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RESEARCH ARTICLE

A novel reuptake inhibitor, IP2015, induces erection by increasing central dopamine and peripheral nitric oxide release

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Background and Purpose: An estimated 40% of patients with erectile dysfunction have a poor prognosis for improvement with currently available treatments. The present study investigated whether a newly developed monoamine transport inhibitor, IP2015, improves erectile function.

Experimental Approach: We investigated the effects of IP2015 on monoamine uptake and binding, erectile function in rats and diabetic mice and the effect on corpus cavernosum contractility.

Key Results: IP2015 inhibited the uptake of 5-HT, noradrenaline and dopamine by human monoamine transporters expressed in cells and in rat brain synaptosomes. Intracavernosal pressure measurement in anaesthetized rats revealed that IP2015 dose-dependently increased the number and the duration of spontaneous erections. Whereas pretreatment with the dopamine D_2-like receptor antagonists, clozapine and (−)-sulpiride, or cutting the cavernosal nerve inhibited IP2015-induced erectile responses, the phosphodiesterase type 5 inhibitor sildenafil further enhanced the IP2015-mediated increase in intracavernosal pressure. IP2015 also increased the number of erections in type 2 diabetic db/db mice. Direct intracavernosal injection of IP2015 increased penile pressure, and in corpus cavernosum strips, IP2015 induced concentration-dependent relaxations. These relaxations were enhanced by sildenafil and blunted by endothelial cell removal, a nitric oxide synthase inhibitor, N^G-nitro-L-arginine and a D_1-like receptor antagonist, SCH23390. Quantitative polymerase chain reaction (qPCR) showed the expression of the dopamine transporter in the rat corpus cavernosum.

Conclusion and Implications: Our findings suggest that IP2015 stimulates erectile function by a central mechanism involving dopamine reuptake inhibition and direct NO-mediated relaxation of the erectile tissue. This novel multi-modal mechanism of action could offer a new treatment approach to erectile dysfunction.

Abbreviations: DAT, dopamine transporter; ICP, intracavernosal pressure; L-NOARG, N^G-nitro-L-arginine; MAP, mean arterial pressure; NAT, noradrenaline transporter; PDE5, phosphodiesterase type 5; SERT, serotonin transporter.

KEYWORDS
dopamine, dopamine transporter, endothelium, erection, IP2015, mice, rats

1 | INTRODUCTION

Patients with erectile dysfunction with co-morbidities may be resistant to the currently recommended treatments with phosphodiesterase type 5 (**PDE5**) inhibitors and/or combinations with other oral treatments (Hatzimouratidis et al., 2016; Munk et al., 2019; Nehra et al., 2012). Although PDE5 inhibitors are effective in many patients with erectile dysfunction, 40% of afflicted men attain sub-optimal treatment (Goldstein et al., 2012; Porst et al., 2013). A higher success rate can be obtained by injection therapy with a combination of drugs capable of relaxing penile smooth muscle, but currently, there is no alternative efficacious peroral treatment to PDE5 inhibitors (Hatzimouratidis et al., 2016; Munk et al., 2019). Accordingly, restoring altered signal transduction and underlying pathophysiological mechanisms in the genital tissue can effectively treat erectile dysfunction (Comerma-Steffensen, Carvacho, et al., 2017; Gruenwald et al., 2012; Haahr et al., 2018; Luttrell et al., 2008). However, comorbid neurological disease, diabetes mellitus and severe vascular disease may also negatively affect the central pathways modulating erectile function (Gratzke et al., 2010). Moreover, various medications used for treating CNS disorders, such as antidepressants, antipsychotics, anti-epileptics and anxiolytics, can negatively alter erectile function and libido (Gratzke et al., 2010; Murru et al., 2015) with a predisposition to affect more men than women (Young et al., 2015). Therefore, selectively targeting perturbed central pathways or the development of molecules which can act on central pathways and erectile tissue may uniquely counteract erectile dysfunction.

Dopaminergic neurotransmission is required for attaining or maintaining a penile erection sufficient for successful intercourse (Melis et al., 2022; Zhang et al., 2021). Dopamine is the main catecholamine of the CNS and plays an important role in regulating the activity of different anatomical areas in the brain, including the medial preoptic area, supraoptic and paraventricular nucleus, which are key anatomical components of sexual function (Melis et al., 2022; Zhang et al., 2021). There are two major dopamine receptor families, D_1-like (**D_1**, **D_5**) and D_2-like (**D_2**, **D_3**, **D_4**) receptors, both of which are associated with the regulation of erectile function (d'Emmanuele di Villa Bianca et al., 2005; Matsumoto et al., 2005; Sibley, 1999). Accordingly, systemic administration of the non-selective dopamine receptor agonist **apomorphine**, when administered systemically to male rats, induces penile erection (Sanna et al., 2011). However, it has a narrow therapeutic window and is often associated with side effects such as nausea in patients (Heaton, 2001). These latter issues have been mitigated using subtype-selective compounds, and a role for central dopamine D_2 and D_4 receptors that mediate erection in rats has been described (Brioni et al., 2004; Melis et al., 2022; Sanna et al., 2011, 2015). In contrast, D_1 receptor activation shows relaxant effects in human corpus cavernosum strips (d'Emmanuele di Villa Bianca et al., 2005; Matsumoto et al., 2005).

What is already known

- An estimated 40% of patients with erectile dysfunction have no improvement with currently available treatments.

What does this study add

- The monoamine reuptake inhibitor, IP2015, stimulates erection via a central dopaminergic mechanism and NO-mediated peripheral relaxation.

What is the clinical significance

- The perspective is that this novel drug approach can be used to treat erectile dysfunction.

The antidepressant drug **bupropion** not only inhibits the dopamine uptake transporter (**DAT**) but also inhibits the noradrenaline uptake transporter (**NAT**) and is a nicotinic receptor antagonist (Stahl et al., 2004). Bupropion is recommended for treatment-emergent sexual dysfunction induced by opiates and anti-depressive drugs (Taylor et al., 2013). In contrast, other antidepressant drugs that inhibit NAT or the 5-HT transporter (**SERT**) are associated with sexual dysfunction (Taylor et al., 2005) Moreover, DAT knockout leads to faster mounting and intromission in rats (Sanna et al., 2020). Therefore, the present study hypothesized that a drug that preferentially inhibited DAT would have pro-erectile effects. IP2015 (pudafensine, NS18313) is a newly developed monoamine transport inhibitor that also inhibits dopamine uptake (Peters et al., 2011). Here we have evaluated the effects of this compound on monoamine uptake and behavioural locomotor measurements in mice, erectile responses in anaesthetized rats and diabetic mice, and isometric tension in penile erectile tissue from rats.

2 | METHODS

2.1 | Animal care and preparation

All animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US

National Institutes of Health (NIH Publications No. 85-23, revised 1996) and followed the ARRIVE guidelines (Lilley et al., 2020; Percie du Sert et al., 2020), and they were performed with permission from the Danish authorities (permissions 2011/561-2011, 2014-15-2934-01059, and 2019-15-0201-00009).

Male Wistar rats (150–250 g; Taconic M&B, Ry, Denmark) were used for in vitro uptake and in vivo 5-HT and noradrenaline binding studies. Female NMRI mice (22–25 g; Harlan, Netherlands) were used for in vivo [^{3}H]**WIN35428** binding studies and assessing effects of IP2015 on locomotor activity. Animals were allowed at least 7 days of acclimatization to the laboratories before use. NMRI mice were housed in groups of eight, 20-week diabetic db/db mice were in groups of two and rats were in groups of four in Macrolon-III cages contained in Scantainers (Scanbur A/S, Denmark) under a 12 h light/dark cycle (lights on 06:00 h) with free access to food (standard laboratory pellets) and tap water. Experiments were performed between 9:00 and 16:00 h in temperature and humidity-regulated rooms (22–24°C, relative humidity: 60% to 70%).

Adult male Wistar rats (12–15 weeks old) were used for all remaining experiments. The animals were killed humanely by cervical dislocation and exsanguinated by decapitation. They were kept and cared for in standard cages under clean conditions in separate quarters in a 12–12 h light-to-darkness cycle with free access to water and chow pellets.

2.2 | In vivo functional experiments

2.2.1 | Mouse locomotor activity

Female mice were used for locomotor studies, as they are sensitive and require less exposure to molecules that can modulate brain dopaminergic transmission than male animals (Lynch, 2018). Moreover, they can be group-housed for longer periods than male mice. For the experiment, mice ($n = 6$–7 per group) were placed individually in transparent cages (30 × 20 × 25 cm) for 30 min. The activity chambers had infrared sensors (6 × 2) arranged along the bottom of each arena wall (TSE Systems, Bad Homburg, Germany). Locomotor activity was monitored automatically in the chambers and was measured as the interruption of two consecutive infrared sensors. Interruptions of infrared sensor pairs were detected by a control unit and registered by a computer equipped with the relevant software (ActiMot, TSE Systems, Bad Homburg, Germany). Each mouse was placed in the locomotor chamber immediately after peroral or subcutaneous administration of IP2015.

2.2.2 | Measurements of intracavernous pressure (ICP) and mean arterial pressure (MAP)

The surgery was non-recovery with a duration of approximately 3 h, and the anaesthetized animals were killed by cutting the carotid artery at the end of the experiment. Surgical technique and placement of systemic and corporeal pressure monitoring catheters for in vivo erectile physiology studies were performed as previously described for rats and mice (Comerma-Steffensen et al., 2022; Kun et al., 2009). Briefly, rats were anaesthetized with buffered pentobarbital sodium (Sygehus Apoteket, Aarhus, Denmark; 50 mg·kg^{-1}) given intraperitoneally. During the experiment, rats breathed spontaneously, and body temperature was monitored continuously and maintained at 37°C. With a midline incision in the perineum, the base of the penis, enclosed by striated muscles, was exposed. The ischiocavernosus muscle covering the crus corpus cavernosum was divided on one side, allowing entrance to the underlying tunica albuginea. A 25-gauge needle attached to a heparinized (100 IE·mL^{-1}) polyethylene catheter was inserted into the crus corpus cavernosum to measure ICP. A heparinized polyethylene catheter (PE 50) was introduced into the carotid artery to measure MAP. Continuous direct measurements of MAP and ICP were performed with transducers (Disposable BP Transducer, ADInstruments, UK) and registered and analysed on a computerized data acquisition system (PowerLab, ADInstruments). A stabilizing period of 20–30 min was allowed before registration of basal ICP and MAP.

Through a lower abdominal incision, the cavernous nerve was isolated at the lateral aspect of the prostate, and electrical stimulation was performed with a slender bipolar platinum electrode, which was connected to a S48 stimulator (Grass Instrument Co., Boston, MA, USA). To measure the maximum amplitude of the erectile response, a first stimulation of the cavernous nerve (square wave pulses of 6 V, 10 Hz, 1 ms pulse duration for 30 s) was performed (Kun et al., 2009).

IP2015, **fluoxetine**, **sildenafil**, **clozapine**, **(−)-sulpiride** or vehicle were administered by jugular vein injection in maximum volumes of 200 µL. IP2015 and fluoxetine were injected at doses of 0.1 and 1 mg·kg^{-1} intravenously. The observation period of spontaneous erection after injection of IP2015 or fluoxetine was 30 min. Clozapine (1 mg·kg^{-1}), (−)-sulpiride (20 mg·kg^{-1}) or sildenafil (1 mg·kg^{-1}) were administered 30 or 10 min prior to 1 mg·kg^{-1} IP2015 injection, respectively. One group was injected with a vehicle alone.

Mechanical denervation was performed to investigate the involvement of proximal neuronal pathways in mediating the effect of IP2015. The isolated cavernous nerve was cut distal to the major pelvic ganglion for mechanical denervation. The absence of erectile response to electrical stimulation verified the efficacy of mechanical denervation. The vehicle control experiments for clozapine and mechanical denervation were the same, as the animals were randomly allocated to infusion of vehicle, mechanical denervation or clozapine.

2.3 | In vitro functional experiments

2.3.1 | Monoamine uptake and binding assays

Three independent experiments were performed in human embryonic kidney (HEK293, ATCC, #Cat CRL-1573, RRID:CVCL_0045) cells stably expressing the 5-HT transporter (hSERT) and noradrenaline transporter (hNAT), respectively. The dopamine transporter (hDAT) was

overexpressed in Chinese hamster ovary cells (ATCC, #Cat CCL-61, RRID:CVCL_0214). A neurotransmitter transporter assay was used following the providers' instructions (Molecular Devices, San Jose, California, USA). The cells were seeded in 96-well plates, and the uptake of fluorescence in each cell line was measured in the presence of specific inhibitors of SERT, NAT and DAT, respectively, **citalopram hydrobromide**, **nisoxetine** and benzothiophenylcyclohexylpiperidine (BTCP) each 1 μM and fourfold serial dilutions, 10 points in duplicate. To measure the effect of IP2015, the plated cells were exposed to 10 μM IP2015 or vehicle in fourfold serial dilution with 10 points in duplicate.

SERT-, NAT- and DAT-containing synaptosomes were prepared from cerebral cortices, hippocampi and striatum, respectively, freshly dissected out from eighteen 8-week-old male Wistar rats (150–200 g; Taconic, Ejby, Denmark). In addition to the effect of IP2015, the effect of **fluoxetine**, **reboxetine** and **duloxetine** on monoamine reuptake was examined. The methods followed the protocols described elsewhere (Andreasen et al., 2013), described in detail in the Supporting Information.

2.3.2 | Functional studies in isolated erectile tissue

The penis was removed by cutting the crura corpora cavernosa at the point of adhesion to the lower pubic bone, and the corpora cavernosa were then dissected free. The penis was submerged immediately in ice-cold (4°C) PSS. The tunica albuginea was carefully opened from its proximal extremity of the corpus cavernosum towards the penile shaft, and the erectile tissue within the corpus cavernosum was microsurgically dissected free. Preparations with and without endothelium were investigated. As previously described, the endothelium was removed mechanically by initially rubbing the strips between the thumb and the index finger for 20 s (Comerma-Steffensen, Carvacho, et al., 2017). After rinsing in chilled PSS, the corpus cavernosum strips were gently rolled across a dry paper towel to generate shear forces across the endothelial surfaces of the lacunar spaces and then mounted. Changes in isometric tension of corpus cavernosum strips ($0.5 \times 0.5 \times 3$ mm) were investigated in a tissue organ bath system (750TOBS, Danish Myotechnology, Aarhus, Denmark). Silk ligatures were applied at both ends of the strip preparations, which were then suspended between two L-formed metal prongs in thermostatically controlled organ baths (10 mL, 37°C) containing PSS aerated with a mixture of 5% CO_2 in air (pH 7.4). The bath fluid was routinely changed every 20 min and replaced with fresh PSS, also kept at 37°C. During an equilibration period of 60 min, tension was adjusted until a mean stable tension of 1.2 mN and 1.5 mN was obtained for rat and mouse corpus cavernosum, respectively, as described earlier (Comerma-Steffensen, Kun, et al., 2017; Kun et al., 2009).

Electrical field stimulation (EFS) was performed with platinum electrodes (J.P. Trading, Aarhus, Denmark), secured in plastic heads on both sides of the mounted segment, placed approximately 2 mm from the strips. The electrodes were connected to an electrical stimulator (CS200, Danish Myotechnology, Aarhus, Denmark) with submaximal constant current output adjusted only to provide **tetrodotoxin** (10^{-6} M)-sensitive responses (Simonsen et al., 2008). The cumulative effect of drugs was investigated in strips contracted with 16 Hz EFS (100 mA, 1 ms pulse duration, 20 s) applied with 5 min intervals.

To test the contractility of the preparations, they were exposed to a potassium physiological saline solution (KPSS) of 125 mM and **phenylephrine** (10^{-6} M). The absence and the presence of the endothelium were evaluated by adding **acetylcholine** (10^{-6} M) in phenylephrine-contracted preparations.

To investigate the effect of IP2015 on corpus cavernosum strips, the drug (10^{-9}–3×10^{-4} M) was administered to preparations maintained at baseline tension or contracted with phenylephrine (10^{-6} M) in the absence or presence of N^G-nitro-L-arginine (L-NOARG, 10^{-4} M); a NO synthase inhibitor, sildenafil (10^{-7} M); a PDE5 inhibitor or **guanethidine** (10^{-5} M); a blocker of adrenergic neurotransmission; a dopamine D_1 receptor antagonist, **SCH23390**; and a dopamine D_2-like receptor antagonist, clozapine (10^{-6} M). The effect of vehicles was also investigated (results not shown).

To investigate whether there is a vasodilatory effect of IP2015 in genital arteries, rat pudendal arteries were mounted in microvascular myographs using a protocol previously described for intracavernosal arteries (Kun et al., 2009; see Supporting Information).

2.4 | Tissue expression studies

Tissue was collected from the cerebellum, substantia nigra and corpus cavernosum tissue, and it was stored in RNA later (Sigma-Aldrich) until extraction and purification of total RNA were performed using the RNeasy Mini Plus Kit (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies).

The quantitative polymerase chain reaction (qPCR) was performed in a MX3005 qPCR system (Agilent Technologies). The sequence of primers and probes for SERT, NAT and DAT are provided in Table S1. The samples were run for a 40-cycle protocol. Ct values for the gene of interest were normalized against Ct values for the housekeeping gene (GAPDH), after quantification with the program MxPro v.4.10 (Stratagene, Agilent Technologies). Values are expressed as a ratio of GAPDH.

2.5 | Data analysis and statistics

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). The declared group size is the number of independent values, and statistical analysis was done using these independent values in the present study. The animals were randomized to generate equal group sizes, and for studies of erectile function allocated to vehicles or increasing doses of IP2015, only two doses were administered, starting with the lowest dose followed by a higher one, which excluded blinding. The animals were randomized to vehicles or active antagonists for the antagonist studies, and the observer was blinded.

For the erectile response, the ratio of peak intracavernosal pressure (PICP) (mmHg)/MAP (mmHg) × 100 was measured, with PICP being the peak value reached by ICP during the erectile response and MAP during the erectile response. A spontaneous erection was identified when the ICP exceeded 25 mmHg and expressed as frequency, while the duration of the response was measured from the start till the end of the spontaneous erection. The magnitude, frequency and duration of the erectile responses were calculated and presented.

The mechanical responses of the corpus cavernosum strips were measured as force and expressed in mN. The magnitude of in vitro responses is given as a percentage of the contraction or relaxation level without or with the addition of phenylephrine. The effects of IP2015 on EFS-evoked contractions are expressed as percentages of previous control contractions.

All results are expressed as means ± SE means. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. The declared group size is the number of independent values, and statistical analysis was done using these independent values in the present study. Data reported for in vitro uptake and binding studies are means ± SEM ($n = 3$–4, independent experiments). For the in vivo binding studies, data are presented as a mean of one to two independent experiments with groups of three mice in each experiment. Drug effects on erectile responses and effects of IP2015 on locomotor activity were tested using one-way ANOVA followed by Dunnett's and Bonferonni's tests, respectively. Post hoc tests were conducted only if F in ANOVA achieved the necessary level of statistical significance and there was no significant variance inhomogeneity. The models' assumptions were investigated by inspecting Q–Q plots, and data were logarithmically transformed when necessary to generate a Gaussian-distributed data set. Sample size calculations based on a pilot study considering drug intervention on the effects of 1 mg·kg^{-1} IP2015 on erectile function ($\alpha = 0.05$; $\beta = 0.8$) estimated that three animals in each group would be needed for a 20% change. Outliers were included in the data analysis and presentation. The concentration–response curves are presented on a semi-logarithmic scale from each concentration or by obtained E_{max} or pD_2 of IP2015. Differences in concentration–response relationships between treatments were analysed using two-way ANOVA or paired Student's t test for E_{max} or pD_2. Differences at the $P < 0.05$ level were considered statistically significant. All statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, CA).

2.6 | Materials

For in vitro, in vivo, and ex vivo uptake studies, [^{3}H]5-HT (27.7 Ci·mmol^{-1}), [^{3}H]noradrenaline (13.8 Ci mmol^{-1}), [^{3}H]dopamine (38.7 Ci mmol^{-1}), and [^{3}H]WIN35,428 (85.9 Ci mmol^{-1}), [^{3}H]5-HT (21 Ci mmol^{-1}), [^{3}H]5-NA (36 Ci· mmol^{-1}), and [^{3}H]dopamine (9.4 Ci·mmol^{-1}) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Benztropine and desipramine were purchased from Sigma-Aldrich (Vallensbæk Strand, Denmark) and Research Biochemicals International (Natick, MA, USA), respectively. Citalopram was purchased from Actavis (Gentofte, Denmark). IP2015 (7-[(exo-8-azabicyclo[3.2.1]octan-3-yl)oxy]-3-methoxy-chromen-2-one) was designed by Dan Peters and purchased from Syngene International Limited (Bengaluru, Karnataka, India) (Figure 1). Acetylcholine, clozapine, dopamine, fluoxetine, noradrenaline HCl, L-NOARG, SCH23390, and (−-)-sulpiride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The composition of physiological salt solution (PSS) was NaCl 119 mM, $NaHCO_3$ 25 mM, glucose 5.5 mM, $CaCl_2$ 1.6 mM, KH_2PO_4 1.18 mM, $MgSO_4$ 1.17 mM and EDTA 0.027 mM. Potassium physiological salt solution (KPSS) was PSS with NaCl exchanged for KCl on equimolar basis.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos et al., 2023; Alexander, Fabbro et al., 2023).

3 | RESULTS

3.1 | IP2015 inhibits dopamine uptake and binds to DAT

IP2015 (NS18313) has a coumarin structure (Figure 1a). In three independent experiments in duplicate, we measured activity at rat and human monoamine transporters to assess the in vitro uptake profile of IP2015. The inhibition of hSET, hNAT and hDAT was measured as the effect of IP2015 on fluorescence uptake in HEK293 and CHO cells expressing the respective human transporters. The results showed a concentration-dependent inhibition of DAT and SERT followed by NAT (Figure 1b and Table 1).

In rat synaptosomes prepared from cerebral cortices, hippocampi and striatum, respectively, the uptake of [^{3}H]5-HT and [^{3}H]noradrenaline was inhibited with low nanomolar potency, and the uptake of dopamine in the order of 10-fold lower potency (Figure 1c and Table 1).

To elaborate on the monoamine profile of IP2015 in animals, we used complementary ex vivo and in vivo assays. For ex vivo uptake, IP2015 was administered to mice, and the removed tissue was incubated with the competing radioligand in vitro. For in vivo binding, IP2015 was administered to mice in combination with the competing radioligand before tissue was removed, as this latter approach has been reported to provide a more robust measure of receptor occupancy (Kupar et al., 2001). In these exploratory data, IP2015 inhibited uptake, ex vivo, of 5-HT and noradrenaline to SERT and NET with ED_{50} values = 49 and >45 mg·kg^{-1}, respectively. However, [^{3}H]WIN35428 binding revealed that IP2015 binding to DAT in vivo was 10-fold more potent than effects at SERT and NET ex vivo (Tables 1

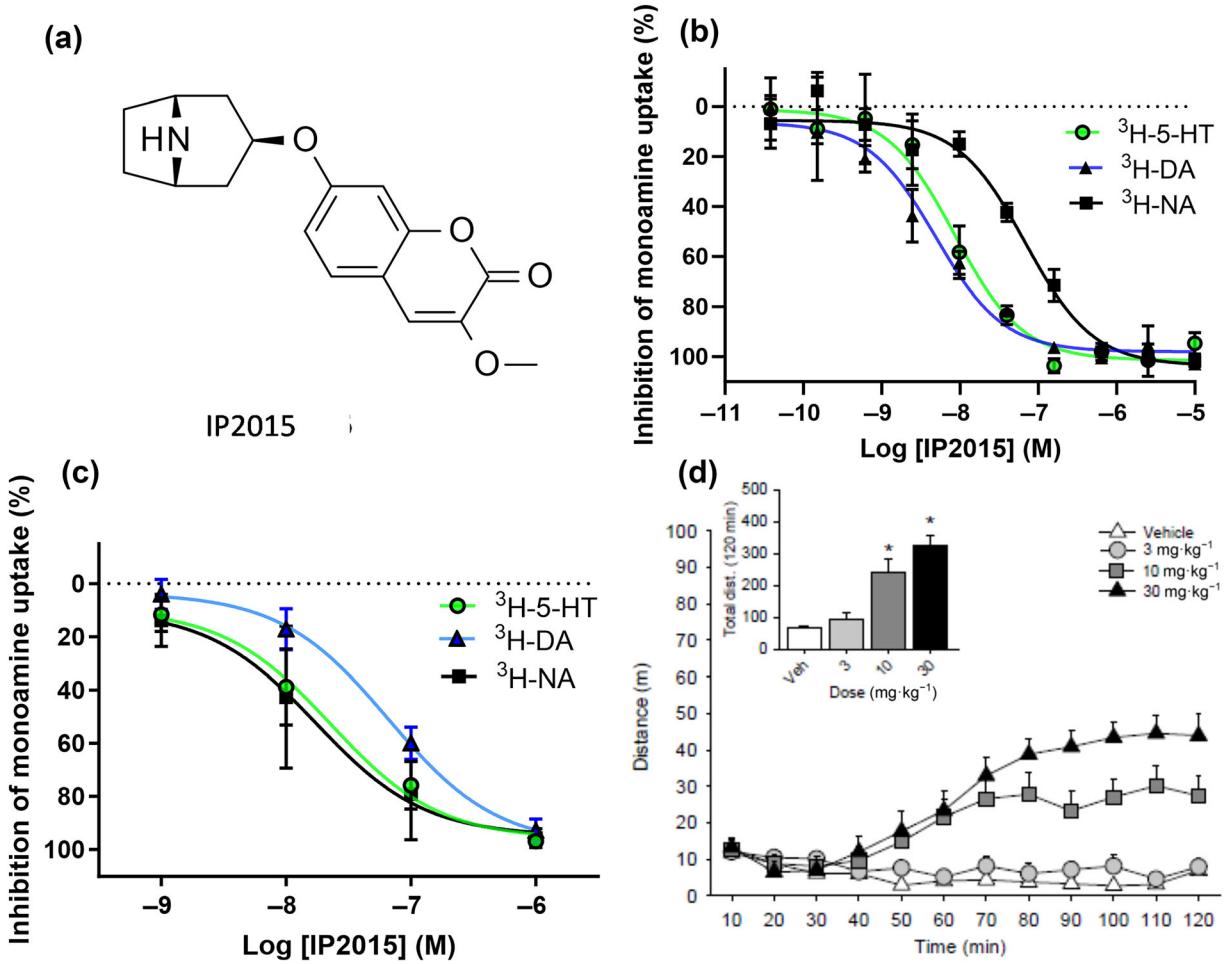

FIGURE 1 Structure, inhibition of monoamine uptake and effect on locomotion in mice of IP2015. (a) Structure of IP2015 (NS18313, H exo-7-[(-8-azabicyclo[3.2.1]octan-3-yl)oxy]-3-methoxy-chromen-2-one hydrochloride). (b) Measurement of IP2015 inhibition of fluorescence uptake in cells overexpressing, respectively, human SERT, NAT and DAT ($n = 3$ independent experiments each in duplicate). (c) Inhibition of 5-HT, noradrenaline and dopamine (^{3}H-5-HT, ^{3}H-NA and ^{3}H-DA) in rat brain synaptosomes ($n = 6$ independent experiments). (d) Normal, uninjured mice were administered peroral vehicle ($n = 7$), IP2015 (3 mg·kg^{-1}, $n = 6$; 10 mg·kg^{-1}, $n = 7$; 30 mg·kg^{-1}, $n = 7$) immediately before being individually placed in motility cages. Automated activity recording was measured as distance travelled (m) every 10 min over a 120 min period. Inset: total distance travelled. Data are presented as means ± SEM. *$P < 0.05$, significantly different from control (Veh); one-way ANOVA followed by Bonferroni t test.

TABLE 1 Inhibition of in vitro and ex vivo monoamine uptake and in vivo dopamine receptor binding by IP2015.

Transporter	In vitro uptake inhibition in cells (nM)	In vitro uptake inhibition in synaptosomes (nM)	Ex vivo inhibition (mg·kg^{-1})
SERT	9.5 ± 1.7	5.1 ± 1.5	49 ± 23
NAT	72.0 ± 21.4	6.2 ± 2.9	>45 ± 21
DAT	3.8 ± 1.7	64 ± 14	4.5[a]

Note: Activity at the 5-HT transporter (SERT), noradrenaline transporter (NAT) and dopamine transporter (DAT) was measured in cells transfected with respective human cDNA and in rat brain synaptosomes. Data reported for in vitro uptake studies in human cells are based on three independent experiments in duplicate, while the uptake in rat synaptosomes ($n = 6$), ex vivo ($n = 2$) and in vivo ($n = 1$) binding studies mean ± SEM.
[a]In vivo study.

and S2). The effects of IP2015 were compared with other monoamine uptake inhibitors, including duloxetine, venlafaxine, fluoxetine and reboxetine (Table S2). The results show that IP2015 differs from these drugs by binding to DAT in vivo at low doses (Table S2). Based on these results, we decided to administer IP2015 up to a maximal dose of 10 mg·kg^{-1} in subsequent in vivo pharmacodynamic studies.

To further confirm the selectivity of IP2015, it was also screened at a concentration of 10^{-5} M in binding assays against a standard

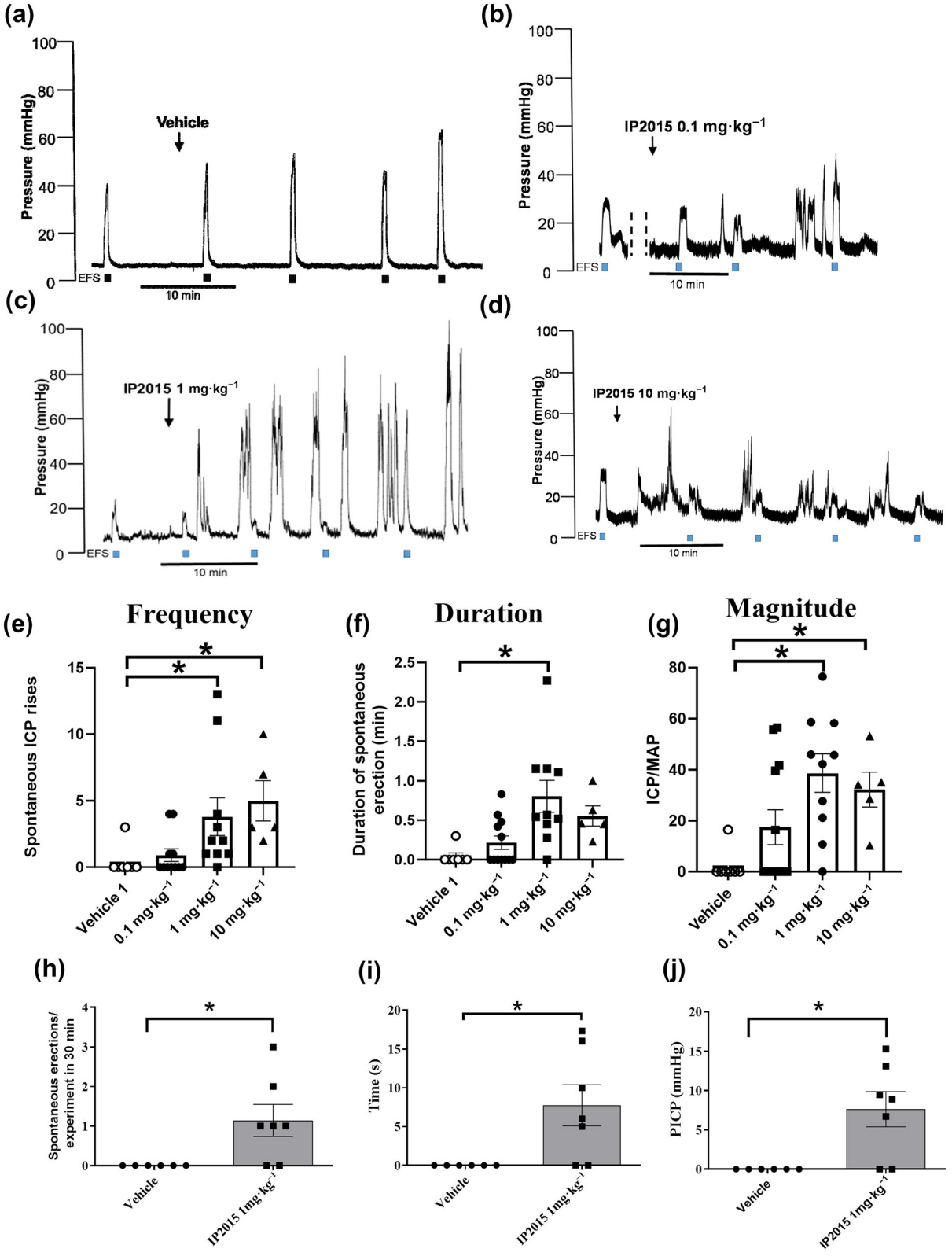

FIGURE 2 Legend on next page.

FIGURE 2 IP2015 increases intracavernous pressure and induces erection in rats and Type 2 diabetic (db/db) mice. (a) Original traces show increased intracavernosal pressure induced by electrical field stimulation (EFS) of the cavernous nerve. Administration of (a) vehicle followed by submaximal EFS 4 min later. Vehicle does not change the pressure, while (b–d) IP2015 administration induces dose-dependent increases in intracavernous pressure (spontaneous erections [SE]) without changing the responses to EFS (3, 13 and 23 min after infusion of drug). (e–g) Frequency, duration and magnitude of spontaneous erections after intravenous infusion of vehicle, IP2015 0.1, 1 or 10 mg·kg^{-1}. The effect on EFS is reported in the supplementary data file. (h–j) Intravenous vehicle infusion and IP2015 (1 mg·kg^{-1}) in diabetic db/db mice. IP2015 increased (h) frequency, (i) duration and (j) the magnitude of spontaneous erectile responses in db/db mice ($n = 7$). The results shown are individual values with means ± SEM. *$P < 0.05$, significantly different as indicated.

panel of G protein-coupled receptors, ion channels and transporters. As expected, IP2015 was found to bind to SERT, NAT and DAT and, to a lesser degree, to the human **α7-nicotinic acetylcholine receptors** (Table S3).

To initially assess the pharmacodynamic actions in conscious animals that were likely to recruit involvement of dopaminergic neurotransmission, IP2015 was assessed in conscious male rats exposed to hormonally primed female rats. As with the dopaminergic agonist, apomorphine, IP2015 decreased rearing and increased immobility frequency (Table S4). Next, microdialysis studies showed that IP2015 increased dopamine, noradrenaline and 5-HT in the cortex and striatum, which indicates that IP2015 is a monoamine reuptake inhibitor in vivo (Figure S1). Finally, we assessed the effects of IP2015 on spontaneous locomotor activity in naïve mice. Hyperlocomotion in this model has been suggested to be a predictor of abuse liability linked to increased dopaminergic neurotransmission in humans (Paterson et al., 2010). Both peroral and subcutaneous administration of IP2015 (3–30 mg·kg^{-1}) produced a marked increase in locomotor behaviour (Figures 1d and S2). A notable difference between the two experiments was that the onset of action was distinctly faster when administered subcutaneously rather than peroral, following a slower entry of IP2015 into the peripheral circulation when administered via the latter route. The minimum effective dose of IP2015 in each case was 10 mg·kg^{-1}, with the total distance travelled by mice treated with the highest dose of IP2015 regardless of whether it was administered peroral (324.4 ± 29.9 m) or subcutaneously (384.8 ± 73.9 m).

3.2 | IP2015 induces spontaneous erections in rats

A mean basal intracavernosal pressure (ICP) of 10.2 ± 1.0 mmHg ($n = 24$) and a mean arterial blood pressure of 117.12 ± 3.2 mmHg ($n = 24$) were recorded at the beginning of the experiments. The maximal amplitude of erection evoked by electrical stimulation of the cavernous nerve at the beginning of the experiments was 71.2 ± 1.5 mmHg ($n = 24$).

In contrast to the vehicle infusion (Figure 2a), administration via the jugular vein of 0.1, 1 and 10 mg·kg^{-1} IP2015 induced transient increases in ICP corresponding to erections (Figure 2b–d). The frequency, duration and magnitude of these responses were increased dose-dependently (Figure 2e–g). The magnitude of erectile responses was characterized by measuring ICP increases expressed as a percentage of MAP and showed they were significantly increased compared with vehicle (Figure 2g). Fluoxetine (1 mg·kg^{-1}), a selective 5-HT uptake inhibitor, was only associated with one spontaneous erection during the observation period in one out of five rats (Figure S3). Fluoxetine and IP2015 did not facilitate erectile responses induced by electrical stimulation of the cavernous nerve in healthy rats (Figures S4). These findings suggest IP2015 induces erection by inhibition of DAT. Infusion of vehicle, IP2015, and fluoxetine did not change MAP (Figure S4).

To further investigate the effects of IP2015 in a simple model of erectile dysfunction, before dissecting its underlying pharmacology in vivo, we assessed its effects in db/db mice, a model of Type 2 diabetes. The db/db mice have decreased erectile function compared with normal C57BL/6 mice and heterozygous db/+ control mice (Comerma-Steffensen et al., 2022). Infusion of IP2015 (1 mg·kg^{-1}) in diabetic db/db mice significantly increased the frequency, duration and magnitude of erectile responses (Figure 2h–j).

To investigate the dopamine receptor subtypes involved in the erectile responses induced by IP2015, the dopamine D_1-like receptor antagonist SCH23390 (0.8 mg·kg^{-1}) was infused but did not significantly alter the effect of IP2015 (Figure 3a–c). However, there was a tendency to reduce the frequency of spontaneous pressure increases (Figure 3a). Infusion of the dopamine D_2 receptor-like antagonist, clozapine (1 mg·kg^{-1}), significantly reduced the frequency and magnitude, while there was no effect on the duration of erectile responses induced by IP2015 (Figure 3d–f). Another D_2 receptor antagonist, (−)-sulpiride, significantly inhibited the frequency of spontaneous erections, while the magnitude and duration were unaltered (Figure 3g–i). Mechanical denervation mediated by cutting the cavernous nerves decreased the magnitude and duration of spontaneous erections observed after IP2015 administration (Figure 3j–l). However, some responses were still present in three out of five rats (Figure 3j–l). These findings suggest that inhibition of DAT by IP2015, followed by activation of central dopamine D_2 receptors, leads to erectile responses, although there can also be a contribution from peripheral dopamine receptors. Upon intracavernosal injection of the vehicle, the penile pressure was unaltered after 10 min (19.6 ± 3.6 vs. 21.6 ± 4.6 mmHg, $n = 4$). The local administration was not associated with a full erection. Injection of IP2015 (0.1 mg·kg^{-1}) in the rat penis increased the ICP from 21.8 ± 2.3 to 33.9 ± 4.5 mmHg ($P < 0.05$, $n = 5$) and was not further increased by the injection of 1 mg·kg^{-1} IP2015.

The phosphodiesterase inhibitor sildenafil improves erection by facilitating erectile responses (Kun et al., 2009). To investigate the effect of sildenafil on IP2015 administration, IP2015 was infused and induced erectile responses, and treatment with sildenafil markedly

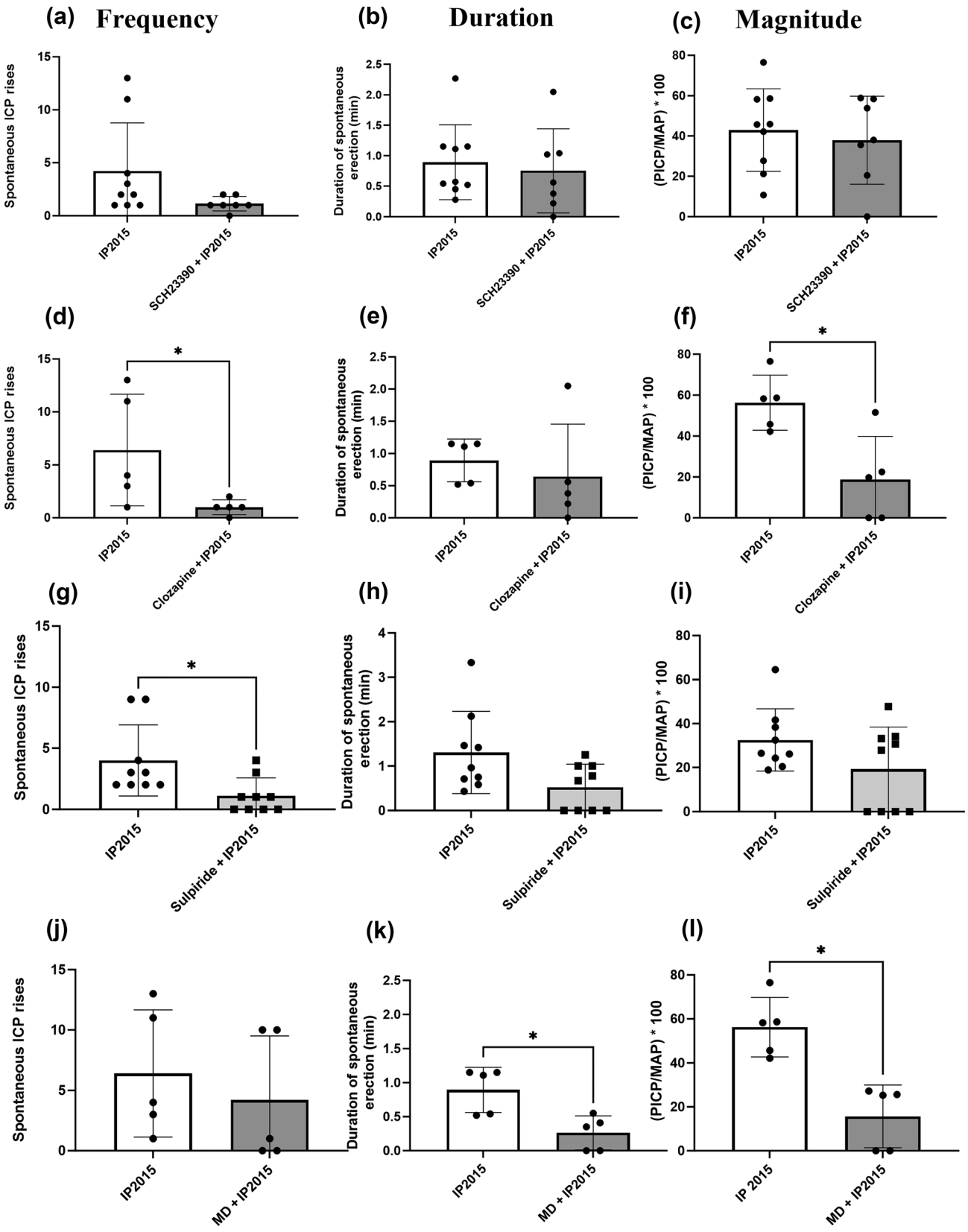

FIGURE 3 Legend on next page.

FIGURE 3 IP2015 increases intracavernous pressure and induces erection in rats by a mechanism involving central dopamine D_2 receptors. Average increases in (a) frequency, (b) duration and (c) intracavernous pressure in response to IP2015 1 mg·kg^{-1} in the absence ($n = 9$) and the presence of the dopamine D_1 receptor antagonist, SCH23390 0.8 mg·kg^{-1} ($n = 7$). Average rises in (d) frequency, (e) duration and (f) intracavernous pressure in response to IP2015 1 mg·kg^{-1} in the absence ($n = 5$) and the presence of clozapine 1 mg·kg^{-1} ($n = 5$). Average rises in (g) frequency, (h) duration and (i) intracavernous pressure in response to IP2015 1 mg·kg^{-1} in the absence ($n = 9$) and the presence of (−)-sulpiride 1 mg·kg^{-1} ($n = 9$). Following mechanical denervation (MD) of the cavernous nerve, average rises in (j) frequency, (k) duration and (l) intracavernous pressure in response to IP2015 1 mg·kg^{-1} are shown. The results shown are individual values with means ± SEM. *$P < 0.05$, significantly different from control.

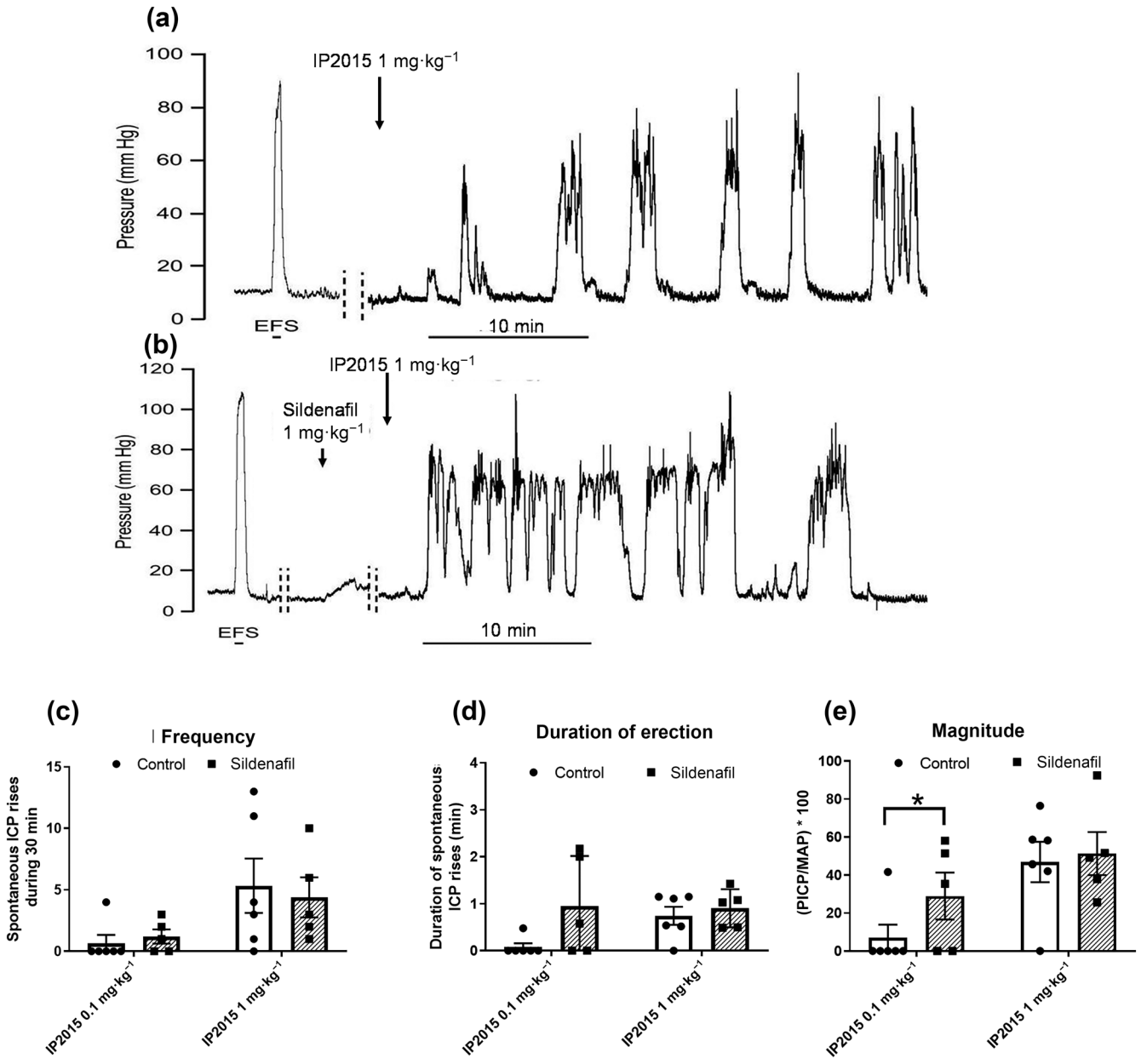

FIGURE 4 Effect of IP2015 and synergy with the phosphodiesterase type 5 inhibitor, sildenafil, on erections. (a) Original traces showing in the upper trace stimulation of the cavernous nerve with maximal stimulation (6 V, 10 Hz) followed by administration of IP2015 (1 mg·kg^{-1}), which 10 min later is followed by rises in intracavernous pressure (ICP). (b) In the lower trace, sildenafil 1 mg·kg^{-1} is administered without inducing spontaneous rises in ICP, while adding IP2015 induces rises in ICP. Below average rises in (c) frequency, (d) duration and (e) magnitude of erectile responses induced by IP2015 in the absence ($n = 6$) and the presence ($n = 5$) of the phosphodiesterase type 5 inhibitor, sildenafil (1 mg·kg^{-1}), in rats. The results shown are individual values with means ± SEM. *$P < 0.05$, significantly different from control.

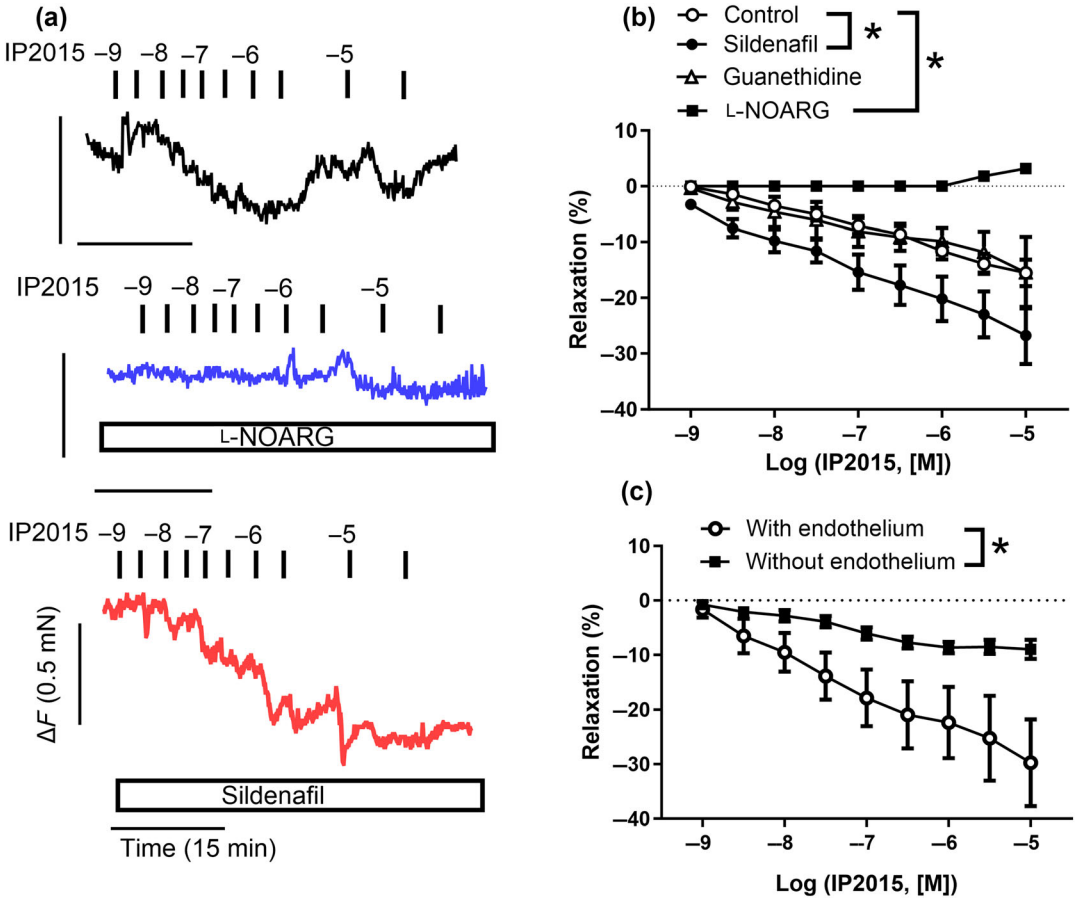

FIGURE 5 IP2015 induces endothelium-dependent relaxations mediated by nitric oxide in isolated rat corpus cavernosum strips. (a) Traces showing that increasing concentrations of IP2015 induce relaxation at baseline tension (upper trace). Responses were also obtained in the presence of N^G-nitro-L-arginine (L-NOARG, 10^{-4} M, middle trace) and sildenafil (10^{-7} M, lower trace). (b) The average concentration–response curves for IP2015 in the absence ($n = 8$) presence of L-NOARG (10^{-4} M, $n = 5$), guanethidine (10^{-5} M, $n = 5$) and sildenafil (10^{-7} M, $n = 8$) at baseline tension. (c) Average relaxations induced by IP2015 in preparations with ($n = 5$) and without endothelium ($n = 5$). The results are means ± SEM. *$P < 0.05$, significantly different from control.

increased the magnitude of erectile responses induced by IP2015 (0.1 mg·kg^{-1}) (Figure 4e). In the presence of sildenafil, the frequency of the erectile responses was difficult to measure (Figure 4), as shown by the original traces (Figure 4a,b). These findings suggest that sildenafil potentiates the effect on the erection of low doses of IP2015.

3.3 | Effect of IP2015 on isolated erectile tissue from rats

After mechanical denervation, the persisting effect of IP2015 on the erectile response prompted further investigation of its actions in erectile tissue. Added at basal tension, IP2015 concentration-dependently induced relaxation in corpus cavernosum strips (Figure 5a). Incubation with the NO synthase inhibitor, L-NOARG (10^{-4} M), abolished the relaxant effect of IP2015, while pretreatment with sildenafil (10^{-7} M) resulted in an enhanced relaxation to IP2015 (Figure 5a,b). Incubation with guanethidine (10^{-5} M) failed to modulate IP2015 relaxations (Figure 5b). IP2015 relaxation was observed in preparations with endothelium but not in preparations without endothelium (Figure 5c). IP2015 relaxations were unaltered in the presence of clozapine but converted to small contractions induced by IP2015 in the presence of the dopamine D_1 receptor antagonist, SCH23390 (Figure 6). These findings suggest that endothelial dopamine D_1-like receptors followed by NO release are involved in the IP2015-induced relaxation of the corpus cavernosum. In rat pudendal arteries, IP2015 induced concentration-dependent relaxations (Figure 7a). IP2015 relaxations were reduced by each of the dopamine receptor antagonists, SCH23390 and clozapine (Figure 7b,c), and abolished in the presence of SCH23390 plus clozapine (Figure 7d,e). IP2015 relaxations of rat pudendal arteries were inhibited in the presence of L-NOARG and indomethacin (Figure S5C,D). These results suggest an involvement of both dopamine D_1- and D_2-like receptors in IP2015 relaxations and that NO mediates these in the pudendal artery.

Dopamine concentration-dependently induced relaxations in corpus cavernosum strips (3×10^{-10}–3×10^{-7} M). Incubation with the NO synthase inhibitor, L-NOARG (10^{-4} M), or a dopamine D_1-like receptor antagonist, SCH23390 (10^{-7} M), abolished the relaxant effect of dopamine (Figure S6).

In preparations contracted with phenylephrine, IP2015 (10^{-9}–10^{-7} M) increased contraction further compared with vehicle-treated

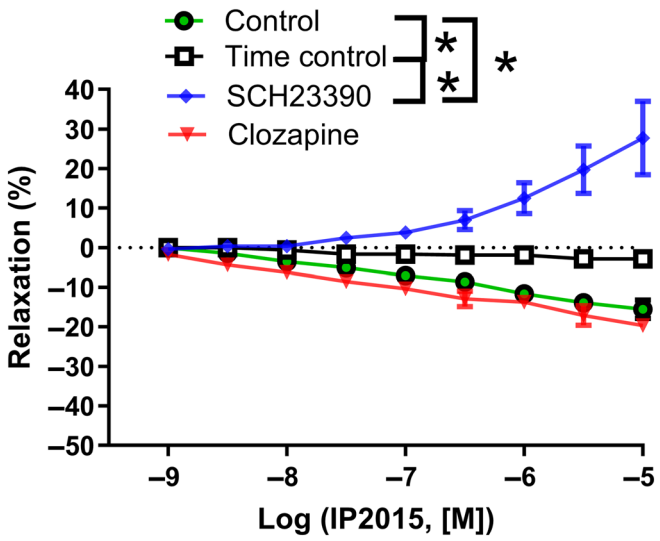

FIGURE 6 IP2015 relaxations are mediated by dopamine D_1 receptors in rat corpus cavernosum. Average IP2015 relaxations were inhibited in the presence of the dopamine D_1 receptor antagonist, SCH23390 ($n = 6$), and unaltered in the presence of the dopamine D_2 receptor antagonist, clozapine ($n = 6$). The results are means ± SEM. *$P < 0.05$, significantly different from control.

preparations, while higher concentrations of IP2015 (3×10^{-7}–3×10^{-5} M) relaxed the preparations back to baseline. Neither L-NOARG nor sildenafil changed these responses (Figure S7).

In phenylephrine-contracted preparations, EFS (16 Hz) induced contraction followed by relaxation, responses that were inhibited in the presence of L-NOARG, while guanethidine inhibited the neurogenic contractions. IP2015 (10^{-9}–10^{-5} M) and sildenafil failed to change the magnitude of these responses to EFS (Figure S8).

To investigate the expression of DAT, NAT and SERT in erectile tissue, we performed qPCR. qPCR revealed the expression of DAT in all isolated tissue, with the most pronounced expression in rat substantia nigra and corpus cavernosum (Figure 8a). SERT and NAT were less expressed in the corpus cavernosum (Figure 8b,c).

4 | DISCUSSION

The novel findings of the present study indicate that IP2015 is a monoamine reuptake inhibitor that induces spontaneous erections in rats. This effect could be blocked by cutting the cavernous nerve and by co-administering the centrally acting dopamine D_2-like receptor

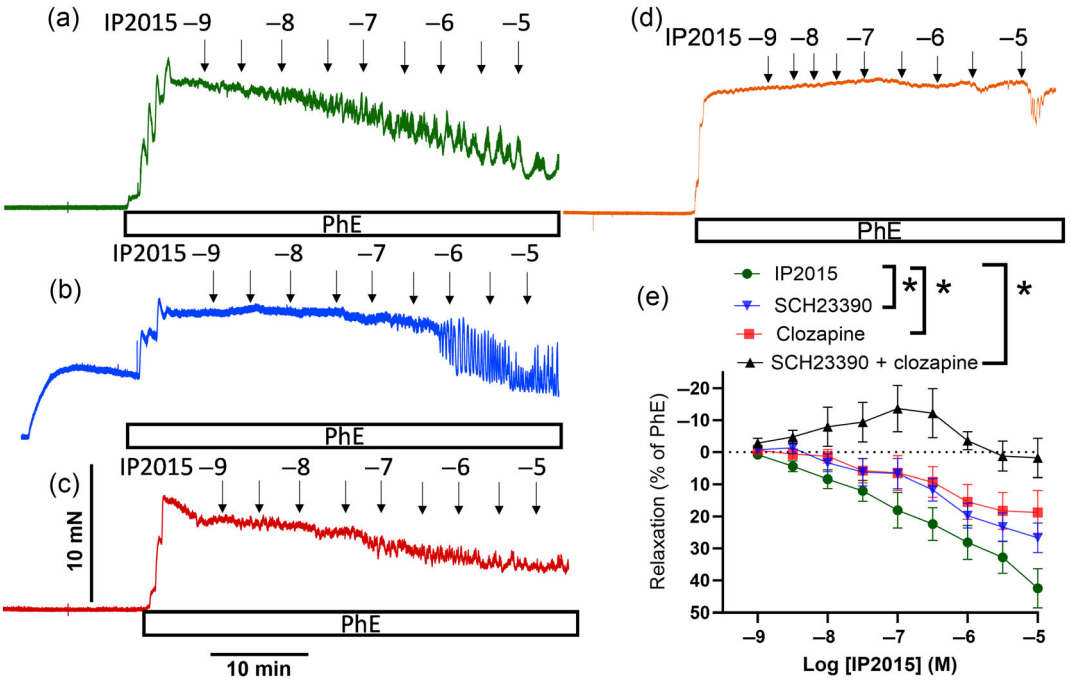

FIGURE 7 IP2015 relaxations are mediated by D_1- and D_2-like receptors in the rat pudendal artery. (a) Trace showing IP2015 induces concentration-dependent relaxations in phenylephrine (3×10^{-6} M PhE)-contracted rat pudendal arteries. (b) The preparation was incubated with the dopamine D_1 receptor antagonist SCH23390 (10^{-7} M), (c) incubated with the dopamine D_2-like receptor antagonist, clozapine (10^{-6} M), and (d) with SCH23390 plus clozapine, then contracted with PhE, and increasing concentrations of IP2015 were added. The vertical scale indicates an increase in force (10 mN), and the horizontal bar indicates time (10 min). (e) The average relaxations induced by IP2015 in the absence ($n = 8$) and the presence of SCH23390 ($n = 8$), clozapine ($n = 6$) and SCH23390 and clozapine ($n = 5$). The results are means ± SEM. *$P < 0.05$, significantly different from the control curve for IP2015.

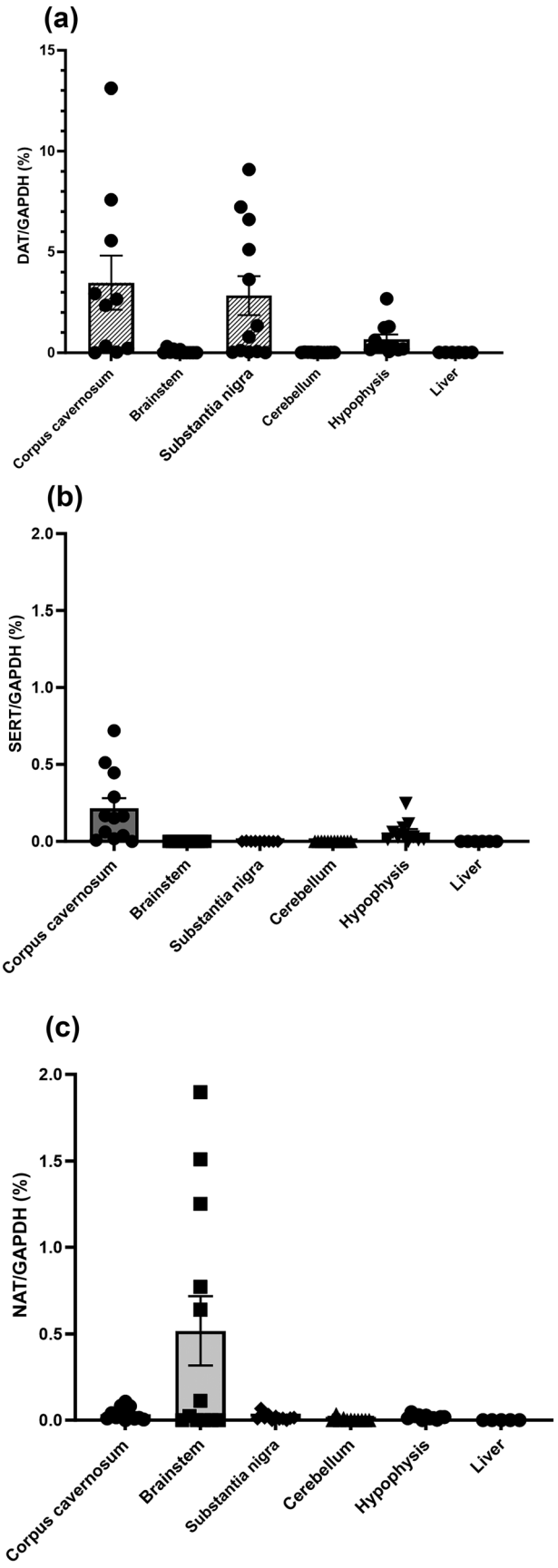

FIGURE 8 qPCR shows the expression of monoamine transporters in the brain and erectile tissue from rats. (a) qPCR for dopamine transporter (DAT), (b) 5-HT transporter (SERT) and (c) noradrenaline transporter (NAT) in different tissues isolated from rats. The results shown are individual values with means ± SEM.

antagonist, clozapine. In isolated corpus cavernosum strips, IP2015 caused relaxations antagonized by the dopamine D_1-like receptor antagonist, SCH23390. IP2015 relaxation was also sensitive to inhibition of NO synthase at baseline tension, while it failed to change neurogenic contractions and relaxations. These results suggest that IP2015 stimulates erectile function by dopamine reuptake inhibition, which then contributes to the stimulation of dopamine D_2-like receptors in the brain and the activation of dopamine D_1-like receptors, followed by the release of NO in the isolated corpus cavernosum.

Inhibitors of monoaminergic transporters are often categorized based on their selectivity for a specific transporter(s) (Aggarwal & Mortensen, 2017). In cell lines overexpressing the human monoamine transporters, the potency of IP2015 was greatest at DAT and SERT, compared with NAT. In contrast, in rat synaptosomes, IP2015 inhibited 5-HT and noradrenaline uptake similarly with nM potency while robustly inhibiting dopamine uptake. Differences in species, the cellular context, transporter localization, expression levels and intracellular trafficking can all contribute to variations in the pharmacological profile of monoaminergic drugs in cells overexpressing transporters versus native tissues such as brain synaptosomes. To help mitigate this discrepancy, we compared our synaptosome data obtained for IP2015 and a number of other reference compounds with that obtained ex vivo/in vivo in rats (see Table S2). We found that reboxetine preferentially inhibits the NAT, fluoxetine the SERT, venlafaxine and duloxetine the SERT and NAT in accordance with their described reuptake profiles (Aggarwal & Mortensen, 2017). We also noted that duloxetine displayed a minor effect on DAT in vitro. IP2015 displayed a similar balanced profile at the SERT and NAT to duloxetine in synaptosomal preparations and ex vivo. Notably, only IP2015 displayed efficacy on the in vivo ^{3}H-WIN35428 binding, used as a marker of DAT inhibition. Together with the observations from the microdialysis studies, where IP2015 increased dopamine, noradrenaline and 5-HT in the cortex and striatum, these findings indicate that IP2015 is a monoamine transport inhibitor with a more dopaminergic profile compared with the other transport inhibitors, fluoxetine, reboxetine, venlafaxine and duloxetine.

Occupancy of brain DAT in the region of 40% to 60% elevates extracellular dopamine levels, with increases in the striatum, including the nucleus accumbens, particularly linked to reporting of drug-induced euphoria in human volunteers (Volkow et al., 2009). In studies in naïve mice, we observed IP2015 (10 and 30 mg·kg^{-1}) induced a similar magnitude of hyperlocomotion. The amount of IP2015 required to cause half-maximal inhibition (ED_{50}) ^{3}H-WIN35428 binding was measured as 4.5 mg·kg^{-1}. These findings agree with the expected DAT occupancy leading to hyperlocomotion and raise the

possibility of abuse liability. However, because the lowest dose of IP2015 (3 mg·kg^{-1}) tested on locomotor activity was inactive, this suggests that DAT occupancy levels are considerably less than those associated with abuse liability. Moreover, the onset of the increase in locomotor behaviour in mice treated even with the highest IP2015 dose (30 mg·kg^{-1}) was slow. These findings suggest that IP2015 should possess a relatively benign adverse effect profile when administered orally. However, the general potential abuse liability aspect for drugs acting on the CNS would need to be addressed as part of the future clinical development of IP2015.

Dopamine is involved in sexual behaviour and controls genital reflexes through dopaminergic projections to the medial optical area (MPOA), the supraoptic and paraventricular nuclei (Andersson, 2011; Baskerville et al., 2009; Melis & Argiolas, 2011). Dopaminergic projections also travel from the caudal hypothalamus with the diencephalospinal dopamine pathway to the lumbosacral spinal cord (Skagerberg & Lindvall, 1985). Moreover, dopamine receptor agonists, such as apomorphine, induce penile erection mainly by activating central dopamine receptors of the D_2-like subtype, including D_2 and D_4 receptors (Brioni et al., 2004; Melis & Argiolas, 2011). In the present study, IP2015 induced dose-dependent increases in intracavernosal pressure, which would appear to be centrally mediated because mechanical cutting of the cavernous nerve markedly decreased the magnitude and the number of spontaneous erections. In contrast to the dopamine D_1 receptor antagonist SCH23390, clozapine, which antagonizes dopamine D_2 and D_4 receptors with high affinity (Meltzer, 1994), markedly reduced the frequency and magnitude of the increases in intracavernosal pressure. The structurally different dopamine D_2 receptor antagonist, (−)-sulpiride (Giuliano et al., 2002), also inhibited the erectile response. These findings suggest that dopamine D_2-like receptors mediate the effect of IP2015. Notably, IP2015 failed to inhibit binding to dopamine receptors (Table S3), confirming a lack of direct dopaminergic receptor modulation. Together with the observations of increased dopamine levels in microdialysis studies, our findings indirectly suggest that IP2015 increases dopaminergic neurotransmission at the supraspinal level by inhibiting DAT, leading to spontaneous erections in rats.

Erectile dysfunction occurs in 46% of men with Type 2 diabetes (Gratzke et al., 2010). Type 2 diabetic db/db mice have the most severe erectile dysfunction compared with heterozygous control and normal C57BL/6 mice (Comerma-Steffensen et al., 2022). Our findings that IP2015 could induce erectile responses reflected by increases in intracavernosal pressure and duration in diabetic mice suggest that IP2015 may improve erectile function in diabetic patients.

After mechanical denervation, infusion of IP2015 still induced some increases in intracavernosal pressure of short duration. These findings suggest a peripheral and direct effect on erectile tissue. When added on baseline tension, IP2015 induced relaxations of the cavernosal strips. Dopamine D_1 and D_2 receptors are expressed in rat corpus cavernosum (Hyun et al., 2002), and apomorphine at high concentrations relaxes corpus cavernosum strips mainly by activating D_1 and D_2 receptors (d'Emmanuele di Villa Bianca et al., 2005). In the present study, IP2015 caused relaxations, which were inhibited by the dopamine D_1-like receptor antagonist, SCH23390, while they were unaltered in the presence of clozapine. The observation that the effect is mediated through dopamine D_1-like receptors is also supported by the observation that SCH23390 also inhibited dopamine relaxation in the corpus cavernosum. Moreover, IP2015 and dopamine relaxations were inhibited in preparations without endothelium and in the presence of an NO synthase inhibitor. Taken together with the observations that IP2015 failed to change the magnitude of neurogenic contractions and relaxations induced by EFS, these findings strongly suggest that IP2015, through inhibition of the DAT in erectile tissue, increases dopamine followed by activation of dopamine D_1-like receptors and the endothelial L-arginine/NO pathway resulting in relaxation of corpus cavernosum.

PDE5 inhibitors, including sildenafil, are the preferred drugs for the treatment of erectile dysfunction (Andersson, 2011; Hatzimouratidis et al., 2010). In the present study, sildenafil markedly enhanced both the magnitude of the spontaneous increases in intracavernosal pressure and the duration of these responses mediated by IP2015. A limitation is that the current data does not allow us to distinguish whether this positive interaction happens at a central level (e.g., in the medial preoptic area) or in the peripheral erectile tissue. To address an interaction at the medial preoptic area, more sophisticated approaches, for example, optogenetics, will be required (Zhang et al., 2021). The enhancement of IP2015-induced relaxation in isolated corpus cavernosum tissue nevertheless suggests that a peripheral mechanism is likely. Our findings suggest that a combination of IP2015 and sildenafil may markedly improve the increase in intracavernosal pressure required to facilitate erectile function.

The main adverse effects of the treatment of erectile dysfunction with sublingual apomorphine are nausea and emesis. Patients rarely suffer syncope, but otherwise, there are no major effects on the cardiovascular system (Heaton, 2001). The PDE5 inhibitors may be associated with lowering systemic blood pressure (Vardi et al., 2002). In contrast to sildenafil, IP2015 in the present study did not change mean arterial blood pressure and heart rate in the rats.

In conclusion, our present findings suggest that IP2015 stimulated erectile function through inhibition of dopamine reuptake, followed by stimulation of central dopamine D_2-like receptors and via dopamine D_1-like receptors in the corpus cavernosum. We propose that this unique multi-modal mechanism and site of action could translate clinically to initiate erection or to support the effect of drugs with a peripheral mechanism of action in patients with erectile dysfunction.

AUTHOR CONTRIBUTIONS

S. Comerma-Steffensen: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **A. Kun:** Data curation (equal); investigation (equal); methodology (equal); writing—review and editing (equal). **J. Prat-Duran:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing—review and editing (equal).

S. Mogensen: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); writing—review and editing (equal). **E. Alan Albayrak:** Data curation (equal); investigation (equal); methodology (equal); writing—review and editing (equal). **R. Fais:** Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **G. Munro:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing—original draft (equal); writing—review and editing (equal). **D. Peters:** Conceptualization (equal); funding acquisition (equal); resources (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **U. Simonsen:** Conceptualization (equal); funding acquisition (lead); investigation (equal); project administration (lead); resources (equal); supervision (lead); validation (equal); writing—original draft (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

Dan Peters and Ulf Simonsen are consultants, and Simon Comerma-Steffensen, Dan Peters and Ulf Simonsen own shares in biotech companies. All the other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and the scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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