Noradrenergic terminals are the primary source of α2-adrenoceptor mediated dopamine release in the medial prefrontal cortex

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Abstract

In various psychiatric disorders, deficits in dopaminergic activity in the prefrontal cortex (PFC) are implicated. Treatments involving selective augmentation of dopaminergic activity in the PFC primarily depend on the inhibition of α2-adrenoreceptors singly or in combination with the inhibition of the norepinephrine transporter (NET). We aimed to clarify the relative contribution of dopamine (DA) release from noradrenergic and dopaminergic terminals to DA output induced by blockade of α2-adrenoreceptors and NET. To this end, we assessed whether central noradrenergic denervation modified catecholamine output in the medial PFC (mPFC) of rats elicited by atipamezole (an α2-adrenoreceptor antagonist), nisoxetine (an NET inhibitor), or their combination. Intraventricular administration of anti-dopamine-beta-hydroxylase-saporin (aDBH) caused a loss of DBH-positive fibers in the mPFC and almost total depletion of tissue and extracellular NE level; however, it did not reduce tissue DA level but increased extracellular DA level by 70% in the mPFC. Because noradrenergic denervation should have caused a loss of NET and reduced NE level at α2-adrenoceptors, the actual effect of an aDBH-induced lesion on DA output elicited by blockade of α2-adrenoceptors and NET was evaluated by comparing denervated and control rats following blockade of α2-adrenoceptors and NET with atipamezole and nisoxetine, respectively. In the control rats, extracellular NE and DA levels increased by approximately 150% each with 3 mg/kg atipamezole; 450% and 230%, respectively, with 3 mg/kg nisoxetine; and 2100% and 600%, respectively, with combined atipamezole and nisoxetine. In the denervated rats, consistent with the loss of NET, nisoxetine failed to modify extracellular DA level, whereas atipamezole, despite the lack of NE-induced stimulation of α2-adrenoceptors, increased extracellular DA level by approximately 30%. Overall, these results suggest that atipamezole-induced DA release mainly originated from noradrenergic terminals, possibly through the inhibition of α2-autoreceptors. Furthermore, while systemic and local administration of the α2-adrenoceptor agonist clonidine into the mPFC of the controls rats reduced extracellular NE level by 80% and 60%, respectively, and extracellular DA level by 50% and 60%, respectively, it failed to reduce DA output in the denervated rats, consistent with the loss of α2-autoreceptors. To eliminate the possibility that denervation reduced DA release potential via the effects at dopaminergic terminals in the mPFC, the effect of systemic administration of the D2-DA antagonist raclopride (0.5 mg/kg IP) on DA output was analyzed. In the control rats, raclopride was found to be ineffective when administered alone, but it increased extracellular DA level by 380% following NET inhibition with nisoxetine. In the denervated rats, as expected due to the loss of NET, raclopride—alone or with nisoxetine—increased DA release to approximately the same level as that observed in the control rats after NET inhibition. Overall, these results suggest that noradrenergic terminals in the mPFC are the primary source of DA released by blockade of α2-adrenoreceptors and NET and that α2-autoreceptors, and not α2-heteroreceptors, mediate DA output induced by α2-adrenoceptor blockade.

Keywords	Anti-DBH-saporin; co-release; D2-antagonist; microdialysis; norepinephrine transporter
Taxonomy	Systems Neuroscience, Molecular Neuroscience, Neuroscience

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Dear Prof. Gendron:

Thank you for reviewing our study and providing constructive feedback. As per the reviewer's comments, we have revised the manuscript entitled "Noradrenergic terminals are the primary source of α_2-adrenoceptor-mediated dopamine release in the medial prefrontal cortex." Please find attached our responses to the reviewers' comments.
First, based on the suggestion from you and the reviewers, a professional editing service has revised the manuscript. We hope the content is now easier to understand, even by non-specialist readers, thanks to the stylistic improvements.
We acknowledge the importance of the studies that Reviewer 2 requested to be cited. One author of the present manuscript is also a co-author of a requested reference and wishes to thank the reviewer for her/his interest in the study. However, these two references are not strictly related to the matter at hand. Moreover, fundamental methodological differences, especially in terms of administration routes (IV vs. IP), species (mice vs. rat), and neurotoxins (DSP-4 vs. aDBH-saporin), make the comparison difficult. Regarding the number of our articles cited, we believe that they constitute the foundation on which the present article has been built as the latest product of a research stream with a history lasting more than 20 years.
We hope that we have addressed all concerns and that you will find the revised manuscript to be suitable for publication in *Progress in Neuropsychopharmacology & Biological Psychiatry*.

Sincerely,
Paola Devoto

Highlights

In the medial prefrontal cortex, central noradrenergic denervation

- suppressed dopamine (DA) output induced by blockade of α_2-adrenoceptor and norepinephrine transporter;
- suppressed DA output inhibition induced by α_2-adrenoceptor agonists; and
- augmented DA output induced by D_2-receptor antagonists

NORADRENERGIC TERMINALS ARE THE PRIMARY SOURCE OF α_2-ADRENOCEPTOR MEDIATED DOPAMINE RELEASE IN THE MEDIAL PREFRONTAL CORTEX

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ABSTRACT

In various psychiatric disorders, deficits in dopaminergic activity in the prefrontal cortex (PFC) are implicated. Treatments involving selective augmentation of dopaminergic activity in the PFC primarily depend on the inhibition of α_2-adrenoreceptors singly or in combination with the inhibition of the norepinephrine transporter (NET).

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In the control rats, extracellular NE and DA levels increased by approximately 150% each with 3 mg/kg atipamezole; 450% and 230%, respectively, with 3 mg/kg nisoxetine; and 2100% and 600%, respectively, with combined atipamezole and nisoxetine. In the denervated rats, consistent with the loss of NET, nisoxetine failed to modify extracellular DA level, whereas atipamezole, despite the lack of NE-induced stimulation of α_2-adrenoceptors, increased extracellular DA level by approximately 30%. Overall, these results suggest that atipamezole-induced DA release mainly originated from noradrenergic terminals, possibly through the inhibition of α_2-autoreceptors.

Furthermore, while systemic and local administration of the α_2-adrenoceptor agonist clonidine into the mPFC of the controls rats reduced extracellular NE level by 80% and 60%, respectively, and extracellular DA level by 50% and 60%, respectively, it failed to reduce DA output in the denervated rats, consistent with the loss of α_2-autoreceptors.
To eliminate the possibility that denervation reduced DA release potential via the effects at dopaminergic terminals in the mPFC, the effect of systemic administration of the D_2-DA antagonist raclopride (0.5 mg/kg IP) on DA output was analyzed. In the control rats, raclopride was found to be ineffective when administered alone, but it increased extracellular DA level by 380% following NET inhibition with nisoxetine. In the denervated rats, as expected due to the loss of NET, raclopride—alone or with nisoxetine—increased DA release to approximately the same level as that observed in the control rats after NET inhibition.
Overall, these results suggest that noradrenergic terminals in the mPFC are the primary source of DA released by blockade of α_2-adrenoreceptors and NET and that α_2-autoreceptors, and not α_2-heteroreceptors, mediate DA output induced by α_2-adrenoceptor blockade.

Key words: Anti-DBH-saporin; co-release; D_2-antagonist; microdialysis; norepinephrine transporter

Abbreviations: ANOVA, Analysis of variance; aDBH, anti-dopamine-ß-hydroxylase-saporin; DA, dopamine; DBH, dopamine-ß-hydroxylase; IP, intraperitoneal; LC, locus coeruleus; mPFC, medial prefrontal cortex; NE, norepinephrine; NET, norepinephrine transporter; PFC, prefrontal cortex; VTA, ventral tegmental area.

1. INTRODUCTION

Dopaminergic and noradrenergic projections from the ventral tegmental area (VTA) and locus coeruleus (LC), respectively, converge to the prefrontal cortex (PFC), where they play key roles in cognitive, emotional, and motivational functions (Descarries et al., 1987; Van Eden et al., 1987; Seguela et al., 1990). In humans, impaired catecholamine transmission in the PFC has been implicated in the cognitive and emotional deficits observed in schizophrenia (Bird et al., 1979; Jobe et al., 1994; Slifstein et al., 2015), depressive disorders (Kapur and Mann, 1992; Invernizzi and Garattini, 2004; Dunlop and Nemeroff, 2007; Belujon and Grace, 2017), drug addiction (Everitt et al., 2007; Volkow et al., 1993), attention deficit/hyperactivity disorder (Bymaster et al., 2002; Arnsten and Pliszka, 2011), and impulsivity disorder (Crews and Boettinger, 2009; Kayser et al., 2012).

Conversely, the efficacy of atypical antipsychotics, including clozapine, olanzapine, and risperidone, in the treatment of the negative symptoms and cognitive deficits in schizophrenia has been partially attributed to the enhancement of prefrontal cortical neurotransmission via α_2-adrenoceptors inhibition (Hertel et al., 1999a; Devoto et al., 2001 and 2003b; Svensson, 2003; Brosda et al., 2014).

Moreover, animal studies indicate that α_2-adrenoreceptor antagonists enhance dopamine (DA) and norepinephrine (NE) outputs in the medial prefrontal cortex (mPFC) and that this effect is potentiated by the inhibitors of the NE transporter (NET) (Gobert et al., 1997; Masana et al., 2011), antipsychotics (Hertel et al., 1999a; Marcus et al., 2010), antidepressants (Invernizzi and Garattini, 2004), and antiparkinsonian treatment (Langer, 2015). α_2-Adrenoceptor antagonists possibly enhance NE output via α_2-autoreceptor inhibition (Langer, 1981). It has been hypothesized that α_2-adrenoceptor blockade withdraws tonic NE α_2-heteroreceptor-mediated inhibitory control on DA release from dopaminergic terminals, resulting in enhanced DA output (Gresch et al., 1995; Gobert et al., 1997; Ihalainen and Tanila, 2002). Moreover, α_2-adrenoceptor blockade may reduce DA clearance from extracellular space by increasing extracellular NE level, which competes with DA for the same transporter (Carboni et al., 1990; Gresch et al., 1995; Yamamoto and Novotney, 1998; Moron et al., 2002).

However, previous results from our laboratory suggest that extracellular DA in the cerebral cortex not only originates from dopaminergic terminals but is also co-released with NE from noradrenergic terminals, where DA acts as a precursor and co-transmitter, and is controlled by the α_2-autoreceptors present on noradrenergic neurons (Devoto et al., 2001; Devoto et al., 2004; Devoto and Flore, 2006; Devoto et al., 2008; Devoto et al., 2015).

In support of the co-release hypothesis, electrical stimulation of the LC reportedly produces concomitant release of DA and NE in the mPFC (Devoto et al., 2005a and 2005b; Masana et al., 2011). Moreover, depletion of cortical NE, induced by the neurotoxin DSP-4, terminated DA output elicited in the mPFC by local perfusion of the NET and dopamine transporter inhibitor nomifensine (Masana et al., 2011), whereas noradrenergic denervation, induced by bilateral injection of the neurotoxin 6-OHDA into the LC, suppressed DA output elicited in the mPFC by the NET inhibitor desipramine (Pozzi et al., 1994).

Consistent with these findings, Smith and Green (2012) provided evidence in an *in vitro* slice preparation from mice that noradrenergic fibers from the LC are the major source of DA release in the hippocampus and mediate synaptic transmission attributed to DA. Kempadoo et al. (2016) found that photostimulation of LC axons increased DA release from the noradrenergic nerve terminals in the dorsal hippocampus, suggesting that DA release from noradrenergic terminals mediates synaptic transmission in this region.

The hypothesis that noradrenergic terminals are the main source of DA output elicited by α_2-adrenoceptor blockade in the mPFC does not conflict with the notion that DA is released from dopaminergic terminals in this region by different stimuli, including physiological stimuli.

The possibility that DA is released from noradrenergic terminals poses the important question of its physiological role, which is possibly distinct from that of “orthodox” DA originating from dopaminergic terminals. A better understanding of this issue could suggest the use of highly selective pharmacological tools for controlling DA release in the PFC.

We aimed to clarify the relative contribution of noradrenergic and dopaminergic terminals to α_2-adrenoreceptor-mediated DA output in the mPFC. Therefore, we examined whether central noradrenergic denervation modified the ability of the α_2-adrenoreceptor antagonist atipamezole—singly or combined with nisoxetine (an NET inhibitor)—to enhance DA output in the mPFC. Moreover, to clarify the relative involvement of α_2-autoreceptors and α_2-heteroreceptors in α_2-adrenoceptor blockade, we examined if noradrenergic denervation modified the inhibitory effect of clonidine (an α_2-adrenoceptor agonist) on DA release in the mPFC.

Central noradrenergic denervation was induced by intraventricular injection of the immunotoxin anti-dopamine-ß-hydroxylase saporin (aDBH), which is known to specifically target noradrenergic neurons, unlike other catecholamine neurotoxins (Wrenn et al., 1996; Rohde and Basbaum, 1998). Furthermore, because aDBH-induced destruction of noradrenergic terminals is expected to eliminate NET, which is the principal mechanism

believed to control DA clearance from extracellular fluid in the mPFC (Carboni et al., 1990; Pozzi et al., 1994; Moron et al., 2002), it should also be able to remove putative α_2-adrenoreceptor-mediated inhibitory control by NE on DA release (Gresch et al., 1997; Gobert et al., 1997; Pozzi et al., 1994). Therefore, we also assessed the effect of lesioning noradrenergic neurons on α_2-adrenoceptor-mediated DA output in the mPFC by comparing denervated and control rats after pharmacological blockade of NET and α_2-adrenoreceptors.

2. METHODS

2.1 Animals. All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the animal policies approved by the local Ethical Committee for Animal Experiments (CESA, University of Cagliari). All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects. Male Sprague-Dawley rats (Envigo, S. Pietro al Natisone, Italy), weighing 175–200 g on arrival, were housed in groups of 5 per cage for at least 7 d before use, with standard conditions of temperature and humidity and artificial light from 8 am to 8 pm; food and water were available ad libitum. Experiments were conducted from 9 am to 5 pm. In total, 76 animals were used.

2.2 Noradrenergic denervation. Rats were deeply anesthetized with Equithesin (containing, per 100 ml, 0.97 g pentobarbital, 2.1 g MgSO , 4.25 g chloral hydrate, 42.8 ml propylene glycol, 11.5 ml 90% ethanol; 5 ml/kg, IP) and placed in a Kopf stereotaxic apparatus. The skull was exposed and a hole was drilled aimed to the lateral ventricle (AP -1.0, L ± 1.5 from the bregma, V -4.3 from skull, coordinates according to Paxinos and Watson, 1997) for administration of immunotoxin or control solutions. The aDBH immunotoxin was diluted in a sterile-filtered phosphate buffered saline (vehicle: 140 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4) and injected intra-cerebroventricularly (ICV) in a volume of 5 µl with a 10 µl syringe operated by a CMA/100 microinjection pump (CMA Microdialysis, Stockholm, Sweden) at 1 µl/min during 5 min, followed by 2 min pause before slowly withdrawing the needle. Injections were randomly distributed into either the left and right lateral ventricles. Control rats received the corresponding volume of vehicle, as previous experiments indicated that tissue and extracellular DA and NE values from vehicle-treated rats were not different from values obtained from rats injected icv with vehicle containing saporin or IgG-saporin, or from intact rats (Devoto et al., 2015).

2.3 Histology. Rats were deeply anesthetized with Equithesin and perfused intracardially with 0.9% saline solution (400 ml) followed by 4% paraformaldehyde (pH 7.4) (200 ml). Brains were carefully removed from skull and post-fixed in same fixative overnight at 4°C. Brains were cut in 50 µm thick coronal slices using a cryostat (Microm Cryo-Star HM 560, Walldorf, Germany). Slices were collected in PB for the following free-floating immunostaining. Slices were pre-incubated in 10% normal donkey serum, 1% BSA, and 0.2% Triton X-100 in PBS overnight at 4°C. Mouse anti-DBH primary antibody (Chemicon) (1:800) containing 0.2% Triton X-100, 0.1% BSA, and1% NGS was used, for 48 h at 4°C. After washing, sections were incubated with the secondary antibody donkey anti-mouse Alexa Fluor 594 (1:200) (Molecular Probes) for 1h in the dark at RT. Observation were made using a Leica DMRB microscope.

2.3 Catecholamine detection. aDBH- and vehicle-treated rats were given antibiotic therapy (enrofloxacin, Bayer HealthCare, Shawnee Mission, KS) for 5 days and allowed to recover in their home cages for 15 to 18 days, then they were stereotaxically implanted with vertical microdialysis probes (membrane AN 69-HF, Hospal-Dasco, Bologna, Italy; cut-off 40,000 Daltons, 4 mm active membrane length), in the mPFC [AP +3.0, L ± 0.6, V -6.5 from the bregma, according to the coordinates of Paxinos and Watson (1997)], under Equithesin anesthesia. The day after probe implantation, artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6-6.5) was pumped through the dialysis probes at a constant rate of 1.1 µl/min via a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden) in freely moving animals, and dialysate samples were collected every 20 min. NE and DA were simultaneously analyzed by HPLC with electrochemical detection, by HPLC systems equipped with 3.0 x 150 mm C18 (3.5 µ) Symmetry columns (Waters, Milan, Italy), maintained at 40°C by Series 1100 thermostats (Agilent Technologies, Waldbronn, Germany), and ESA Coulochem II detectors (Chelmford, MA, USA) with microdialysis cells mod. 5014B. The mobile phase consisted of 80 mM Na_2HPO_4, 0.27 mM EDTA, 0.6 mM sodium octyl sulfate, 8% methanol, 3% acetonitrile, pH 2.6 with H_3PO_4, delivered at 0.3 ml/min; the Coulochem analytical cell first electrode was set at +200 mV, the second at -200 mV. Quantification was performed by recording the second electrode signal. Under these conditions, NE and DA detection limits (signal to noise ratio 3:1) were 0.3 pg per injection on column. In 24 out of 32 aDBH injected rats extracellular NE levels fell below the HPLC detection limit. In these animals the actual NE value was therefore in the range of 0 - 0.3 pg. To limit bias due to attribution of a theoretical value to these samples, data were analyzed 3 times with undetectable values set to either 0, 0.15 or 0.3 pg.

Statistical analysis of data indicated the same significant differences in the three simulations, thus we reported data calculated with the 0.15 pg theoretical value. Indeed, according to McKittrick and Abercrombie (2007), the latter approximation is the most realistic, taking into account that the actual value may range between the 2 extremes. Extracellular catecholamine values were expressed as pg per 20 µl sample, not corrected for probe recovery, or as percent of mean basal value.

To evaluate tissue contents of NE and DA, rats were sacrificed by decapitation at least 24 h after the microdialysis experiment (n= 10 denervated and 10 control rats); brains were rapidly removed and placed on a brain cutting block maintained on ice. The mPFC was dissected out from 2 mm slices, immediately frozen on dry ice, and stored at –80° C until processing for catecholamine content. Briefly, tissues were weighed, homogenized by sonication in 0.1 M $HClO_4$ (1:20 weight tissue per solvent volume), centrifuged at 10,000 x g, the supernatant filtered using microspin centrifuge tubes (0.22 µm nylon filter), and 20 µl directly injected into the HPLC, under analytical conditions described for microdialysis experiments. Data were expressed as pg neurotransmitter per mg tissue.

2.4 Drugs. Immunotoxin aDBH was purchased from Advanced Targeting System (San Diego, CA, USA); atipamezole hydrochloride, clonidine hydrochloride and nisoxetine hydrochloride were from Tocris (Bristol, UK), raclopride tartrate was from Sigma-Aldrich. The doses were chosen according to our previous experiments and the data reported in literature, to obtain a full effect (clonidine: Gresch et al., 1995; Devoto et al., 2003; atipamezole: Lapiz et al., 2007; raclopride: Timmerman et al., 1995; nisoxetine: Rothman et al., 2003). All drugs were dissolved in sterile distilled water, and IP administered in a volume of 1 ml/kg body weight.

2.5 Statistical analysis. To compare catecholamine levels in denervated and control animals, significances of inter-group differences were calculated using the unpaired two-tailed Student's t test with Welch's correction, or repeated measure two-way analysis of variance (ANOVA) with lesion as between-subject factor and time as within-subject (repeated measure) factor. Sidak's multiple comparison test was applied as post hoc, when more than two data groups were compared, as detailed in Results. Statistical significance was set at $P< 0.05$. Statistical elaboration of data employed the program Prism 7.0b for Mac (GraphPad Software Inc., San Diego, CA, USA).

2. RESULTS

DBH immunostaining in the frontal cortex revealed almost total loss of DBH immunoreactivity in lesioned vs. vehicle-treated rats (Sup. Fig. 1).

Supplementary Figure 1

Representative microphotograph of DBH-positive fibers in the prelimbic cortex in (a) vehicle- and (b) aDBH-treated rats (obj. 20×).

Table I shows that aDBH treatment depleted tissue and extracellular basal NE level to approximately 5% and 10% of the control value, respectively, but failed to modify tissue DA content. It also increased extracellular basal DA level to approximately 170% of the control value (Table I). Values for tissue NE and dialysate NE dropped below the detection limit in 5 of 10 (50%) and 24 of 32 (75%) aDBH-treated rats, respectively; both values were calculated to be 0.15 pg/sample, as explained in the Methods. Statistical analysis (two-tailed, unpaired Student's *t*-test with Welch correction) demonstrated a significant difference between the control and lesioned animals in terms of extracellular basal NE ($t = 8.452$, $df = 41.72$, $P < 0.0001$), basal DA ($t = 4.361$, $df = 58.29$, $P < 0.0001$), and tissue NE ($t = 42.12$ $df = 9.155$, $P < 0.0001$) levels but not in terms of tissue DA level ($t = 0.6793$ $df = 15.43$, $P = 0.5070$).

TABLE I.

Treatment	Tissue		Dialysate	
	Norepinephrine	Dopamine	Norepinephrine	Dopamine
Control	484.6 ± 11.4 (10)	98.3 ± 5.0 (10)	2.54 ± 0.27 (41)	1.73 ± 0.15 (44)
aDBH	2.8 ± 1.1* (10)	92.1 ± 7.7 (10)	0.25 ± 0.04* (32)	2.9 ± 0.22* (32)

Tissue and extracellular basal catecholamine levels in the medial prefrontal cortex of sham-operated (control) and lesioned (aDBH) rats. Tissue values are expressed in pg/mg tissue and are the mean ± SEM values of 10 rats. Dialysate

levels are the mean ± SEM values of basal values obtained from the number of rats specified in parentheses and are expressed as pg/sample.
* $P < 0.0001$ vs. control (two-tailed, unpaired Student's *t*-test with Welch correction).

The effects of nisoxetine and atipamezole on extracellular NE and DA levels in the control and denervated rats were then examined.
Nisoxetine (3 mg/kg IP) increased extracellular NE and DA levels by approximately 400% and 200%, respectively, in the control rats (n = 6; Fig. 1a and b), whereas it showed no effect in the denervated rats (n = 5; Fig. 1a and b). Two-way repeated measures analysis of variance (ANOVA), using lesion as a between-subject factor and time as a within-subject factor, indicated a significant effect of lesion for NE ($F_{(1, 9)} = 10.2$, $P = 0.0110$) and DA ($F_{(1, 9)} = 13.9$, $P = 0.0047$) and a significant time × lesion interaction (NE: $F_{(5, 45)} = 4.73$, $P = 0.0015$; DA: $F_{(5, 45)} = 3.53$, $P = 0.009$).

Figure 1

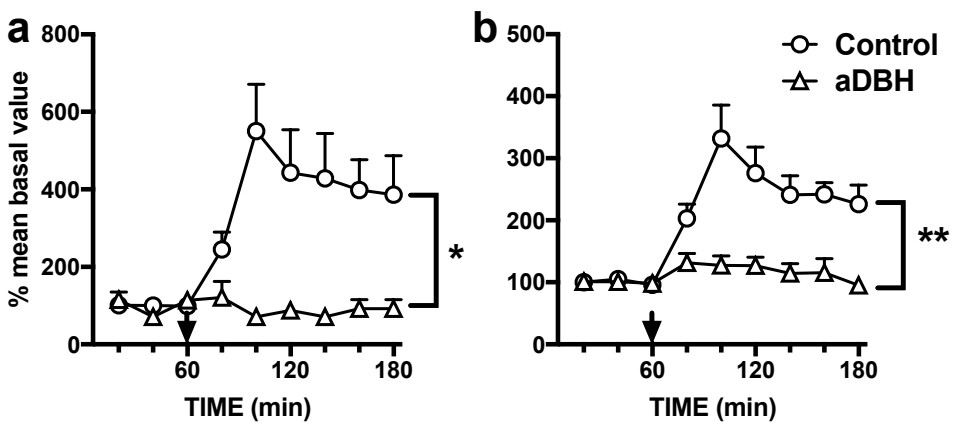

Figure 1. Effect of nisoxetine (3 mg/kg IP) on extracellular norepinephrine (a) and dopamine (b) levels in vehicle-treated (control) and antiDBH-saporine-lesioned (aDBH) rats
The drug was administered at the time indicated by arrows. Values (mean ± SEM) are expressed as the percentage of the mean basal level.
* $P < 0.05$; ** $P < 0.01$ (two-way ANOVA)

The effects of atipamezole (3 mg/kg IP) on catecholamine levels were different in the intact and denervated rats (Fig. 2). Two-way ANOVA for repeated measures showed a significant effect of lesion for NE ($F_{(1, 10)} = 21.6$, $P = 0.0009$) and DA ($F_{(1, 10)} = 6.61$, $P = 0.0278$), a insignificant time × lesion interaction for NE ($F_{(5, 50)} = 1.03$, $P = 0.411$), and a significant time × lesion interaction for DA ($F_{(5, 50)} = 3.54$, $P = 0.0081$).

Indeed, atipamezole increased extracellular NE and DA levels by approximately 150% each in the control rats (n = 7) (one-way ANOVA, NE: $F_{(1.91, 11.46)} = 10.96$, $P = 0.0024$; DA: $F_{(2.165, 12.99)} = 10.64$, $P = 0.0016$) but did not affect extracellular NE level (one-way ANOVA, $F_{(1.869, 7.477)} = 8.836$, $P = 0.0114$), whereas they produced a slight (approximately 30%–50%) but significant increase in DA level in the denervated rats (n = 5).

Figure 2

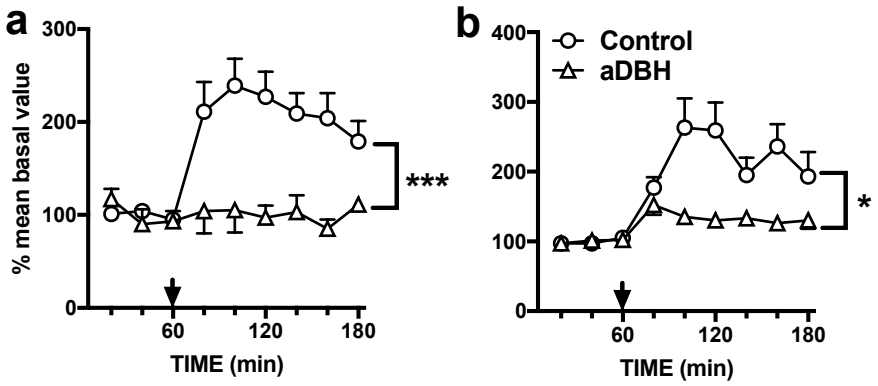

Figure 2. Effect of atipamezole (3 mg/kg IP) on extracellular norepinephrine (a) and dopamine (b) levels in vehicle-treated (control) and antiDBH-saporine-lesioned (aDBH) rats

The drug was administered at the time indicated by arrows. Values (mean ± SEM) are expressed as the percentage of the mean basal level.

* $P < 0.05$ (two-way ANOVA)

In the control rats, the combined administration of nisoxetine and atipamezole (3 mg/kg each, IP) produced a 2100% and 600% increase of NE and DA levels, respectively, (n = 7), whereas in the denervated animals (n = 6), the catecholamine levels were slightly affected (Fig. 3). Two-way ANOVA for repeated measures, using lesion as a between-subject factor and time as a within-subject factor, indicated a significant effect of lesion for NE ($F_{(1, 11)}$ =

8.49, $P = 0.0141$) and DA ($F_{(1, 11)} = 9.59$, $P = 0.0102$) and a significant time × lesion interaction (NE: $F_{(5, 55)} = 6.68$, $P < 0.0001$; DA: $F_{(5, 55)} = 6.81$, $P < 0.0001$).

Figure 3

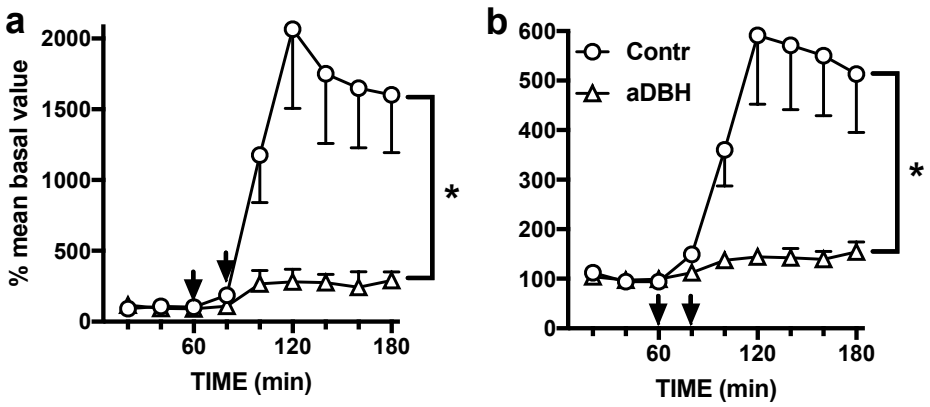

Figure 3. Effect of nisoxetine (3 mg/kg IP, first arrow) plus atipamezole (3 mg/kg IP, second arrow) on extracellular norepinephrine (a) and dopamine (b) levels in vehicle-treated (control) and antiDBH-saporine-lesioned (aDBH) rats
Values (mean ± SEM) are expressed as the percentage of the mean basal level.
* $P < 0.05$ (two-way ANOVA)

To clarify the relative contribution of α_2-autoreceptors and α_2-heteroreceptors to the atipamezole-induced increase of extracellular DA level, the effect of clonidine on extracellular DA level in the denervated and control rats was compared. In the control rats, systemically and locally administered clonidine (0.15 mg/kg IP, n = 7) infused through a dialysis probe into the mPFC (0.1 μM, n = 7) reduced extracellular NE level by 90% and 60%, respectively, and extracellular DA level by 50% and 60%, respectively (Fig. 4a and b). By contrast, in the denervated rats (n = 5), IP and locally administered clonidine infused into the mPFC failed to modify extracellular DA level (Fig. 4a and b). Because extracellular basal NE level was below the detection limit, no effect of clonidine could be detected in these animals (data not shown). Two-way ANOVA of DA results demonstrated no time × lesion interaction for the IP administered ($F_{(3, 36)} = 2.47$, $P = 0.0778$) and perfused groups ($F_{(3, 27)} = 1.48$, $P = 0.243$), but there was a significant effect of the lesion in the IP ($F_{(1, 12)} = 14.5$, $P = 0.0025$) and locally administered animals ($F_{(1, 9)} = 20.5$, $P = 0.0014$).

Figure 4

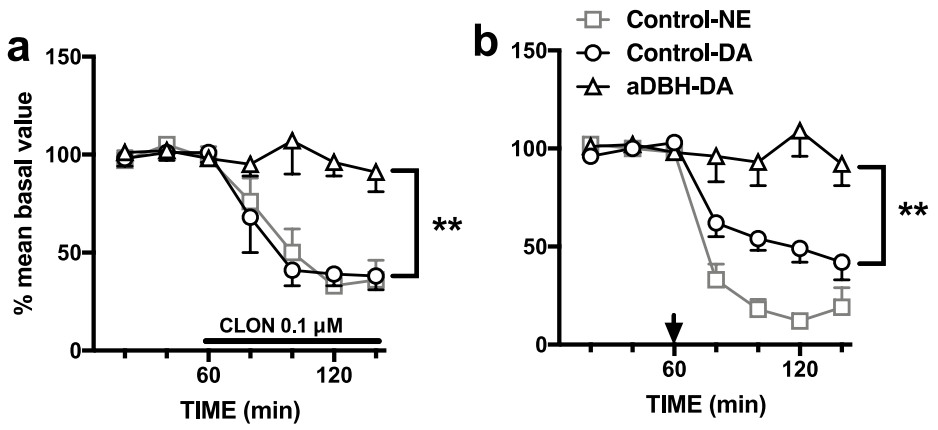

Figure 4. Effect of clonidine locally infused into the mPFC (0.1 µM, a) or systemically injected (0.15 mg/kg IP, b) on extracellular dopamine levels in vehicle-treated (control) and antiDBH-saporine-lesioned (aDBH) rats and on extracellular norepinephrine level in the control rats

The horizontal bar represents the time of clonidine perfusion, and the arrow indicates the time of IP administration. Values (mean ± SEM) are expressed as the percentage of the mean basal level.

** $P < 0.01$ (two-way ANOVA)

To evaluate the possibility that noradrenergic denervation reduced the capacity of dopaminergic neurons to release DA, the effect of the D_2-DA receptor antagonist raclopride (Racl; 0.5 mg/kg, IP) in the control and denervated rats was assessed. As previously shown (Linner et al., 2002; Masana et al., 2011), raclopride alone had no effect in the control rats (n = 4), but it increased DA output by 380% following NET inhibition with nisoxetine (n = 6, Fig. 5). By contrast, in the denervated rats (n = 6), consistent with the loss of NET, raclopride increased extracellular DA level by 230%, i.e., the levels were slightly, but not significantly, lower than those in the control rats following NET blockade (Fig.5). Indeed, two-way ANOVA demonstrated a significant interaction ($F_{(10, 65)} = 7.26$, $P < 0.0001$) and effect of lesion ($F_{(2, 13)} = 8.73$, $P = 0.0039$), and post hoc Sidak's test indicated a significant difference between Control-Racl and aDBH-Racl ($P < 0.05$) and Control-Nis+Racl ($P < 0.01$) curves, but not between aDBH-Racl and Control-Nis+Racl curves.

Figure 5

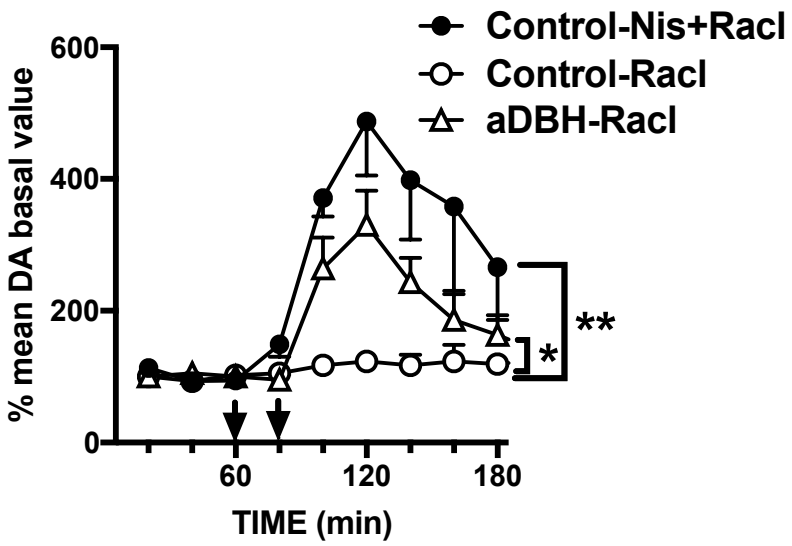

Figure 5. Effect of raclopride (Racl, 0.5 mg/kg IP, second arrow) alone or with nisoxetine (Nis, 3 mg/kg IP, first arrow) on extracellular dopamine in vehicle-treated (control) and antiDBH-saporine-lesioned (aDBH) rats
Values (mean ± SEM) are expressed as the percentage of the mean basal level.
* $P < 0.05$, ** $P < 0.01$ vs. Control-Racl (Sidak's multiple comparison test).

3. DISCUSSION

In apparent contrast to our hypothesis that DA is co-released with NE from noradrenergic terminals, representing the majority of extracellular DA concentration in the mPFC (Devoto et al., 2001; Devoto and Flore, 2006; Devoto et al., 2008; Devoto et al., 2015), loss of noradrenergic neurons failed to reduce tissue DA level but increased extracellular DA level by approximately 70% in the mPFC.
However, because denervation should have eliminated both NET and the presence of NE at α_2-adrenoceptors and, extracellular DA level in the denervated rats should be compared with that in control rats after concomitant blockade of α_2-adrenoceptors and NET. Accordingly, while in the control rats the concomitant inactivation of NET and α_2-

adrenoceptors produced a remarkable increase in NE and DA output (Gobert et al., 1997; Wortley et al., 1999; Masana et al., 2011), in denervated rats nisoxetine failed to modify extracellular DA level, and atipamezole produced only a modest increase in extracellular DA level, consistent with the loss of NET and the lack of NE at α_2-adrenoceptors.

The 10-fold difference in extracellular DA level between the denervated and control rats after blockade of NET and α_2-adrenoceptors possibly represents the contribution to DA output from noradrenergic terminals. Accordingly, the finding that atipamezole-induced DA output was substantially reduced by noradrenergic denervation suggests that noradrenergic terminals are the primary source of DA release after α_2-adrenoceptor block, and that α_2-autoreceptors, not α_2-heteroreceptors, mediate DA output induced by α_2-adrenoceptor antagonists in the mPFC. Consistent with this hypothesis, clonidine, which has been shown to predictably reduce extracellular DA level in the mPFC of control rats (Gresch et al., 1995; Devoto et al., 2004), had no effect in the denervated rats in which α_2-autoreceptors had been eliminated.

However, this interpretation does not eliminate the possibility that α_2-heteroreceptors, which are highly expressed in mesoprefrontal DA neurons (Castelli et al., 2016), could exert state-dependent modulation of cortical DA release from dopaminergic terminals. Indeed, as mentioned above, the effect of atipamezole on cortical DA release was not completely suppressed by noradrenergic fiber lesioning, suggesting that atipamezole removed an inhibitory action by DA on α_2-heteroreceptors, for which DA presents high affinity (Cornil et al., 2002; Zhang et al., 2004; Cornil and Ball, 2008; Guiard et al., 2008). Future experiments should verify whether clonidine and atipamezole modify DA release in denervated rats when dopaminergic neurons are stimulated by raclopride.

Notably, the hypothesis that noradrenergic fibers provide the major contribution of DA release induced by α_2-adrenoceptor antagonists does not preclude the possibility that DA output from mesocortical dopaminergic neurons is elicited by other stimuli, such as blockade of D2-DA autoreceptors. Accordingly, NE denervation did not inhibited DA output elicited by raclopride. Actually, the D2-DA antagonist increased extracellular DA level only after NET inhibition in the control rats, while, given alone, it increased extracellular DA in the denervated rats to the same level as in the control rats after NET inhibition. Remarkably, the apparent failure of the D2-DA antagonist to increase DA output in the control rats when administered alone implies that increases in DA level actually released from dopaminergic terminals in the mPFC are not detected in the dialysate owing to the effective DA uptake by NET.

An alternative interpretation of our results is suggested by the reports from other laboratories showing that noradrenergic denervation of the PFC reduces DA release in the nucleus accumbens elicited by amphetamine in rats (Lategan et al., 1990), by amphetamine and morphine in mice (Ventura et al., 2003; Ventura et al., 2005), by milk chocolate in food-restricted mice (Latagliata et al., 2018), and by restraint stress in rats (Pascucci et al., 2007). These results could be explained by the hypothesis that noradrenergic transmission in the mPFC is requested to facilitate different stimuli to activate DA release by mesolimbic neurons into the nucleus accumbens. Thus, the possibility that noradrenergic depletion in the mPFC can silence mesoprefrontal dopaminergic neurons by inhibiting their DA release capacity cannot be completely disregarded.

Contrary to this possibility, Ventura et al. (2003) found that noradrenergic denervation in the mPFC, while suppressing amphetamine-induced DA release in the nucleus accumbens, failed to modify amphetamine effect in the mPFC. Moreover, Guiard et al. (2008b) found that a selective loss of NE neurons plus 6-OHDA injection in the LC enhanced the firing activity of VTA neurons in rats, consistent with a net inhibitory control by NE input on VTA DA neurons. Notably, the present finding that denervation did not inhibit but rather enhanced DA output elicited by raclopride support the idea that noradrenergic denervation does not reduce the DA release potential of dopaminergic terminals in the mPFC.

Although numerous studies have addressed the modulatory influence of LC noradrenergic projections on midbrain dopaminergic activity (Grenhoff et al., 1995; Paladini and Williams, 2004; Guiard et al., 2008; Goertz et al., 2015; Park et al., 2017), no study, to the best of our knowledge, has specifically analyzed the effect of LC lesion or stimulation on the electrophysiological activity of mesoprefrontal dopaminergic neurons, a distinct neuronal population with unique molecular and electrophysiological properties (Lammel et al., 2008). Future research is needed to address this issue. A better understanding of the relative contribution of DA release from noradrenergic and dopaminergic terminals in the action of α_2-adrenoceptor agonists and antagonists may highlight novel strategies for controlling dopaminergic function in the PFC.

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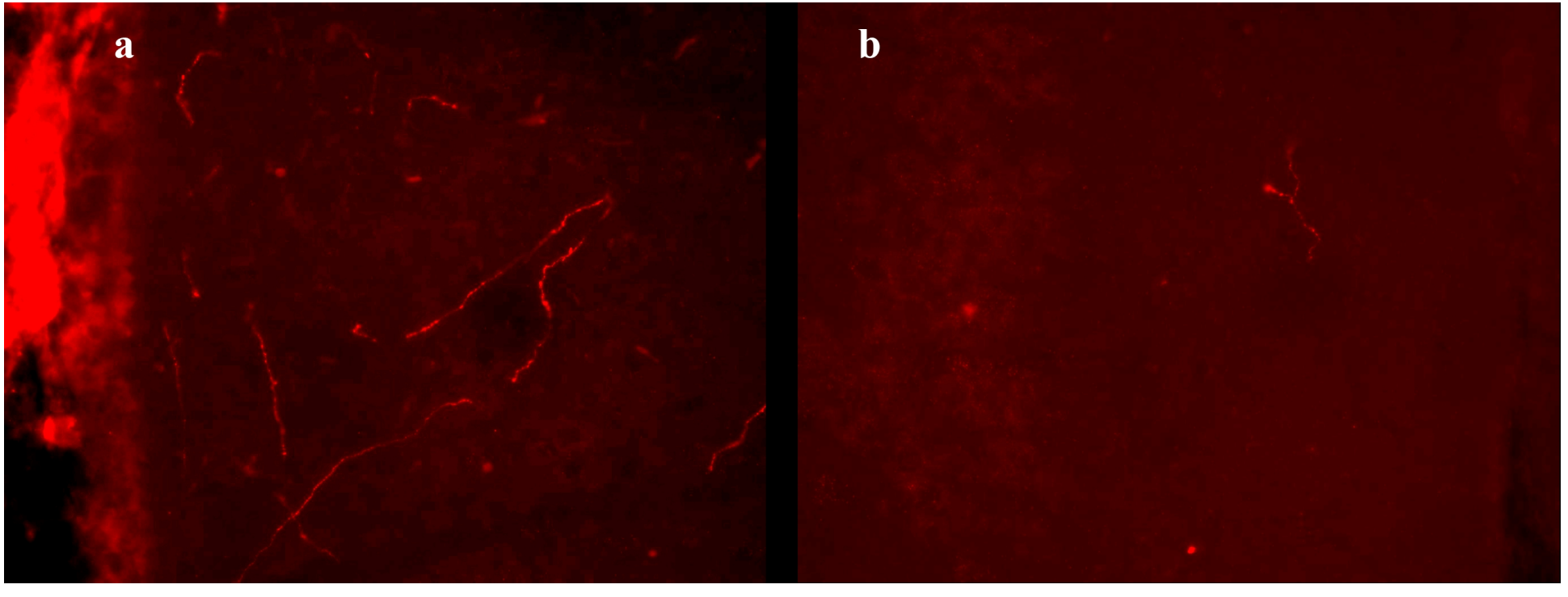
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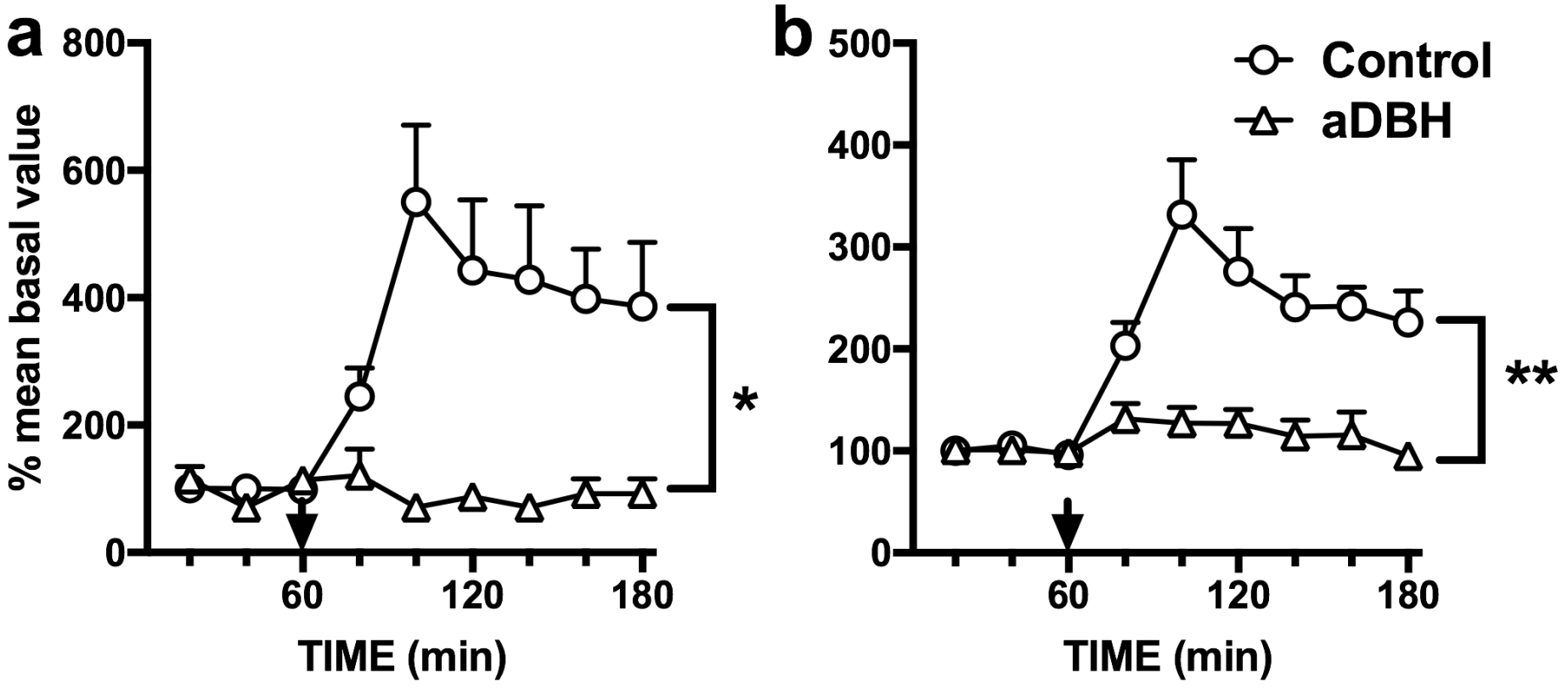
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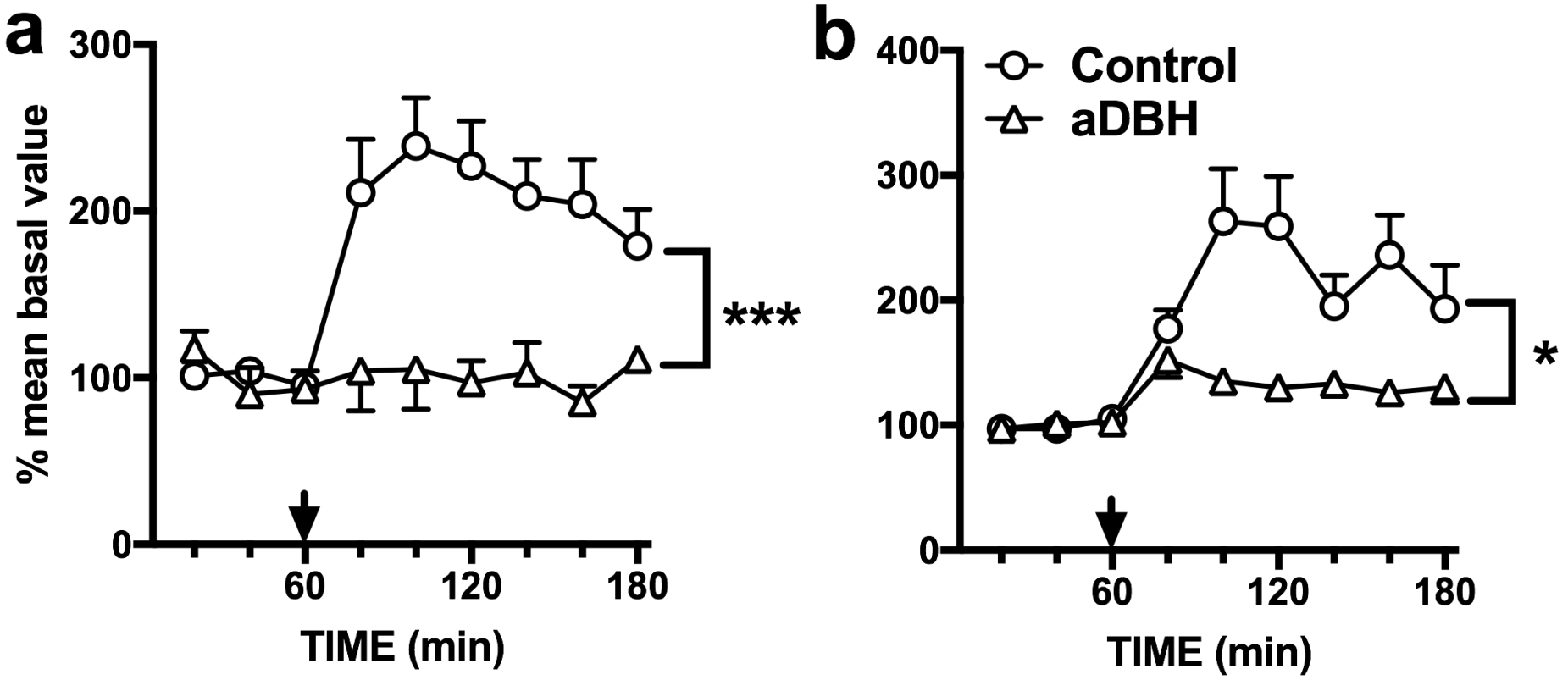
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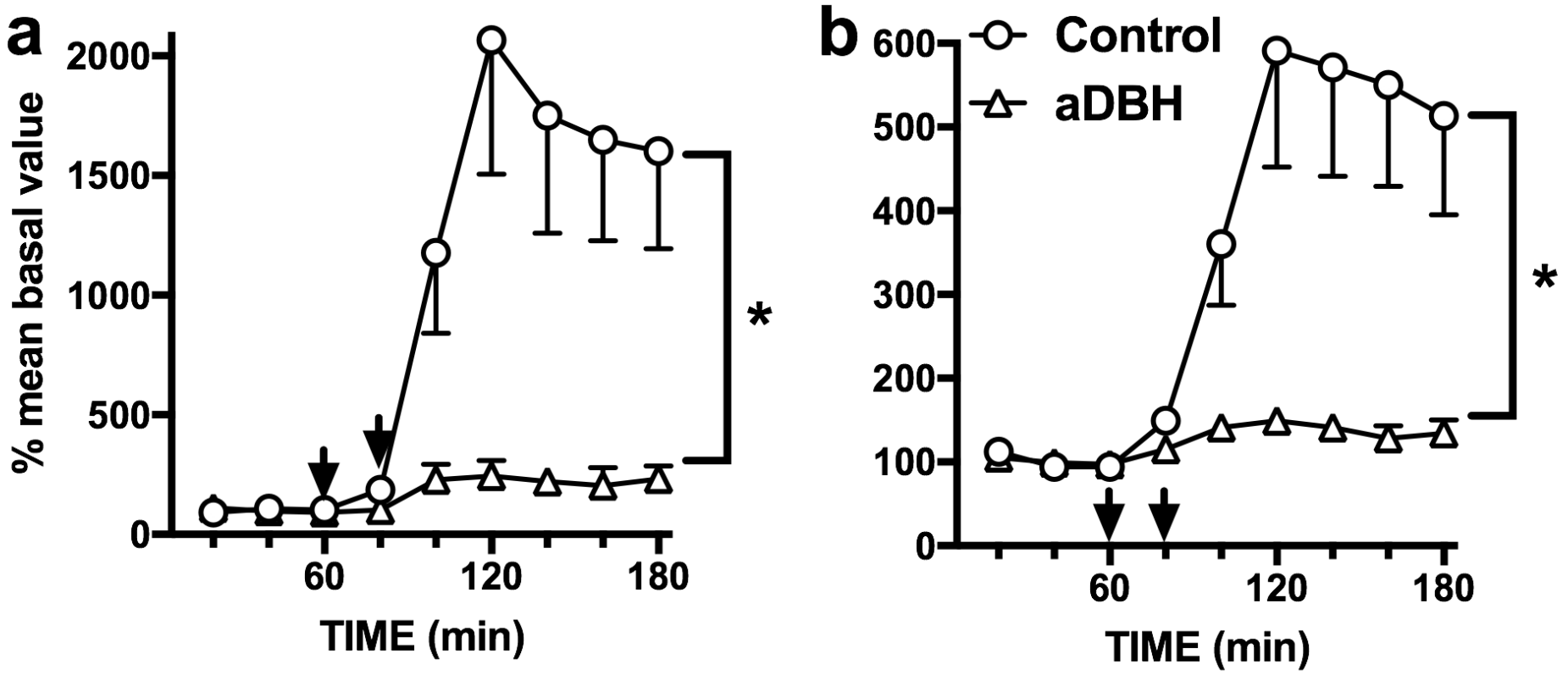
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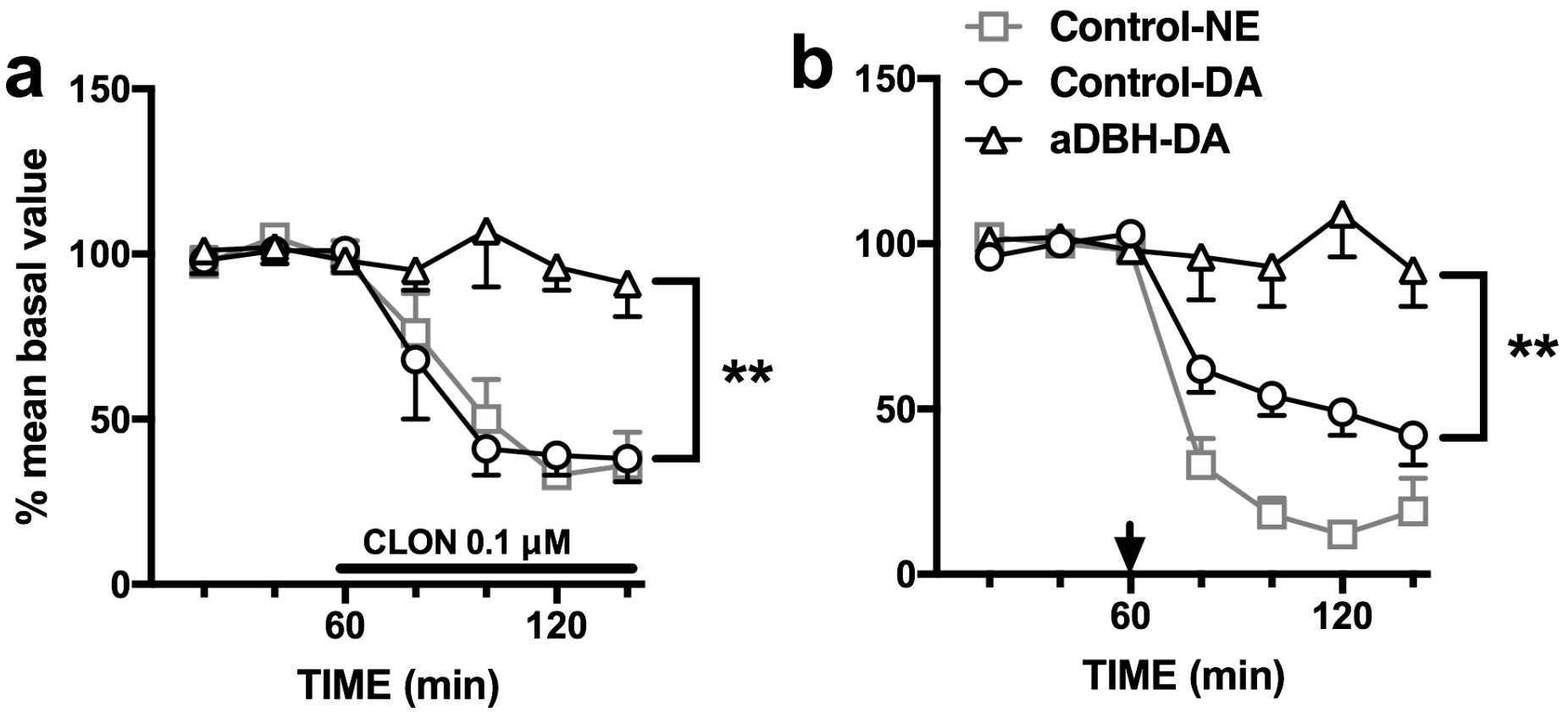
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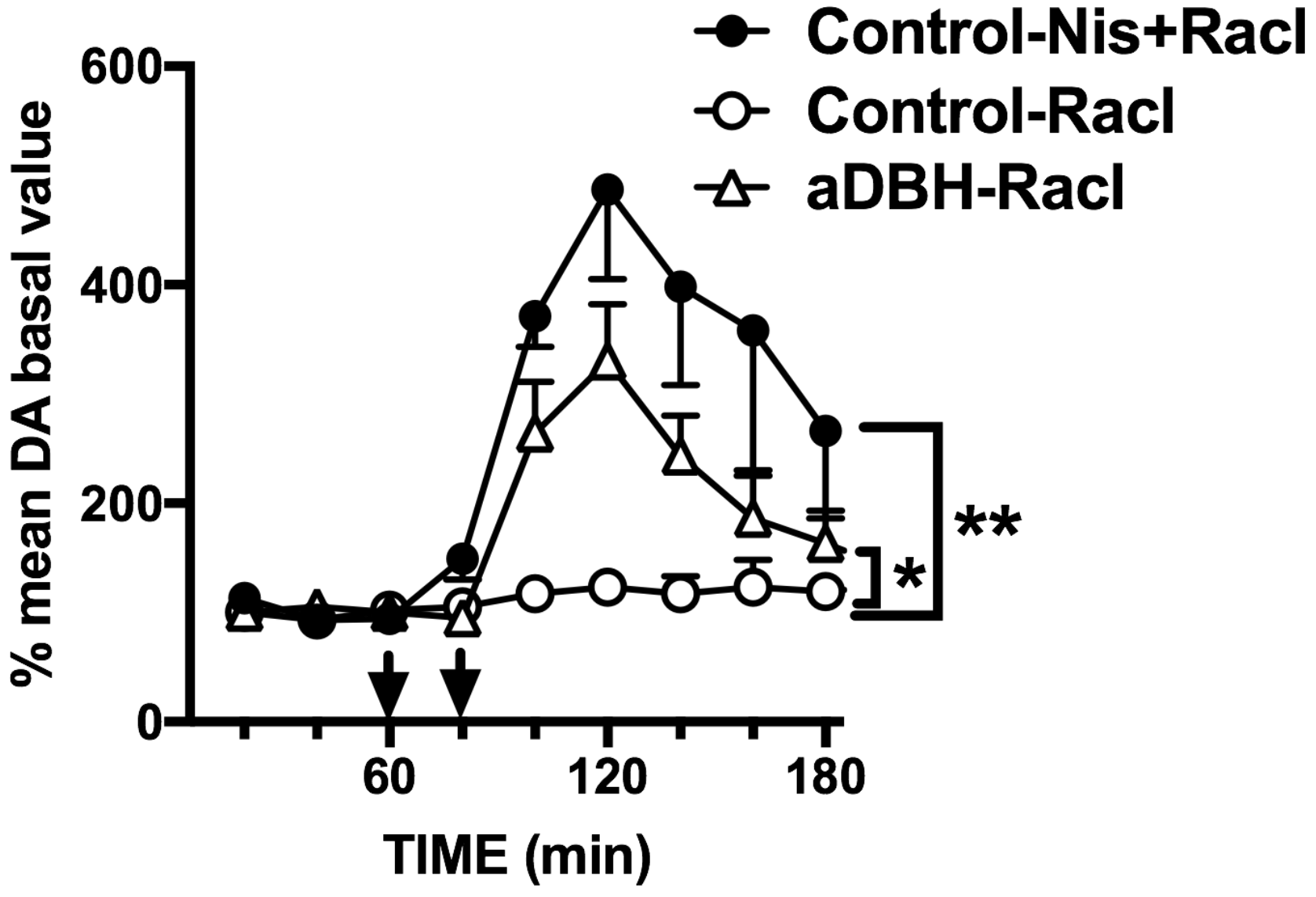
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Ethical statement

All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the animal policies approved by the local Ethical Committee for Animal Experiments (CESA, University of Cagliari). All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.