cells (Fig. 3).

Direct activation of inward current in HEK 293 cells transfected with α1β2γ2 $GABA_A$ receptor subunits

Perfusion of HEK 293 cells expressing α1β2γ2 $GABA_A$ receptor subunits with 1 to 300 μM androstenol (in the absence of GABA) resulted in activation of long-lasting inward current responses (Fig. 6). Because of the concern that these high concentrations of androstenol would modify the response to subsequent GABA applications, the responsiveness to 5 μM GABA was assayed in 2 to 3 cells in the culture dish prior to application of androstenol. The androstenol response amplitudes were normalized to the mean amplitude of the GABA response in the corresponding dish. As shown in the graph of Fig. 6, increasing concentrations of androstenol produced a concentration-dependent increase in the peak current elicited. The maximal amplitudes of direct androstenol responses were approximately 30% of the 5 μM GABA response amplitude obtained from a sample of cells in the same cultures, indicating that androstenol is less efficacious in activating $GABA_A$ receptors than is GABA (Fig. 6).

Androstenol prolongs spontaneous and miniature IPSCs

Spontaneous IPSCs were recorded from DIV 7 cultured cerebellar granule cells. As shown in Fig. 7, application of 100 nM androstenol caused a prolongation of the decay of the IPSCs as

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reflected by the increase in τ_w. There was only a small increase in the IPSC amplitude that did not reach statistical significance. Miniature IPSCs (mIPSCs) were recorded from cultured cerebellar granule cells as in the experiments studying spontaneous IPSCs except that 0.5 μM tetrodotoxin was included in the bathing solution. As for spontaneous IPSCs, androstenol did not influence the mIPSC peak amplitude but did cause a concentration-dependent increase in mIPSC duration (Fig. 8).

Effects of androstenol on spontaneous motor behavior

The effects of androstenol were examined on several measures of spontaneous motor behavior, including spontaneous locomotor activity (horizontal behavior); repetitive head, limb or body movements (stereotyped behavior); and open field activity, which is considered a measure of anxiety level. Fig. 9 summarizes the results of experiments comparing a single 10, 30, 50 or 100 mg/kg injection of androstenol with vehicle. As shown in the upper panels of Fig. 9, animals exhibited a reduction in horizontal and stereotyped behaviors over the 60 min observation period, reflecting normal habituation. Two-way repeated measures ANOVA demonstrated a significant effect of post-treatment time on these two parameters (respectively, $F_{5,191}$= 96.8; $p < 0.001$ and $F_{5,191}$ = 100.9; $p < 0.001$). Androstenol at doses of 10 to 50 mg/kg did not affect either of these measures of activity. Thus, for horizontal activity, the treatment effect ($F_{3,191} = 0.36$ $p > 0.05$) and treatment vs. time interaction ($F_{15,191} = 1.4$; $p > 0.05$) were not statistically significant. However, when the data from the 100 mg/kg dose was included in the analysis, the treatment effect ($F_{4,221} = 5.87$) and treatment vs. time interaction ($F_{20,221} = 5.26$) were significant ($p < 0.001$), indicating that at the highest dose there is an effect on horizontal activity. For stereotyped behavior the treatment effect ($F_{3,191} = 0.527$; $p > 0.05$) and treatment vs. time interaction ($F_{15,191} = 1.2$; $p > 0.05$) for doses of 10 to 50 mg/kg were not statistically significant. Including the data from the 100 mg/kg dose in the analysis did not alter the outcome of the statistical analysis (treatment effect: $F_{4,221} = 1.49$; treatment

vs. time interaction: $F_{20,221} = 1.12$; $p > 0.05$). Thus, at doses of 50 mg/kg and below, androstenol did not affect these spontaneous motor behaviors and their habituation. However, at the 100 mg/kg dose, there was an effect on horizontal activity but not sterotypy.

In contrast to the lack of effect of androstenol on gross motor activity at doses of 50 mg/kg and below, the steroid at these doses did increase in a dose-dependent fashion the fraction of time spent in the center of the arena (Fig. 9, lower-left panel), indicating an anxiolytic-like action. Two-way repeated measures ANOVA revealed a significant effect of time spent in center of the arena for both treatment ($F_{3,191} = 3.2$; $p < 0.05$) and time ($F_{5,191} = 8.2$; $p < 0.001$), but no treatment vs. time effect ($F_{15,191} = 0.8$; $p > 0.05$). We assume that the significant time effect is due to the rise and fall in plasma levels of the steroid during the 60 min observation period. The various steroid doses were compared by determining the mean area-under-the-curve values for percent time in center at each dose. As shown in the lower-right panel of Fig. 9, androstenol doses of 30 and 50 mg/kg were associated with significant increases in the mean area-under-the-curve values. Because the 100 mg/kg dose of androstenol was associated with an overall reduction in motor behavior, percent of time in center values are not meaningful and are therefore not presented.

Effects of androstenol in the horizontal screen test

The horizontal screen test was used to further characterize the sedative/motor impairing action of high doses of androstenol. The steroid was administered 15 min before testing to groups of 6 mice at doses of 100–300 mg/kg. None of the 6 animals receiving the 100 mg/kg dose of androstenol scored positive in the test, whereas at 200 and 300 mg/kg, 4 and 5 animals, respectively, showed impairment. The TD_{50} (95% confidence limit) value was 181 (108–303) mg/kg.

Effects of androstenol in elevated zero-maze

We used the elevated zero-maze as an alternative to the open field test for assessing the anxiolytic activity of androstenol. As shown in Fig. 10, androstenol caused a dose-dependent reduction in the latency to first open arm entry, a dose-dependent increase in the number of open arm entries and a dose-dependent increase in the percent time in open arms; significant effects were observed at doses of 10–50 mg/kg, depending upon the measure. Similar reductions in latency to first open arm entry and increases in percent time in open arm have been observed for anxiolytic drugs (Mombereau et al., 2004).

Effects of androstenol in the forced swim test

At low doses (5 and 10 mg/kg), androstrostenol reduced immobility times in the forced swim test compared with the vehicle-treated group indicating that it has an antidepressant-like effect (Fig. 11). However, no such effect was obtained with a higher dose (30 mg/kg).

Anticonvulsant activity of androstenol in 6 Hz and PTZ seizure models

As shown in Fig. 12, androstenol (10–100 mg/kg) conferred protection against seizures in the 6 Hz and PTZ models. The ED_{50} (95% confidence limit) values in the two models were 21.9 (11.4–42.2) and 48.9 (33.0–72.6) mg/kg. The 3β-epimer at a dose of 100 mg/kg failed to protect any animals in the PTZ model.

Discussion

The pheromone androstenol is a 16–17-unsaturated steroid that is structurally similar to $GABA_A$ receptor modulating neurosteroids. We now show that androstenol has $GABA_A$ receptor modulating activity comparable to such neurosteroids. Since androstenol is synthesized endogenously, it too can be considered to be a neurosteroid. Neurosteroids are conventionally viewed as hormones, that is, they are produced endogenously and convey a biological signal between separate cells of the same organism, either by blood-borne or local transmission. Androstenol has not previously been considered to be a hormone, although it is now apparent that it could act in this fashion. Rather, androstenol is well recognized to be a pheromone, which serves as a signaling molecule between individuals of the same species. Our results raise the possibility that an interaction with $GABA_A$ receptors could play a role in the signaling function of this steroid.

A critical structural characteristic of endogenous $GABA_A$ receptor modulating neurosteroids is that they are reduced at the 5- and 3-positions of the A-ring (Rogawski and Reddy, 2004) (Fig. 1). Among such structures, maximal potency is obtained when the substituents at the 3- and 5-positions are in the α-configuration. Altering the stereochemistry at these critical positions dramatically influences activity. In particular, the 3β-epimers all largely lack effects on $GABA_A$ receptors (Peters et al., 1998; Gee et al., 1988; Kokate et al., 1994). Androstenol shares an A-ring structure with these other endogenous neurosteroids. Thus, our first objective was to demonstrate that androstenol exhibits functional actions on $GABA_A$ receptors similar to that of A-ring reduced neurosteroids. We found that the steroid markedly potentiates responses to GABA in granule cell cultures in a concentration-dependent manner (EC_{50}, 0.4 μM), with up to a four-fold increase in current amplitude at the highest concentration tested (3 μM). A similar action was observed in granule cell slices at modestly higher concentrations (EC_{50}, 1.4 μM), presumably because access to $GABA_A$ receptors is more restricted in the slices. Comparison with the prototypic 5α,3α-reduced neurosteroid

allopregnanolone in granule cell cultures suggested that androstenol is less potent (see also Bolger et al., 1996; Fodor et al., 2005), although it produces comparable maximal potentiation to that reported for allopregnanolone and allotetrahydrodeoxycorticosterone in various in vitro preparations, and thus has similar efficacy (Peters et al., 1988; Puia et al., 1990; Wohlfarth et al., 2002). In addition, we found that androstenol produces a concentration-dependent prolongation of the decay time of spontaneous inhibitory postsynaptic currents without a significant alteration in the amplitude of the currents. These actions are similar to those of other $GABA_A$ receptor modulating neurosteroids which have generally been found to enhance the amplitude of currents induced by exogenously applied GABA, but mainly influence the decay of synaptically-generated currents (Zhu and Vicini, 1997; Cooper et al., 1999). Androstenol is produced in the body and is able to access the brain. Thus, like these other steroids, it could have a hormonal role in the modulation of CNS neuronal excitability. In contrast to androstenol, the 3β-epimer failed to potentiate responses to exogenous GABA even when applied at high (1 and 3 μM) concentrations, as expected from the aforementioned structure–activity relationship for neurosteroid stereoisomers.

Our results with native $GABA_A$ receptors in granule cells were corroborated in studies with recombinant $GABA_A$ receptors in a mammalian cell expression system. We found that GABA-evoked responses of $GABA_A$ receptors composed of α1β2γ2 and α2β2γ2 subunits, which are abundant subunit combinations in many brain regions (Mehta and Ticku, 1999; Sieghart, 1995) including the olfactory bulb (Laurie et al., 1992; Pirker et al., 2000), were potentiated by the steroid to a similar extent as native receptors. In addition, androstenol at higher concentrations (1–300 μM) directly gated the recombinant $GABA_A$ receptors, as has previously been observed for other neurosteroids (Kokate et al., 1994; Reddy and Rogawski, 2002; Wohlfarth et al., 2002).

Having found androstenol to be active in vitro we sought to determine if systemic administration of the steroid has behavioral actions expected of $GABA_A$ receptor modulating

neurosteroids, including anxiolytic-like, antidepressant-like, and anticonvulsant activities. In the open field test, we observed that androstenol increased the time spent by mice in the center of the arena at doses that did not alter spontaneous locomotor activity, which suggests that it has anxiolytic properties. There is a large body of evidence indicating that neurosteroids, like other $GABA_A$ receptor modulators, have such anxiolytic actions (Bitran et al., 1991; Rodgers and Johnson, 1998; Finn et al., 2003). At higher doses (≥ 100 mg/kg), androstenol inhibited locomotor activity and caused gross impairment of motor function, as is also typical of neurosteroids (Kokate et al., 1994).

There is also evidence that neurosteroids have antidepressant-like activity in the forced swim test (Khisti et al., 2000). In this regard, neurosteroids are similar to $GABA_A$ receptor agonists or drugs that elevate brain GABA levels (Borsini et al., 1986), although it is not clear that such drugs have true antidepressant actions. We examined androstenol in the forced swim test and found that low doses (5–10 mg/kg) cause a significant reduction in immobility time, an effect that is produced by some drugs with antidepressant properties (Porsolt et al., 1997, 2001). A higher dose of androstenol (30 mg/kg) was inactive. Anxiolytic drugs that lack intrinsic antidepressant activity such as benzodiazepines can reverse the reduction in immobility produced by antidepressants (Flugy et al., 1992). Thus, it seems plausible that, at higher doses, the anxiolytic activity of androstenol could counteract the antidepressant-like reduction in immobility times.

Neurosteroids that act as positive $GABA_A$ receptor modulators have anticonvulsant activity in various animal seizure models, including the 6 Hz and PTZ models (Kaminski et al., 2004, 2005; Kokate et al., 1994, 1996; Reddy and Rogawski, 2002). In the present study, we found that androstenol similarly is protective in these two seizure models. The potency in the 6 Hz model was greater than in the PTZ model, as observed previously for other neurosteroids. In accordance with the stereospecificity for potentiation of $GABA_A$ receptor currents, the 3β-epimer of androstenol was devoid of anticonvulsant activity. Thus, it is likely that modulation of $GABA_A$ receptors accounts for

the in vivo anticonvulsant activity and, indeed, there is extensive evidence that the target site for anticonvulsant effects of neurosteroids in the 6 Hz and PTZ models is the $GABA_A$ receptor (Rogawski and Reddy, 2004; Kaminski et al., 2004). The potency of androstenol in these models was similar to that of its 16-keto analog androsterone (Kaminski et al., 2005). However, androstenol was relatively less potent in the two seizure models than other neurosteroids, such as allopregnanolone, despite comparable potency on $GABA_A$ receptor currents in vitro, suggesting that the bioavailability with parenteral administration may be lower.

The key question raised by our studies is whether the $GABA_A$ potentiating activity of androstenol contributes to its pheromonal properties. We did not discern any differences between androstenol and other endogenous neurosteroids with respect to their effects on $GABA_A$ receptors. However, androstenol has certain physiochemical properties that make it better suited to serve as a pheromone than other neurosteroids. The calculated vapor pressure of androstenol at 37 ºC is 254 μTorr, which is markedly lower than other neurosteroids (values for allopregnanolone, allotetrahydrodeoxycorticosterone, androstanediol, and androsterone are 26, 4, 55, 60 μTorr, respectively). Thus, androstenol is more volatile than other neurosteroids and is better able to serve as an airborne vapor-phase chemical messenger. By the universal gas law, the concentration of androstenol in air at 37 ºC is estimated to be 13 nmol/L. Interestingly, this value is comparable to the basal serum concentrations of allopregnanolone (3–10 nM) (Paul and Purdy, 1992), indicating that whether neurosteroids are delivered as blood-borne hormones or as pheromones carried in a vapor, the concentrations in the delivery medium are remarkably similar. The estimated vapor phase concentration of androstenol is below the concentration active on $GABA_A$ receptors (threshold concentration, ~100 nM). However, it is likely that the steroid would reach the required concentration in mucus within seconds or less (T.H. Morton, personal communication). A second characteristic of androstenol that makes it well suited as a pheromone is its hydrophobicity.

Androstenol has a calculated log *P* (octanol-water partition coefficient) value of 5.9. Thus, it has significantly greater hydrophobicity than other neurosteroids (comparable values, 3.8–4.9), which would promote accumulation in mucus and transcellular delivery following exposure to a vapor.

The receptive organ for the pheromonal activity of androstenol is not known, although the olfactory system is an obvious possibility, particularly since androstenol is detected as an odorant by ~70 percent of humans (Gower and Ruparelia, 1993) and possibly all non-human primates (Laska et al., 2005). Although functional GABA neurons are present transiently in the nose during embryonic development (Tobet et al., 1996; Wray et al., 1996), as of yet, $GABA_A$ receptors have not been identified in olfactory receptor cells situated in the adult olfactory epithelium. However, virtually every type of neuron in the olfactory bulb receives GABAergic input. The glomerular layer in the most superficial zone of the bulb has among the highest densities of $GABA_A$ receptors of any region in the brain (Palacios et al., 1981; Bowery et al., 1987; Laurie et al., 1992). Within the glomerular layer, periglomerular cells inhibit mitral cells through GABA-mediated dendrodendritic inhibition (Shepherd et al., 1998). There is extensive evidence that hydrophobic, small molecular weight substances can be transported from the nasal cavity into the olfactory bulb and other brain structures (Illum, 2004). Thus, inhaled androstenol could access $GABA_A$ receptors which are present in high abundance in superficially located mitral cell dendrites as well as $GABA_A$ receptors in deeper structures in the bulb and elsewhere in the brain. In addition to environmental and blood-borne delivery, the olfactory system may also be exposed to locally synthesized androstenol. In this regard it is noteworthy that the nasal mucosa contains many of the enzymes required for androstenol synthesis, including 3α-hydroxysteroid oxidoreductase (Brittebo et al., 1984), which can synthesize androstenol from androstenone, a companion steroidal pheromone to androstenol. Androstenol and androstenone are among the best-accepted mammalian pheromones (Grammer et al., 2005). The

present results raise the possibility that effects on $GABA_A$ receptors could contribute to their pheromonal activity.

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Footnotes

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Address correspondence to: Michael A. Rogawski, M.D., Ph.D., Epilepsy Research Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 35 Convent Drive MSC 3702, Bethesda, MD 20892-3702. E-mail: michael.rogawski@nih.gov

[1]H. Marini's present address is Faculty of Medicine and Surgery, A.O.U. G. Martino, University of Messina, Messina, Italy.

Legends for Figures

Figure 1. Structural similarity between the androstenol epimers (16-17 unsaturated) and A-ring reduced (16-17 saturated) $GABA_A$ receptor modulating neurosteroids, including the testosterone metabolites androstanediol and androsterone, the progesterone metabolite allopregnanolone and the deoxycorticosterone metabolite allotetetrahydrodeoxycorticosterone.

Figure 2. Androstenol reversibly enhances GABA activated whole-cell currents in DIV 7 cultured mouse cerebellar granule cells. Black bars indicate period of application of 0.25 μM GABA; grey bars indicate preapplication/coapplication of androstenol at the concentrations noted. Recovery was obtained at 100 s with 300 nM androstenol and at 800 s with 1000 nM androstenol. Upper and lower panel represent different neurons. The holding potential was –60 mV.

Figure 3. Effects of androstenol on GABA activated currents in cultured mouse cerebellar granule cells at DIV 7 (A) and granule cells in cerebellar slices from 11–12 day-old mice (B). Top panels in A and B show representative whole-cell currents elicited by 0.25 μM GABA (top black bars) during the coapplication of androstenol at the indicated concentration (in nM, bottom grey bars). Androstenol was preapplied for 15–20 s before the combined androstenol and GABA applications. (Full duration of preapplication not shown.) Each trace is from a separate cell. Bottom panels show concentration-response curves from experiments similar to those illustrated. Percent of control values were calculated from the peak current values with respect to the peak response to GABA alone in the same cell. Each data point represents the mean ± S.E.M of data from 4 to 18 cultured granule cells and 4 to 5 granule cells in slice recordings. The curves represent logistic fits to the data points. The EC_{50} values for cultured granule cells and granule cells in slices were 382 and 1400 nM, respectively.

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The mean ± S.E.M peak amplitude values of GABA responses in absence of androstenol for granule cells and granule cells in slices were 48.0 ± 6.5 pA (*n*=18) and 38.4 ± 3.7 pA (*n*=11), respectively. Small closed and open squares are mean values from experiments of Fig. 5 for HEK 293 cells transfected with α1β2γ2 and α2β2γ2 subunit combinations, respectively.

Figure 4. Stereoselective effects of the 3-hydroxy epimers of androstenol on GABA-activatived currents. Recordings were obtained from DIV 7 cultured cerebellar granule cells. Representative current traces shown at the top compare the effects of 1000 nM 3β-androstenol (5α,3β-a) with the natural 3α-isomer (5α,3α-a) at the same concentration. The bar graph below shows percent of control values calculated from peak current values obtained in a series of similar experiments. The percent of control values were calculated from the peak response to 0.25 μM GABA in the presence of an androstenol epimer at a concentration of either 1000 or 3000 nM with respect to the peak response to GABA alone in the same cell. Each bar represents the mean ± S.E.M of values from 4–16 granule cells. 3α-Androstenol produced a significant potentiation at both concentrations ($p < 0.05$; two-tailed *t*-test) whereas 3β-androstenol did not.

Figure 5. Androstenol potentiates GABA currents in human embryonic kidney cells (HEK 293) transfected with α1β2γ2 and α2β2γ2 $GABA_A$ receptor subunit combinations. Representative current traces shown at the top illustrate the effects of 100, 300 and 1000 nM androstenol (grey bars) on currents evoked by 1 or 3 μM GABA (black bars). Bar chart below summarizes results from 6–9 cells transfected with α1β2γ2 subunits and 4–6 cells with α2β2γ2 subunits. Bars represent mean ± S.E.M. percent peak amplitude values with respect to the amplitude of GABA alone in the same cell. The mean ± S.E.M. peak amplitude values for GABA in the absence of androstenol in α1β2γ2 and α2β2γ2 transfected cells were 60 ± 11.7 pA (*n*=11) and 59 ± 11.6 pA (*n*=9), respectively.

Figure 6. Direct activation of inward current in HEK 293 cells expressing $\alpha1\beta2\gamma2$ $GABA_A$ receptors subunits. Traces (left) compare responses to 5 μM GABA and 100 μM androstenol in the same cell. The interval between the GABA and androstenol perfusions was 30 s. Graph (right) summarizes results from a series of similar experiments. In each culture dish, the response to 5 μM GABA was determined in 2–3 cells before application of androstenol. The data points indicate the peak direct androstenol current expressed as a fraction of the mean current amplitude evoked by 5 μM GABA in the dish. The average response to 5 μM GABA in 2 dishes was 381 ± 53 pA (n=5).

Figure 7. Effects of 100 nM androstenol on spontaneous inhibitory postsynaptic currents (sIPSCs) in cultured cerebellar granule cells. Top left, sample recordings before (control) and after application of androstenol. Currents are presumed to be mediated by $GABA_A$ receptors; no fast AMPA receptor mediated currents are present in the traces shown. Bottom left, superimposed traces show representative averaged sIPSCs from control (black) and androstenol (grey) recordings scaled to the same peak amplitude and displayed on a faster time scale. The weighted time constant (τ_w) values for each trace are indicated. The chart on right plots mean ± S.E.M. values of peak amplitude (left two bars) and τ_w (right two bars) values from 24 control cells and 9 cells exposed to androstenol. For each cell, a minimum of 30 sIPSCs were analyzed and the peak amplitude and weighted time constant values were averaged. *, $p < 0.01$.

Figure 8. Effects of androstenol on miniature inhibitory synaptic currents (mIPSCs) in cultured cerebellar granule cells. Recordings were carried out in the presence of 0.5 μM tetrodotoxin. Top left, sample recordings before (control) and after application of 300 nM androstenol. Bottom left, superimposed traces show representative averaged mIPSCs from control (black) and androstenol

(grey) recordings scaled to the same peak amplitude and displayed on a faster time scale. Mean peak amplitude and mean weighted time constant values during application of androstenol at various concentrations are plotted in the graph as percent of control (prior to androstenol application). The control mean ± S.E.M. peak amplitude and weighted time constant values are –45.5 ± 7.0 pA and 46.8 ± 3.3 ms (*n*=9). Each data point represents data from recordings from 4–8 cells. In each recording, a minimum of 30 mIPSCs were analyzed and the peak amplitude and weighted time constant values were averaged.

Figure 9. Effects of androstenol on spontaneous motor behavior in mice. Graphs in upper panel show horizontal activity counts and stereotypy counts for the preceding 10 min period after injection of vehicle or the indicated androstenol doses at zero time. The time in the center of the recording area (expressed as a percentage of total time) is plotted in the lower-left graph. The data points represent the mean ± S.E.M. of values from 11 vehicle-treated control mice or 6–7 androstenol-treated mice. The bar chart in lower right shows area-under-curve values from the time in center of the recording area plots. Androstenol was injected i.p. just before the placement of the animals in the open field arena. *, $p < 0.05$; **, $p < 0.01$ versus vehicle; Bonferroni's *t*-test except AUC where Student's *t*-test was used.

Figure 10. Effects of androstenol on anxiety-related behaviors in the elevated zero-maze. Androstenol or vehicle was administered i.p. 15 min before testing. Top panel, latency to first open area entry; middle panel, number of open area entries; bottom panel, time spent in open areas. The bars represent the mean ± S.E.M. of values from 8 vehicle-treated control mice or 6–7 androstenol-treated mice. *, $p < 0.05$; **, $p < 0.01$ versus vehicle; Student's *t*-test.

Figure 11. Immobility times in the forced swim test following treatment with vehicle or different doses of androstenol. Androstenol was administered i.p. 15 min before the beginning of the test. *$p <$ 0.05, Student's *t*-test.

Figure 12. Dose-response relationships for protection by androstenol and 5α-androst-16-en-3β-ol (β) against 6 Hz and pentylenetetrazol (PTZ) induced seizures in mice. Steroids were administered i.p. 15 min before electrical stimulation or injection of PTZ. Data points indicate percentage of animals protected against seizures. Each point represents 6 to 8 mice. The smooth curves represent logistic fits to the data.

Figure 1

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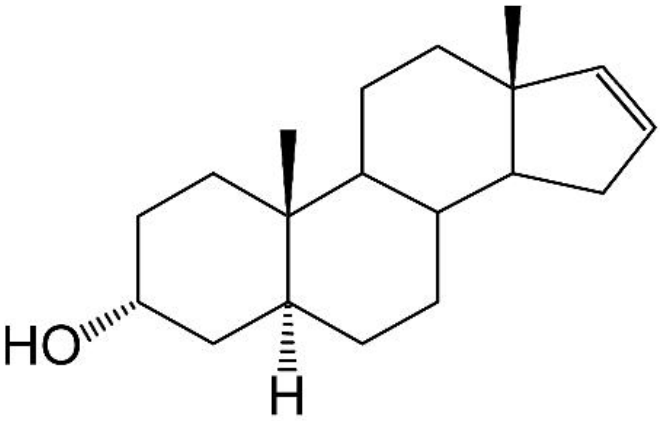

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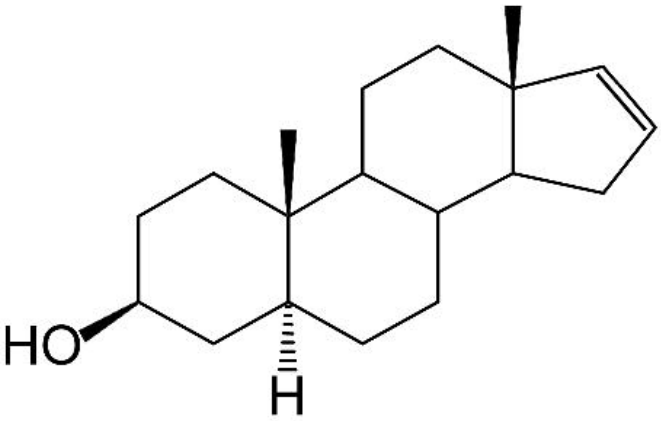

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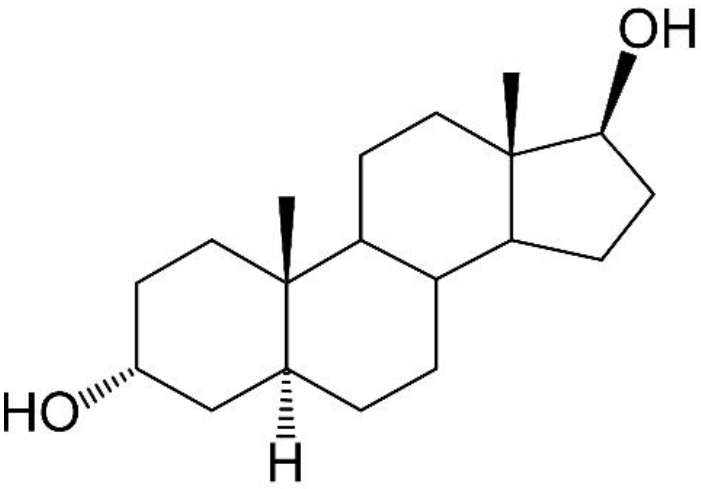

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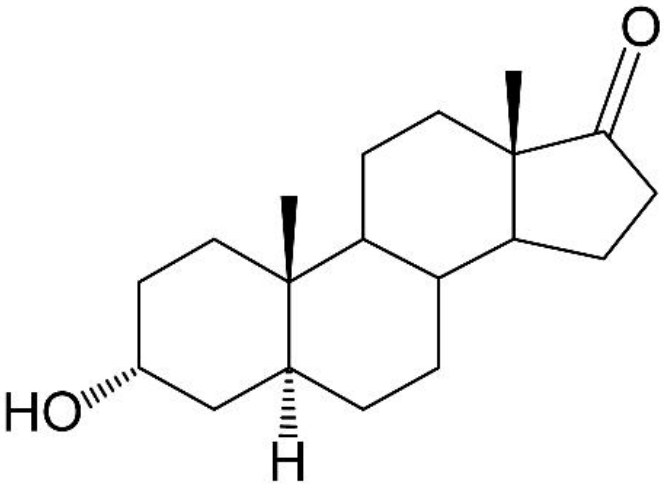

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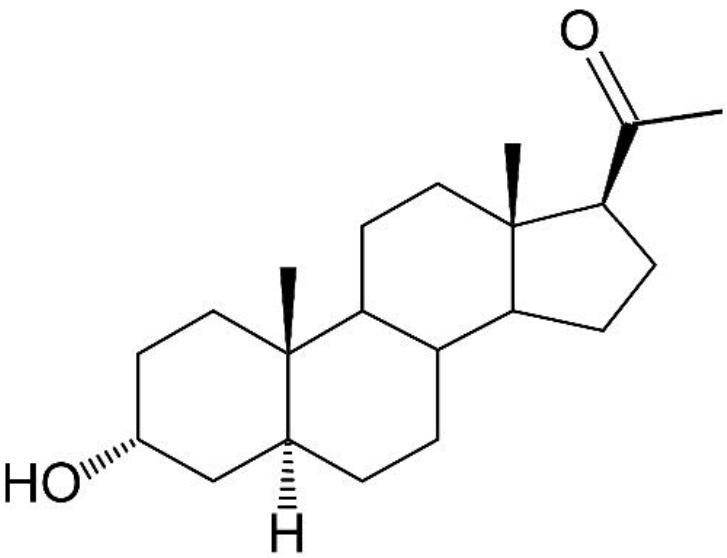

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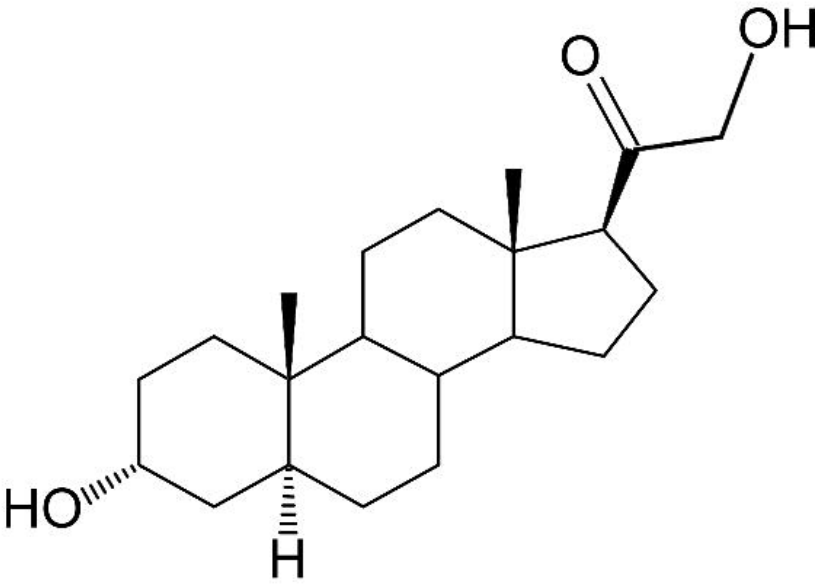

Allotetrahydrodeoxycorticosterone

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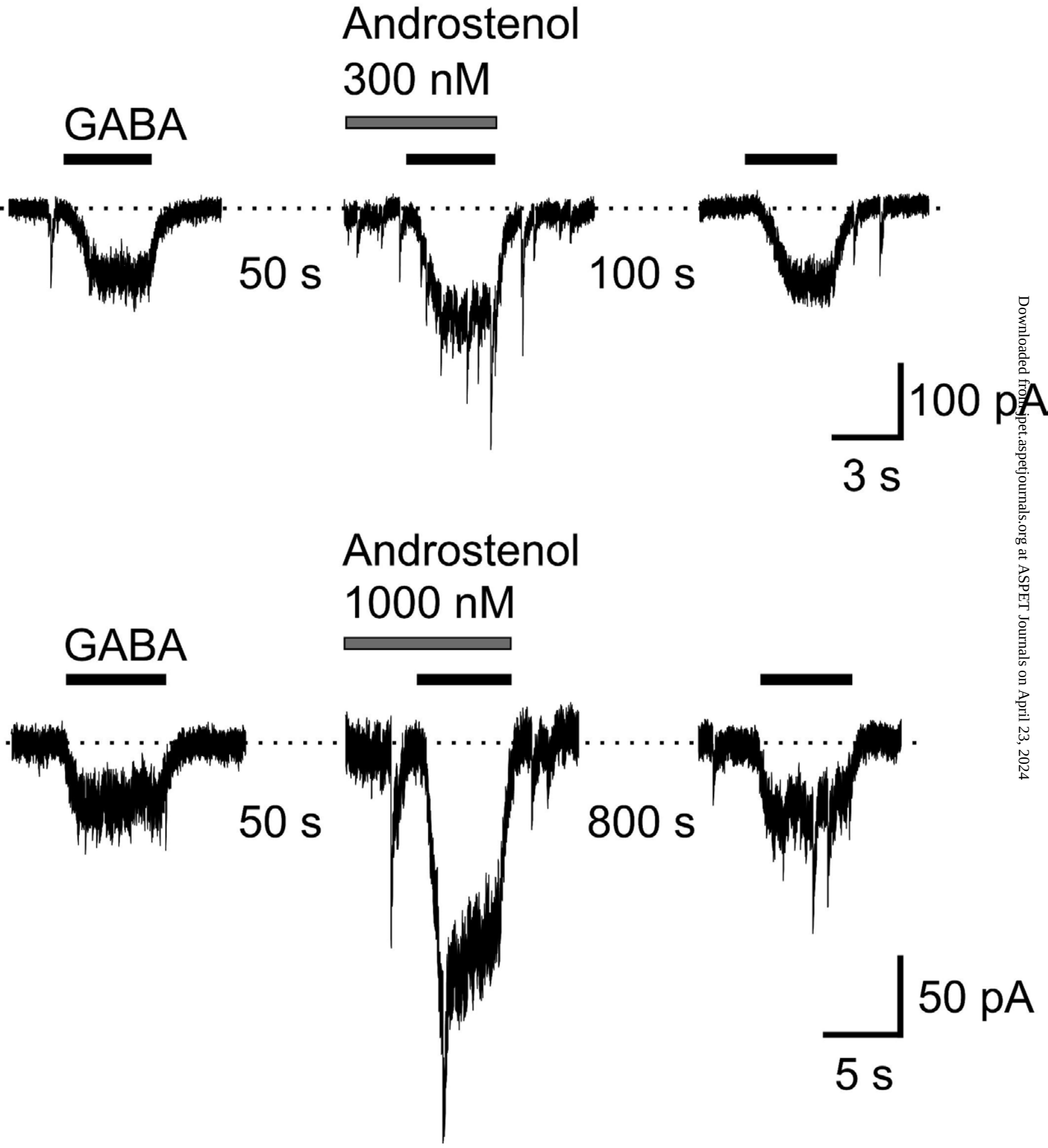
Figure 2



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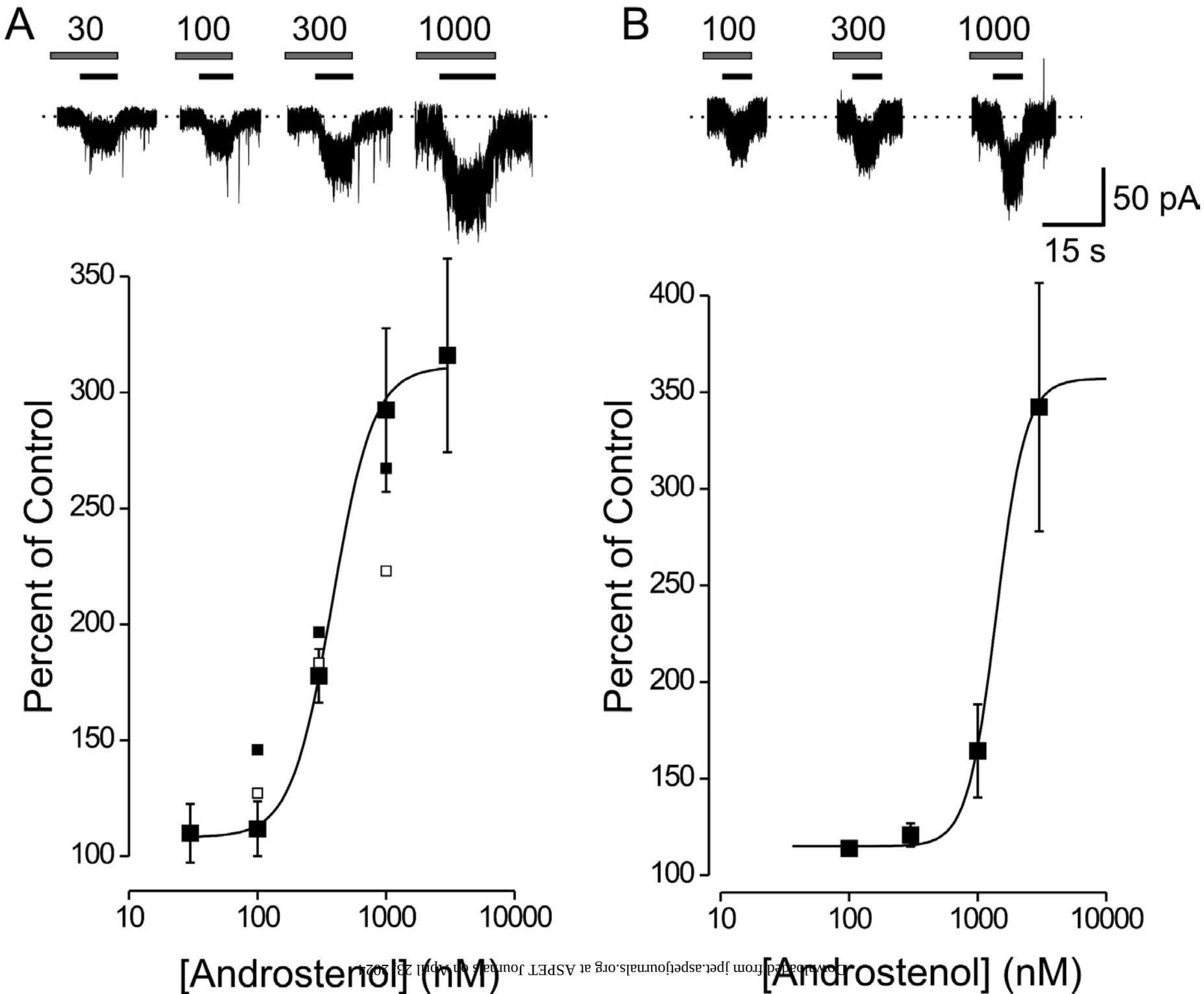

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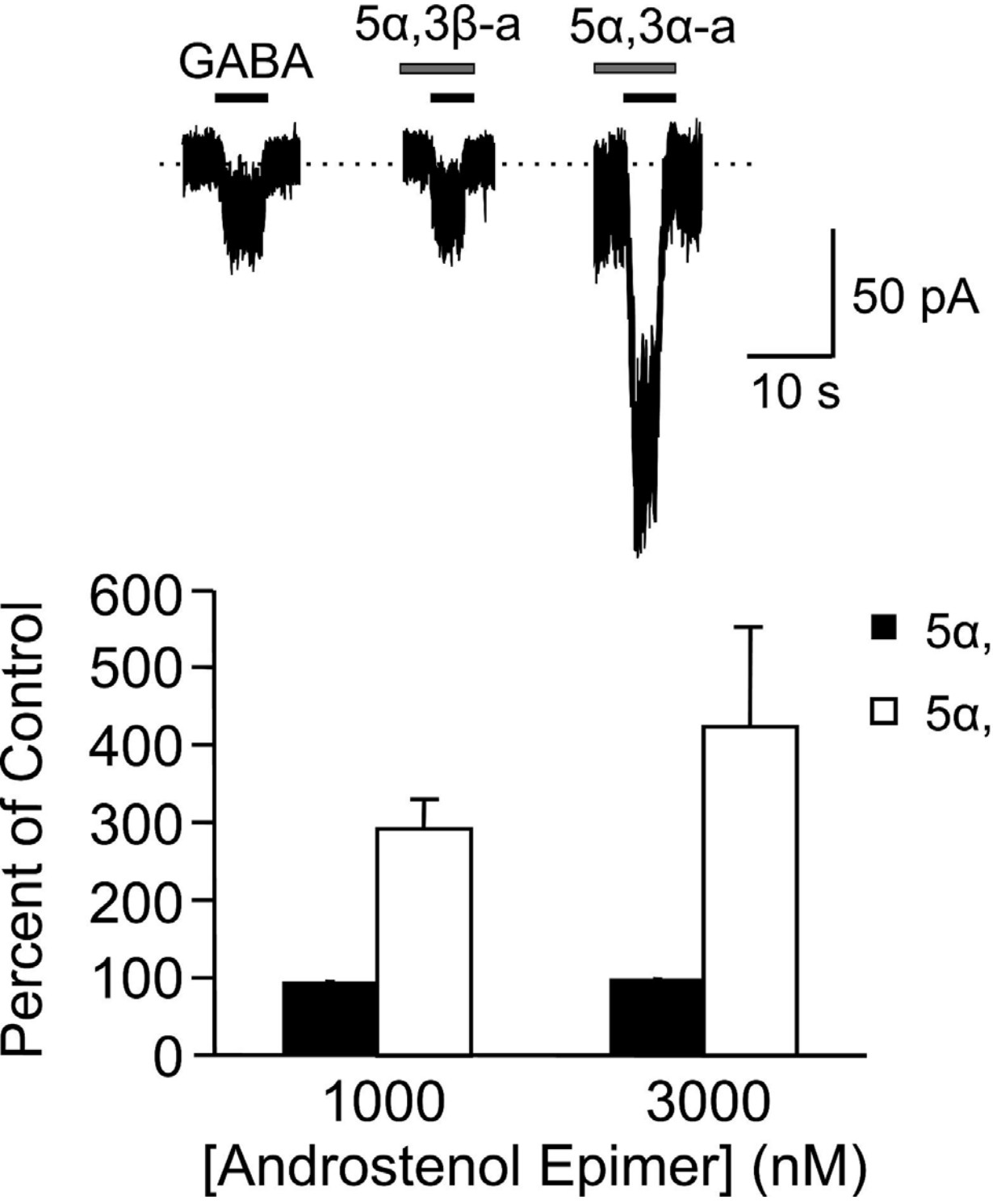

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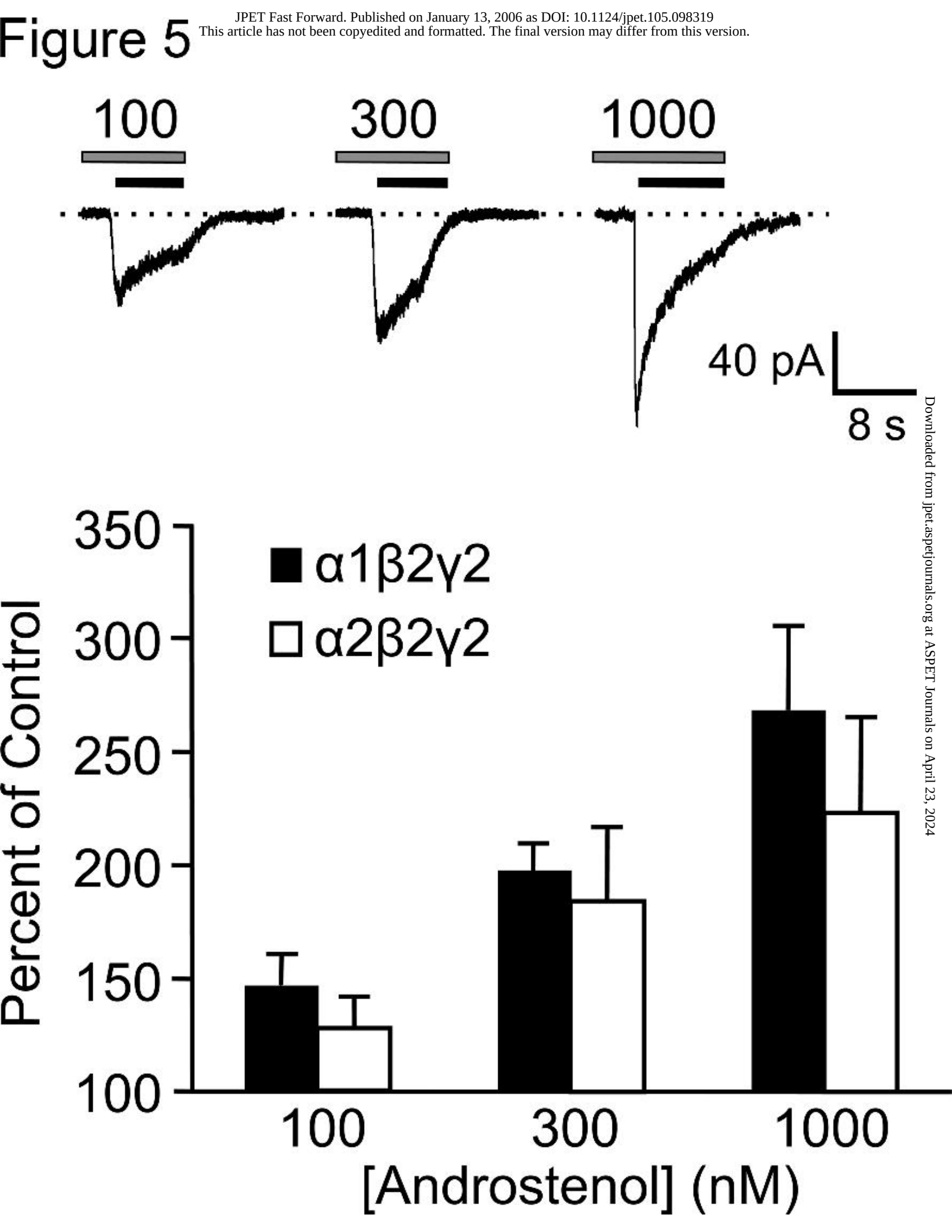

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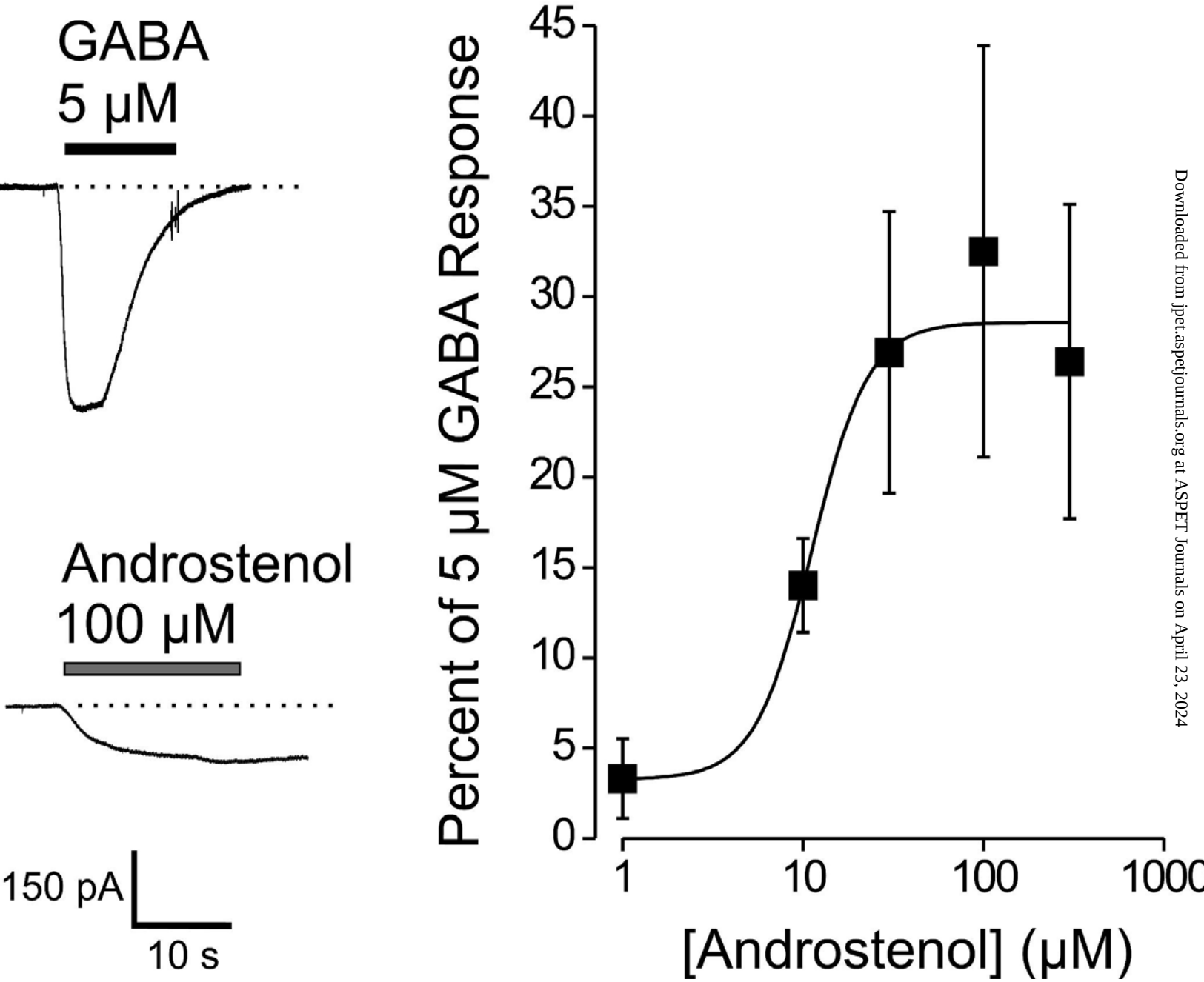

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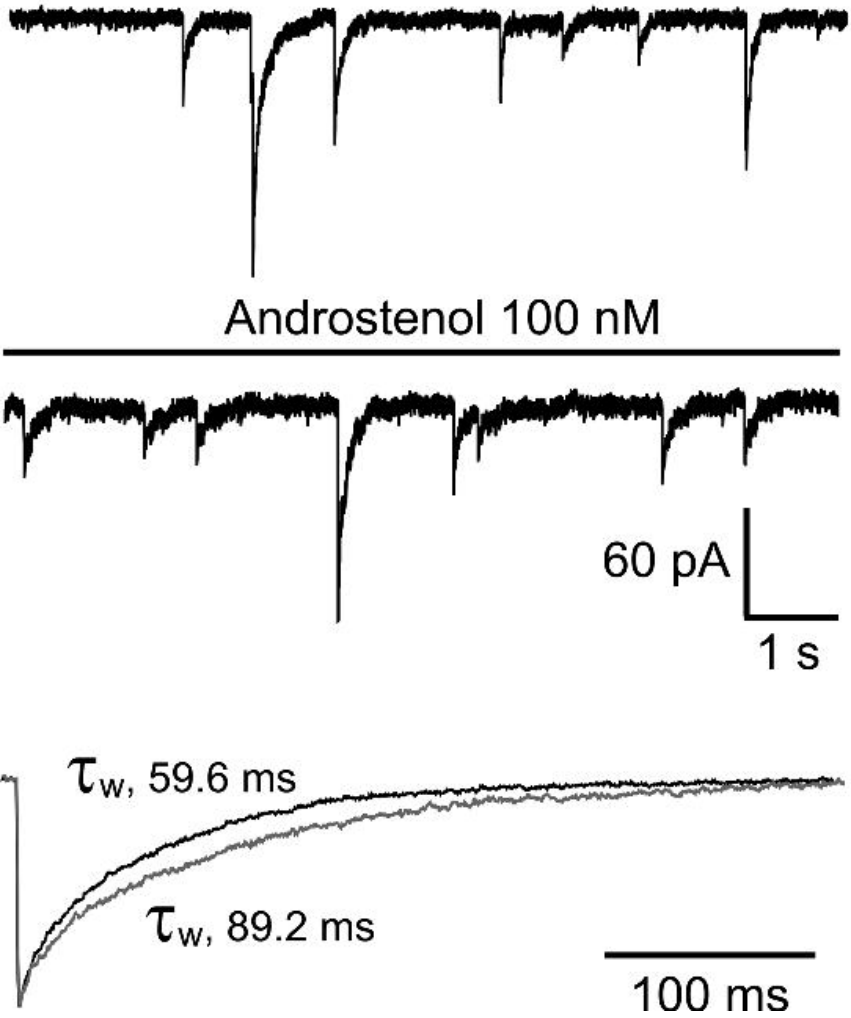

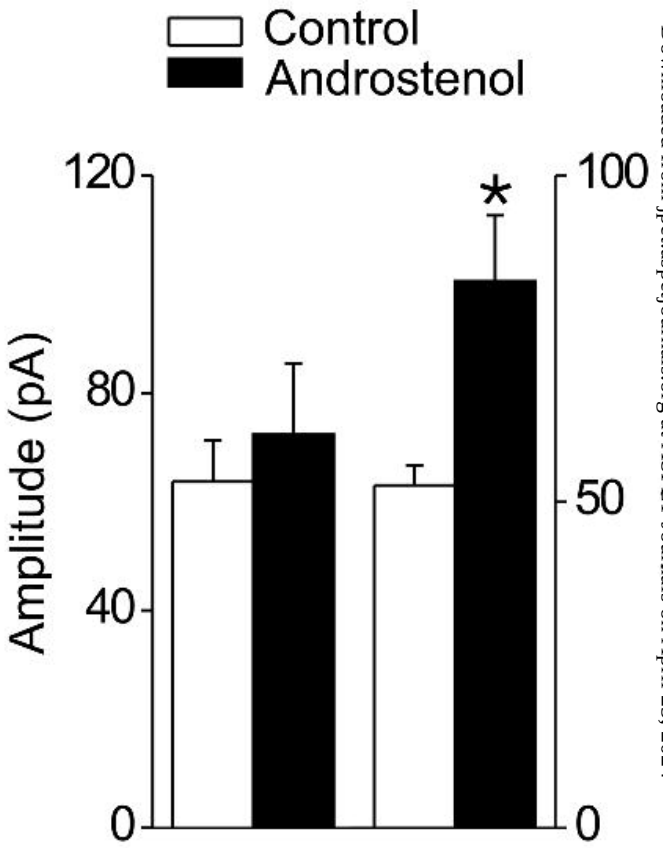

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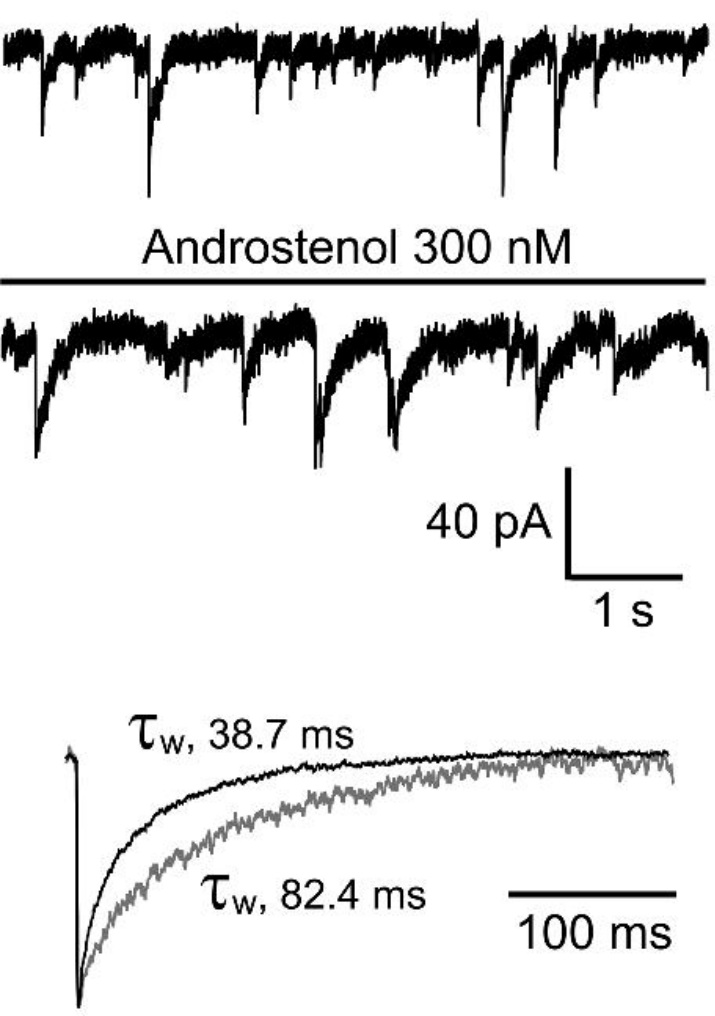

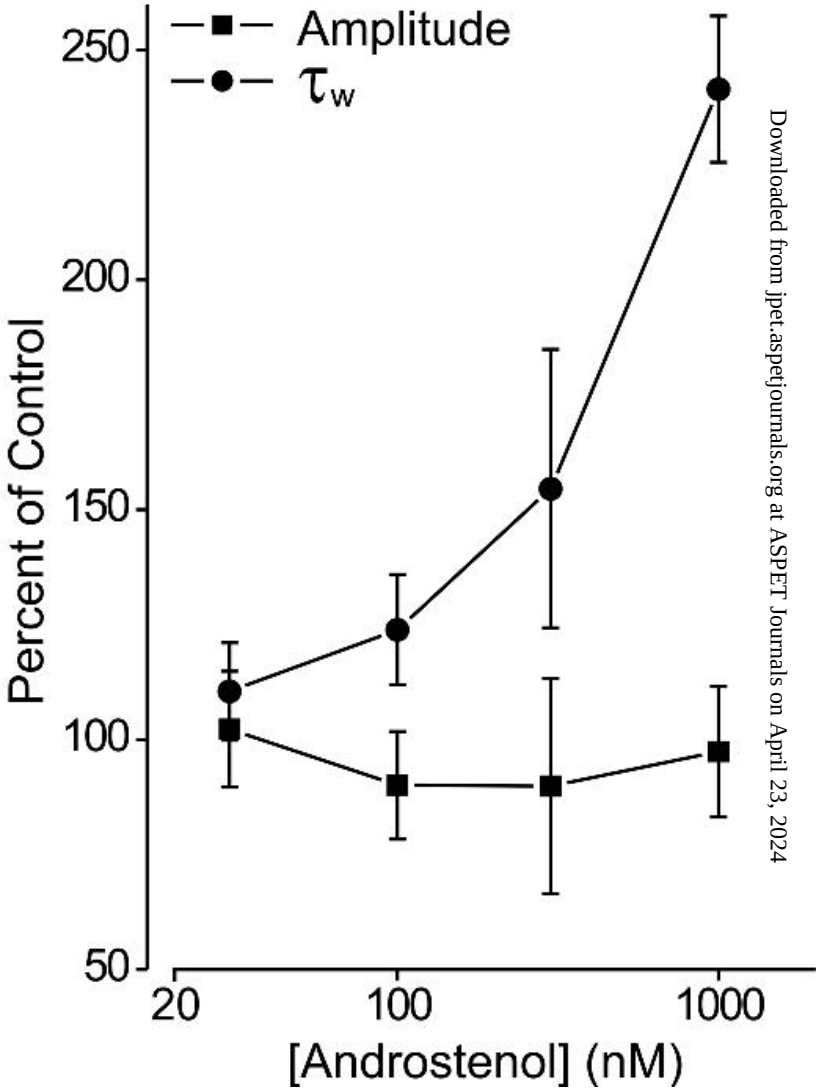

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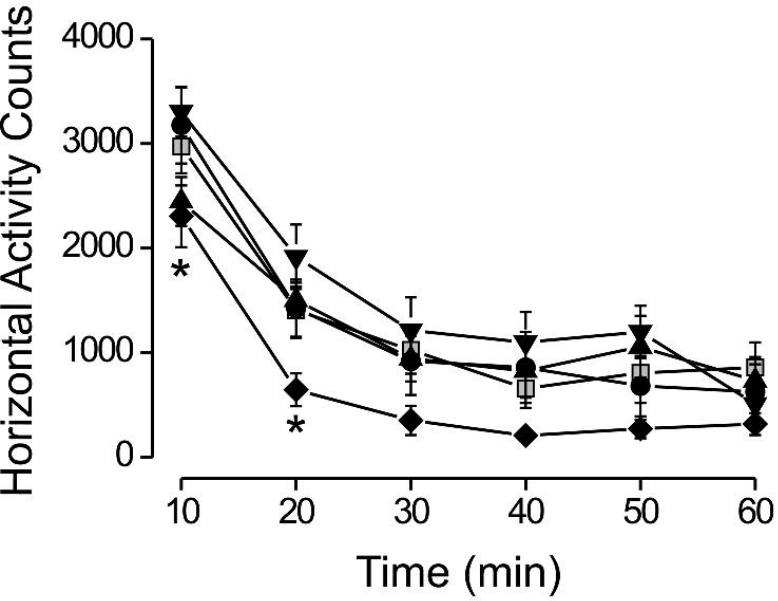

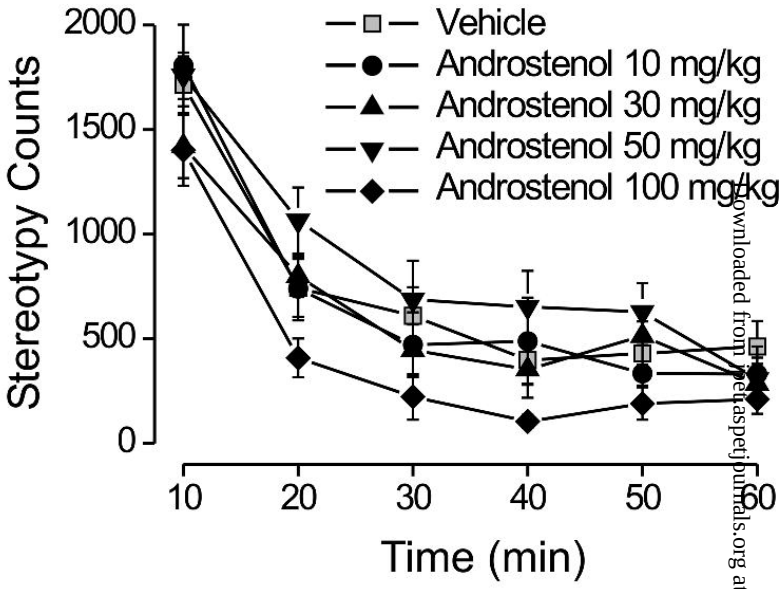

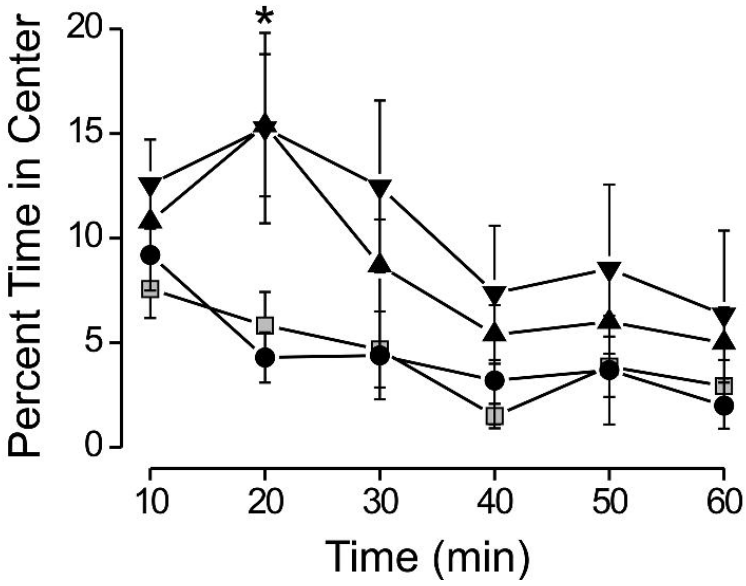

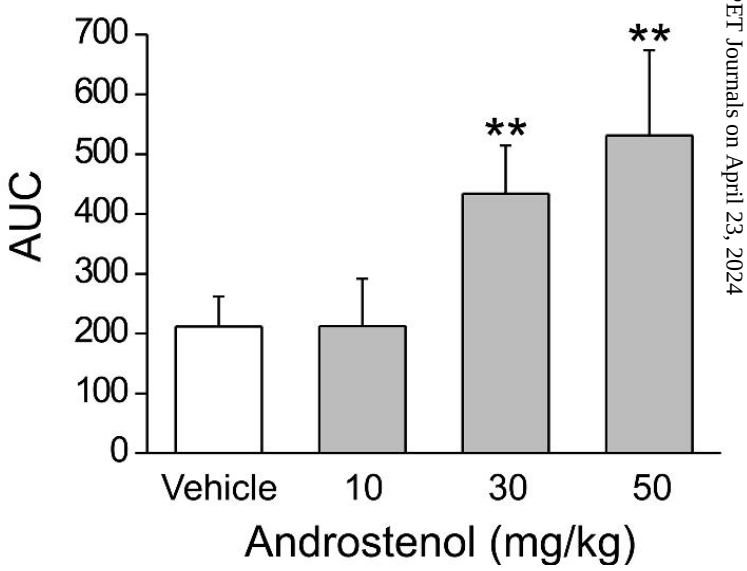

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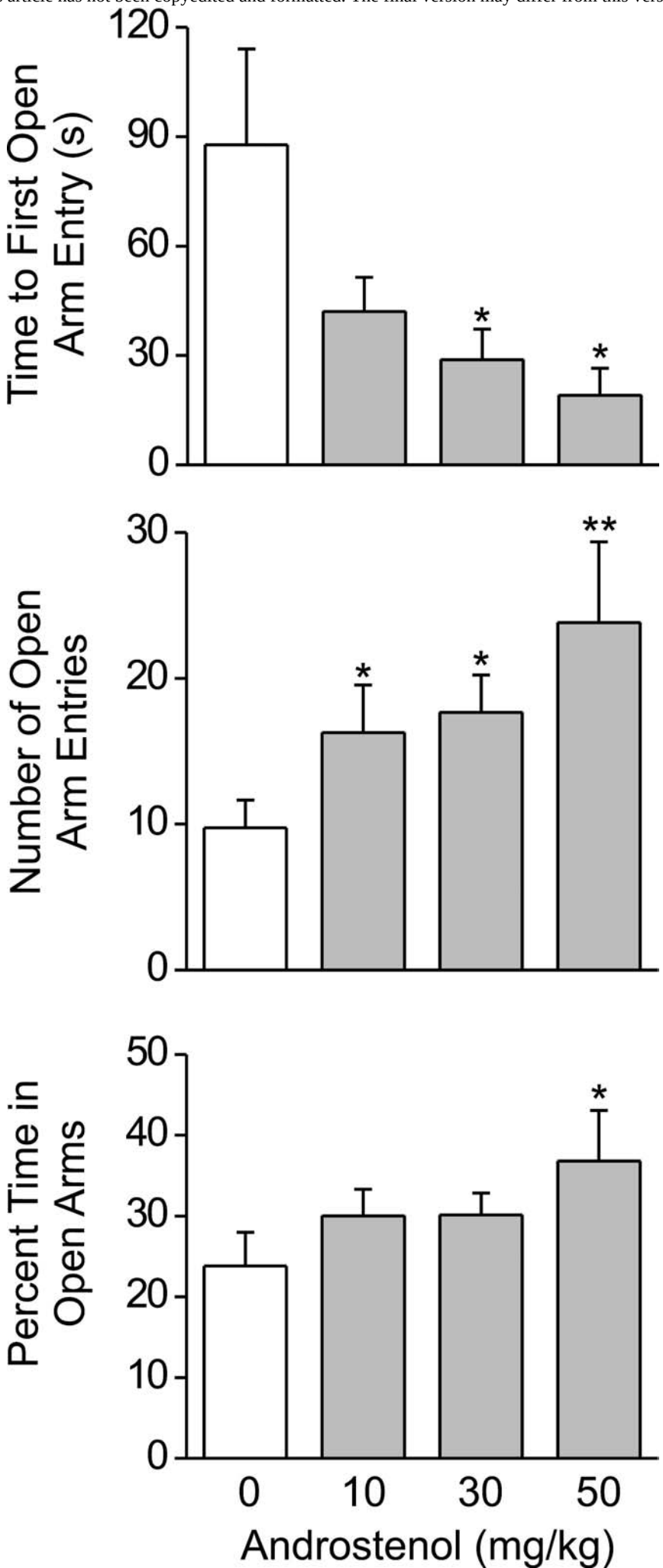

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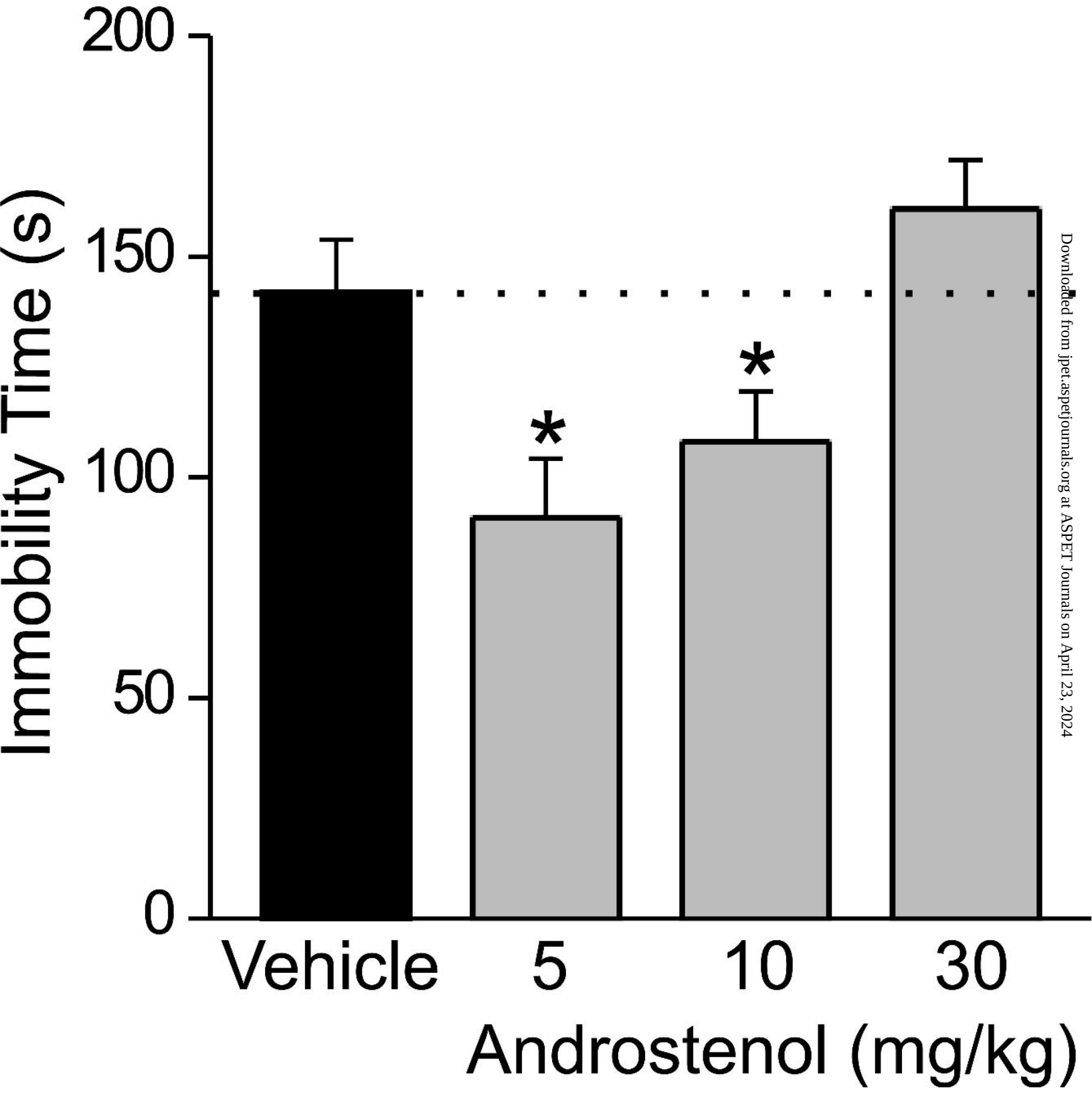

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Figure 12