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Prenatal exposures to paracetamol/acetaminophen and precursor aniline impair masculinisation of male brain and behaviour

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Keywords: Paracetamol, acetaminophen, aniline, testosterone, prostaglandin, sexual development, behaviour

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Abstract

Paracetamol/acetaminophen (N-Acetyl-p-Aminophenol; APAP) is the preferred analgesic for pain relief and fever during pregnancy. It has therefore caused concern that several studies have reported that prenatal exposure to APAP results in developmental alterations in both the reproductive tract and the brain. Genitals and nervous system of male mammals are actively masculinized during foetal development and early postnatal life by the combined actions of prostaglandins and androgens, resulting in the male-typical reproductive behaviour seen in adulthood. Both androgens and prostaglandins are known to be inhibited by APAP. Through intrauterine exposure experiments in C57BL/6 mice, we found that exposure to APAP decreased neuronal number in the sexually dimorphic nucleus (SDN) of the preoptic area (POA) in the anterior hypothalamus of male adult offspring. Likewise, exposure to the environmental pollutant and precursor of APAP, aniline, resulted in a similar reduction. Decrease in neuronal number in the SDN-POA is associated with reductions in male sexual behaviour. Consistent with the changes, male mice exposed *in uteri* to APAP exhibited changes in urinary marking behaviour as adults and had a less aggressive territorial display towards intruders of the same gender. Additionally, exposed males had reduced intromissions and ejaculations during mating with females in oestrus. Together these data suggest that prenatal exposure to APAP may impair male sexual behaviour in adulthood through disrupting the sexual neurobehavioral programming. These findings add to the growing body of evidence suggesting the need to limit the widespread exposure and use of APAP by pregnant women.

Introduction

The foetal brain is bipotential in its ability to adopt a masculine or feminine phenotype; a property lost after a foetal and perinatal sensitive period (McCarthy, 2008). The foetal male brain requires gonadal hormones, such as testosterone, to differentiate into the male configuration resulting in male reproductive behaviour later in life (Juntti *et al.*, 2010). It is known that testosterone in the hypothalamus through its conversion to estradiol increases prostaglandin E2 (PGE2) by up-regulating cyclooxygenase-2 (COX-2) resulting in masculinisation (Amateau and McCarthy, 2004). Accordingly, perinatal exposure to the COX enzyme inhibitor, acetylsalicylic acid (aspirin), results in impairment of male sexual behaviour (Amateau and McCarthy, 2004). It is also known that intrauterine exposure to phthalates (like dibutyl phthalate), which also are inhibitors of COX enzymes with potency in the same range as mild analgesics (Kristensen *et al.*, 2011), can result in feminised play behaviour among young boys (Swan *et al.*, 2010). At the same time, it is becoming increasingly clear that mild analgesics (hereafter termed analgesics) are potent inhibitors of foetal androgen production and through this mechanism can result in congenital malformations (reviewed in Kristensen *et al.*, 2016). Hence, it seems possible that analgesics can affect brain masculinisation through both blocking production of testosterone in testicular Leydig cells and prostaglandins in the brain. Moreover, previous studies have shown that postnatal exposure (on postnatal day 10) to paracetamol/acetaminophen (N-Acetyl-p-Aminophenol; APAP) can induce long-lasting effects on e.g. cognitive function in the adult mouse (Viberg et al., 2014).

In the present report, we therefore examined mouse brain morphology and behaviour following prenatal exposure to APAP, used widely among pregnant women (Kristensen *et al.*, 2016), and the industrial pollutant and APAP precursor, aniline (Modick *et al.*, 2014; Holm *et al.*, 2015). We investigated changes in the sexually dimorphic nucleus (SDN) of the preoptic area (POA) in the anterior hypothalamus of male adult offspring after intrauterine exposure. The SDN

77 has been shown to be 2.5-7 times larger in male than in female rodents, with the difference being
78 dependent on induced levels of PGE2 resulting from the action of testosterone (Amateau and
79 McCarthy, 2004). Similar sex differences in size of this nucleus have been described in other
80 mammals (Morris *et al.*, 2004; Gilmore *et al.*, 2012). The calcium binding protein calbindin-D28 is
81 one of the best markers for identifying the anatomical borders of the SDN-POA in mice (Sickel and
82 McCarthy, 2000; Gilmore *et al*., 2012). The protein belongs to a family of low molecular weight
83 calcium-binding proteins that has been hypothesised to play a role in neuroprotection against
84 excitatory neurotransmitter and calcium induced cell death (Sickel and McCarthy, 2000). In
85 rodents, neurogenesis of SDN neurons begins around embryonic day 14 and proceeds until about
86 embryonic day 18, and all the neurons in the surrounding area of the POA have been created by
87 embryonic day 16 in the rodent (Jacobson and Gorski, 1981). Our findings indicate a potential
88 effect of APAP on intrauterine brain development, and suggest that these changes may have a direct
89 effect on male sexual behaviour.
90

Materials and Methods

Mating and dosing

The local Danish ethical committees approved all animal experiments. Mice (*Mus musculus*) dams with a C57BL/6JBomTac background (n = 10 per group, purchased from Taconic, Denmark) were fed on a phytoestrogen-free diet. The dams were randomly caged pairwise with a male during mating. Pregnant dams were subsequently treated every morning at the end of the dark period from 7 days post coitum (DPC) to delivery following dosing protocols also described in Holm *et al.* (2015 & 2016). Compounds were administered in 0.5 ml water by gavage in the following groups: (*i*) water control, (*ii*) 50 mg/kg/day APAP, (*iii*) 150mg/kg/day APAP, (*iv*) 30 mg/kg/day aniline and (*v*) 90 mg/kg/day aniline. APAP doses were chosen to be nontoxic and the lower to be equal to the maximum human exposure of 50 mg/kg/day. The higher dose of 150 mg/kg/day was chosen so it also was in range of the human exposure when a system of allometry based on the body surface area difference between mouse and human was applied dividing the mice dose with a factor of 12.33 (Reagan-Shaw *et al*., 2008). Aniline doses was given in equi-molar amounts to the APAP doses and were, after adjusting for difference in body size between mouse and human, also within the human exposure range (Holm *et al.*, 2015, 2016).

Analysis of the sexually dimorphic nucleus of the preoptic area

After termination at PNW 12-13, brains were carefully dissected from the skull and examined to explore any changes in SDN-POA. Brains were fixed in PBS (phosphate buffered saline) with 4 % paraformaldehyde and subsequently split in half in the coronal plane (where the midbrain and the thalamus meet) and submerged in 30% sucrose to avoid freezing damage. From each of the treatment groups brains were chosen at random. Coronal sections of each brain were cut into four

series at 40 µm on a sledge microtome. Series were stored at -20°C in cryoprotectant with sodium azide (NaAZ).

Before immunohistochemical staining, free-floating sections were rinsed three times for 10 min in PBS and subsequently incubated for 15 min in 0.5 % perhydrol (H_2O_2) to block endogenous peroxidase that could otherwise lead to false-positive results in the non-specific background. Sections were rinsed in PBS again before being pre-incubated with human serum albumin (HSA) in PBS-T (1% HSA, PBS-T, 0.1% NaAZ) for 30 min to block unspecific absorbance to tissue or to Fc receptors and permeabilize the tissue. After this, the sections were exposed to primary rabbit anti-Calbindin-D-28K (1:2000; Sigma-Aldrich; EG-20) antibody overnight in 1% HSA-PBST-NaAZ. Next morning, the sections were rinsed in PBS-T and incubated with secondary donkey anti-rabbit antibody for 1 hr in 1% HSA-PBST-NaAZ. This was followed by another rinse in PBS and incubation with an avidin-biotin (AB) complex (1:100; Vector-labs) in PBS for 1 hr. The sections were then rinsed in PBS and incubated in Vector SG-peroxidase HRP substrate kit (Vector-labs) for 15 min for development. Finally, the sections were rinsed twice in MilliQ ultrapure water and mounted in chrome-gelatine onto slides.

Defining the area of the SDN-POA

The procedure described by Gilmore *et al.* 2012 to distinguish the calbindin D28 immunoreactive part of the SDN (CALB-SDN) from other calbindin immunoreactive (CALB-ir) cells found in the C57BL/6 mouse brain was used. The CALB-SDN is defined as a distinctive cluster that is ellipsoidal in shape and centred at about 675 µm dorsal to the optic chiasm (OC) and 275 µm lateral to the third ventricle. It is angled away from the third ventricle *dorsolaterally* and the cluster spans between 90 µm to 180 µm *rostrocaudally* in males (Gilmore *et al.*, 2012).

Depending on the exact plane of sectioning, the cluster was present in sections in which the anterior commissure (AC) crossed the midline or, more often, just caudal to this point. Using these landmarks to pinpoint the correct location of CALB-SDN, sections were chosen with the aid of the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). Especially in specimens where the calbindin reactive cells did not form a distinct cluster or form a very small cluster (Gilmore *et al.*, 2012), the presence of these landmarks was the determining factor in the identification of the correct location.

Quantifying the CALB-SDN

The tissue sections were examined using an Evolution MP Color camera from Media Cybernetics connected to a light microscope and analysed with the aid of Image-Pro Plus and ImageJ software. All measurements were made on images coded to conceal the sex and treatment of the animals. For every brain in the experiment, one section was chosen that best represented the CALB-SDN. The correct location was determined as described in the above section, and if the CALB-SDN was visible in multiple sections, the section was chosen that had the most CALB-ir cells in the CALB-SDN. For the images in the group where a CALB-SDN was discernible, an ellipse of a fixed size was placed on both sides of the third ventricle in approximately the same location in all images relative to two markers, the third ventricle and the optic chiasm. The placement differed from what is described by Gilmore *et al.* (2012) in that it was consistently closer to the optic chiasm, approximately 400 μm instead of 675 μm. The cells within the elliptical border were subsequently counted independently by two investigators. Importantly, if a CALB-ir cell was lying at the edge of the ellipse and was too far from the cluster of the other cells, it was counted as a background staining and not as part of the CALB-SDN. The number of cells from counts on both sides of the

160 third ventricle was noted and added together for a representative CALB-SDN cell number from that
161 brain.
162
163
164
165 *Behavioural testing:*
166 From postnatal week eight (PNW 8), one male was selected at random from each of the 8 litters that
167 were prenatally exposed to 150 mg/kg/day APAP during intrauterine life and compared to an equal
168 number of similarly chosen controls to explore (*i*) urinary behaviour, (*ii*) aggression and (*iii*) sexual
169 behaviour. All behavioural assays were conducted 1 hr after initiation of the dark cycle and the
170 protocols used were adapted from Juntti *et al.*, 2010 and Yang *et al.*, 2013 with males single caged
171 and with 2 ≥ days separating each of the experiments. The tests were videotaped and subsequently
172 analysed blindly by two investigators.
173 The urinary behaviour in males and females differ by the size of the urine spots,
174 numbers and spread. Hence, a territory-marking male mouse will be more prone to marking their
175 territory with urine scent and leave multiple, widespread spots, while a female will more commonly
176 choose one or few areas where she will leave fewer, bigger droplets (Juntti *et al.*, 2010). Males were
177 tested by transferring the animal for 1 hr to a cage freshly lined with Whatman paper and then
178 returned to their home cage. The marking pattern was visualized with UV transillumination and the
179 imaging program ImageJ (NIH, USA).
180 Inter-male aggression was provoked by the introduction of an intruder male (Barkley
181 and Goldman, 1977). A singly-housed intruder mouse was introduced to the home cage of a singly-
182 housed resident mouse (prenatally treated). The intruder test was conducted for 15 minutes and
183 sniffs, attacks, tail rattles and bites of the resident mouse were counted.

184 Male mating/sexual behaviour was tested for 30 min with a receptive intruder female
185 in oestrous. During mating, the male mouse behaviour was documented by quantifying approach
186 and olfactory investigation of the female (anogenital sniffing), mounting, intromission (penetration
187 identified as thrusting), and ejaculation (Swaney *et al.*, 2012).
188
189 *Statistics*
190 A one-way ANOVA follow by *post hoc* Dunnett's tests were used to determine significance
191 between outcomes in the neuronal examinations. Unpaired *t*-tests were performed between
192 outcomes in behavioural trials.
193

Results

Intrauterine exposure to APAP and aniline decreased cell number in the CALB-SDN

There was no difference between animals at the lowest dose of APAP and control; mean numbers of CALB-ir cells were 44 and 41 in control and APAP treated animals, respectively. In contrast, exposure to the highest dose (150 mg/kg/day) significantly decreased cell number in the SDN by approximately 50% with a mean cell number of 21 (Fig. 1). The decrease seen for aniline-exposed males was similar to that seen for the highest dose of APAP. Hence, both aniline doses resulted in a decrease in CALB-ir cell number in the CALB-SDN with significant decrease for the highest dose resulting in a mean cell number of 21. These data suggest that the intrauterine exposure may have impaired the brain masculinisation process.

Intrauterine exposure to APAP changed urinary marking behaviour

Aniline is known to be converted nearly completely in the liver of the mouse to APAP (Holm *et al.*, 2015). We therefore performed behavioural experiments with animals exposed to the highest dose of APAP (150 mg/kg/day), which had resulted in a significant effect on masculinization of the SDN-POA. The area covered by urine markings in the cages did not differ between control and APAP exposed animals, as both covered approximately 13% of the cage with urinary marks (Fig. 2). In contrast, APAP exposed animals had marked the cages with significantly larger droplets. Quantifying the number of droplets from each animals using a "cut off" of >2000 pixels per droplet showed that the intrauterine exposed males had marked their cages with significantly fewer but bigger droplets (Fig. 2B-C). These data suggest that the APAP exposure may have resulted in a less masculinized territorial marking of the cages.

Intrauterine exposure to APAP resulted in reduced territorial display

The male mouse is characterized by fierce aggression towards foreign males. We therefore introduced male intruders into the cages of the intrauterine exposed animals. Following the males interaction for 30 min, we observed a significantly reduced number of sniffs among exposed animals (150 mg/kg/day) and a similar trend in tail rattles (Fig. 3A & C). While the number of direct attacks did not differ between exposed and control animals, the number of attacks that resulted in bites was markedly different; none of the APAP exposed males bit their intruder (Fig. 3B & D). This data suggest that the intrauterine exposure to APAP may have moderated male-typical aggression during territorial display.

Intrauterine exposure to APAP resulted in reduced mating

The male mouse begins sexual behaviour immediately when introduced to a female in oestrus. As the urine marking and aggressiveness towards an intruding male had indicated that the male specific behaviour could be reduced in the APAP (150 mg/kg/day) exposed males, we next introduced a female in oestrus to males for 30 min to investigate the mating behaviour. Examining the behaviour, there were no changes between APAP and control mice in sniffing or mounting (Fig. 4A-B). However, moving from mounting to intromission there was a clear decline in APAP mice (Fig. 4C). This was further supported when examining the females for vaginal plugs. 50% of the females encountering a control mouse had vaginal plugs after the 30 min experiment lasted, while none of the females introduced to an APAP male had plugs (Fig. 4D). This data suggest that the intrauterine exposure to APAP may have reduced the final stages of male sexual behaviour.

Discussion

Recent years have seen several rodent and human studies reporting connections between intrauterine exposure to APAP and male congenital reproductive malformations and other morphological changes. Accordingly, APAP has been found to increase the risk of cryptorchidism and reduced anogenital distance through its anti-androgenic effects; both associated with later life reproductive disorders (reviewed in Kristensen *et al.*, 2016).

With this exploratory study, we now report that the anti-androgenic effect of APAP on reproduction may not be restricted to urogenital development but could also include an effect on the masculinisation processes of the brain. APAP is clinically the preferred compound to treat pain and fever during pregnancy (Kristensen *et al.*, 2016). The actions of APAP remains partly elusive, but one mechanism is through its inhibition of prostaglandin synthesis (Kristensen *et al.*, 2011). Prostaglandins are oxylipins that both induce pain and fever, while also being intrinsically involved in the brain masculinisation process during foetal life through translating the testosterone signal originating from testis to the SDN-POA (Amateau and McCarthy, 2004). The convergence of both prostaglandin and testosterone inhibitory effects are likely the reason for the reduction in CALB-ir cell number in the SDN-POA. However, we cannot in the present report distinguish between the effect on testosterone and prostaglandin, as evidence also suggests a direct role for androgens in the brain masculinisation process. Thus, androgen receptor (AR) deficient mice exhibit results in reduced CALB-SDN cell number and an intermediate phenotype not significantly different from females (Edelmann *et al.*, 2007).

In our behaviour experiments, the pattern of urine marking appeared to be independent of the intrauterine APAP exposure as the exposed males distributed their urine marks across the cage floor similarly to control males. However, the exposed males did have fewer and larger urine marks compared to control males. Interestingly, a previous report, using a brain specific

deletion of AR in mice, has shown that androgens are not required to masculinize urine marking (Juntti *et al.*, 2010). Rather, AR enhances the male display of urine marking, increasing the number of urine marks and the male pattern. With these data in mind, the anti-androgenic effect of APAP could account for the fewer and larger markings among the intrauterine exposed males.

Both male mice with lesions in the POA and rats with decreased SDN-POA cell number have impaired mounting behaviour and copulation (Lin *et al.*, 2011; Swaney *et al.*, 2012; Houtsmuller *et al.*, 1994; Morris *et al.*, 2004). Furthermore, SDN-POA cell number has been reported significantly reduced in prenatally stressed rat males that did not copulate, and random control males with similar lack of copulation had similar reductions in cell number (Rhees *et al.*, 1999). Moreover, specific lesions in the SDN-POA results in similar reductions in copulatory behaviour (De Jonge *et al.*, 1989; Morris *et al.*, 2004). It is therefore not surprising that the reduction in CALB-ir cell number in the SDN-POA reported here is correlated with a reduction in male sexual behaviour and lack of ejaculation. This data is also in accordance with a former rat study that showed that intrauterine and perinatal exposure to acetylsalicylic acid, an analgesic not recommended during pregnancy (Kristensen *et al.*, 2016), resulted in reduced copulatory behaviour (Amateau and McCarthy, 2004).

As several dimorphic regions have been described in the brain, a limitation of the present report is that we cannot argue that the differences found in aggression and sexual behaviour is exclusively due to changes in the SDN-POA. Even the POA is known to harbour another site of sexual dimorphism termed the anteroventral periventricular (AVPV) nucleus where increased cell number is associated with feminine behaviour (Simerly, 1989). The regions associated with mating and aggression do, however, exhibit spatial overlap in the brain (Lin *et al.*, 2011). In some instances these areas respond during both mating and aggression (Lin *et al.*, 2011), showing that the behavioural schemes are both morphologically and functionally linked. Another limitation of the

study is that the activities of these regions are likely also dependent of gonadal-pituitary axis e.g. the testosterone-LH ratio in adulthood, which was not investigated.

We included aniline in our experiments to explore both the pharmacological and the environmental exposure. Hence, it is becoming increasing clear that APAP is presenting itself as a compound with a dual exposure pattern, including high doses through pharmaceutical use and lower doses through the environment possibly through the conversion of ubiquitous pollutant aniline and its derivatives (Kristensen *et al*., 2016). Exposure to aniline resulted in a similar reduction in CALB-ir cell number of the SDN-POA as APAP. The reason for this is likely that aniline is metabolized to APAP in the liver of the pregnant dam (Holm *et al.*, 2015). We used an aniline exposure level corresponding to the amount of APAP allowed to be used by pregnant women (50 mg/kg/day) together with 3 times this amount, reaching a level known from rodent studies to inflict anti-androgenic effects (Kristensen *et al.*, 2011). The lowest level of aniline exposure was 31 mg/kg/day, which we have previously shown to result in mean urinary concentrations of 309.9 mg/l APAP after 4 h in C57Bl/6 (Holm *et al.*, 2015). In humans occupationally exposed to aniline, the range of APAP found in the urine is 4.2–10.9 mg/l (Dierkes *et al.*, 2014). The exposure is therefore in proximity to the exposure observed among occupationally exposed humans.

In common practice, a system of allometry based on body surface area is used to translate animal to human doses. In this system, the mouse dose is divided by a factor of 12.33 to reach appropriate human levels (Reagan-Shaw *et al.*, 2008). According to this estimate, the doses in this report resulting in significant effects of 150 mg/kg/day are well below the APAP doses used by pregnant women across Europe and the North America of 50 mg/kg/day. Consequently, these exploratory experiments have relevance to human health and follow previous reports suggesting an impact of APAP on brain development resulting in e.g. hyperkinetic disorders (Liew *et al.*, 2014; Hoover *et al.*, 2015; Brandlistuen et al 2013).

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Declaration of Interests:

No funding bodies or other agencies have had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Legends:

Figure 1: Prenatal exposure to paracetamol (APAP) and aniline from 7 days post coitum to delivery decrease neuronal number in the in the sexually dimorphic nucleus (SDN) of the preoptic area (POA) in the anterior hypothalamus of male adult offspring.

(A-B) Decrease in the size of the SDN-POA (shown by arrows on the right side of the third ventricle) detected by staining for calbindin D28 (calbindin-ir) in adult male mouse. The study found no difference in SDN-POA size between controls and animals exposed to 50 mg/kg/day APAP. Micrographs therefore here show the difference between exposure for (A) 50 and (B) 150 mg/kg/day APAP during prenatal life.

(C) Decrease in the size of the SDN-POA detected by staining for calbindin D28 (calbindin-ir) in adult male mouse after exposure to vehicle/water (n = 7), 50 mg/kg/day APAP (n = 6), 150 mg/kg/day APAP (n = 7), 31 mg/kg/day aniline (n = 6) and 93 mg/kg/day aniline (n = 9). Results are depicted as mean ± SEM and evaluated with a one-way ANOVA follow by *post hoc* Dunnett's test; * $p < 0.05$.

Figure 2: Intrauterine exposure to paracetamol 150 mg/kg/day (APAP) changes male territorial urine marking.

(A) No change was found between APAP males and controls in area covered by urine marking.

(B) APAP males deposited fewer urine marks compared to controls.

(C) APAP males deposit larger urine marks compared to controls.

Results are depicted as mean ± SEM and evaluated with an unpaired t-test; $n = 8$; * $p < 0.05$.

Figure 3: Intrauterine exposure to 150 mg/kg/day paracetamol (APAP) moderates male territorial display and fighting.

(A) APAP males have fewer aggressive sniffs compared to controls.

(C) APAP males have tendency to less tail rattles compared to controls.

(B) No change was found between APAP males and controls in number of attacks.

(D) APAP males did not bite intruding males in contrast to controls.

Results are depicted as mean ± SEM and evaluated with an unpaired t-test; $n = 8$; * $p < 0.05$.

Figure 4: Intrauterine exposure to 150 mg/kg/day paracetamol (APAP) decreased male mating behaviour.

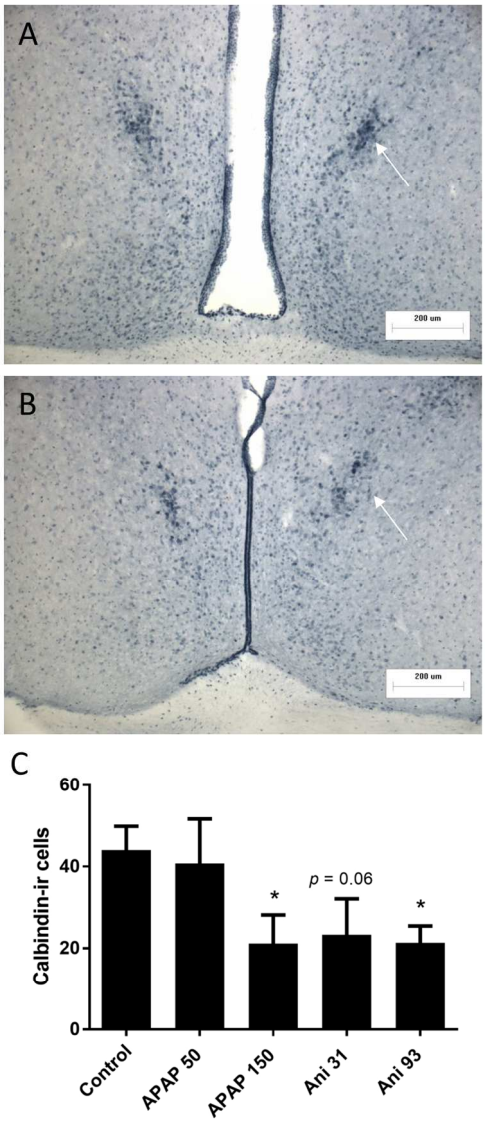
(A) No change was found between APAP males and controls in mating sniffs.

(B) No change was found between APAP males and controls in mounting females.

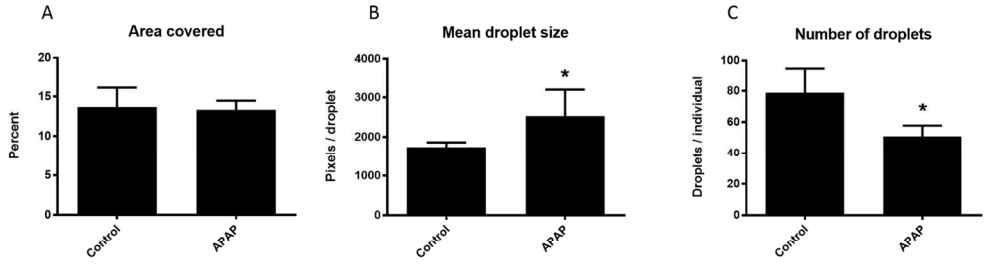
(C) APAP males had a tendency to fewer intromissions with females in oestrus compared to controls.

(D) APAP males did not ejaculate during intromission in contrast to controls.

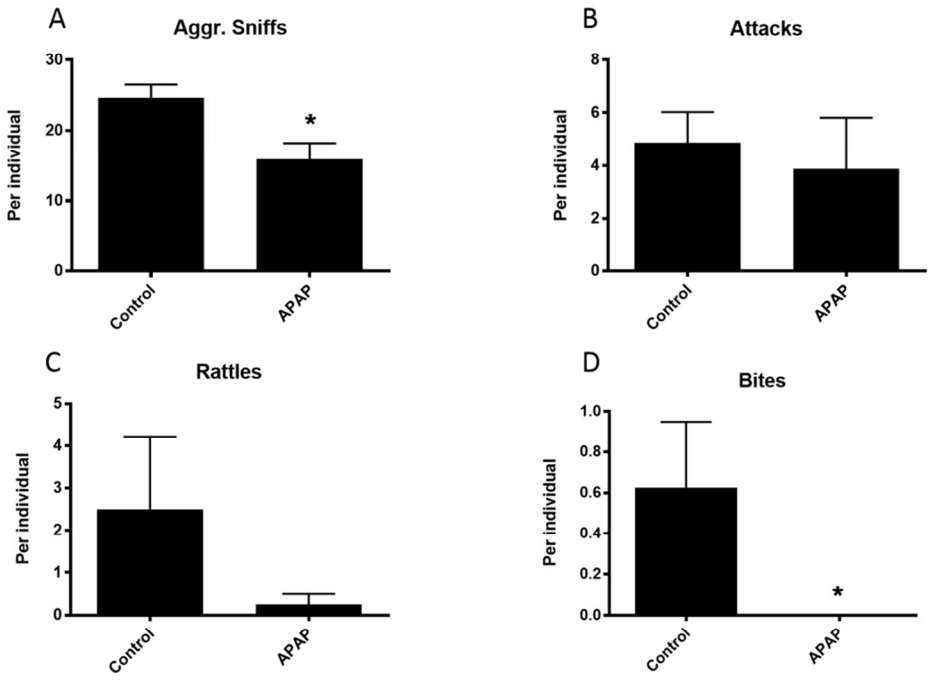
Results are depicted as mean ± SEM and evaluated with an unpaired t-test; $n = 8$; * $p < 0.05$.



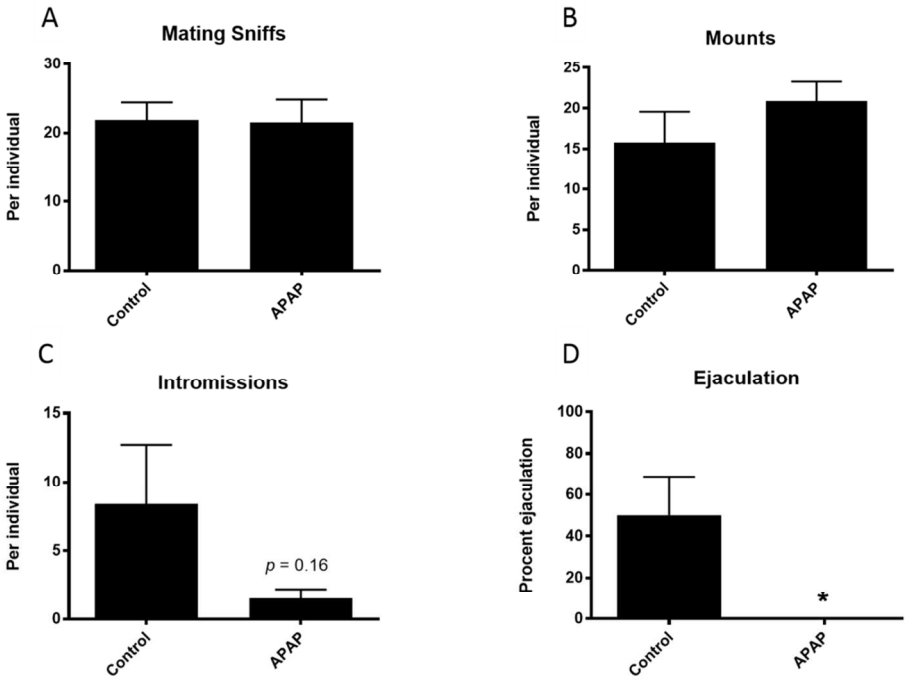
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185x64mm (300 x 300 DPI)



136x104mm (300 x 300 DPI)



140x103mm (300 x 300 DPI)