MR. FELIX EFFAH (Orcid ID : 0000-0001-5623-914X)

Article type : Research Article

Research Article

REGION-SPECIFIC SEX MODULATION OF CENTRAL OXYTOCIN RECEPTOR BY GUT MICROBIOTA: AN ONTOGENIC STUDY

Felix Effah[1], Nívea Karla de Gusmão Taveiros Silva[2], Rosana Camarini[2], Fatima Joly[3], Sylvie Rabot[3], Vincent Bombail[4*], Alexis Bailey[1*]

[1]Pharmacology Section, St George's University of London, Cranmer Terrace, SW17 0RE, London, UK

[2] Pharmacology Department, Universidade de Sao Paulo, São Paulo, Brazil

[3] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350 Jouy-en-Josas, France

[4] UMR PNCA, AgroParisTech, INRAE, Université Paris-Saclay, Paris, France

***Equal contributions and correspondent authors**

Correspondent authors:

Alexis Bailey, Pharmacology Section, St George's University of London, Cranmer Terrace, SW17 0RE, London, UK. Email: abailey@sgul.ac.uk

Vincent Bombail, UMR PNCA, AgroParisTech, INRAE, Université Paris-Saclay, Paris, France, Email : vincent.bombail@inrae.fr

Acknowledgements

Claire Le Poupon and Gaëlle Champeil-Potokar helped with brain sample processing. Aurélien Raynaud and Patrice Dahirel provided expert assistance with animal care. The research was funded by St. George's

University of London (SGUL) and by the MRes Translational Medicine pioneering scholarship offered to Felix Effah by SGUL.

Conflict of interest statement

No conflict of interest to declare

Data Statement:

All data generated or analysed during this study are included in this published article.

ABSTRACT

Oxytocin (OT) is a developmentally important neuropeptide recognised to play a dominant role in social functioning and stress-related behaviours, in a sex-dependent manner. Nonetheless, the underlining factors driving OT and OT receptor (OTR) early brain development remain unclear. Recent evidence highlight the critical influence of gut microbiota and its bidirectional interaction with the brain on neurodevelopment via the gut microbiota-brain axis. Therefore, we aimed to determine the impact of gut microbiota on the OTR system of the rat brain at different developmental stages in a pilot study. Quantitative OTR [^{125}I]-OVTA autoradiographic binding was carried out in the forebrain of male and female conventional (CON) and germ-free (GF) rats at postnatal days (PND) 8, 22 and 116-150. OTR binding was also assessed in the eyes of PND 1 and PND 4 GF female rats. Significant 'microbiota x sex x region' interaction and age-dependent effects on OTR binding were demonstrated. Microbiota status influenced OTR levels in males but not females with higher levels of OTR observed in GF vs CON rats in the cingulate, prelimbic and lateral/medial/ventral orbital cortex, and septum across all age groups, while sex differences were observed in GF, but not in CON rats. Interestingly, OTRs present in the eyes of CON rats were abolished in GF rats. This is the first study to uncover a sex-specific role of gut microbiota on the central OTR system, which may have implications in understanding the developmental neuroadaptations critical for behavioural regulation and the aetiology of certain neurodevelopmental disorders.

Keywords: microbiota, oxytocin receptor, receptor ontogeny, germ-free, rat brain, quantitative autoradiography

INTRODUCTION

Oxytocin (OT) is a neuropeptide hormone that mediates a broad spectrum of sexual, reproductive, emotional, and social functioning in mammals (Caldwell *et al.*, 1986; Lee *et al.*, 2009; Tamma *et al.*, 2009; Vaidyanathan and Hammock, 2017) and is critical for normal postnatal neurodevelopment such as sensory processing and social bonding (for review see Muscatelli *et al.*, 2018). Studies performed in OT or OT receptor (OTR) knockout (KO) mice revealed deficits in social memory (Ferguson *et al.*, 2001) and social interaction (Pobbe *et al.*, 2012), increased anxiety and stress responses to psychogenic and certain physiological stimuli (Amico *et al.*, 2004; Mantella *et al.*, 2003). Many of these behaviours were reversed by the administration of OT in OT-deficient mice (Mantella *et al.*, 2003) highlighting a pivotal role for OT in modulating a range of behaviours associated with social functioning and stress regulation. Interestingly, differences in the effects of OT on several social behaviours including social avoidance, social recognition, partner preference, social play and social interest in males and females have been consistently reported across several species indicating a profound sexual dimorphism effect (Dumais and Veenema, 2016). Many of these sex differences have been documented following OT administration during early life development (Bales *et al.*, 2007; Bales and Carter, 2003) and persist in adulthood (Yamamoto *et al.*, 2004), indicating that manipulation of the OT system during developmentally sensitive periods may have long-lasting effects. Although more research in this area is warranted, it appears that while OT is involved in most of these social behaviours in both sexes, females may be more sensitive to some of the effects of OT than males (Dumais and Veenema, 2016). For instance, in prairie voles, while in females, partner preference behaviour was developed upon OT infusion, in males, no OT-induced pair-bonding behaviour was detected (Insel and Hulihan, 1995). Similar effects were observed in other species, including humans (Campbell, 2010).

With respect to OTR brain distribution, while some sex differences identified in central OTR levels appear to be species and region-dependent with males overall showing higher levels of OTR than females in specific brain regions, the majority of studies did not reveal dimorphic sexual effect on OTR binding in most regions analysed (for extensive reviews on the subject see Caldwell *et al.*, 1986; Dumais and Veenema, 2016). Nonetheless, whether sex differences appear during early development or whether sex differences influence behaviour and how these may develop over time remains elusive.

Similar to many other receptors, OTR undergoes profound ontogenic development in the brain. Shapiro and Insel, (1989) demonstrated developmental variations that occur in OTR in the rat brain over the first 60 days from birth with regions such as the nucleus accumbens, thalamus, posterior cingulate and dorsal subiculum showing an increase in OTR binding which peaked at postnatal day (PND) 20, followed by a decrease after that till PND60 (Shapiro and Insel, 1989). The significance of these ontogenic variations on

brain function and behaviour is not entirely clear. However, given the central role of OT on neurophysiological functions and behaviours intrinsically associated to neurodevelopment and mental health well-being (Grinevich *et al.*, 2015), it is highly likely that these variations may play a vital role in the developmental pattern of certain behaviours. Manipulation of this ontogenic variation may have a profound effect on mental health well-being in later life (Cirulli *et al.*, 2009). Therefore, identifying the nature of these developmental variations of the central OTR system and the factors influencing them may be critical for our understanding of specific neurodevelopmental disorders, such as autism, as well as neurobehavioural development.

Emerging evidence suggests that gut microbiota plays a pivotal role in brain function and behavioural modulation via the so-called gut-brain axis (Cryan and O'Mahony, 2011). The gut microbiota play a key role in neuro-endocrinal signalling pathways (Cryan and Dinan, 2012; Nicholson *et al.*, 2012). They are capable of metabolising endogenous metabolites derived from the host as well as nutrients into small molecules (e.g. serotonin (5-HT), short-chain fatty acids (SCFAs), gamma-aminobutyric acid (GABA)). These, in turn, may activate the enteric nervous system in the gut to cause alterations in various neurotransmitter systems in the brain, thus impacting on behaviour (Dinan and Cryan, 2016). Some human but mostly animal studies have identified early postnatal microbiota colonisation as critical for healthy neurodevelopment; and disruption of that colonisation has been linked to neuropsychiatric disorders (Warner, 2019). Concerning OT, intriguingly, there is evidence that *Lactobacillus reuteri*, probiotic strain (ATCC PTA 6475) can increase brain OT levels via the hypothalamic-pituitary axis (HPA) dependent mechanism (Erdman and Poutahidis, 2014). Indeed, *Lactobacillus reuteri* increased social behaviours in mouse models of autism by incrementing OT levels in neuronal regions involved in reward processing (Sgritta *et al.*, 2019). This suggests that specific strains of gut microbiota may play a key role in central OT physiology. Nonetheless, the impact of gut microbiota on OT system development during a developmentally sensitive period characterised by profound neuroadaptations remains elusive.

Given the critical role of OT in neurodevelopment and the evidence that gut microbiota can affect the central OT system and hence behaviour, we hypothesise that they are also involved in the ontogenic development of the central OT system. Thus, we carried out quantitative OTR autoradiographic binding with the use of [^{125}I]-OVTA on coronal brain cryosections from germ-free rats (GF) and conventional (CON) rats at different developmental ages [PND 8, 22 and 116-150 (adult)] in a pilot study in order to assess the influence of gut microbiota on OTR ontogeny. Early postnatal (PND 8) and weaning ages (PND 22) were selected as they constitute critical developmental windows where early postnatal colonisation takes place, which in turn is known to influence early behavioural outcomes (Warner, 2019). Adult rats were selected in order to assess whether potential alterations in OTR binding during early development

persist into adulthood. Due to the aforementioned sexually dimorphic nature of OT, we assessed the effect of gut microbiota on OTR ontogeny in both male and female rats. We hypothesised the presence of a gender x microbiota status interaction across and within brain regions and age groups.

In addition, in an attempt to assess the role of microbiota on OTR expression within the eye, we also investigated OTR binding patters in the eyes of CON and GF rats at PND1 and PND4. The role of OT in the eyes remains to be extensively investigated, but there is evidence to suggest that OTRs are present in the eye at birth (Greenwood and Hammock, 2017) and OT activation of the OTR in the posterior retina may play a key role in the communication between the cone photoreceptor and the the retinal pigment epithelium (RPE) (Halbach *et al.*, 2015).

GF rats, also known as gnotobiotic rats have no internal or external microorganism (Martín *et al.*, 2016). They were chosen as the preferable animal model to compare against CON rats in this study for several reasons. This study aimed to unravel the impact of gut microbiota on brain development and more specifically on the ontogeny of the OTR system. One method for obliterating gut microbiota is the antibiotic-treated model. This model is obtained as a result of antibiotic cocktail administration, which broadly depletes rat gut microbiota. However, this method is incapable of depleting the gut microbiota thoroughly (Kennedy *et al.*, 2018) and therefore, there would be some bacteria still present that could have impacted on the outcome of this study. Had antibiotic-treated models been used for this investigation, it would have been difficult to determine at what developmental stage the absence of the gut microbiota initiates impact on brain OTR neurochemistry. Also, rats are highly susceptible to antibiotic-induced diarrhoea, which may have impacted on the OTR expression due to the off target/non specific effects of the antibiotics. Therefore, the most suitable animal model to achieve this aim is the GF model.

This is the first study to uncover a gender-specific role of gut microbiota on central OTRs , which may have implications in the understanding of crucial neurobehavioural development as well as neurodevelopmental disorders aetiology.

MATERIALS AND METHODS

2.1 Animals

Male and female germ-free (GF) and conventional (CON) Fischer rats (Fischer 344; age ranges from 1 to 150 days old) were used. GF rats were obtained from the breeding unit of Anaxem, the GF facility of the Micalis Institute (INRAE, Jouy-en-Josas, France) and CON rats were purchased from Charles River

Laboratories (L'Arbresle, France). All standardised procedures, including the breeding of GF animals, were carried out in France in licensed animal facilities (Anaxem license number: B78-33-6). GF and CON rat litters were kept with their lactating mothers until weaning at 21 days (litter size 6-8), and after individuals of the same sex were kept in pairs. To maintain axenic status, the GF rats were grown in sterile isolators and every week; their sterile conditions were monitored by microscopic examination and screening cultures in their faeces. Makrolon cages containing sterile beddings made of wood shavings hosted the GF animals within the isolators. The CON rats were kept under standard laboratory environment (Bombail *et al.*, 2019). GF rats were given free access to autoclaved tap water and a gamma-irradiated (45 kGy) standard diet (R03; Scientific Animal Food and Engineering, Augy, France). CON rats were exposed to regular tap water and the same diet (non-irradiated). The animal room was maintained on a 12 hours light-dark cycle (lights switched on at 7:30 AM – 7:30 PM). On different days, the rats were sacrificed by decapitation, and their brains were rapidly removed, frozen in isopentane then stored at -80 °C. GF and CON rat brains were processed for quantitative receptor autoradiographic analysis.

2.2 OTR autoradiography

General methods for autoradiographic binding were carried out as previously described by (Farshim *et al.*, 2016; Georgiou *et al.*, 2016; Rae *et al.*, 2018; Zanos *et al.*, 2014b). Brains of male and female GF and CON rats at PND age of 8, 22 and 116-150 (adult) days were removed from a -80 °C freezer and sectioned using a cryostat apparatus (Thermoscientific, UK) set at -21°C. Heads containing eyes and olfactory nuclei of female GF and CON rats at PND 1 and PND 4 were sectioned. Adjacent coronal brain sections of 20 μm thick cut at 400 μm intervals were thaw-mounted onto gelatine-coated ice-cold microscope slides. Sections cut range from the level of the olfactory bulb (Bregma 4.20 mm) to the forebrain (Bregma 1.20 mm). Brain slides were conserved at -40 °C in airtight containers containing a layer of anhydrous calcium sulphate (Drierite-BDH chemicals, Dorset, UK) for a minimum of one week to dry before usage. Quantitative OTR autoradiographic binding was carried out on those brain sections. Sections were rinsed for 10 minutes in a pre-incubation buffer solution (50 mM Tris-HCl pH 7.4 at room temperature) to washout endogenous OT. Total binding was determined by incubating the prepared sections with 50 pM [^{125}I]-Ornithine vasotocin analog [d(CH2)5[Tyr(Me)2,Thr4,Orn8,[125I]Tyr9-NH2]-vasotocin] ([^{125}I]-OVTA), in an incubation buffer medium (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% w/v bovine serum albumin, 0.05% w/v bacitracin, pH 7.4 at room temperature) for 60 min. For the non-specific binding, adjacent sections were incubated with [^{125}I]-OVTA (50 pM) for 60 minutes in the presence of 50 µM of OT ligand, (Thr4, Gly7)-oxytocin. When the incubation was completed, slides were rinsed three times for five minutes in ice-cold rinse buffer solution (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4 at 0ºC) followed by a 30-minute wash in the ice-cold rinse buffer, and a subsequent 2- second wash in ice-cold distilled water. Slides were then dried

under a stream of cool air for 2 hours and stored in sealed containers with anhydrous calcium sulphate for two days. The slide sections were placed side by side to Kodak MR-1 films in hyper cassettes with autoradiographic [^{14}C] microscales of known radioactive concentration for three days (Zanos *et al.*, 2015). Sections for the same developmental groups (CON and GF, males and females) were arranged in parallel, processed and apposed to the same film at the same time to avoid inter-experimental variations. Film development was conducted in a dark room using red-filter light. The films were developed by immersing them individually one at a time into a tray containing 50% Kodak D19 developer for three minutes. The films were then immersed in a second tray containing distilled water and three drops of glacial acetic acid solution for 30 seconds to stop the development reaction. A two-minute at least fixation step followed the step above by immersing the films into a third tray containing Kodak rapid fix solution. Ultimately, the films were thoroughly rinsed under cold running water for 20 minutes and left to dry on hanging clips in a fume hood.

2.3 MCID image analysis

Quantitative analysis of autoradiographic films was carried out aided by video-based, computerised densitometry using an MCID image analyser as previously described by Kitchen and co-workers (Kitchen *et al.*, 1997). Optical density values were quantified from the [^{14}C]-microscale standards of known radioactive concentration, and cross-calibrated with [^{125}I] and then were entered into a calibration table on MCID. Specific binding was calculated by subtraction of non-specific binding from total binding and expressed as fmol/mg tissue equivalents. The 16 brain regions where OTR binding was analysed, were selected based on literature and the involvement of OT/OTR system in these regions in regulating certain behaviours such as social functioning, mood, sexual behaviour stress related emotional behaviours (Neumann and Landgraf, 2012). Brain structures were identified by reference to the rat atlas of Paxinos and Watson, (2013). Motor cortex (M2), prelimbic cortex (PrL), lateral/medial/ventral-olfactory cortex (LOMOVO),medial anterior olfactory (AOM), ventral anterior olfactory (AOV)and lateral anterior olfactory nucleus (AOL) were analysed from Bregma 4.20 mm. Theucleus accumbens shell (AcbSh), nucleus accumbens core (AcbC), caudate putamen (CPu), cingulate cortex (Cg), septum (SEP), superficial primary and secondary motor cortex (M1+M2 SUP), deep primary and secondary motor cortex (M1+M2 DEEP), superficial somatosensory cortex (S1+S2 SUP), deep somatosensory cortex (S1+S2 DEEP) anndolfactory tubercle (TU) were analysed from Bregma 1.20 mm.

2.4 Data analysis for quantitative receptor

The mean (and standard error of the mean, SEM), n=3-4 (3 only for the day 8 GF females) of specific radioligand binding was determined for all brain structures analysed from male and female CON and GF rat

groups for OTR binding. Linear mixed model analysis with Sex, microbiota status, age, Brain Region x microbiota status, brain region x Sex, Brain Region x Age, microbiota status x Sex, microbiota status x Age, Sex x Age, Brain Region x microbiota status x Sex, Brain Region x microbiota status x Age, Brain Region x Sex x Age, microbiota status x Sex x Age, Brain Region x microbiota status x Sex x Age as fixed factor variables, "brain region" as repeated measures and rat ID as random effect factor followed by Bonferroni *post hoc* test corrected for multiple comparisons was performed for the determination of the effect of these factors and their two, three and four-way interactions on OTR binding. Bonferroni *post hoc* test selected to correct for type I error following multiple comparison testing was only performed if the linear mixed model revealed a significant factorial or interaction effect. Changes in OTR density in the eye and olfactory nuclei of PND 1 and PND 4 CON and GF female animals were analysed employing a Mann-Whitney U test (n=3-4). Linear model analysis was carried out using SPSS and all other statistical analyses were performed using GraphPad Prism 8.

RESULTS

3.1 Effect of microbiota on OTR binding in the eyes of CON and GF rats at PND1 and 4

Analysis of the eyes of CON and GF female rats at PND 1 and PND 4 revealed that while significant OTR binding was observed in the CON rats, no OTRs were detected in GF rats (**Figure 1**). No alterations in OTR binding were detected in olfactory nuclei of GF rats vs CON (**Figure 1**)

3.2 Ontogenic variation in OTR receptor binding

Significant 'age', 'brain region', 'sex x microbiota status', 'brain region x microbiota status', 'brain region x age', and 'sex x microbiota status x brain region' interaction effects on OTR binding were demonstrated (**Table 1**). 'Sex x microbiota status x age x brain region' interaction was not statistically significant (**Table 1**).

The pairwise comparison revealed striking developmental variations of OTR levels across all forebrain regions, sex and microbiota status groups over the first 150 days from birth (age effect, $p<0.001$; **Table 1**). A significant transient increase in OTR binding was detected across all regions at PND 22 vs PND 8 rats ($p<0.001$) which significantly declined ($p<0.001$) to PND 8 levels in adulthood ($p<0.001$) (Bonferroni correction *post-hoc* comparison; supplementary **Table 1**). Significant developmental variations within forebrain regions were observed (age x region interaction, $p<0.001$; **Table 1**). Eight out of the sixteen brain regions analysed; AOM, AOV, AOL, Cg, SEP, CPu, AcbC, Tu showed a significant ontogenic variation (**Figure 2, 3**). In the Cg, high levels of OTR binding were detected at PND 8, which significantly declined

thereafter at PND 22 and adulthood (**Figure 2**). In the AOL, AOV, AOM, SEP and AcbC a significant transient increase in OTR was observed at PND 22 when compared to PND 8, which declined thereafter in adulthood (**Figure 2**). In the CPu, OTR binding levels were significantly reduced in adult rats compared to PND 8 and PND 22 old rats (**Figure 2**). In the Tu, a significant increase in OTR levels was detected in PND 22 vs PND 8 only (**Figure 2**). No difference in OTR binding throughout the 3 developmental stages was observed in M2, PrL, LOMOVO, AcbSh, M1+M2 SUP and Deep and S1+S2 SUP ands DEEP ($p>0.05$; see **Supplementary Figure 1**).

3.3 Effect of microbiota, sex and their interaction on OTR binding

Although neither factors 'sex' or 'microbiota status' were significant (though microbiota status was near significant $p<0.069$), a significant "sex x microbiota status" interaction was detected across all regions and age groups (**Table 1**). While significantly higher levels of OTR were detected in the female CON vs male CON rats, the gender effect disappeared in GF rats (**Table 2**). Moreover, the microbiota status effect was restricted to male rats with higher levels of OTR binding detected in GF male compared to CON male rats (**Table 2**). No alteration in OTR binding was detected between female CON and GF rats. Interestingly, significant "sex x microbiota status" interactions were detected within brain regions across all age groups (sex x microbiota status x brain region interaction $p<0.01$) (**Table 1**). The microbiota status effect was restricted to male rats with higher levels of OTR binding detected in GF male compared to CON male rats in the PrL, LOMOVO, Cg and SEP (**Table 3**). No alteration in OTR binding was detected between female CON and GF rats in any brain regions analysed. Moreover, while no significant gender effect was detected in CON rats in any regions analysed, significantly higher levels of OTR were observed in male compared to female GF rats in the PrL, LOMOVO and Cg (**Table 3**). No other gender or microbiota status effect across all age groups were detected in any other regions analysed.

As 'sex x microbiota status x age x brain region' interaction was not statistically significant (**Table 1**), multiple comparisons between male and female, CON and GF rats within each region in each age group was not permitted.

4. DISCUSSION

This study reveals a profound sex-dependent and region-specific influence of microbiota on OTR levels across developmental ages in the rat forebrain. To our knowledge, this is the first study to investigate the role of microbiota on ontogenic receptor development. These findings will pave the way for future studies focusing on the understanding of the role of microbiota on brain development and hence behaviour, which may have implications in the aetiology of specific neurodevelopmental disorders.

The neuroanatomical distribution of OTR in the CON rat forebrain as detected with the use of [^{125}I]-OVTA autoradiographic binding is in line with previous studies showing OTR expression in specific olfactory nuclei, CPu, SEP and regions of the neocortex in two different rat strains: Sprague-Dawley (Shapiro and Insel, 1989) and Wistar (Smith *et al.*, 2017). An interesting pattern of ontogenic variation of OTR levels was observed across all brain regions but also within several forebrain regions over the first 150 days from birth with profound transient increases of OTR levels detected in specific olfactory nuclei (AOM, AOV, AOL), the SEP and the AcbC at PND 22, which declines significantly in adulthood. Similar pattern of ontogenic variation was reported by Newmaster *et al.*, (2020) in the subcortical regions of an OTR reporter mice while Hammock and Levitt (2013) reported similar pattern in the neocortex of C57BL/6J mice suggesting that this pattern of OTR ontogenic variation is conserved among different rat and mice strains and possibly species, at least in rodents. The Cg and the CPu showed a different pattern of ontogenic variation in our study with high OTR levels observed at PND 8, followed by a decline into adulthood, which was observed to be steeper in Cg as opposed to CPu. No overall developmental changes in OTR levels were observed in the M1 and M2 superficial and deep, S1 and S2 superficial and deep and M2, PrL, and LOMOVO in our study. The mechanism underlying these ontogenic variations is unclear; however, it is likely to reflect the enormous amount of synaptic wiring and pruning taking place during that early developmental age (Levitt, 2003; Li *et al.*, 2010). Further studies are warranted to determine the significance of these developmental changes in OTR on behavioural development, albeit during a sensitive developmental period. Interestingly, the lack of significant interactions between 'age and sex', 'age x microbiota status', 'age x microbiota status x sex' and 'age x microbiota status x sex x region' may signify that the ontogenic patterns of variation of OTR, at least at those 3 developmental ages, may not be affected by sex and microbiota status or their interaction across and within brain regions. Nonetheless, considering the relatively low n number, caution should be taken with this observation as the lack of effect may reflet the low statistical power.

Given the vast body of evidence highlighting the sexually dimorphic nature of OT effects on certain behaviours (Caldwell, 2018), we expanded our study to determine the likelihood of a gender effect on forebrain OTR density across and within different developmental stages and brain regions. Interestingly, while significantly lower OTR levels were detected in male CON rats vs female across all brain regions and age groups, when conducting the analysis within each forebrain region, we failed to identify a significant sex effect in any of the specific forebrain brain regions analysed across the three age groups. The lack of brain-specific gender effect in CON rats is in agreement with the general consensus that the expression of OTRs in brain regions do not appear to be sexually dimorphic across several species (Cushing and Kramer, 2005) although some studies have revealed higher or lower OTR levels in specific brain regions of male rodents vs female (Dumais *et al.*, 2013; Mitre *et al.*, 2017; Newmaster *et al.*, 2020). Species, strain, age and

brain region differences where OTR density was analysed are likely to account for these discrepancies. Nonetheless, the fact that a significant sex effect was observed across all brain regions and age groups of CON rats is reflective of a common "trend" of higher OTR levels in female vs males in forebrain regions which may suggest a common mechanism underlying this trend across the brain. It is likely that estrogen, through its effect on the estrogen alpha receptors, may explain this sexual dimorphic trend, as estrogen is known to upregulate OTRs in the brain by activating the estrogen response elements located on the promoter region of the OTR gene to modulate gene transcription (Ivell and Walther, 1999; Young *et al.*, 1998). Whether these sexual dimorphic "trends" contribute to the profound sexual dimorphic behavioural responses of OT remains to be determined.

Given the emerging evidence demonstrating an essential contribution of the gut microbiome to neurobehavioural development and neuropsychiatric disorders (Warner, 2019), we assessed with the use of GF rats, the impact of microbiota on the ontogenic expression OTR in forebrain regions at different developmental period, including early life where significant neuroadaptations are known to take place. While only a near significant effect of "microbiota status" ($p<0.069$) was detected across all regions, age groups and genders, a significant "sex x microbiota status" interaction was detected across and within brain regions, across all three age groups. Microbiota status affected solely male rats with higher OTR binding detected in GF male rats vs CON across all brain region. This effect was confined to the PrL, LOMOVO, Cg and SEP. No microbiota status effect was observed in females in any regions analysed**.** Moreover, unlike CON rats, where no regions specific significant dimorphic sexual effect was observed, significant sex differences in OTR density were revealed in the PrL, LOMOVO and Cg of GF rats across all developmental ages with significantly higher levels of OTR observed in male compared to female GF rats. Overall, these findings clearly demonstrate for the first time a sex-dependent region-specific contribution of microbiota on central OTR levels, with microbiota reducing OTR levels in the male but not female rat in specific brain regions. This adds to the growing literature demonstrating a pivotal role for gut microbiota on brain neurodevelopment, which may impact on behaviour and performance (Dinan and Cryan, 2016; Warner, 2019) and expands it to the central OTR system. In support of our findings, Erdman and Poutahidis, (2014) reported that a *Lactobacillus reuteri* probiotic strain, can increase OT levels via an HPA axis mechanism suggesting that specific gut bacteria species may contribute to the regulation of central OT system.

Although the molecular mechanism underpinning the upregulation of OTR in certain brain regions of male GF rats cannot be determined from this study, it is likely that this may reflect a compensatory consequence of alterations in central OT levels. Several studies have reported low levels of central OT go hand in hand with high OTR density in the brain of the same animals (Lee *et al.*, 2007; Zanos *et al.*, 2014a)).

Interestingly, this central oxytocinergic dysregulation has been shown to be concomitant with the emergence of social deficit and emotional impairment, behaviours which were reversed by administration of the OT or OT analogue (Lee *et al.*, 2007; Zanos *et al.*, 2014a), pointing towards a causal relationship between central oxytocinergic dysregulation and socio-emotional impairment. Therefore, we can hypothesise that the increased OTR binding observed in male GF rats in the present study is caused by a reduction in OT peptide levels in the brain of these animals as a compensatory neuroadaptive mechanism. Such mechanism may then contribute to the behavioral phenotype of GF rats, which notably display impairments in social behaviour (Warner, 2019).

Of particular interest is the fact that the microbiota effect on OTR binding was restricted to male rats pointing to the presence of sex differences in the microbiome-gut-brain axis, which is in agreement with multiple studies (Coretti *et al.*, 2017; Davis *et al.*, 2017; Leclercq *et al.*, 2017; Sylvia *et al.*, 2017). The mechanism underlying these sex differences on the effect of gut microbiota status remains to be elucidated, but it may reflect changes in circulating gonadal hormone levels or/and sex-specific differences in gut microbiota profiles in CON rats. Both estrogen and testosterone are known to modulate OTR expression (Cushing and Kramer, 2005; Tribollet *et al.*, 1990) although it is unclear if endogenous hormonal levels reach the threshold necessary to induce changes in OTR levels. As discussed above, estrogen appears to directly regulate OTR gene expression through binding to the estrogen receptor alpha, which in turn interacts with the estrogen response elements located on the promoter region of the OTR gene to modulate gene transcription (Ivell and Walther, 1999; Young *et al.*, 1998). Estrogen or testosterone administration in neonatal female rats has been shown to upregulate OTR binding in specific brain regions (Uhl-Bronner *et al.*, 2005). In contrast, gonadectomy decreased OTR binding in both male and females brain regions (Tribollet *et al.*, 1990). Levels of estrogen and testosterone may differ profoundly in female and male GF rats which, as a result, may impact on the observed differential OTR regulation in the two sexes. Although levels of gonadal hormones in GF rats are not known, there is evidence to suggest that gut microbiome is a crucial regulator of estrogen and testosterone levels (Baker *et al.*, 2017) in mice (Kamimura *et al.*, 2019; Markle *et al.*, 2013). Therefore, it is highly likely that the elimination of microbiota in GF rats would cause a profound disruption of gonadal hormone levels which in turn would affect OTR. This may explain the sexual dimorphism observed in OTR binding observed in GF rats.

Whether and to what extent the impact of gut microbiota on central OTRs in male rats detected in our study influences behaviour remains to be elucidated but given the key role of oxytocin on social behaviour this is likely. Interestingly, GF rodents exhibit deficits in social behaviour **(**Desbonnet *et al.***,** 2014**;** Warner**,** 2019) and altered anxiety-like behaviour (Crumeyrolle-Arias *et al.*, 2014; Neufeld *et al.*, 2011) and exhibit increased repetitive stereotypic behaviours which are reminiscent of autistic spectrum disorder phenotype

(Desbonnet *et al.*, 2014). Intriguingly, this social deficit and concomitant alterations in neurochemistry were found to be much more pronounced in male germ-free mice (Clarke *et al.*, 2013; Hoban *et al.*, 2016) compared to females which in line with the higher incidence of ASD in males among the human population. Future research should focus on investigating the potential behavioural consequence of this sex difference of microbiota effect on central OTR to determine its role in the aetiology and development of neurodevelopmental disorders such as ASD which is higher among males.

Apart from the brain, the peripheral OT system also undergoes developmental changes at early postnatal age. In mice, OTRs are present in several peripheral tissues, including the eyes, olfactory nuclei and teeth as early as at their day of birth (Greenwood and Hammock, 2017). In agreement, we demonstrated high levels of OTR in the eye and olfactory nuclei in females of a different rodent species (the rat) at PND 1 and PND 4, suggesting that ontogenic development of OTR in the eyes and olfactory nuclei takes place prenatally and is conserved in different rodent species. Interestingly, while OTR binding was retained in the olfactory nuclei of GF rats at both PND 1 and PND 4, OTR binding in the eyes was abolished entirely in GF rats at both postnatal developmental ages, revealing a profound influence of microbiota on the OTR development in the eye, at a very early postnatal age or even prenatally. To the best of our knowledge, this is the first study to report a significant influence of microbiota on OTR development in the eye. The role of OTR in the eyes remaines unclear although there is evidence to suggest that it is involved in eye physiology. OT is located in the cones of the retina and is involved in paracrine retinal signaling between the cone photoreceptor and the RPE where OTRs are located (Halbach *et al.*, 2015). It is not possible to distinguish whether the OTR binding identified in CON rats in our study represents solely retina OTRs but is highly likely that retina OTRs account for big proportion of the OTR binding. Considering the critical role of OT-OTR signalling in the posterior retina for vision, it would be interesting to assess the impact of the role of gut microbiota on retina function development in light of our current findings and thus, further studies are warranted to understand the role of gut microbiota on developmental vision physiology.

One ought to point out the limitations of this study. The low sample number of rats allocated to each age, sex, microbiota status group resulted in lower statistical power which may lie behind the lack of significant 4-way (sex x age x microbiota status x region) as well as some 3-way and 2-way interactions and as such, this study may be considered as a pilot study. While GF rodents are considered a useful model to investigate the impact of microbiota on brain neurochemistry and behaviour, one has to be cautious in extrapolating these findings to human physiology and pathology as this model has its limitations. GF mice exhibit alterations in gut morphology, and there are differences concerning their immune system (Rooks and Garrett, 2016; Smith *et al.*, 2007). Nonetheless, our study provides a clear indication toward a direct causal link between gut microbiota and cerebral OTR regulation in males which may impact on behaviour.

Healthy postnatal development of the central OT/OTR system is thought to be critical for social functioning and emotional regulation; as such, any manipulation of this system during this developmentally sensitive periods may contribute towards the causation of neuropsychiatric disorders later on in life. Here we demonstrate for the first time that gut microbiome colonisation affects the regulation of OTR density in a region-specific and sex-dependent manner. This may have implications in the understanding of the forces driving developmental neuroadaptations critical for neuro-behavioural functioning as well as neurodevelopmental disorders such as autism.

AUTHOR CONTRIBUTION

Felix Effah – carried out the experiments, acquisition of data and data analysis, wrote the manuscript

Nivea Karla de Gusmao Taveiros Silva – data analysis, preparation of figures and tables and contributed to the writing of the manuscript.

Rosana Camarini – contributed to the study design, data analysis and interpretation and written part of the manuscript.

Fatima Joly – Grew the animals that were utilised in this study and contributed with the study design.

Sylvie Rabot – Grew the animals that were utilised in this study, contributed with the data interpretation and writing of the manuscript.

Vincent Bombail – Correspondent author who planned study design, contributed to the data analysis, interpretation and writing of the manuscript.

Alexis Bailey – Correspondent author who together with Vincent planned the study design, contributed to the data analysis, data interpretation and writing of the manuscript.

REFERENCE

Amico, J. A., Mantella, R. C., Vollmer, R. R. and Li, X. (2004) Anxiety and stress responses in female oxytocin deficient mice, *Journal of Neuroendocrinology*, 16 (4), pp. 319–324. DOI:10.1111/j.0953-8194.2004.01161.x.

Baker, J. M., Al-Nakkash, L. and Herbst-Kralovetz, M. M. (2017) Estrogen-gut microbiome axis: Physiological and clinical implications, *Maturitas*, 103, pp. 45–53. DOI:10.1016/j.maturitas.2017.06.025.

Bales, K. L. and Carter, C. S. (2003) Developmental exposure to oxytocin facilitates partner preferences in male prairie voles (Microtus ochrogaster)., *Behavioral Neuroscience*, 117 (4), pp. 854–859. DOI:10.1037/0735-7044.117.4.854.

Bales, K. L., van Westerhuyzen, J. A., Lewis-Reese, A. D., Grotte, N. D., Lanter, J. A. and Carter, C. S. (2007) Oxytocin has dose-dependent developmental effects on pair-bonding and alloparental care in female prairie voles, *Hormones and Behavior*, 52 (2), pp. 274–279. DOI:10.1016/j.yhbeh.2007.05.004.

Bombail, V., Jerôme, N., Lam, H., Muszlak, S., Meddle, S. L., Lawrence, A. B. and Nielsen, B. L. (2019) Odour conditioning of positive affective states: Rats can learn to associate an odour with being tickled, *PLOS ONE*, 14 (6), pp. e0212829. DOI:10.1371/journal.pone.0212829.

Caldwell, H. K. (2018) Oxytocin and sex differences in behavior, *Current Opinion in Behavioral Sciences*, 23, pp. 13–20. DOI:10.1016/j.cobeha.2018.02.002.

Caldwell, J. D., Hruby, V. J., Hill, P., Prange, A. J. and Pedersen, C. A. (1986) Is oxytocin-induced grooming mediated by uterine-like receptors?, *Neuropeptides*, 8 (1), pp. 77–86. DOI:10.1016/0143-4179(86)90068-5.

Campbell, A. (2010) Oxytocin and Human Social Behavior, *Personality and Social Psychology Review*, 14 (3), pp. 281–295. DOI:10.1177/1088868310363594.

Cirulli, F., Francia, N., Berry, A., Aloe, L., Alleva, E. and Suomi, S. J. (2009) Early life stress as a risk factor for mental health: Role of neurotrophins from rodents to non-human primates, *Neuroscience & Biobehavioral Reviews*, 33 (4), pp. 573–585. DOI:10.1016/j.neubiorev.2008.09.001.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., Dinan, T. G. and Cryan, J. F. (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner, *Molecular Psychiatry*, 18 (6), pp. 666–673. DOI:10.1038/mp.2012.77.

Coretti, L., Cristiano, C., Florio, E., Scala, G., Lama, A., Keller, S., *et al.* (2017) Sex-related alterations of gut microbiota composition in the BTBR mouse model of autism spectrum disorder, *Scientific Reports*, 7 (1), pp. 45356. DOI:10.1038/srep45356.

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L. and Rabot, S. (2014) Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats, *Psychoneuroendocrinology*, 42, pp. 207–217. DOI:10.1016/j.psyneuen.2014.01.014.

Cryan, J. F. and Dinan, T. G. (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour, *Nature Reviews Neuroscience*, 13 (10), pp. 701–712. DOI:10.1038/nrn3346.

Cryan, J. F. and O'Mahony, S. M. (2011) The microbiome-gut-brain axis: from bowel to behavior: From bowel to behavior, *Neurogastroenterology & Motility*, 23 (3), pp. 187–192. DOI:10.1111/j.1365-2982.2010.01664.x.

Cushing, B. and Kramer, K. (2005) Mechanisms underlying epigenetic effects of early social experience: The role of neuropeptides and steroids, *Neuroscience & Biobehavioral Reviews*, 29 (7), pp. 1089–1105. DOI:10.1016/j.neubiorev.2005.04.001.

Davis, D. J., Hecht, P. M., Jasarevic, E., Beversdorf, D. Q., Will, M. J., Fritsche, K. and Gillespie, C. H. (2017) Sex-specific effects of docosahexaenoic acid (DHA) on the microbiome and behavior of socially-isolated mice, *Brain, Behavior, and Immunity*, 59, pp. 38–48. DOI:10.1016/j.bbi.2016.09.003.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G. and Cryan, J. F. (2014) Microbiota is essential for social development in the mouse, *Molecular Psychiatry*, 19 (2), pp. 146–148. DOI:10.1038/mp.2013.65.

Dinan, T. G. and Cryan, J. F. (2016) Mood by microbe: towards clinical translation, *Genome Medicine*, 8 (1), pp. 36. DOI:10.1186/s13073-016-0292-1.

Dumais, K. M., Bredewold, R., Mayer, T. E. and Veenema, A. H. (2013) Sex differences in oxytocin receptor binding in forebrain regions: Correlations with social interest in brain region- and sex- specific ways, *Hormones and Behavior*, 64 (4), pp. 693–701. DOI:10.1016/j.yhbeh.2013.08.012.

Dumais, K. M. and Veenema, A. H. (2016) Vasopressin and oxytocin receptor systems in the brain: Sex differences and sex-specific regulation of social behavior, *Frontiers in Neuroendocrinology*, 40, pp. 1–23. DOI:10.1016/j.yfrne.2015.04.003.

Erdman, S. E. and Poutahidis, T. (2014) Probiotic 'glow of health': it's more than skin deep, *Beneficial Microbes*, 5 (2), pp. 109–119. DOI:10.3920/BM2013.0042.

Farshim, P., Walton, G., Chakrabarti, B., Givens, I., Saddy, D., Kitchen, I., R. Swann, J. and Bailey, A. (2016) Maternal Weaning Modulates Emotional Behavior and Regulates the Gut-Brain Axis, *Scientific Reports*, 6 (1). DOI:10.1038/srep21958.

Ferguson, J. N., Aldag, J. M., Insel, T. R. and Young, L. J. (2001) Oxytocin in the Medial Amygdala is Essential for Social Recognition in the Mouse, *The Journal of Neuroscience*, 21 (20), pp. 8278–8285. DOI:10.1523/JNEUROSCI.21-20-08278.2001.

Georgiou, P., Zanos, P., Garcia-Carmona, J.-A., Hourani, S., Kitchen, I., Laorden, M.-L. and Bailey, A. (2016) Methamphetamine abstinence induces changes in μ-opioid receptor, oxytocin and CRF systems: Association with an anxiogenic phenotype, *Neuropharmacology*, 105, pp. 520–532. DOI:10.1016/j.neuropharm.2016.02.012.

Greenwood, M. A. and Hammock, E. A. D. (2017) Oxytocin receptor binding sites in the periphery of the neonatal mouse, *PLOS ONE*, 12 (2), pp. e0172904. DOI:10.1371/journal.pone.0172904.

Grinevich, V., DesarmÃ©nien, M. G., Chini, B., Tauber, M. and Muscatelli, F. (2015) Ontogenesis of oxytocin pathways in the mammalian brain: late maturation and psychosocial disorders, *Frontiers in Neuroanatomy*, 8. DOI:10.3389/fnana.2014.00164.

Halbach, P., Pillers, D.-A. M., York, N., Asuma, M. P., Chiu, M. A., Luo, W., Tokarz, S., Bird, I. M. and Pattnaik, B. R. (2015) Oxytocin Expression and Function in the Posterior Retina: A Novel Signaling Pathway, *Investigative Ophthalmology & Visual Science*, 56 (2), pp. 751–760. DOI:10.1167/iovs.14-15646.

Hammock, E. A. D. and Levitt, P. (2013) Oxytocin receptor ligand binding in embryonic tissue and postnatal brain development of the C57BL/6J mouse, *Frontiers in Behavioral Neuroscience*, 7, pp. 195. DOI:10.3389/fnbeh.2013.00195.

Hoban, A. E., Stilling, R. M., Ryan, F. J., Shanahan, F., Dinan, T. G., Claesson, M. J., Clarke, G. and Cryan, J. F. (2016) Regulation of prefrontal cortex myelination by the microbiota, *Translational Psychiatry*, 6 (4), pp. e774–e774. DOI:10.1038/tp.2016.42.

Insel, T. R. and Hulihan, T. J. (1995) A gender-specific mechanism for pair bonding: oxytocin and partner preference formation in monogamous voles, *Behavioral Neuroscience*, 109 (4), pp. 782–789. DOI:10.1037//0735-7044.109.4.782.

Ivell, R. and Walther, N. (1999) The role of sex steroids in the oxytocin hormone system, *Molecular and Cellular Endocrinology*, 151 (1–2), pp. 95–101. DOI:10.1016/S0303-7207(99)00025-8.

Kamimura, I., Watarai, A., Takamura, T., Takeo, A., Miura, K., Morita, H., Mogi, K. and Kikusui, T. (2019) Gonadal steroid hormone secretion during the juvenile period depends on host-specific microbiota and contributes to the development of odor preference, *Developmental Psychobiology*, 61 (5), pp. 670–678. DOI:10.1002/dev.21827.

Kennedy, E. A., King, K. Y. and Baldridge, M. T. (2018) Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria, *Frontiers in Physiology*, 9, pp. 1534. DOI:10.3389/fphys.2018.01534.

Kitchen, I., Slowe, S. J., Matthes, H. W. D. and Kieffer, B. (1997) Quantitative autoradiographic mapping of μ-, δ- and κ-opioid receptors in knockout mice lacking the μ-opioid receptor gene, *Brain Research*, 778 (1), pp. 73–88. DOI:10.1016/S0006-8993(97)00988-8.

Leclercq, S., Mian, F. M., Stanisz, A. M., Bindels, L. B., Cambier, E., Ben-Amram, H., Koren, O., Forsythe, P. and Bienenstock, J. (2017) Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior, *Nature Communications*, 8 (1), pp. 15062. DOI:10.1038/ncomms15062.

Lee, H.-J., Macbeth, A. H., Pagani, J. H. and Young, W. S. (2009) Oxytocin: the great facilitator of life, *Progress in Neurobiology*, 88 (2), pp. 127–151. DOI:10.1016/j.pneurobio.2009.04.001.

Lee, P. R., Brady, D. L., Shapiro, R. A., Dorsa, D. M. and Koenig, J. I. (2007) Prenatal stress generates deficits in rat social behavior: Reversal by oxytocin, *Brain Research*, 1156, pp. 152–167. DOI:10.1016/j.brainres.2007.04.042.

Levitt, P. (2003) Structural and functional maturation of the developing primate brain, *The Journal of Pediatrics*, 143 (4), pp. 35–45. DOI:10.1067/S0022-3476(03)00400-1.

Li, M., Cui, Z., Niu, Y., Liu, B., Fan, W., Yu, D. and Deng, J. (2010) Synaptogenesis in the developing mouse visual cortex, *Brain Research Bulletin*, 81 (1), pp. 107–113. DOI:10.1016/j.brainresbull.2009.08.028.

Mantella, R. C., Vollmer, R. R., Li, X. and Amico, J. A. (2003) Female Oxytocin-Deficient Mice Display Enhanced Anxiety-Related Behavior, *Endocrinology*, 144 (6), pp. 2291–2296. DOI:10.1210/en.2002-0197.

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., *et al.* (2013) Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity, *Science*, 339 (6123), pp. 1084–1088. DOI:10.1126/science.1233521.

Martín, R., Bermúdez-Humarán, L. G. and Langella, P. (2016) Gnotobiotic Rodents: An In Vivo Model for the Study of Microbe–Microbe Interactions, *Frontiers in Microbiology*, 7. DOI:10.3389/fmicb.2016.00409.

Mitre, M., Kranz, T. M., Marlin, B. J., Schiavo, J. K., Erdjument-Bromage, H., Zhang, X., *et al.* (2017) Sex-Specific Differences in Oxytocin Receptor Expression and Function for Parental Behavior, *Gender and the Genome*, 1 (4), pp. 1–25. DOI:10.1089/gg.2017.0017.

Muscatelli, F., Desarménien, M. G., Matarazzo, V. and Grinevich, V. (2018) Erratum to: Oxytocin Signaling in the Early Life of Mammals: Link to Neurodevelopmental Disorders Associated with ASD, in: Hurlemann, R. and Grinevich, V. (eds.) *Behavioral Pharmacology of Neuropeptides: Oxytocin*. Cham: Springer International Publishing,35, pp. E3–E3.

Neufeld, K. M., Kang, N., Bienenstock, J. and Foster, J. A. (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice: Behavior in germ-free mice, *Neurogastroenterology & Motility*, 23 (3), pp. 255-e119. DOI:10.1111/j.1365-2982.2010.01620.x.

Neumann, I. D. and Landgraf, R. (2012) Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors, *Trends in Neurosciences*, 35 (11), pp. 649–659. DOI:10.1016/j.tins.2012.08.004.

Newmaster, K. T., Nolan, Z. T., Chon, U., Vanselow, D. J., Weit, A. R., Tabbaa, M., *et al.* (2020) Quantitative cellular-resolution map of the oxytocin receptor in postnatally developing mouse brains, *Nature Communications*, 11 (1), pp. 1885. DOI:10.1038/s41467-020-15659-1.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W. and Pettersson, S. (2012) Host-gut microbiota metabolic interactions, *Science (New York, N.Y.)*, 336 (6086), pp. 1262–1267. DOI:10.1126/science.1223813.

Paxinos, G. and Watson, C. (2013) *The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition.* Burlington: Elsevier Science. Available from: http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=777409

Pobbe, R. L. H., Pearson, B. L., Defensor, E. B., Bolivar, V. J., Young, W. S., Lee, H.-J., Blanchard, D. C. and Blanchard, R. J. (2012) Oxytocin receptor knockout mice display deficits in the expression of autism-related behaviors, *Hormones and Behavior*, 61 (3), pp. 436–444. DOI:10.1016/j.yhbeh.2011.10.010.

Rae, M., Zanos, P., Georgiou, P., Chivers, P., Bailey, A. and Camarini, R. (2018) Environmental enrichment enhances conditioned place preference to ethanol via an oxytocinergic-dependent mechanism in male mice, *Neuropharmacology*, 138, pp. 267–274. DOI:10.1016/j.neuropharm.2018.06.013.

Rooks, M. G. and Garrett, W. S. (2016) Gut microbiota, metabolites and host immunity, *Nature Reviews Immunology*, 16 (6), pp. 341–352. DOI:10.1038/nri.2016.42.

Sgritta, M., Dooling, S. W., Buffington, S. A., Momin, E. N., Francis, M. B., Britton, R. A. and Costa-Mattioli, M. (2019) Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder, *Neuron*, 101 (2), pp. 246- 259.e6. DOI:10.1016/j.neuron.2018.11.018.

Shapiro, L. E. and Insel, T. R. (1989) Ontogeny of oxytocin receptors in rat forebrain: A quantitative study, *Synapse*, 4 (3), pp. 259–266. DOI:10.1002/syn.890040312.

Smith, C. J. W., Poehlmann, M. L., Li, S., Ratnaseelan, A. M., Bredewold, R. and Veenema, A. H. (2017) Age and sex differences in oxytocin and vasopressin V1a receptor binding densities in the rat brain: focus on the social decision-making network, *Brain Structure and Function*, 222 (2), pp. 981–1006. DOI:10.1007/s00429-016-1260-7.

Smith, K., McCoy, K. D. and Macpherson, A. J. (2007) Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota, *Seminars in Immunology*, 19 (2), pp. 59–69. DOI:10.1016/j.smim.2006.10.002.

Sylvia, K. E., Jewell, C. P., Rendon, N. M., St. John, E. A. and Demas, G. E. (2017) Sex-specific modulation of the gut microbiome and behavior in Siberian hamsters, *Brain, Behavior, and Immunity*, 60, pp. 51–62. DOI:10.1016/j.bbi.2016.10.023.

Tamma, R., Colaianni, G., Zhu, L., DiBenedetto, A., Greco, G., Montemurro, G., *et al.* (2009) Oxytocin is an anabolic bone hormone, *Proceedings of the National Academy of Sciences*, 106 (17), pp. 7149–7154. DOI:10.1073/pnas.0901890106.

Tribollet, E., Audigier, S., Dubois-Dauphin, M. and Dreifuss, J. J. (1990) Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study, *Brain Research*, 511 (1), pp. 129–140. DOI:10.1016/0006-8993(90)90232-Z.

Uhl-Bronner, S., Waltisperger, E., Martínez-Lorenzana, G., Condes Lara, M. and Freund-Mercier, M. J. (2005) Sexually dimorphic expression of oxytocin binding sites in forebrain and spinal cord of the rat, *Neuroscience*, 135 (1), pp. 147–154. DOI:10.1016/j.neuroscience.2005.05.025.

Vaidyanathan, R. and Hammock, E. A. D. (2017) Oxytocin receptor dynamics in the brain across development and species, *Developmental Neurobiology*, 77 (2), pp. 143–157. DOI:10.1002/dneu.22403.

Warner, B. B. (2019a) The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders, *Pediatric Research*, 85 (2), pp. 216–224. DOI:10.1038/s41390-018-0191-9.

Warner, B. B. (2019b) The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders, *Pediatric Research*, 85 (2), pp. 216–224. DOI:10.1038/s41390-018-0191-9.

Yamamoto, Y., Cushing, B. S., Kramer, K. M., Epperson, P. D., Hoffman, G. E. and Carter, C. S. (2004) Neonatal manipulations of oxytocin alter expression of oxytocin and vasopressin immunoreactive cells in the paraventricular nucleus of the hypothalamus in a gender-specific manner, *Neuroscience*, 125 (4), pp. 947–955. DOI:10.1016/j.neuroscience.2004.02.028.

Young, L. J., Wang, Z., Donaldson, R. and Rissman, E. F. (1998) Estrogen receptor α is essential for induction of oxytocin receptor by estrogen:, *NeuroReport*, 9 (5), pp. 933–936. DOI:10.1097/00001756-199803300-00031.

Zanos, P., Georgiou, P., Metaxas, A., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2015) Region-specific up-regulation of oxytocin receptor binding in the brain of mice following chronic nicotine administration, *Neuroscience Letters*, 600, pp. 33–37. DOI:10.1016/j.neulet.2015.05.054.

Zanos, P., Georgiou, P., Wright, S. R., Hourani, S. M., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2014a) The Oxytocin Analogue Carbetocin Prevents Emotional Impairment and Stress-Induced Reinstatement of Opioid-Seeking in Morphine-Abstinent Mice, *Neuropsychopharmacology*, 39 (4), pp. 855–865. DOI:10.1038/npp.2013.285.

Zanos, P., Wright, S. R., Georgiou, P., Yoo, J. H., Ledent, C., Hourani, S. M., Kitchen, I., Winsky-Sommerer, R. and Bailey, A. (2014b) Chronic methamphetamine treatment induces oxytocin receptor up-regulation in the amygdala and hypothalamus via an adenosine A2A receptor-independent mechanism, *Pharmacology, Biochemistry, and Behavior*, 119, pp. 72–79. DOI:10.1016/j.pbb.2013.05.009.

Table 1. Linear mixed model analysis with brain region, gender, microbiota status and age as fixed factor variables

SOURCE	Numerator df	Denominator df	F	Sig.
INTERCEPT	1	74.082	777.302	0.000
BRAIN REG	15	45.641	100.150	0.000
MICROBIOTA STATUS	1	73.788	3.411	0.069
SEX	1	73.641	0.001	0.973
AGE GRP	2	72.611	33.243	0.000
BRAIN REG * GF STATUS	15	45.280	2.105	0.028
BRAIN REG * SEX	15	45.324	1.368	0.204
BRAIN REG * AGE GRP	30	45.456	11.860	0.000
MICROBIOTA STATUS * SEX	1	72.944	12.708	0.001
MICROBIOTA STATUS * AGE GRP	2	73.493	0.915	0.405
SEX * AGE GRP	2	73.446	1.909	0.156
BRAIN REG * MICROBIOTA STATUS* SEX	15	45.439	2.076	0.030
BRAIN REG * MICROBIOTA STATUS * AGE GRP	30	45.335	1.194	0.290
BRAIN REG * SEX * AGE GRP	30	47.108	0.582	0.941
MICROBIOTA STATUS * SEX * AGE GRP	1	72.944	0.049	0.825
BRAIN REG * MICROBIOTA STATUS * SEX * AGE GRP	15	45.439	1.466	0.159

Abbreviations: Reg, regions; GRP, group; GF, germ-free.

Table 2. Linear mixed model analysis with microbiota status and sex as fixed factor variables.

GF STATUS * SEX					
MICROBIOTA STATUS	**Sex**	**Mean**	**Std. Error**	**df**	**95% Confidence Interval**

					Lower Bound	Upper Bound
CON	Female	0.423	0.026	75.502	0.370	0.476
	Male	0.311#	0.026	70.866	0.259	0.363
GF	Female	0.366	0.025	70.284	0.315	0.416
	Male	0.424*	0.035	77.138	0.353	0.494

Abbreviations: CON, conventional; GF, germ-free. * p<0.05 vs Male CON; # p<0.05 vs Female Con

Table 3. Linear mixed model analysis with brain region, microbiota status and sex as fixed factor variables.

BRAIN REG * GF STATUS * SEX

BRAIN REG	MICROBIOTA STATUS	Sex	Mean	Std. Error	df	95% Confidence Interval	
						Lower Bound	Upper Bound
M2	**CON**	**Female**	0.121	0.033	47.879	0.054	0.188
		Male	0.063	0.032	48.515	0.000	0.128
	GF	**Female**	0.070	0.031	48.515	0.007	0.133
		Male	0.195	0.045	47.688	0.106	0.285
PRL	**CON**	**Female**	0.166	0.036	49.121	0.094	0.239
		Male	0.080	0.035	50.037	0.010	0.150
	GF	**Female**	0.097	0.034	50.037	0.029	0.165
		Male	0.251*#	0.048	48.862	0.154	0.347

LOMO	**CON**	**Female**	0.175	0.038	48.182	0.098	0.252
VO		**Male**	0.082	0.037	49.249	0.008	0.156
	GF	**Female**	0.077	0.036	49.249	0.005	0.149
		Male	0.266*#	0.051	47.891	0.163	0.369
AOM	**CON**	**Female**	1.443	0.153	34.129	1.132	1.753
		Male	0.871	0.145	34.255	0.576	1.165
	GF	**Female**	1.079	0.141	34.255	0.793	1.366
		Male	1.025	0.205	34.098	0.609	1.441
AOV	**CON**	**Female**	0.812	0.089	36.362	0.63	0.993
		Male	0.613	0.085	36.732	0.44	0.785
	GF	**Female**	0.643	0.083	36.732	0.476	0.811
		Male	0.530	0.120	36.269	0.287	0.773
AOL	**CON**	**Female**	0.559	0.050	43.442	0.459	0.659
		Male	0.464	0.048	44.461	0.368	0.559
	GF	**Female**	0.400	0.046	44.461	0.307	0.493
		Male	0.397	0.066	43.181	0.263	0.531
ACBSH	**CON**	**Female**	0.655	0.084	31.34	0.483	0.827
		Male	0.493	0.077	32.005	0.335	0.650
	GF	**Female**	0.576	0.074	32.202	0.426	0.726
		Male	0.620	0.115	31.121	0.385	0.855
ACBC	**CON**	**Female**	0.977	0.124	29.502	0.724	1.230
		Male	0.805	0.113	29.802	0.574	1.037
	GF	**Female**	1.031	0.108	29.891	0.812	1.251
		Male	0.948	0.169	29.402	0.602	1.295
TU	**CON**	**Female**	0.300	0.049	47.785	0.201	0.399
		Male	0.307	0.049	47.785	0.207	0.406
	GF	**Female**	0.344	0.048	47.785	0.247	0.440
		Male	0.217	0.065	47.785	0.085	0.348
CPU	**CON**	**Female**	0.393	0.084	39.187	0.223	0.563
		Male	0.322	0.084	39.187	0.152	0.492
	GF	**Female**	0.436	0.082	39.187	0.271	0.602
		Male	0.609	0.111	39.187	0.384	0.834
CGCX	**CON**	**Female**	0.223	0.055	42.738	0.113	0.333
		Male	0.145	0.057	41.83	0.03	0.261

	GF	**Female**	0.213	0.056	41.782	0.101	0.326
		Male	0.604****###	0.072	42.738	0.459	0.750
M1+M2	**CON**	**Female**	0.101	0.022	39.379	0.057	0.146
SUP		**Male**	0.098	0.022	39.379	0.054	0.143
	GF	**Female**	0.066	0.021	39.379	0.023	0.109
		Male	0.123	0.029	39.379	0.064	0.181
M1+M2	**CON**	**Female**	0.087	0.023	42.969	0.041	0.132
DEEP		**Male**	0.081	0.023	42.969	0.036	0.127
	GF	**Female**	0.057	0.022	42.969	0.013	0.101
		Male	0.116	0.030	42.969	0.056	0.176
S1 SUP	**CON**	**Female**	0.110	0.028	56.369	0.054	0.165
		Male	0.058	0.028	56.369	0.003	0.114
	GF	**Female**	0.092	0.027	56.369	0.038	0.146
		Male	0.096	0.037	56.369	0.022	0.169
S1	**CON**	**Female**	0.103	0.027	55.537	0.049	0.157
DEEP		**Male**	0.050	0.027	55.537	0.000	0.104
	GF	**Female**	0.085	0.026	55.537	0.033	0.137
		Male	0.097	0.036	55.537	0.026	0.168
SEPTU	**CON**	**Female**	0.541	0.048	48.408	0.443	0.638
M		**Male**	0.450	0.048	48.408	0.353	0.547
	GF	**Female**	0.583	0.047	48.408	0.489	0.677
		Male	0.682*	0.064	48.408	0.554	0.811

Abbreviations: CON, conventional; GF, germ-free; AOM, medial anterior olfactory; AOV, ventral anterior olfactory; AOL, lateral anterior olfactory; Cg, cingulate cortex; CPu, Caudate putamen; SEP, septum; AcbC, nucleus accumbens core; Tu, tubercle, M2, motor cortex; PrL, prelimbic cortex; LOMOVO, lateral, medial & ventral ofactory; AcbSh, nucleus accumbens shell; M1 and M2 SUP and Deep, superficial and deep primary motor cortex one and two; S1 and S2 SUP ands DEEP, superficial and deep primary somatosensory cortex.*P< 0.05, ****P<0.0001 vs male CON, and # p<0.05, ###p<0.001 vs female GF.

Figure Legends

Figure 1 | OTR binding density in the eyes and olfactory nuclei of female CON and GF Fischer rats at PND1 and PND4. This figure illustrates [^{125}I]-OVTA (50pM) specific binding in the eyes of CON and GF female rats at **(A)** PND 1 and **(B)** PND 4. Computer-enhanced pseudocolour representative autoradiograms of [^{125}I]-OVTA binding (total and non-specific binding (NSB)) in coronal sections from CON and GF rat heads at the level of the eye at PND 1 (C) and PND 4 (D). The colour bar illustrates a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. [^{125}I]-OVTA (50pM) specific binding in the olfactory nuclei of CON and GF female rats at **(E)** PND 1 and **(F)** PND 4. [^{125}I]-OVTA (50pM) was used for total binding and [^{125}I]-OVTA (50pM) in the presence of 50μM unlabelled oxytocin was used for non-specific binding (NSB) Abbreviations: PND1, postnatal day one; PND 4, postnatal day four: CON, conventional; GF, germ-free. Data are expressed as mean ± S.E.M (n=3-4 per group) specific [^{125}I]-OVTA binding (fmol/mg tissue equivalent). *P* values were set at *$*p<0.05$* (Mann-Whitney U test).

Figure 2 | Significant ontogenic variation in OTR binding in brain regions of male and female CON and GF Fischer rats. This figure illustrates [^{125}I]-OVTA specific binding in brain regions from female and male CON and GF rats at PND 8, 22 and adult. The concentration of [^{125}I]-OVTA used for OTR labelling was 50 pM. Quantitative OTR binding levels are presented in the **(A)** AOM **(B)** AOV **(C)** AOL **(D)** Cg **(E)** CPu (**F**) SEP (**G**) AcbC (**H**) Tu. Data are expressed as mean ± S.E.M (n=3-4 per group) specific [^{125}I]-OVTA binding (fmol/mg tissue equivalent). *$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ vs PND 22, $^{\S}P<0.05$, $^{\S\S\S}p<0.001$, $^{\S\S\S\S}p<0.0001$ vs PND 8* [Bonferroni post hoc analysis corrected for multiple comparisons following a linear mixed model analysis ("brain region x age" interaction p<0.001 see Table 1)]. Abbreviations: AOM, medial anterior olfactory; AOV, ventral anterior olfactory; AOL, lateral anterior olfactory; Cg, cingulate cortex; CPu, Caudate putamen; SEP, septum; AcbC, nucleus accumbens core; Tu, tubercle.

Figure 3. Computer-enhanced representative autoradiograms of OTR binding in coronal forebrain sections of male and female GF and CON rats at PND 8, 22 and adult. The represented images are of total [^{125}I]-OVTA binding at the level of the CPu and SEP (Bregma 1.20 mm) at PND8, 22 and adult. [^{125}I]-OVTA (50pM) was used for total binding. Regions analysed from this bregma have been labelled in CON females of all three developmental stages. The colour bar illustrates a pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. Abbreviations: GF, germ-free; CON, conventional; M1+M2, motor cortex one and two; S1+S2, somatosensory cortex one and two; Cg, cingulate

cortex; CPu, caudate putamen; SEP, septum; AcbSC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Tu, tubercle.

Supplementary Figure 1 | Quantitative binding of OTR in the brain regions of male and female CON and GF Fischer rats where no ontogenic variation was detected. This figure illustrates [^{125}I]-OVTA specific binding in brain sections from female and male CON and GF rats at PND 8, 22 and adult. The concentration of [^{125}I]-OVTA used for OTR labelling was 50 pM. Quantitative OTR binding levels are presented in the **(A)** M2 **(B)** PrL **(C)** LOMOVO **(D)** AcbSh (**E**) M1+M2 SUP (**F)** M1+M2 DEEP (**G**) S1+S2 SUP (**H**) S1+S2 DEEP. Abbreviations: M2, motor cortex; PrL, prelimbic cortex; LOMOVO, lateral/medial/ventral-olfactory cortex; AcbSh, nucleus accumbens shell, superficial primary and secondary motor cortex (M1+M2 SUP), deep primary and secondary motor cortex (M1+M2 DEEP), superficial somatosensory cortex (S1+S2 SUP), deep somatosensory cortex (S1+S2 DEEP). Data are expressed as mean ± S.E.M (n=3-4 per group) specific [^{125}I]-OVTA binding (fmol/mg tissue equivalent). Bonferroni post hoc analysis corrected for multiple comparisons was carried out following a linear mixed model analysis ("brain region x age" interaction $p<0.001$ see table 1). No ontogenic variation was detected in any of the regions analysed.

Figure 1

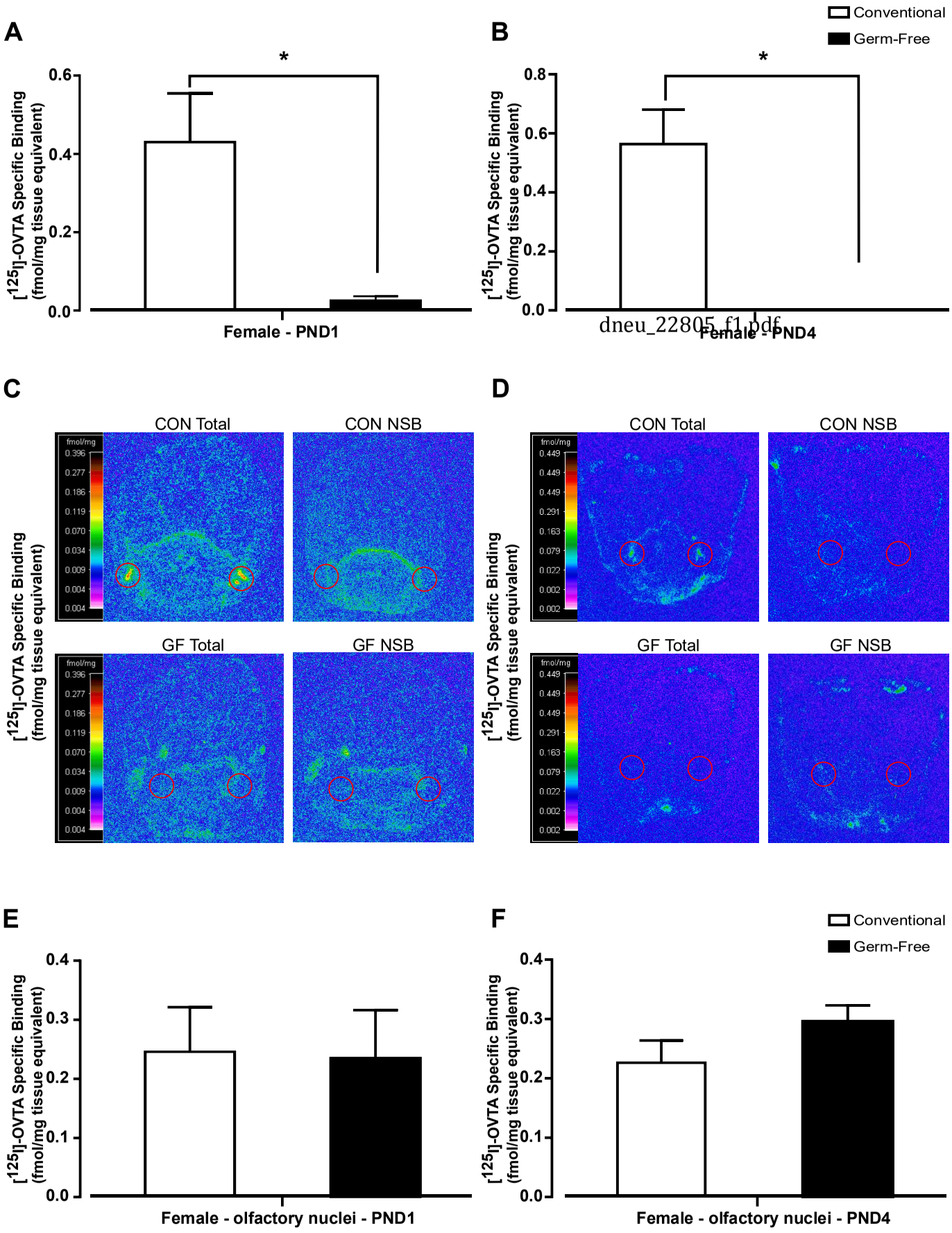


Figure 2

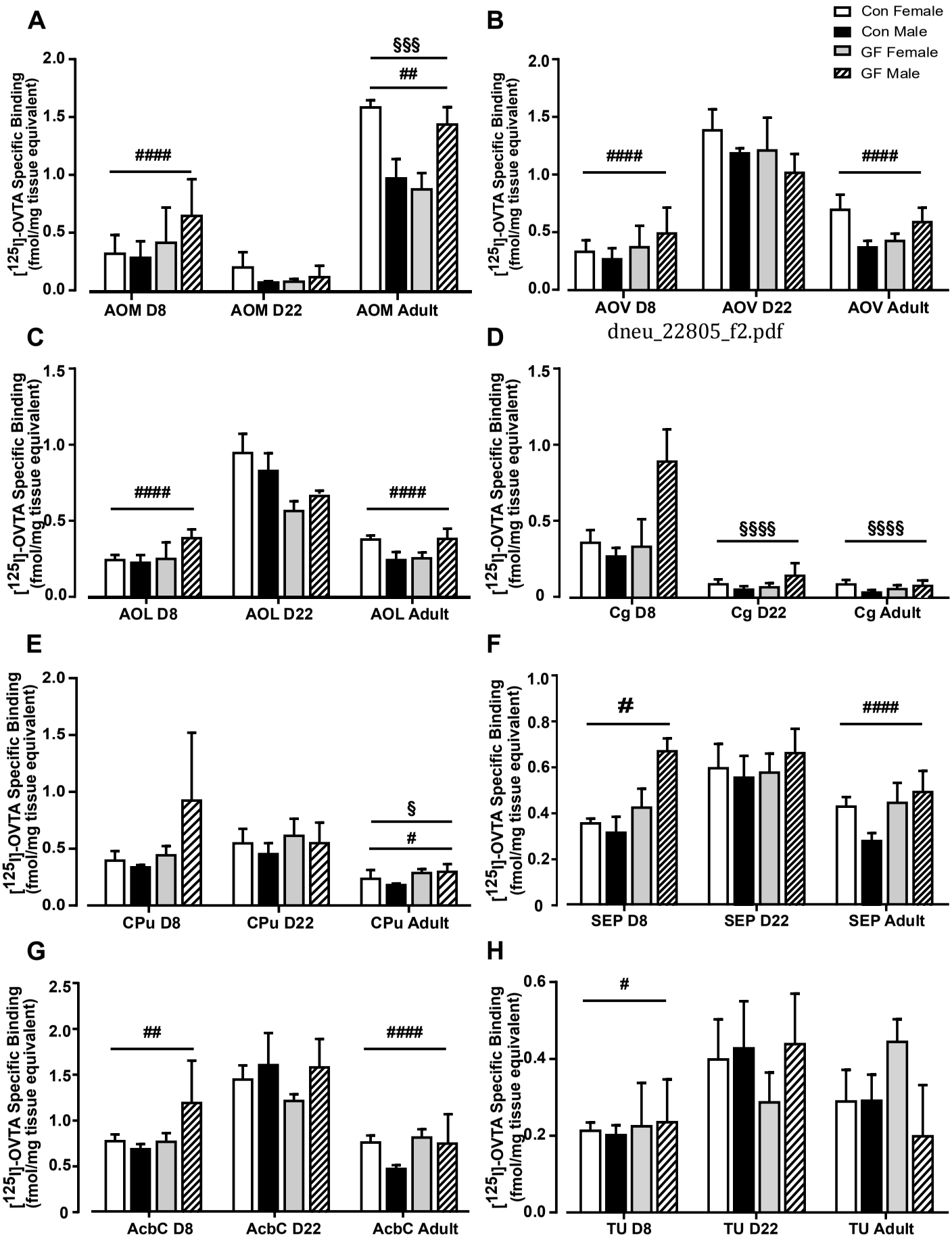
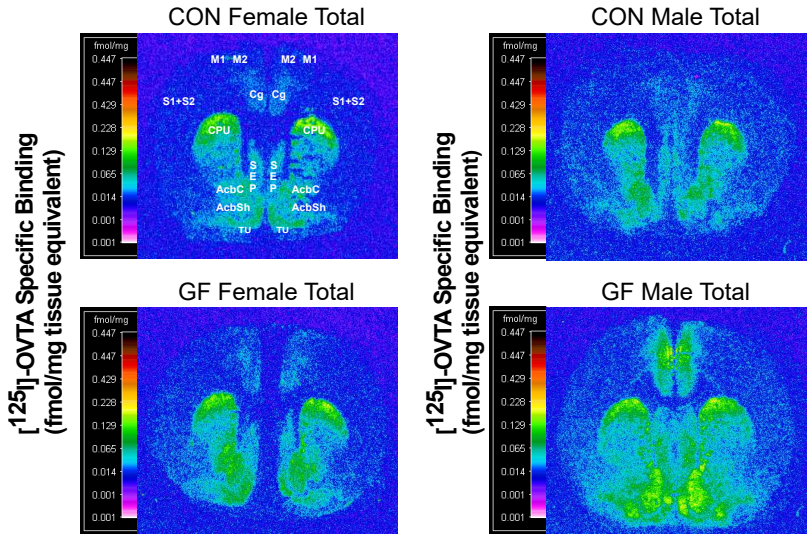
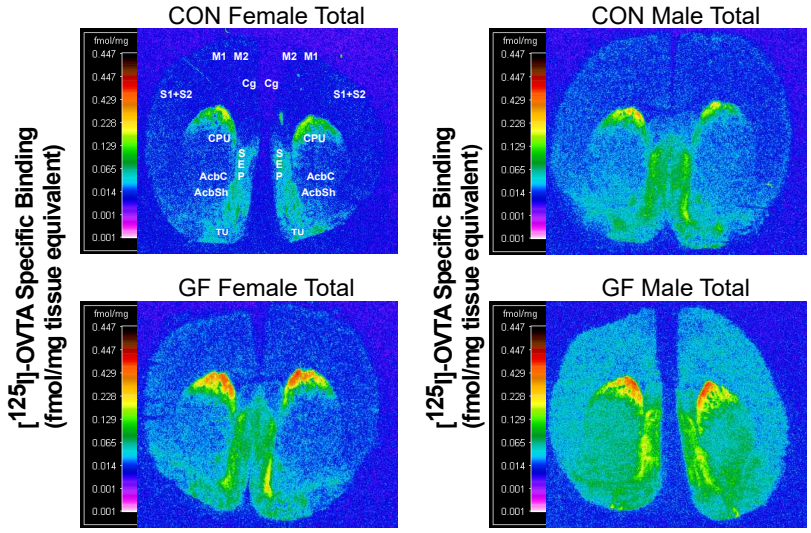


Figure 3

PND 8



PND 22



Adult