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Title: Effect of maternal renin-angiotensin-aldosterone system activation on social coping strategies and gene expression of oxytocin and vasopressin in the brain of rat offspring in adulthood

Running title: Maternal RAAS activation and programming of social coping
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Abstract

The intrauterine condition in which the mammalian foetus develops has an important role in prenatal programming. The aim of this study was to determine the extent to which activation of the maternal renin-angiotensin-aldosterone system (RAAS) could influence social behaviour strategies in offspring via changes in social neurotransmitters in the brain. Pregnant female Wistar rats were implanted with osmotic minipumps which continually released angiotensin II for 14 days at concentration of 2 μg/kg/h. The adult offspring (angiotensin and control groups) underwent a social interaction test. The mRNA expression of vasopressin, oxytocin and the oxytocin receptor in selected brain areas was measured by in situ hybridisation. Prenatal exposure to higher levels of angiotensin II resulted in a strong trend toward decreased total social interaction time and significantly decreased time spent in close proximity and frequency of mutual sniffing. The angiotensin group showed no changes in oxytocin mRNA expression in the hypothalamic paraventricular or supraoptic nuclei, but this group had reduced vasopressin mRNA expression in the same areas. We concluded that maternal activation of RAAS (via higher levels of angiotensin II) caused inhibition of some socio-cohesive indicators and decreased vasopressinergic activity of offspring. Taken together, these results suggest a reactive rather than proactive social coping strategy.

Keywords: renin-angiotensin-aldosterone system; angiotensin II; social coping; prenatal programming

Full title:

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Running head: Maternal RAAS activation and programming of social coping

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Abstract

The intrauterine condition in which the mammalian foetus develops has an important role in prenatal programming. The aim of this study was to determine the extent to which activation of the maternal renin-angiotensin-aldosterone system (RAAS) could influence social behaviour strategies in offspring via changes in social neurotransmitters in the brain. Pregnant female Wistar rats were implanted with osmotic minipumps which continually released angiotensin II for 14 days at concentration of 2 µg/kg/h. The adult offspring (angiotensin and control groups) underwent a social interaction test. The mRNA expression of vasopressin, oxytocin and the oxytocin receptor in selected brain areas was measured by *in situ* hybridisation. Prenatal exposure to higher levels of angiotensin II resulted in a strong trend toward decreased total social interaction time~~, prolonged latency until first contact~~ and significantly decreased time spent in close proximity and frequency of mutual sniffing. The angiotensin group showed no changes in oxytocin mRNA expression in the hypothalamic paraventricular or supraoptic nuclei, but this group had reduced vasopressin mRNA expression in the same areas. We concluded that maternal activation of RAAS (via higher levels of angiotensin II) caused inhibition of some socio-cohesive indicators and decreased vasopressinergic activity of offspring. Taken together, these results suggest a reactive rather than proactive social coping strategy.

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Introduction

Prenatal programming is a very complex process. During a critical developmental period, a stimulus or insult can alter foetal or neonatal physiological processes, which can have a long-lasting or permanent effect on the brain, behaviour and disease risk (Barker, 1998; Bale et al., 2010). The intrauterine conditions in which the mammalian foetus develops has an important role in this process (Barker, 1998).

One of the systems, which can be prenatally programmed, is the renin–angiotensin–aldosterone system (RAAS) (Nuyt, 2008). The RAAS is an important regulator of the blood pressure and the fluid/electrolyte homeostasis (Zhuo and Li, 2011). However, its role in modulating different physiological/pathological functions has also been described. The components of the RAAS are present in the central nervous system modulating sensory information, emotional and behavioural responses, learning, memory, pain, anxiety and stress (Llorens-cortes and Mendelsohn, 2002; Von Bohlen Und Halbach and Albrecht, 2006; Bali et al., 2014). RAAS is necessary for proper development of the foetus and all the components of this system are expressed in early human development (Schutz et al., 1996).

During pregnancy, the maternal RAAS can be influenced by several external and internal factors (O'Regan et al., 2004; Nogueira et al., 2007; Sykes et al., 2014; Xiao et al., 2014), which can alter the level of the individual components of this system. In humans, angiotensin converting enzyme (ACE) inhibitors and AT1 receptor (AT1R) antagonist are widely used in therapy of hypertension and it is not unique that pregnant women have this medication (Cooper et al., 2006). However, foetal exposure to angiotensin converting enzyme inhibitors and/or AT1 receptor antagonist has been associated with multiple developmental defects or even intrauterine foetal death (Quan, 2006; Bullo et al., 2012). On the other hand, very little data has been provided about the effects of increased angiotensin II (Ang II)

concentrations during pregnancy on *in utero* development of the foetus (Svitok et al., in press).

Foetal adaptations to supraoptimal intrauterine conditions (e.g. chronic variable stress) can lead to changes in the physiological mechanisms associated with coping strategies (Mueller and Bale, 2007).

Two stress response patterns have been distinguished based on social stress research in animals and humans - proactive (active) and reactive (passive) coping (Henry and Grim, 1990; Koolhaas et al., 1999, 2010).

Proactive social coping behaviour with higher sympathetic reactivity has been shown to increase the predisposition for ~~developing~~ hypertension, atherosclerosis and tachyarrhythmia (Henry and Grim, 1990; Hessing et al., 1994; Sgoifo et al., 2005). On the other hand, reactive social coping behaviour is associated with higher parasympathetic tone and reactivity, predisposing the individual to sudden cardiac death due to bradyarhythmia (Koolhaas et al., 1999).

Social stressors play an important role during the mammalian life course (Koolhaas et al., 1997; Sgoifo et al., 2005), and prenatal programing influences the adaptation strategy for this type of stress. The oxytocin (OT) and vasopressin (AVP) neuropeptides are involved in inter-neuronal communication within various areas of the brain to modulate social-emotional behavioural and physiological responses, reflective of the coping style (Holmes et al., 2003; Koolhaas et al., 2010). ~~While AVP is generally known to increase anxiety-like behaviour, stress and aggressiveness, OT has the opposite effect, facilitating social attachment, care and relationships (Insel et al., 1998; Heinrichs et al., 2009).~~

Both neuropeptides are produced by magnocellular and parvocellular neurosecretory neurons in the paraventricular (PVN), supraoptical (SPO) and accessory nuclei of the

hypothalamus. AVP is also produced by parvocellular cells in the suprachiasmatic nucleus (SCN) (Carter, 2007).

Increased vasopressinergic activity in discrete brain regions linked to regulation of stress coping behaviour is associated with increased levels of aggressiveness, and perhaps, a more proactive coping style in general (Bult et al., 1992; Compaan et al., 1992; Aubry et al., 1995; Everts et al., 1997; Koolhaas et al., 2010). On the other hand, increased oxytocinergic activity in these very same neural structures associated with facilitating social attachment, care and relationships (Insel et al., 1998; Heinrichs et al., 2009) and may be implicated in more reactive (passive) coping behaviours (de Boer et al., 2003; Koolhaas et al., 2010).

In the current study, we investigated the extent to which the activation of maternal RAAS (via prenatally increased Ang II) could influence social behaviour strategies in adult offspring via changes in the level of social neurotransmitters in the brain.

Material and Methods

Our research is part of a larger project focused on the effects of prenatal activation of regulatory mechanisms of blood pressure. The experiment was performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of Comenius University in Bratislava, Slovak Republic. The experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic.

Animals

Wistar rats (VELAZ Praha, Czech Republic) were used in this study. The parental generation consisted of nine females and four males. Animals were housed in groups of two or three animals in standard light conditions (12:12 hour light-dark cycle; lights on at 6:00), with an average temperature of 21 ± 2°C and 55 ± 5% relative humidity. Water and food

(standard laboratory chow) were available *ad libitum*. After an acclimatisation period of 7 days, female rats were divided into two groups: the control group (Ctrl; n = 4), and the angiotensin II group (AngII; n = 5). The animals were mated overnight. The ovulatory cycle phase and day 0 of gestation (the presence of spermatozoa in a vaginal smear) were identified according to Gleich and Frohberg (1977).

Osmotic minipumps were implanted on the sixth day of gestation (model 2002, Alzet, Canada), placed subcutaneously on the back of dams in the AngII group, through which Ang II (1.36 mg/ml in physiological solution) was continuously released at 2 µg/kg/h for 14 days. In contrast, an oval object of the same shape and size (sham operation) was implanted into Ctrl dams. For the surgery, animals were anesthetised using a solution of ketamine (135 mg/ml; Narketan^{TM} 10, Chassot GmbH, Germany) and xylazine (18 mg/ml; Rometar 2%, Spofa, Czech Republic). The animals were kept under the same conditions as prior to surgery until birth. Following birth, the litters were culled to eight animals per litter (four males and four females). Each dam and litter were housed together in individual cages until weaning at postnatal day 21. After weaning, rats of each gender were housed separately in groups of four animals per cage.

Body weight did not differ between control and AngII dams before, or at the end of pregnancy. Ang II administration had no effects on litter size or birth weight of the offspring. Sex ratio of pups was similar between both groups and the body weight did not differ between groups during the whole course of the experiment. Increased Ang II during pregnancy raised blood pressure in the offspring. Treatment also increased aldosterone and decreased plasma renin activity in the offspring (Svitok et al., in press).

Animals were anaesthetised with CO_2 and decapitated at postnatal day 79–83. Their brains were immediately removed and placed into frozen medium (Cryomount, Histolab AB, Sweden), frozen in dry ice and stored at − 80◦C. For *in situ* hybridisation, brains from the

litters of 4 Ctrl and 5 AngII females (Ctrl n = 20, males n = 10, females n = 10; AngII n = 20, males n = 10, females n = 10) were used. The brains of all animals tested in the social interaction test were also evaluated by *in situ* hybridisation.

Social interaction test

The social interaction test was adapted from File (1980). At 77 days of age, the offspring (Ctrl: male n = 10; female n = 10; AngII: male n = 10, female n = 10) were tested for their social interaction behaviours with an unknown test partner of the same weight (no more than 10 g difference), sex and treatment. Pairs were tested in a random order between 14:00 and 15:00. The dimensions of the test box were 33.5 × 71 cm, with walls 37 cm high.

Animals were acclimatised the day before testing by singly placing each animal in the test box for 20 minutes. Pairs of animals were placed in diagonally opposite corners of the box. The behaviour of the animals was measured over a period of 5 minutes, which was recorded using a digital camera (DCR-DVD 92 E; Sony, Japan).

The latency until first contact (duration in seconds) and total duration and frequency of socio-cohesive (following, chasing, mutual sniffing, genital investigation, climbing over, crawling under, allogrooming contact, time in close proximity) and socio-aversive (escape, mounting, tail biting) interactions were recorded during the time period from the video.

In situ hybridisation

The mRNA expression of vasopressin, oxytocin (neurotransmitters responsible for social activity) in the hypothalamic paraventricular (PVN) or supraoptic (SPO) nuclei (the site of their synthesis) was measured by *in situ* hybridisation.

The *in situ* hybridisation protocol has been previously described elsewhere (Štefánik et al., 2015). Briefly, consecutive frozen cryostat sections of the rat brain (14 µm) were mounted

on adhesion slides (SuperFrost® Plus, MenzelGläser, Thermo Fisher Scientific Inc., Germany) for subsequent hybridisation of the chosen mRNAs. A synthetic 41-base oligonucleotide probe with the sequence 5′-GGG CTC AGC GCT CGG AGA AGG CAG ACT CAG GGT CGC AGG CG-3′ was used, complementary to nucleotides 906–946 of the rat OT mRNA (GenBank accession number K01701) (Patisaul et al., 2003). For hybridisation of AVP mRNA, a 48-base probe with the sequence 5′-GCA GAA GGC CCC GGC CGG CCC GTC CAG CTG CGT GGC GTT GCT CCG GTC-3′ was used (directed against the final 16 amino acids of the glycoprotein that is not shared with oxytocin) (Müller et al., 2000). All sequences were firstly checked for complementarity with non-specific rat RNA using BLAST in the EMBL sequence database. None of the probes recognised different non-specific sequences with sufficient fidelity.

Probes were labelled at the 3′-end with [α-^{35}S] dATP (1200 Ci/mmol; Perkin Elmer Inc., USA) by terminal deoxynucleotidyl transferase (Thermo Fisher Scientific Inc., Canada). Unincorporated radioactivity was removed with Sephadex G-50 micro-columns (GE Healthcare, Little Chalfont, UK). The sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 5 minutes, then rinsed twice in PBS, acetylated in a solution containing 0.25% acetic anhydride in 0.8% triethanolamine hydrochloride and 0.9% sodium chloride for 10 minutes. Sections were dehydrated through graded ethanol solutions, delipidated in chloroform for 5 minutes, then dipped in 100 and 95% ethanol prior to air-drying. Sections were hybridised with the labelled probe in 70 µl of hybridisation buffer per slide (50% formamide, 4× saline-sodium citrate (SSC) buffer, 1× Denhardt's solution, 10% dextran sulphate, 500 µg/ml sheared single-stranded DNA and 250 µg/ml yeast tRNA), which was mixed with 0.1 M dithiothreitol (DTT) and the purified radiolabelled oligoprobe (270,659 cpm/µl for OT and 186,625 cpm/µl for AVP) before use. The radiolabeled probe was diluted to a concentration of 2.7×10^6 counts/min/ml (OT and AVP). The slides were

incubated in humidified boxes for 18 hours at 41°C. Post-hybridisation, the slides were processed by washing with 1× SSC for 5 minutes at room temperature, then in 1× SSC for 1 hour at 55°C, and finally in 1× SSC for 1 hour at room temperature. Afterwards, the slides were dipped in distilled water then 70% ethanol, then left to air-dry.

Slides with probes for OT and AVP were exposed to Hyperfilm-Beta-Max (Eastman Kodak Company, Chalon-sur-Saône, France) for 48 hours. The film was developed using a developer (LQN) solution then fixed in FOMAFIX (FOMA, Hradec Králové, Czech Republic) solution. After exposure, the slides were stained with cresyl violet to determine the position and shape of the neuronal structures.

Autoradiographs were captured using an optical magnifier and digital camera (AM423X Dino-Eye, Dino-Lite, Taiwan) and corresponding software (Dino Capture 2.0, Dino-Lite, Taiwan), then analysed using ImageJ software (version 1.47i; NIH, USA). The relative density of individual structures was measured for each slide. Optical densities of the SON and PVN nuclei were determined for OT and AVP. The optical density of signals was bilateral, but was separately evaluated for each section. As an internal standard, the background density adjacent to each structure was captured and subtracted from the density of the region of interest (performed separately for each side). The mean values from the left and right side were obtained from four to six slides per animal. The rat brain atlas (Paxinos and Watson, 1998) was used to verify the correct locations.

Statistical analysis

All data were analysed using Kolmogorov-Smirnov tests to determine whether they fit a normal distribution. The results were analysed using Statistica version 7.0 software (StatSoft, Inc., Tulsa, OK, USA). Data were analysed by ~~general linear model~~ two-way analysis of variance (ANOVA) with the treatment and ~~sex—gender~~ as co-factors. If the

interaction was significant ($p < 0.05$), differences in behavioural parameters between control and AngII rats were estimated by Fischer LSD *post hoc* test. ~~If data did not fit a normal distribution, the Kruskal-Wallis test was used.~~

Results

Social interaction test

For the latency until first contact, the two-way ANOVA did not reveal any significant effect of treatment ($F_{(1,36)} = 0.001$, $p = 0.97$), sex ($F_{(1,36)} = 0.167$, $p = 0.685$) and interaction treatment vs. sex ($F_{(1,36)} = 2.124$, $p = 0.154$).

For the total time spent participating in socio-cohesive interactions, the two-way ANOVA did not reveal any significant effect of treatment ($F_{(1, 36)} = 2.26$, $p = 0.14$), sex ($F_{(1, 36)} = 1.62$, $p = 0.21$) and interaction treatment vs. sex ($F_{(1,36)} = 1.717$; $p = 0.2$). We recognised only a trend towards a lower number of socio-cohesive interactions in AngII females in comparison with Ctrl females ($p = 0.054$; Fig. 1a).

For the time spent in close proximity, the two-way ANOVA revealed significant effect of treatment ($F_{(1,36)} = 7.487$; $p < 0.05$), sex ($F_{(1,36)} = 6.343$; $p < 0.05$), and interaction treatment vs. sex ($F_{(1,36)} = 10.456$; $p < 0.01$). In total, AngII animals spent less time in close proximity compared to Ctrl animals ($p < 0.05$; Fig. 1b). We observed differences between males and females, such that AngII females spent less time in close proximity to the other animal during this test compared to Ctrl females ($p < 0.001$). Ctrl males also spent less time in close proximity than Ctrl females ($p < 0.001$; Fig. 1b). The two-way ANOVA revealed significant effect of treatment for frequency of sniffing ($F_{(1,36)} = 6.1425$; $p < 0.05$). Animals in the AngII group had a lower frequency of sniffing in comparison with Ctrl males ($p < 0.05$; Fig. 1c).

Socio-aversive interactions were not observed for any animal in either the AngII or Ctrl groups.

242

243 < Please insert Fig. 1 >

244

245 ***In situ hybridisation***

246 The two-way ANOVA revealed significant effect of treatment ($F_{(1,36)}$ = 4.4973; $p <$
247 0.05) for AVP mRNA expression in PVN. AngII animals had lower AVP mRNA expression
248 compared with Ctrl group (Fig. 2).

249 Statistical analysis revealed also significant effect of interaction treatment vs. sex
250 ($F_{(1,36)}$ = 5.637; p = 0.023) for AVP mRNA expression in the SPO. AngII males had lower
251 AVP mRNA expression compared with Ctrl males (Fig. 2).

252

253 < Please insert Fig. 2 >

254

255 There were no significant effect of treatment and/or sex in OT mRNA expression in
256 either the hypothalamic PVN or SPO between.

257

258 **Discussion**

259 In the current study we applied a new model for evaluating developmental
260 programming of cardiovascular disease. This model involved the activation of maternal
261 RAAS via implantation of osmotic minipumps that continually released Ang II during most of
262 the gestational period. The research presented here is part of a larger project focused on the
263 effects of prenatal activation of regulatory mechanisms of blood pressure, part of which has
264 already been published (Svitok et al., in press).

265 We hypothesised that the activation of maternal RAAS (via higher levels of Ang II)
266 would be associated with changes in the social coping behaviour of the offspring. There are

no studies in the literature that have described the prenatal effects of Ang II administration on social behaviour, therefore, we have compared our results with animal models with upregulation (TGR(mRen2)27) and downregulation (TGR(ASrAOGEN6)680) of RAAS.

Studies that have investigated models of downregulated RAAS have reported that rats spent more time in relatively close spatial proximity of the rats without further motor interactions compared with controls in social interaction tests (Voigt et al., 2005; Mayorov, 2011). Rats with upregulated RAAS could have a similar effect to the total social interaction observed in rats with downregulated RAAS (Voigt et al., 2005).

Our results showed a trend toward differences in total socio-cohesive interaction. When we focused on individual parameters (~~latency until first contact,~~ time spent in close proximity and frequency of sniffing), we observed similar results to that reported by Voigt *et al.* (2005), who observed reduced sniffing in rats with downregulated RAAS. The time spent in close proximity was also found to be decreased in AngII treated animals. Rats with downregulated RAAS were not different in their latency until first contact behaviour (Voigt et al., 2005), similarly, in our study, prenatal exposure to Ang II did not change latency period until first contact.

The present study was based on a prenatal intervention to investigate how blood pressure regulation mechanisms influence programming of social coping strategies. ~~We recognised two coping strategies, proactive and reactive (Koolhaas et al., 1999).~~ In regard to behaviour, the proactive strategy is not only characterised by higher levels of aggression, dominance and territoriality, but also by a tendency to develop routine-like, rigid, intrinsically driven behaviours (Sgoifo et al., 2005). In contrast, the reactive strategy is characterised by less aggressive, less territorial and subordinated behaviours, in addition to being more flexible and driven by changes in environmental stimuli (Koolhaas et al., 1999; Sgoifo et al., 2005). These two styles of adaptation (coping strategies) also differ in terms of

292 autonomic/neuroendocrine (re)activity. Proactive animals have higher sympathetic activation
293 and lower (re)activity of the HPA axis, whereas reactive animals are parasympathetically
294 driven and have higher HPA axis (re)activity (Koolhaas et al., 1999; Sgoifo et al., 2005).
295 From our results, maternal RAAS activation (via higher levels of Ang II) caused inhibition
296 some aspects of social behaviour in offspring, which we suggest is associated with reactive
297 rather than proactive social coping strategies. Our suggestion is supported by higher
298 activation of the HPA axis, that leads to increase of aldosterone levels (Kubzansky and Adler,
299 2010). Aldosterone may induce a wide spectrum of behavioural manifestations of
300 psychological stress (e.g., anxiety, depression) (Hlavacova and Jezova, 2008; Hlavacova et
301 al., 2012). This mineralocorticoid plays a key role in modulation of mood through effects on
302 relevant mood-regulating brain areas including nucleus tractus solitarii (Murck et al., 2014).
303 According to Ngarmukos and Grekin (2001), behavioural effect of aldosterone may be
304 mediated by non-genomic mechanism on ionic exchangers or membrane receptors.
305 Aldosterone plays a central role in the regulation of plasma sodium through mineralocorticoid
306 receptors (Bailey et al., 2009). Plasma sodium imbalance can lead to mood disorders and
307 decreased reactivity to psychogenic stressors (Krause et al., 2011; Leshem, 2011).
308 In our study, AngII animals have been reported to have increased plasma aldosterone
309 levels compared to controls (Svitok et al., in press), also observed in transgenic rats
310 (TGR(mRen2)27) with upregulated RAAS. Aldosterone levels were found to be 10-times
311 higher in rats with upregulated RAAS than in control rats, which supports upregulation of the
312 renin-angiotensin system, as it is well known that angiotensin II stimulates aldosterone
313 synthesis (Ferrario, 1983; Zeman et al., 2007).
314 Furthermore, reactivity of the HPA axis to adrenocorticotropic hormone in transgenic
315 rats is higher compared to other normotensive rat strains (Husková, 2006). Higher activation
316 of the HPA axis suggests reactive social coping behaviour.

317 The two main brain neurotransmitters responsible for social activity in animals
318 ~~(individuals)~~ are vasopressin and oxytocin. Generally, increased vasopressinergic activity in
319 discrete brain regions is associated with proactive coping styles, and increased oxytocinergic
320 activity in these very same neural structures is associated with reactive (passive) coping
321 (Koolhaas et al., 2010). In our study, animals in the AngII group did not differ in OT mRNA
322 expression in the hypothalamic PVN or SPO nuclei, however, this group had lower
323 expression of AVP mRNA in the PVN and SPO nuclei of the hypothalamus~~, which is the site~~
324 ~~of AVP and OT synthesis~~. These changes again suggest a reactive rather than proactive social
325 coping strategy.

326 In conclusion, prenatal exposure to higher levels of Ang II caused changes in social
327 behaviour, suggestive of a reactive rather than proactive social coping strategy, ~~in addition~~
328 supported by to altered vasopressinergic activity.

329

330 **Acknowledgments**

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335

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Captions

Fig. 1 Total time spent participating in socio-cohesive interactions (**a**), total time spent in close proximity (**b**), frequency of sniffing (**c**) in angiotensin (AngII: males n = 10, females n = 10) and control (Ctrl: males n = 10; females n = 10) groups. Error bars represent standard error of the mean and the significance of differences is indicated by asterisk * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Fig. 2 *In situ* hybridisation for arginine-vasopressin (AVP) Representative *in situ* photos of AVP mRNA signals in Ctrl (**a**) and AngII (**b**) group. Comparison of optical density signal for AVP of angiotensin (AngII: males n = 10, females n = 10) and control (Ctrl: males n = 10, females n = 10) rats in paraventricular (PVN; **c**) and supraoptical nuclei (SPO; **d**). Error bars represent standard error of the mean, and the significance of differences is indicated by asterisk * $p < 0.05$.

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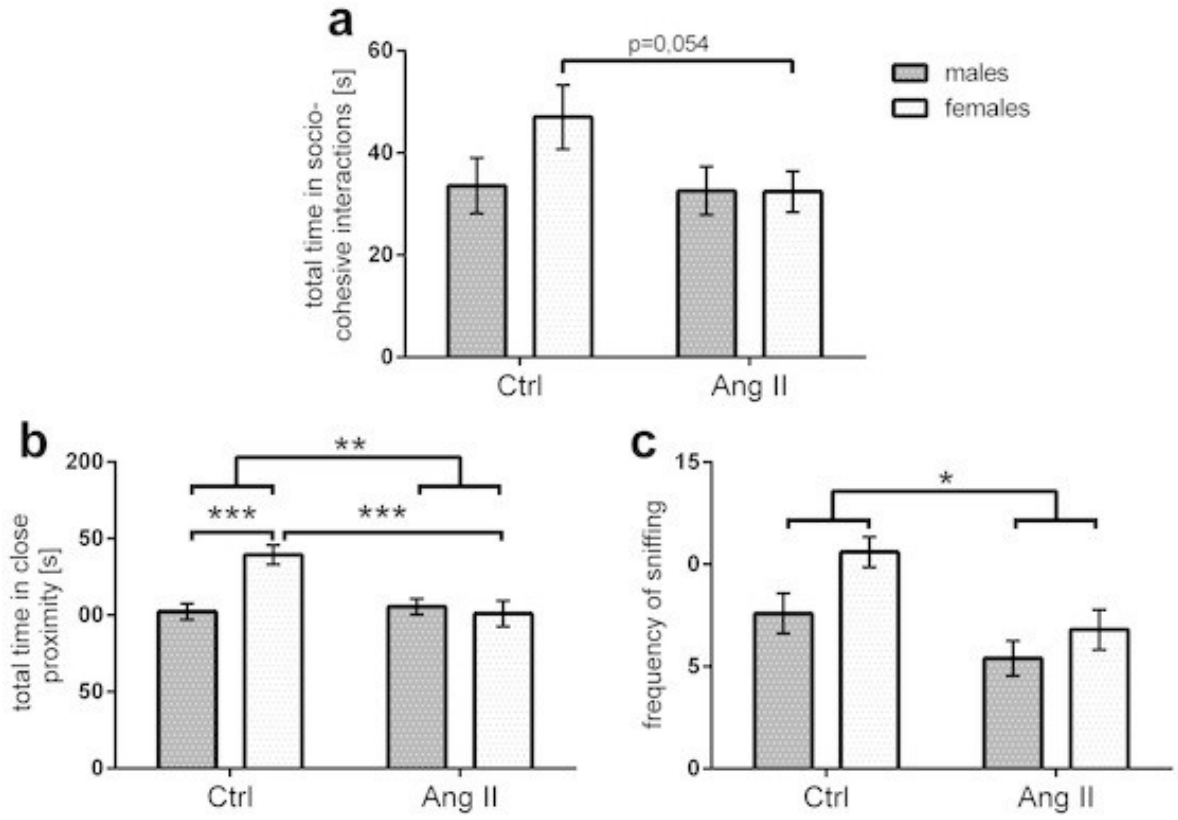

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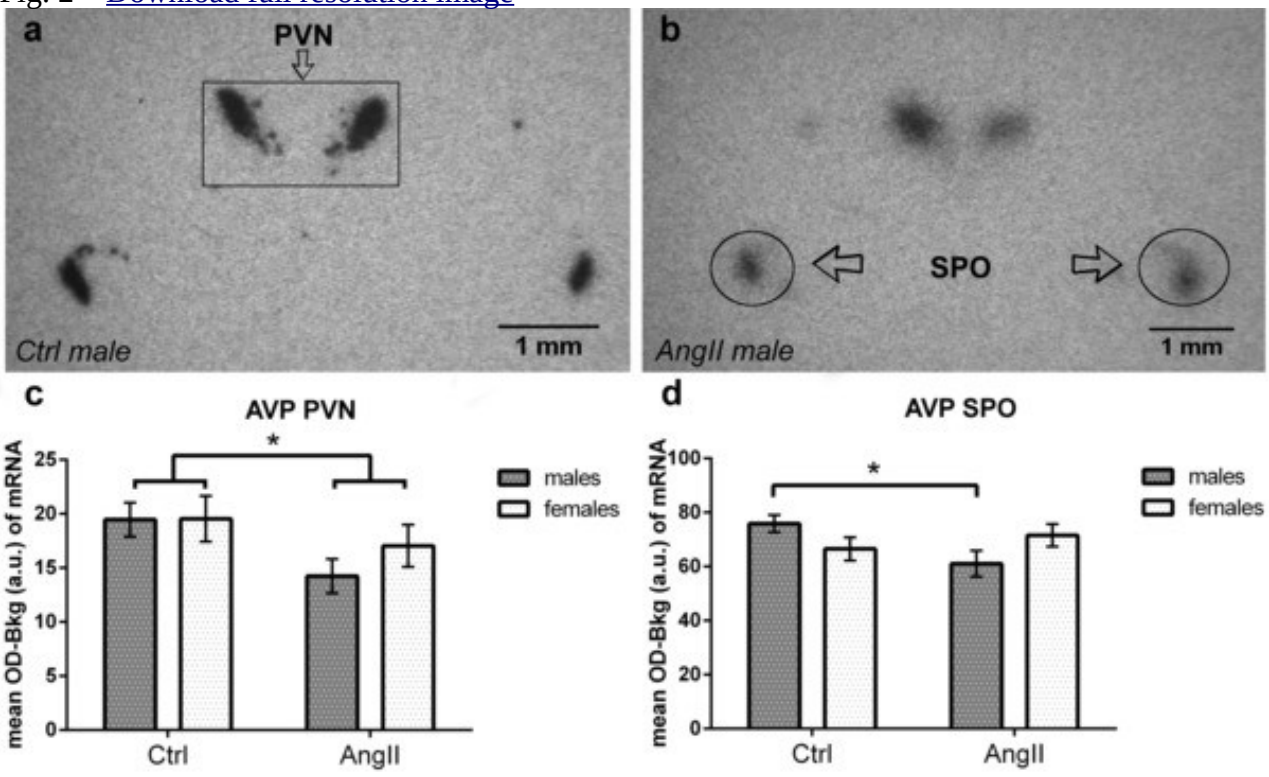

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