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Dopamine and ghrelin receptor co-expression and interaction in the spinal defecation centres

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Abstract:

Background: Dopamine receptor 2 (DRD2) and ghrelin receptor (GHSR1a) agonists both stimulate defecation by actions at the lumbosacral defecation centre. Dopamine is in nerve terminals

surrounding autonomic neurons of the defecation centre, whereas ghrelin is not present anywhere in the spinal cord. Dopamine at D2 receptors generally inhibits neurons, but at the defecation centre the effect is excitatory.

Methods and key results: We investigated whether an interaction between DRD2 and GHSR1a in the defecation centre of rat might explain the excitation by dopamine agonists and the conservation of GHSR1a in the absence of ghrelin. We found that dopamine, and the DRD2 agonist, quinpirole, directly applied to the lumbosacral cord, caused defecation. The effect of intrathecal dopamine was inhibited by the GHSR1a antagonist, YIL781, given systemically, but YIL781 was not an antagonist at DRD2. The DRD2 agonist, pramipexole, administered systemically caused colorectal propulsion that was prevented when the pelvic nerves were cut. *Drd2* and *Ghsr* were expressed together in autonomic preganglionic neurons at the level of the defecation centres in rat. The receptors were also expressed together in neurons in the region of the defection centres in human spinal cord. Behaviourally-induced defecation (caused by water avoidance stress) was reduced by the DRD2 antagonist, sulpiride. We had previously shown it is reduced by YIL781.

Conclusions: Our observations, considered along with previous results, imply that dopamine is a transmitter of the defecation pathways whose actions are exerted through interacting dopamine (D2) and ghrelin receptors on lumbo-sacral autonomic neurons that project to the colorectum.

KEYWORDS: Dopamine, colokinetics, spinal defection centres, ghrelin; G-protein coupled receptors, DRD2, GHSR1a

1 Introduction

Ghrelin receptors (GHSR1a) are expressed by neurons in several central nervous system (CNS) control centres of mammalian species, including rodents and primates.[1, 2] Amongst these are neurons in autonomic nuclei in the spinal cord.[3] When ghrelin receptors in the lumbosacral cord are activated by oral or systemic administration of centrally-penetrant ghrelin receptor agonists, or by direct application of ghrelin or ghrelin receptor agonists, defecation is triggered in humans, dogs, rats and mice.[4-8] In rat, experiments using centrally-penetrant GHSR1a agonists with different chemistries have confirmed the site of action to be the defecation centre in the lumbosacral spinal cord.[4, 9-11] However, the only known endogenous agonist of the receptor, ghrelin, is absent from most regions of the CNS, including the spinal cord, and there is no evidence of another natural agonist, despite considerable effort to find one.[8, 12-16] In the absence of ghrelin in the CNS, another explanation for the conservation of functional GHSR1a was sought.[17] These authors discovered that GHSR1a in appetite-controlling neurons in the hypothalamus is co-expressed with dopamine type2 receptors (DRD2). The selective DRD2 agonist cabergoline, which suppressed feeding in

wild type mice and ghrelin knockout mice, was ineffective in GHSR1a knockout, illustrating dependence on GHSR1a, but not ghrelin. Moreover, evidence for interaction of dopamine type 2 and ghrelin receptors in hypothalamic neurons was found using a combination of FRET confocal microscopy and time-resolved FRET.[17] It has recently been found that DRD2 agonists mimic the effects of GHSR1a agonists to stimulate defecation.[18] Thus it is possible that the effects of ghrelin agonists to stimulate defecation occur because these agonists act at a DRD2/GHSR1a receptor complex, the endogenous agonist of the receptors being dopamine, which occurs in nerve endings that ramify within spinal cord autonomic neuron nuclei.[19, 20]

2 Materials and methods

Experiments were conducted in accordance with the National Health and Medical Research Council guidelines for the care and use of animals and with approval from the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (approval #1714284).

2.1 Recording colorectal propulsion in anesthetized rats

Colorectal propulsion was recorded as previously described.[10] Rats were sedated with ketamine hydrochloride (50-60 $mg.kg^{-1}$, i.m.), following which anesthesia was induced with α-chloralose (60 $mg.kg^{-1}$, i.v.). The femoral artery was then cannulated for the infusion of anesthetic and blood pressure recording, and the femoral vein was cannulated for delivery of drugs. Blood pressure and heart rate were recorded with a Power Lab recording system using Chart 5 software (both from ADInstruments, Sydney, Australia). Anesthesia was maintained by intra-arterial infusion of α-chloralose (12-20 $mg.kg^{-1}.h^{-1}$) plus ketamine (3-5 $mg.kg^{-1}.h^{-1}$) in phosphate buffered saline (PBS; 0.15M NaCl containing 0.01M sodium phosphate buffer, pH 7.2). For colorectal recording, the distal colon was cannulated at the colonic flexure, and a second cannula was inserted into the anus. The colon remained *in situ*, and the muscle and skin were closed around the proximal cannula. The oral cannula was connected to a Mariotte bottle filled with warm PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 7-8 mmHg by adjusting the heights of the Mariotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve, and measured by weighing with a force transducer.

An equilibration time of an hour after completing cannulations was allowed before commencing experiments. Baseline activity was recorded for 30 minutes before drugs were added. Only one agonist at one concentration was used in any experiment. The volume of drug delivered intravenously was 0.32-0.36 mL/rat.

2.2 Multiplex Fluorescent in situ hybridisation (RNAScope)

For rat tissues, we used multiplex fluorescent in situ hybridisation (ISH)[23] to localise neurons expressing *Drd2*, *Ghsr* and *Chat* using RNAscope. Six-week-old Sprague Dawley rats (2 female, 2 male) were anesthetized using isoflurane and euthanised by severing their carotid arteries and spinal cord. The lumbosacral spinal cord was removed quickly (~5 min) and snap frozen on dry ice. Tissue was frozen in optimal cutting temperature compound (OCT) and cryostat sections (12µm) were cut and thaw mounted directly onto Superfrost Plus slides and stored at -20°C. RNA ISH for *Drd2* (Cat No. ADV315641C3, NM_012547.1, target region: 445-1531), *Ghsr* (Cat No. ADV480031, NM_032075.3, target region: 2-742) and *Chat* (Cat No. ADV430111C2, NM_001170593.1, target region: 259-1141) mRNA was performed manually according to the user manual for Fresh Frozen Tissue using RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, Hayward, California, USA). Tissue sections were fixed by immersion in 4% paraformaldehyde for 15 min at 4°C. They were then rinsed twice in PBS and dehydrated in 50% and 70% ethanol solution for 5 min each, followed by dehydration in 100% ethanol twice for 5 min at RT. Slides were transferred to fresh 100% ethanol and kept at -20°C overnight. The slides were dried at room temperature for 10 min. Sections were incubated with a protease solution (Pretreatment IV solution) for 30 min at RT. Excess solution was removed, and slides were washed twice in 1x PBS. Target probes for specific RNAs were applied to the sections and incubated at 40°C for 2 h in the HybEZ Hybridization System (Advanced Cell Diagnostics). Slides were then washed 2 x 2 min in wash buffer solution before amplification. Sections were incubated with preamplifier and amplifier probes by applying AMP1 (40°C for 30 min), AMP2 (40°C for 15 min), and AMP3 (40°C for 30 min), washing slides in wash buffer solution twice for 2 min between addition of solutions. Sections were then incubated with the fluorescently labelled probes by selecting a specific combination of colours associated with each channel: green (Alexa Fluor 488 nm), orange (Atto 550 nm), and far-red (Atto 647 nm). AMP4 AltC-FL was used to detect target probes (Ghsr- Atto550, Atto 647 – Chat, Alexa Fluor 488 – Drd2). Slides were incubated in DAPI for 30 s to stain nuclei and then mounted using Prolong® Gold Antifade Reagent (Life Technologies; P36930). Negative control sections were incubated with RNAscope 3-Plex Negative Control Probe and positive control sections incubated with RNAscope 3-Plex Positive Control Probe. Fluorescent images were captured using a 40x oil objective using a Zeiss Axioimager Z2 (Zeiss, Germany). Images were quantified in a binary fashion, with cells containing single or clustered dots interpreted as positive and those without, as negative, for the target RNA.

Five human lumbosacral spinal cord samples, fixed in 10% neutral buffered formalin, were obtained from AnaBios Corporation (San Diego, CA). Tissues were processed into paraffin using the Leica ASP2560 automated tissue processor (Leica Biosystems, Buffalo Grove, IL). Sections of

sacral spinal cord (5μM) were placed on Superfrost Plus slides and dried overnight at room temperature. Chromogenic in situ hybridization was performed using the RNAscope® 2.5 LS Duplex Reagent Kit (Cat No. 322440, Advanced Cell Diagnostics – ACD) in an automated assay on the Leica BOND RX Advanced Staining System (Leica Biosystems). Variations from the ACD standard protocol include use of protease 4 (Cat No. 300040) and increasing antigen retrieval time to 20 minutes. Quality of RNA in tissue samples, assessed as containing >4 dots/cell on average, was verified for 4 of 5 samples, using a positive control probe for peptidylprolyl isomerase B (Hs-PPIB, Cat. No. 313908). Duplex staining of sacral spinal cord was carried out with a probe for *Ghsr* (Hs-GHSR-O1, transcript variant 1a, Cat No. 544968) in channel 1 (DAB) and a probe for *Drd2* (Hs-DRD2-C2, Cat No. 553998-C2) in channel 2 (Fast Red). Stained slides were imaged with NanoZoomer 2.0-HT slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Images were analysed for evidence of duplex staining; one or more dots of both GHSR and DRD2 probes within a cell was scored as positive for both RNA targets.

2.3 Severing the spinal outflow

Following completion of instrumenting rats for recording and intravascular administration of drugs, rats were placed in a prone position, a dorsal laminectomy was performed to get access to the cauda equina, below the conus medullaris, and the spinal nerves in the spinal canal were completely severed with sharp scissors. The muscle and skin wounds were closed using sterile sutures and the region was covered with gauze. Rats were left undisturbed for 90-120 minutes after the spinal nerves were cut before a compound was administered. After the surgery, the numbers of phasic spontaneous contractile events were reduced. Drugs were not administered until 90-120 minutes later, and after 30 minutes of baseline spontaneous contractile activity, with similar amplitude to that in control rats, had been observed.

2.4 Water avoidance test

Fecal output in response to water avoidance was measured as previously described.[21, 22] Rats were familiarized with handling over a period of 2 weeks. On the experimental day, the rats were injected with the GHSR1a receptor antagonist, YIL781 (Tocris Bioscience, Bristol, UK), 3 mg kg^{-1}, i.p., or vehicle. Ten minutes later the rat was placed on a platform (height 8 cm, length 6 cm and width 6 cm), located in the middle of a large plastic tub that was filled with water up to 7 cm (1 cm below the top of the platform), for a period of 60 min. The numbers of fecal pellets produced by the rat in the first 10, 15, 30, 45 and 60 min on the platform were counted.

2.5 Test compounds

The following compounds were used, their sources and solvents being given in the brackets: capromorelin tartrate (Pfizer Pharmaceuticals, water or buffered saline); dopamine (Sigma-Aldrich,

Castle Hill, New South Wales, Australia; water or buffered saline); YIL781 (Tocris Bioscience, Bristol, UK, water or buffered saline); JMV2959 (Sigma-Aldrich; water or buffered saline); pramipexole (Sigma-Aldrich; water or buffered saline); quinpirole HCl (Bio-strategy, Tingalpa, Queensland, Australia, water or buffered saline); sumanirole maleate (Sigma-Aldrich; water or buffered saline); ropinirole HCl (Sigma-Aldrich; water or buffered saline); sulpiride (Bio-strategy, 1:1:3 DMA: PEG400: dH_2O, then diluted in saline).

2.6 Data and statistics

Data are expressed as mean ± SD or SEM, as indicated. Statistical comparisons are made using Student's t-test, one-way ANOVA with Tukey's multiple comparisons test followed by one-way ANOVA, as indicated in the text. Results were considered to differ significantly when $P \leq 0.05$.

3 Results

3.1 Effects of DRD2 agonists in anesthetized rats

Both dopamine and the dopamine receptor agonist, quinpirole, applied intrathecally directly to the defecation centres of anaesthetized rats, elicited propulsive contractions of the colorectum. Dopamine (0.5 μM in 30 μL) caused an increase in the number of colorectal contractions after a latency of 2-3 min (Fig. 1A). The response peaked at about 10 minutes and waned over a period of about 30 min. Quinpirole (0.5 μM in 30 μL, i.t.) elicited a similar response.

Each of the DRD2 agonists, pramipexole (0.1 to 3.0 mg/kg; Fig. 1B), sumanirole (0.3 – 3 mg/kg) and ropinirole (0.3 to 10 mg/kg), administered systemically (i.v.) to anaesthetized rats, elicited propulsive contractions of the colorectum. The most consistent results were obtained with pramipexole in the dose range 0.1 to 1.0 mg/kg (Fig 1C). The effect of pramipexole was abolished by the DRD2 antagonist, sulpiride (40 mg/kg, i.v.) given 10 min prior to pramipexole. When given during the action of pramipexole, sulpiride truncated its effect (Fig 1B).

To investigate the site of action of the systemically administered DRD2 agonist pramipexole, it was administered after the neural connections between the defecation centres and the colorectum, including L6 to S4 spinal nerves, were interrupted by cutting through the cauda equina, between the end of the spinal cord and the exit of spinal nerves (Fig 2). Pramipexole (0.3 mg/kg) that was administered after cutting the spinal nerves bilaterally failed to elicit propulsive contractions. With the nerves intact, pramipexole increased the number of propulsive contractions (> 6 mmHg) per 30 min from 4.7±0.1 before pramipexole to 25.8±4.1 (n=5) in the 30 min after pramipexole (0.3 mg/kg). The peripherally acting colokinetic, neostigmine (25ug/kg bolus i.v), was equally effective in evoking propulsive contractions in intact rats and rats with the spinal outflow severed. These

data indicate that peripherally applied, centrally penetrant DRD2 agonists activate the spinal defecation centres but do not exert colokinetic effects due to peripheral sites of action.

We then tested whether the colokinetic actions of dopamine agonists are dependent on ghrelin receptors. The GHSR1a antagonist, YIL781, given systemically, blocked the colokinetic effect of intrathecal dopamine (Fig 3). The effect of dopamine partly returned as the effects of YIL781 faded. A second GHSR1a antagonist of different chemistry, JMV2959, also reduced the effect of dopamine applied intrathecally.

3.2 Relationships between DRD2 and GHSR1a expression in autonomic nuclei of the lumbosacral spinal cord

We used RNAscope to investigate the expression of *Drd2* and *Ghsr* in neurons of the autonomic intermediolateral (IML) cell nuclei in the lumbosacral spinal cord of the rat (Fig 4). The preganglionic neurons of these nuclei were identified by their expression of *Chat*. Expression of both *Drd2* and *Ghsr* was found in around 25% of *Chat* expressing neurons and a further ~45% expressed *Ghsr* but not *Drd2*. Roughly 80% of preganglionic neurons exhibiting the D2 receptor gene expression also expressed GHSR1a. About 40% of GHSR1a containing preganglionic neurons contained transcripts for both *Drd2* and *Ghsr* There was no difference in expression levels of RNA between sexes (data not shown).

We also investigated the expression of *Drd2* and *Ghsr* in neurons of human lumbosacral spinal cord, from 4 subjects, using duplex RNAscope with chromogenic development. Dual-positive cells, containing both *Ghsr* and *Drd2* probes, were located in the lateral regions of the intermediate zone of the sacral spinal cord (Fig 5). Single probe-positive cells were also found.

3.3 Physiological involvement of dopamine in defecation

In order to investigate a possible physiological role of DRD2 in the control of defecation, we investigated the effects of the DRD2 antagonist, sulpiride, on fecal output in response to water avoidance stress (WAS). It is known that WAS stimulates brain centres that are responsive to stress and activates pathways that travel down the spinal cord to the defecation centres and thence to the colorectum.[8, 21] Placing the rat on a platform surrounded by water caused substantial fecal output in the first 15 min, with little further output in the two subsequent 15 min periods (Fig 6). Injection of sulpiride (40 mg/kg), intraperitoneal, 20 min before the rat was placed on the platform significantly reduced the stress-induced defecation. At 10 min, the fecal output was less than 10% of that of rats injected with vehicle, and even at 45 min the cumulative output was less than half the vehicle control.

4 Discussion

This study supports the hypothesis that the well conserved effect of ghrelin receptor agonists to stimulate colorectal propulsion and defecation in human, mouse and rat, despite the absence of ghrelin in the spinal cord, could be explained by defecation being physiologically dependent on the ghrelin receptor, whose endogenous activator is dopamine acting through dopamine D2 receptors that are coupled to the ghrelin receptors.

We confirmed a previous observation[18] that both dopamine and the DRD2 selective agonist, quinpirole, applied directly to the spinal cord in the region of the lumbosacral spinal cord, caused propulsive contractile waves in the colorectum. Systemic administration of different DRD2 agonists (pramipexole, sumanirole and ropinirole) also caused colorectal propulsion. We used one of these agonists, pramipexole, to investigate the site of action of the systemically applied agonists, because its effects were the most consistent. We do not know why the effects of pramipexole were the most consistent. It is possible that different individual rats sequester or metabolise the compounds differently. However, pharmacokinetic data in rats that would assist in interpreting the variabilities is not available. The effects of pramipexole, i.v., were prevented when the nerve connections between the defecation centres and the colorectum were severed, supporting the view that DRD2 agonists act in the central nervous system. This is consistent with direct motility stimulating effects of dopamine at the level of the colon being not via DRD2.[24] The effects of pramipexole were quite similar to the effects of ghrelin receptor agonists, whose effects are also blocked when the connections between the defecation centres and the colorectum are severed.[10]

The lumbo-sacral intermediolateral autonomic cell groups that harbour the DRD2/GHSR1a cells are innervated by dopaminergic nerve terminals,[20] but not by ghrelin nerve terminals.[8, 14] It thus seems likely that dopamine is involved in the physiological control of defecation through these receptors. We tested this by eliciting a defecation response to water avoidance stress, an effect that is mediated through the defecation centres.[8] The response was substantially reduced by the intraperitoneal injection of the dopamine receptor antagonist, sulpiride, to conscious rats. In a similar way, colorectal propulsion elicited by intraluminal capsaicin, that is also mediated via the defecation centres, was reduced by the intrathecal injection of the D2 dopamine receptor antagonist haloperidol.[25] We previously showed that the GHSR1a antagonist, YIL781, inhibited defecation in conscious rats, showing that ghrelin receptors, despite not being innervated by ghrelin-expressing nerve terminals, are also physiologically involved in the control of defecation.[8] In cells expressing rat GHSR1a, YIL781 antagonised the action of the GHSR1a agonist, capromorelin, with a pA_2 of 7.55±0.35, but failed to reduce the effect of dopamine at DRD2 (our unpublished data).

Drd2 and *Ghsr* receptor expression were found in about 25% of preganglionic neurons in the intermedio-lateral (IML) cell columns at the level of the lumbo-sacral defecation centres of rat, and

in an equivalent location in human. Ghrelin has excitatory effects on central neurons through GHSR1a,[26] but dopamine acting through DRD2 is inhibitory on central neurons.[27] Thus stimulation of autonomic neurons in the IML by ghrelin is predicted, whereas stimulation by dopamine at DRD2 is unexpected. However, dopamine and quinpirole depolarized and excited neurons within the IML that were labelled by retrograde tracing from the colon.[18] It is hypothesized that interaction with the ghrelin receptor converts activation of DRD2 to excitation.

Taken together, the colocalization of *Drd2* and *Ghsr* receptor gene expression in neurons of the defecation pathways and the excitatory effects of both dopamine and ghrelin receptor agonists suggest that DRD2 and GHSR1a may interact in these cells. Our observation that the stimulant effect of the dopamine receptor agonist, quinpirole, was blocked by the GHSR1a antagonist, YIL781, supports this interpretation. About 25% of the preganglionic autonomic neurons expressed both *Drd2* and *Ghsr* and a further ~45% expressed *Ghsr* without *Drd2*. These preganglionic neurons that express *Ghsr* but not *Drd2* may be involved in other aspects of autonomic regulation such as blood pressure control[3] or urinary bladder function.[28] GHSR1a in these pathways could be coupled to GPCRs other than DRD2; several other heteromers involving GHSR1a have been identified.[29] In preliminary experiments we have found that the dopamine receptor antagonist, sulpiride (40 mg/kg, i.v.) reduced the response to capromorelin (10 µg/kg, i.t.). This may mean that this dopamine antagonist causes a conformational change in a GHSR1a/ DRD2 receptor complex that prevents stimulatory actions by GHSR1a agonists.

DRD2 and GHSR1a receptors have been deduced to form tetrameric complexes in lipid membranes.[30] They are also appear to form heteromers in hypothalamic neurons, in which FRET analysis showed interaction between fluorescently labelled ghrelin and fluorescently labelled anti-DRD2 antibodies.[17] Investigation of cell signalling in transfected HEK cells are consistent with close proximity and interaction between DRD2 and GHSR1a. It was found that dopamine had no effect on cytoplasmic Ca^{2+} in cells expressing only GHSR1a or only DRD2, but in cells expressing both receptors, dopamine elevated cytoplasmic Ca^{2+}, an effect that was antagonised by the ghrelin receptor antagonist, JMV2959.[17] Thus the interactions in transfected cells parallel the interactions that are predicted to occur in our in vivo studies.

We conclude that the most parsimonious explanation of our observations, when considered along with previous results, is that dopamine is a transmitter of the defecation pathways whose actions are exerted through dopamine type 2 and ghrelin receptors that interact in lumbo-sacral autonomic neurons that project to the colorectum. The reduction by dopamine D2 antagonists of colorectal responses to water avoidance or to intraluminal irritation by capsaicin, point to a physiological role of dopamine in the defecation pathways.

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Disclosures

Andrew J Syder, Eun Ji Yoo, Andrea Fanjul and Jill Wykosky are employees of the Takeda Pharmaceutical Company Limited. The other authors declare no conflict of interest.

Author contributions

JBF, JW and RVP conceptualized and designed the study. RVP performed animal studies. MTR, AJS and EJY designed probes and conducted localization studies. AF and EAW characterized ligands. AJS, MTR, LJF, SGBF, RVP and JBF analyzed and integrated data. JBF wrote the manuscript. All authors contributed to discussion and approved the manuscript.

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Figure Descriptions

Fig 1. Colokinetic effects of dopamine, applied directly to the spinal defecation centres, and pramipexole given intravenously. A: A bolus of dopamine, 0. 5 μM in 0.2 mL, injected into the spinal cord at L6-S1, caused phasic increases in intracolonic pressure that caused propulsion of the fluid in the colon (representative trace from 6 experiments in response to 0.3 mg/kg). B: Intravenous injection of pramipexole, 0.3 mg/kg, increased the numbers, amplitudes and propulsive effectiveness of colorectal contractions (representative trace from 3 sulpiride experiments). The record of fluid outflow is cumulative. C: Relation between pramipexole dose, colorectal contractions and fluid propulsion. There was no fluid propulsion in the periods without pramipexole (o). Mean ± SD, individual animal data as points.

Fig 2. Lack of effect of intravenous pramipexole when the connections of the lumbosacral defecation centres with the colon were severed. A: Intravenous administration of pramipexole in an anaesthetised rat caused strong propulsive contractions of the colorectum. B: Pramipexole

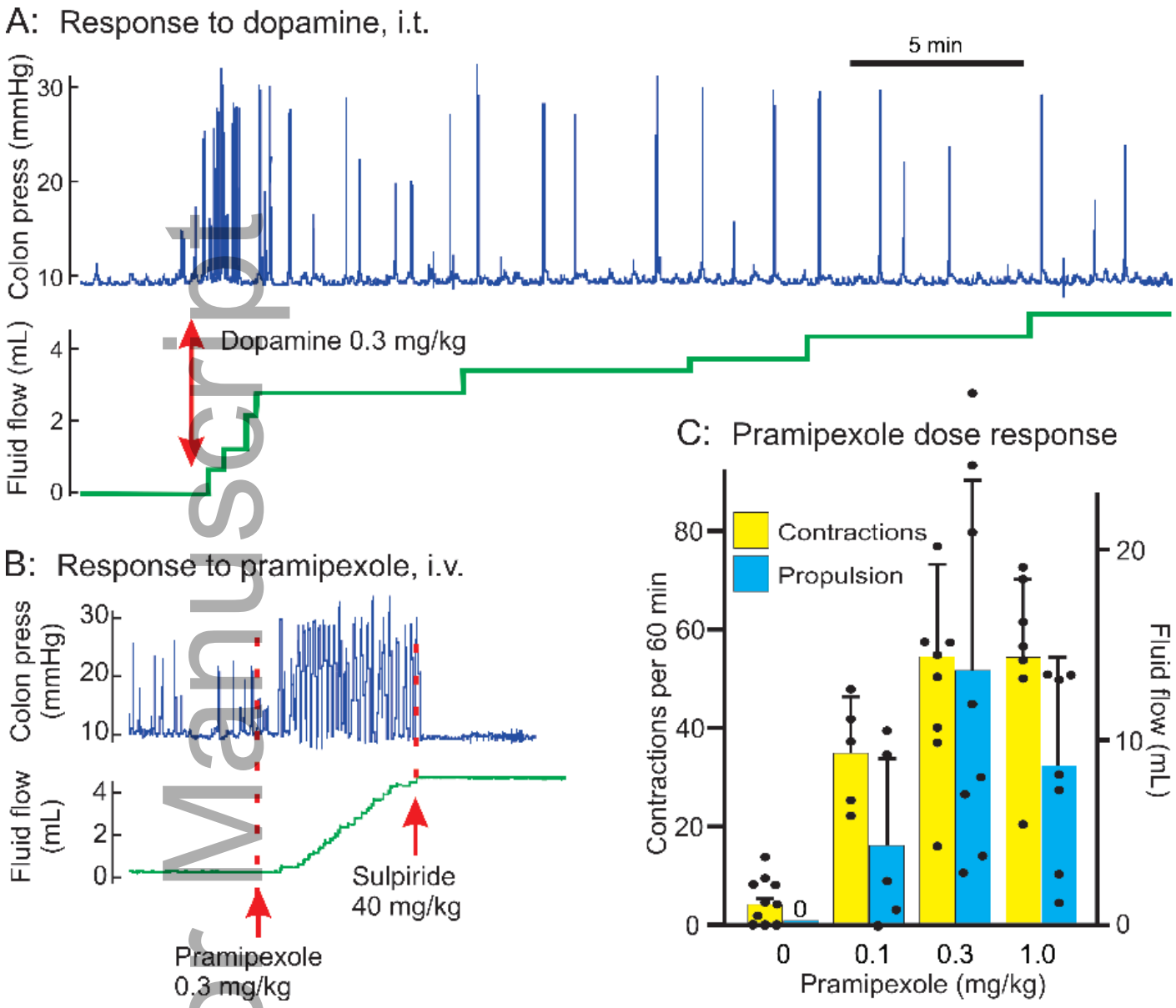
administered intravenously after the spinal outflow was severed had no effect on colorectal contractions. Representative trace from one of 3 experiments.

Fig. 3. Dopamine (0.5μM) injected intra-thecally, at the level of the defecation centre, elicited propulsive contractions. These were blocked by the ghrelin receptor antagonist, YIL781, i.v. (3 mg/kg bolus i.v, followed by 5 mg/kg/h i.v. infusion). Two hours after YIL781, responsiveness to dopamine returned (half-life for YIL781 elimination is ~ 30 min). Representative traces from 3 experiments.

Fig 4. Neurons expressing *Drd2* and *Ghsr* RNA in the lumbosacral spinal cord. A: Nissl staining at the L6 level of mouse spinal cord; region of IML defined by box. B: IML at greater power. C: localisation of *Drd2*, *Ghsr* and *Chat* in the IML. Positive neurons are indicated by the arrow *Drd2*, *Ghsr* and *Chat*. Note that there is bleed-through of nuclear staining in the green channel (*Drd2*). D: Quantitation of the proportional overlaps of gene expression in neurons of the IML. About 25% of preganglionic neurons, identified by *Chat* expression, also expressed *Drd2* and *Ghsr* in the IML of the lumbosacral region (n=4 animals, 300 cells counted). Data represented as mean ± SEM. Abbreviations: IML, Intermediolateral column nucleus; W, white matter.

Fig 5 Localization of DRD2 and GHSR1a RNA in the human sacral spinal cord. A: Locations of neurons expressing both receptors (blue circles) depicted on an image of the human sacral spinal cord in transverse section. The ovals indicate the positions of the IML. B, C, D: Neurons showing localization of Ghsr transcripts (DAB probe, black) and Drd2 (Fast Red probe).

Fig 6: Defecation caused by the physiological activation of the defecation pathway by water avoidance stress is significantly reduced by the dopamine receptor antagonist, sulpiride (40 mg/kg, i.p., 20 minutes before the rat was placed on the water-surrounded platform) compared to the defecation that was observed when vehicle was administered before the rats were placed on the platform. * $P<0.05$; n = 10, mean ± SD, individual animal data as points.



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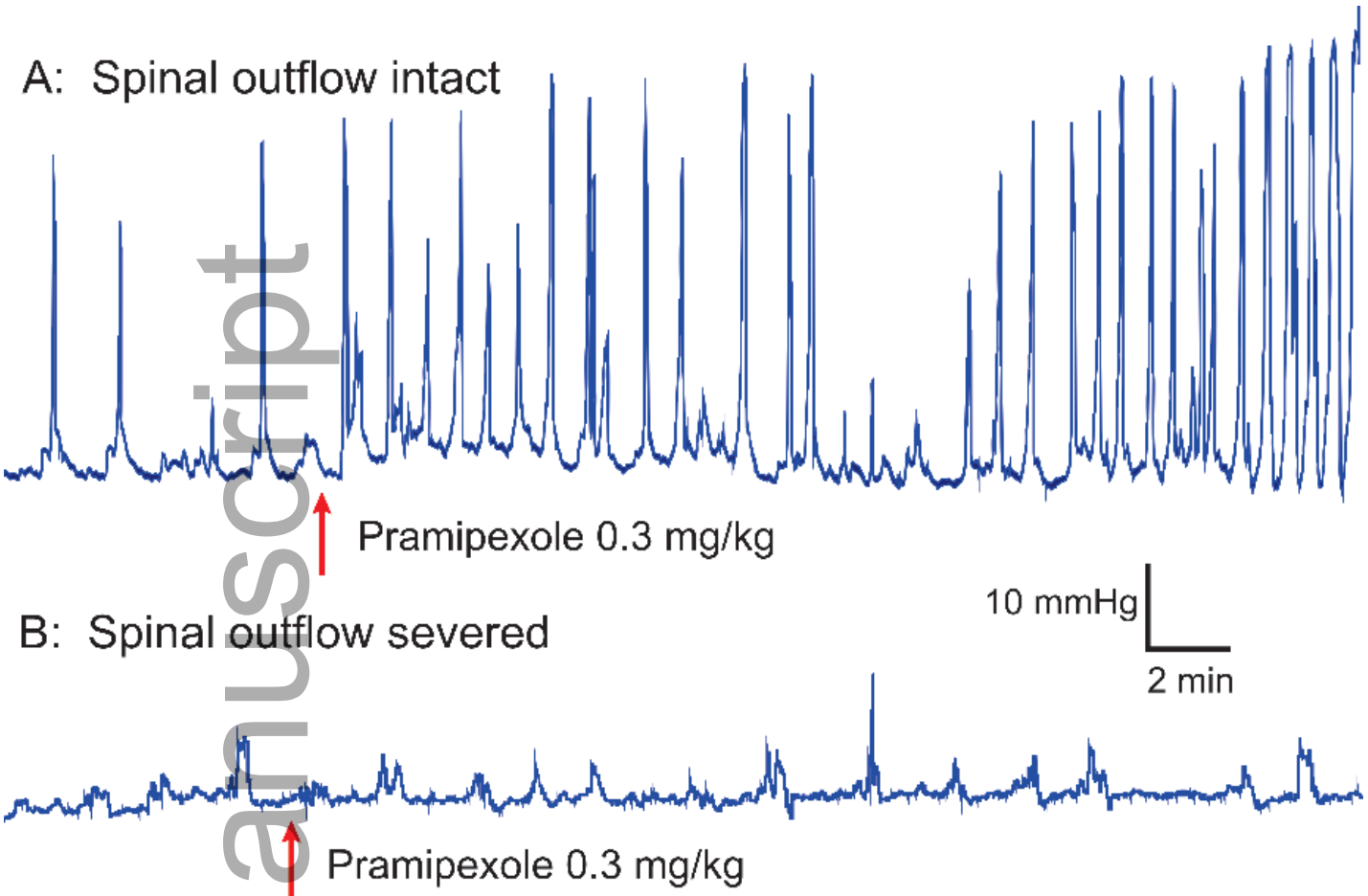

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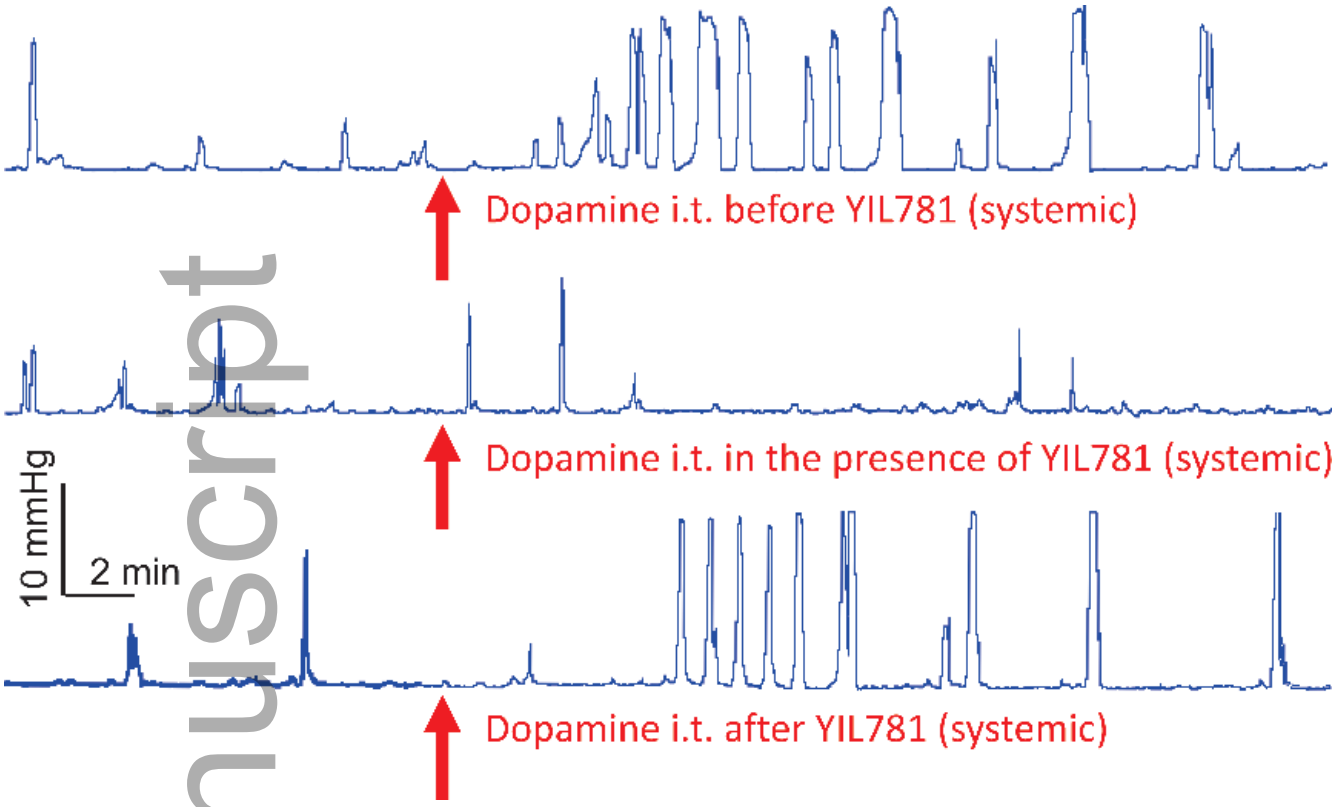

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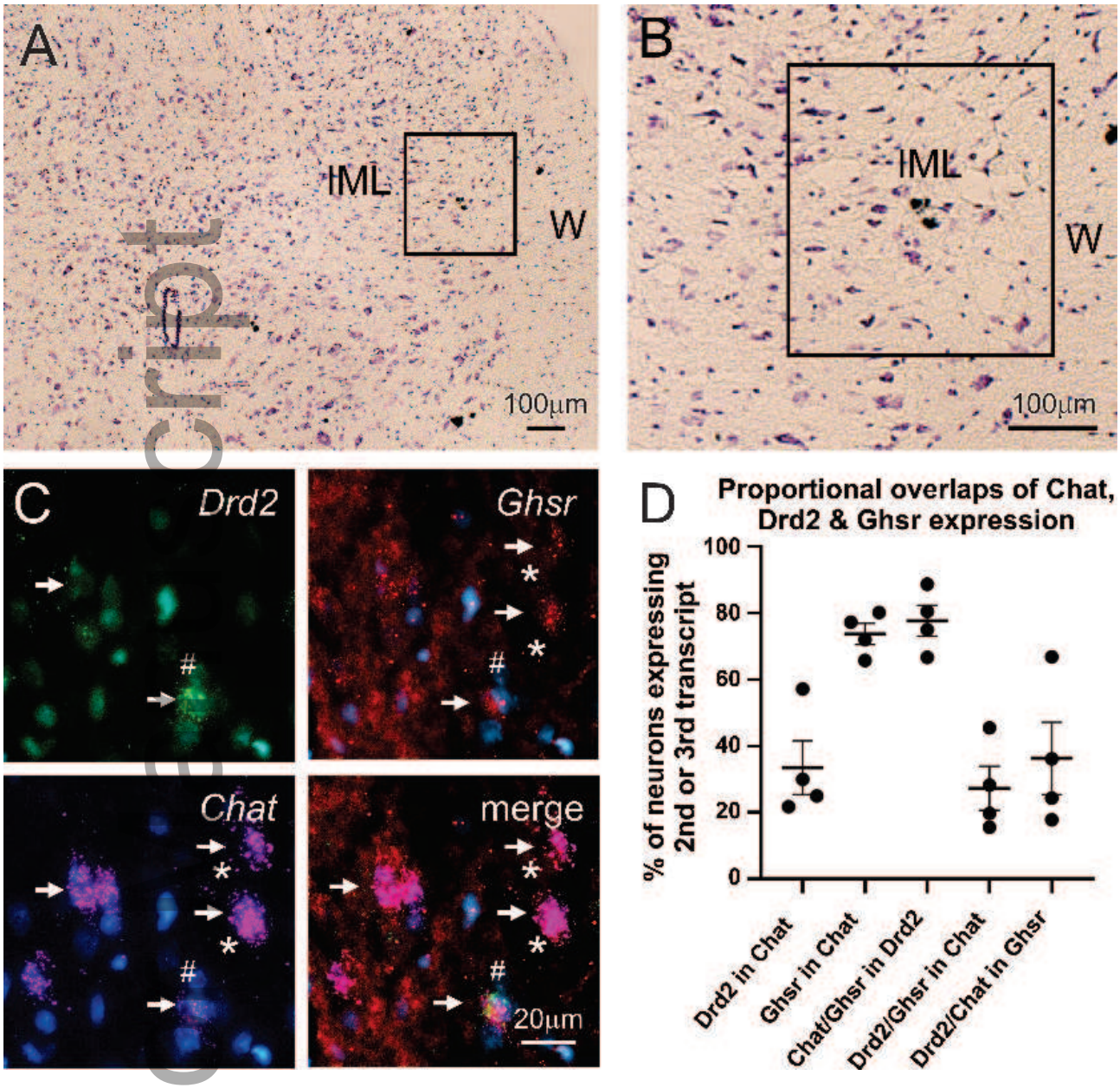

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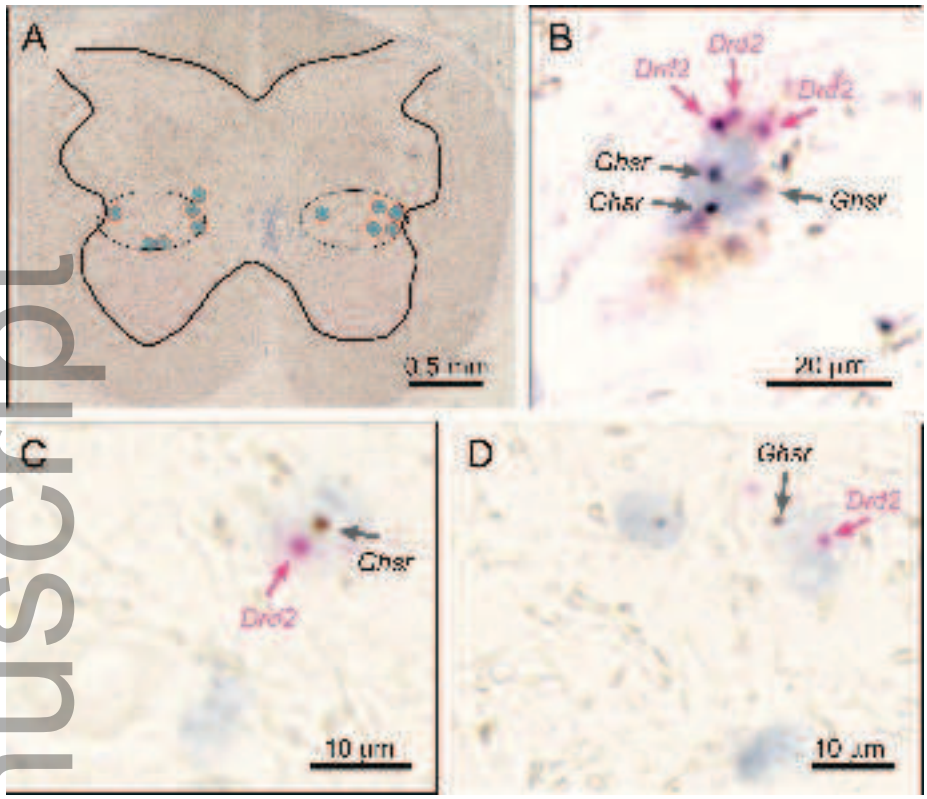

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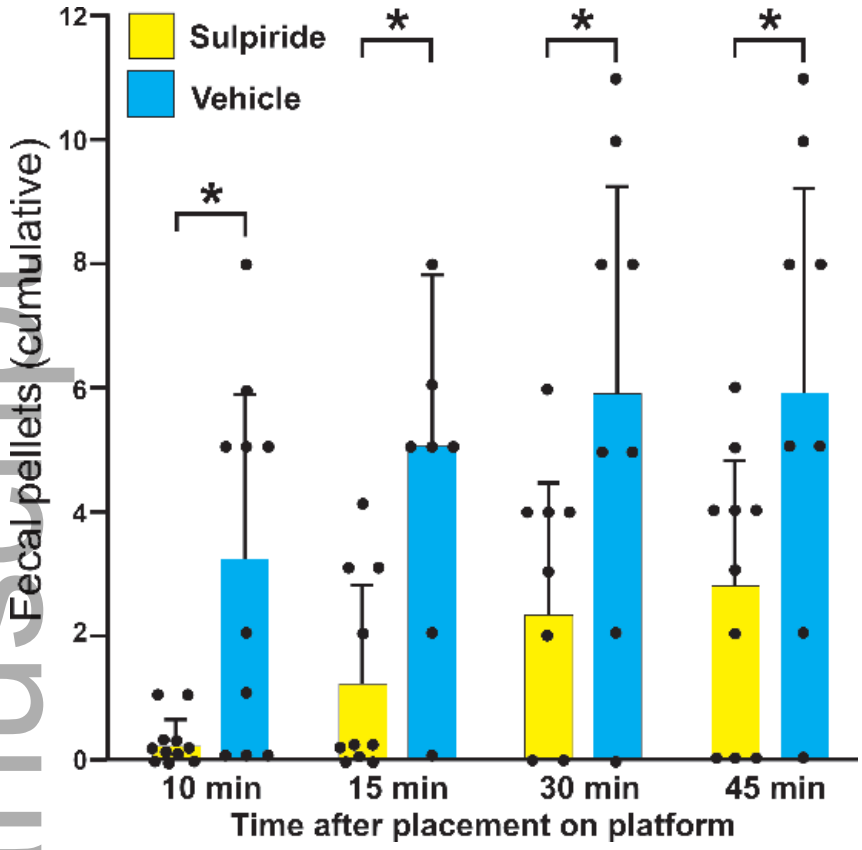

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