



Region-specific sex modulation of central oxytocin receptor by gut microbiota: An ontogenic study

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Abstract

Oxytocin (OT) is a developmentally important neuropeptide recognized to play a dominant role in social functioning and stress-related behaviors, 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 [¹²⁵I]-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 × sex × 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 versus 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 behavioral regulation and the etiology of certain neurodevelopmental disorders.

KEYWORDS

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

Vincent Bombail and Alexis Bailey contributed equally to this study.

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1 | 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 & 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 behaviors 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 behaviors associated with social functioning and stress regulation. Interestingly, differences in the effects of OT on several social behaviors 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 & Veenema, 2016). Many of these sex differences have been documented following OT administration during early life development (Bales & Carter, 2003; Bales et al., 2007) 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 behaviors in both sexes, females may be more sensitive to some of the effects of OT than males (Dumais & Veenema, 2016). For instance, in prairie voles, while in females, partner preference behavior was developed upon OT infusion, in males, no OT-induced pair-bonding behavior was detected (Insel & 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 analyzed (for extensive reviews on the subject see Caldwell et al., 1986; Dumais & Veenema, 2016). Nonetheless, whether sex differences appear during early development or whether sex differences influence behavior 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 PND 60 (Shapiro & Insel, 1989). The significance of these ontogenic variations on brain function and behavior is not entirely clear. However, given the central role of OT on neurophysiological functions and behaviors 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 behaviors. 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 neurobehavioral development.

Emerging evidence suggests that gut microbiota plays a pivotal role in brain function and behavioral modulation via the so-called gut-brain axis (Cryan & O'Mahony, 2011). The gut microbiota play a key role in neuroendocrine signaling pathways (Cryan & Dinan, 2012; Nicholson et al., 2012). They are capable of metabolizing 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 behavior (Dinan & Cryan, 2016). Some human but mostly animal studies have identified early postnatal microbiota colonization as critical for healthy neurodevelopment; and disruption of that colonization 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 & Poutahidis, 2014). Indeed, *L. reuteri* increased social behaviors 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 characterized 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 behavior, we hypothesize 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 [¹²⁵I]-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 colonization takes place, which in turn is known to influence early behavioral 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 hypothesized the presence of a gender \times 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 patterns in the eyes of CON and GF rats at PND 1 and PND 4. 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 & 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 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 that still present 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 diarrhea, which may have impacted on the OTR expression due to the off-target/nonspecific 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 neurobehavioral development as well as neurodevelopmental disorders etiology.

2 | 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 standardized 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 feces. 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 (nonirradiated). The animal room was maintained on a 12 hr light-dark cycle (lights switched on at 7:30 a.m.–7:30 p.m.). 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, Wright, et al., 2014). 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 (Thermo Scientific, 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 gelatin-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 sulfate (Drierite-BDH chemicals, Dorset, UK) for a minimum of 1 week to dry before usage. Quantitative OTR autoradiographic binding was carried out on those brain sections. Sections were rinsed for 10 min in a preincubation 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,[^{125}I]Tyr₉-NH₂]-vasotocin] ([^{125}I]-OVTA), in an incubation buffer medium (50 mM Tris-HCl,

10 mM MgCl₂, 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 nonspecific binding, adjacent sections were incubated with [¹²⁵I]-OVTA (50 pM) for 60 min in the presence of 50 μM of OT ligand (Thr4, Gly7)-oxytocin. When the incubation was completed, slides were rinsed three times for 5 min in ice-cold rinse buffer solution (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4 at 0°C) followed by a 30-min wash in the ice-cold rinse buffer, and a subsequent 2-s wash in ice-cold distilled water. Slides were then dried under a stream of cool air for 2 hr and stored in sealed containers with anhydrous calcium sulfate for 2 days. The slide sections were placed side-by-side to Kodak MR-1 films in hyper cassettes with autoradiographic [¹⁴C] microscalers of known radioactive concentration for 3 days (Zanos et al., 2015). Sections for the same developmental groups (CON and GF, males and females) were arranged in parallel, processed and one film placed on top of slices 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 3 min. The films were then immersed in a second tray containing distilled water and three drops of glacial acetic acid solution for 30 s to stop the development reaction. A 2-min 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 min 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, computerized densitometry using an MCID image analyzer as previously described by Kitchen and coworkers (Kitchen et al., 1997). Optical density values were quantified from the [¹⁴C]-microscale standards of known radioactive concentration, and cross-calibrated with [¹²⁵I] and then were entered into a calibration table on MCID. Specific binding was calculated by subtraction of nonspecific binding from total binding and expressed as fmol/mg tissue equivalents. The 16 brain regions where OTR binding was analyzed, were selected based on literature and the involvement of OT/OTR system in these regions in regulating certain behaviors such as social functioning, mood, sexual behavior stress-related emotional behaviors (Neumann & 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 analyzed from Bregma 4.20 mm. The nucleus 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) and olfactory tubercle (Tu) were analyzed 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 analyzed from male and female CON and GF rat groups for OTR binding. Linear mixed model analysis with Sex, microbiota status, age, Brain Region × microbiota status, brain region × Sex, Brain Region × Age, microbiota status × Sex, microbiota status × Age, Sex × Age, Brain Region × microbiota status × Sex, Brain Region × microbiota status × Age, Brain Region × Sex × Age, microbiota status × Sex × Age, Brain Region × microbiota status × Sex × 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 analyzed 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.

3 | RESULTS

3.1 | Effect of microbiota on OTR binding in the eyes of CON and GF rats at PND 1 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 versus CON (Figure 1).

3.2 | Ontogenic variation in OTR receptor binding

Significant “age,” “brain region,” “sex × microbiota status,” “brain region × microbiota status,” “brain region × age,” and

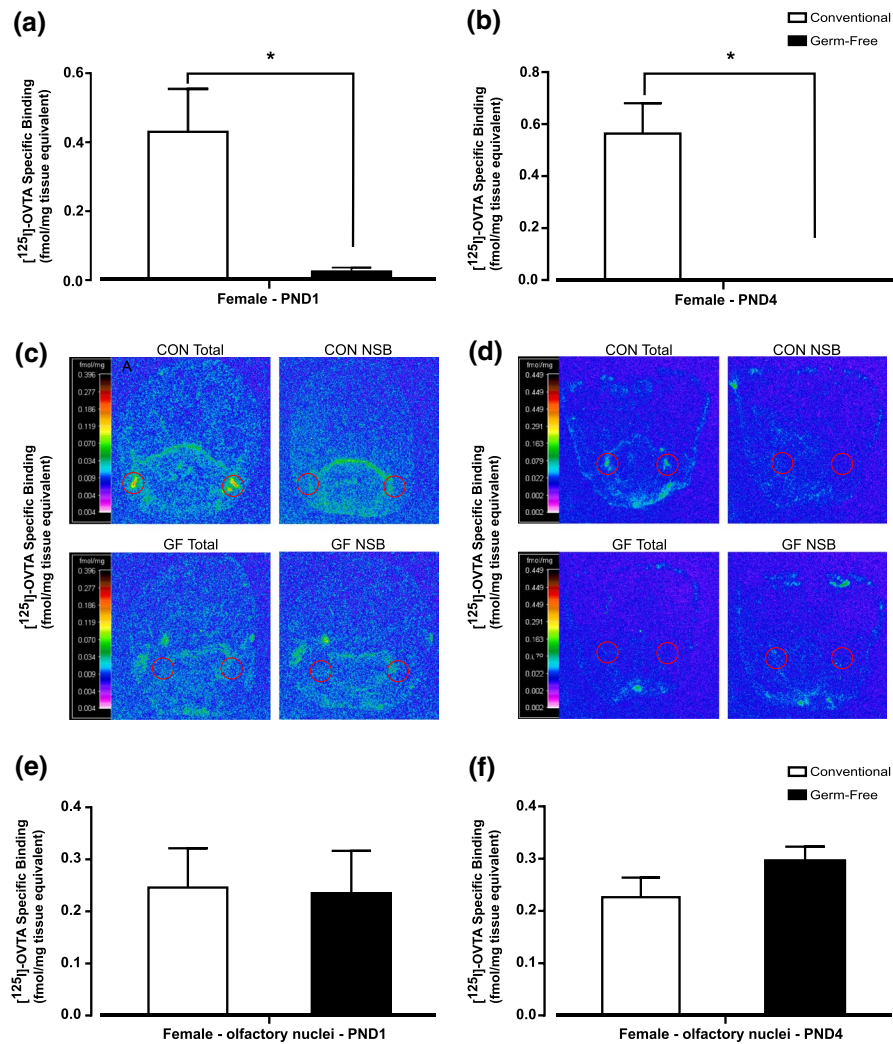


FIGURE 1 OTR-binding density in the eyes and olfactory nuclei of female CON and GF Fischer rats at PND 1 and PND 4. This figure illustrates [^{125}I]-OVTA (50 pM)-specific binding in the eyes of CON and GF female rats at (a) PND 1 and (b) PND 4. Computer-enhanced pseudo-color representative autoradiograms of [^{125}I]-OVTA binding (total and nonspecific 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 color bar illustrates a pseudo-color interpretation of black and white film images in fmol/mg tissue equivalent. [^{125}I]-OVTA (50 pM)-specific binding in the olfactory nuclei of CON and GF female rats at (e) PND 1 and (f) PND 4. [^{125}I]-OVTA (50 pM) was used for total binding and [^{125}I]-OVTA (50 pM) in the presence of 50 μM unlabeled oxytocin was used for nonspecific binding (NSB). CON, conventional; GF, germ-free; PND 1, postnatal day one; PND 4, postnatal day four. Data are expressed as mean \pm SEM ($n = 3\text{--}4$ per group) specific [^{125}I]-OVTA binding (fmol/mg tissue equivalent). p values were set at $*p < .05$ (Mann–Whitney U test)

“sex \times microbiota status \times brain region” interaction effects on OTR binding were demonstrated (Table 1). “Sex \times microbiota status \times age \times 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, $p < .001$; Table 1). A significant transient increase in OTR binding was detected across all regions at PND 22 versus PND 8 rats ($p < .001$) which significantly declined ($p < .001$) to PND 8 levels in adulthood ($p < .001$) (Bonferroni correction post hoc comparison; Table S1).

Significant developmental variations within forebrain regions were observed (age \times region interaction, $p < .001$; Table 1). Eight out of the 16 brain regions analyzed: AOM, AOV, AOL, Cg, SEP, CPu, AcbC, and Tu showed a significant ontogenic variation (Figures 2 and 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).

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

Source	Numerator <i>df</i>	Denominator <i>df</i>	<i>F</i>	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: GF, germ-free; GRP, group; Reg, regions.

In the Tu, a significant increase in OTR levels was detected in PND 22 versus PND 8 only (Figure 2). No difference in OTR binding throughout the three developmental stages was observed in M2, PrL, LOMOVO, AcbSh, M1 + M2 SUP and Deep, and S1 + S2 SUP and DEEP ($p > .05$; see Figure S1).

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 $p < .069$), a significant “sex \times 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 versus 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 \times microbiota status” interactions were detected within brain regions across all age groups (sex \times microbiota status \times brain region interaction $p < .01$) (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 analyzed. Moreover, while no significant gender effect was detected in CON rats in any

regions analyzed, 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 analyzed.

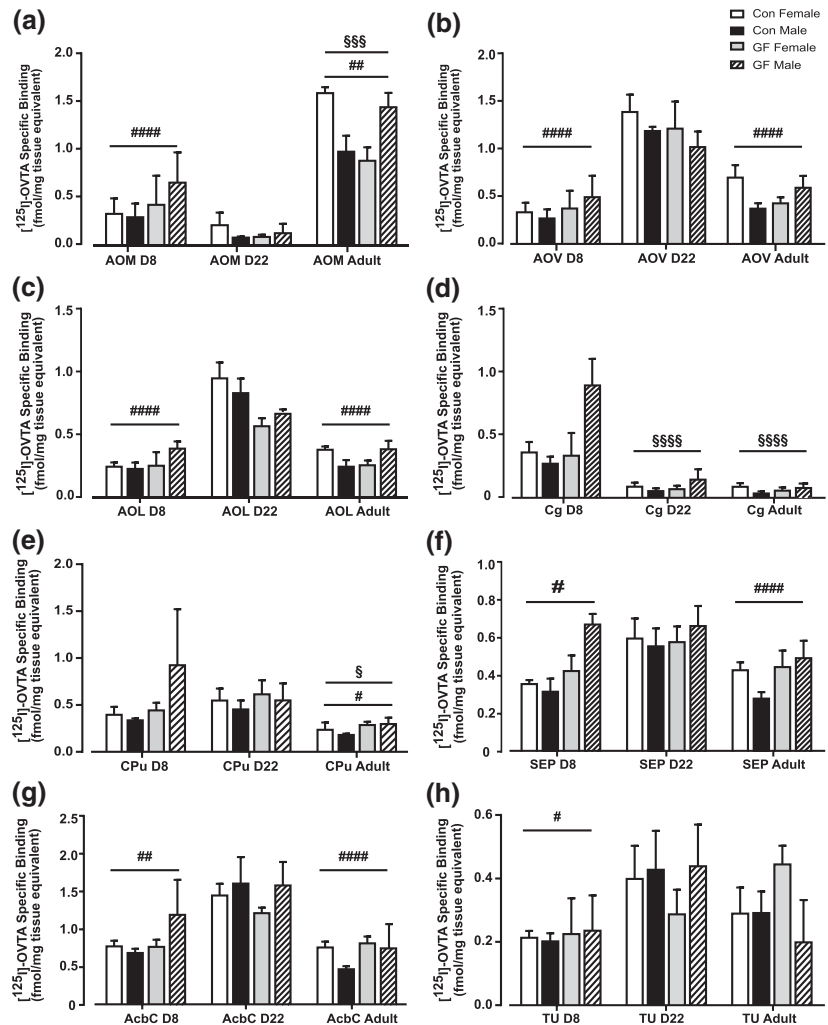
As “sex \times microbiota status \times age \times brain region” interaction was not statistically significant (Table 1), multiple comparisons between male and female, CON and GF rats within each region in each age group was not permitted.

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 behavior, which may have implications in the etiology 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 & Insel, 1989) and Wistar (Smith et al., 2017). An interesting pattern of ontogenic variation of OTR levels was observed not only across all brain regions, but also within

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 labeling 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 \pm SEM ($n = 3-4$ per group) specific [125 I]-OVTA binding (fmol/mg tissue equivalent). # $p < .05$, ## $p < .01$, ### $p < .001$, #### $p < .0001$ versus PND 22, § $p < .05$, §§ $p < .001$, §§§ $p < .0001$ versus PND 8 (Bonferroni post hoc analysis corrected for multiple comparisons following a linear mixed model analysis ["brain region \times age" interaction $p < .001$ see Table 1]). AcbC, nucleus accumbens core; AOL, lateral anterior olfactory; AOM, medial anterior olfactory; AOV, ventral anterior olfactory; Cg, cingulate cortex; CPu, Caudate putamen; SEP, septum; Tu, tubercle

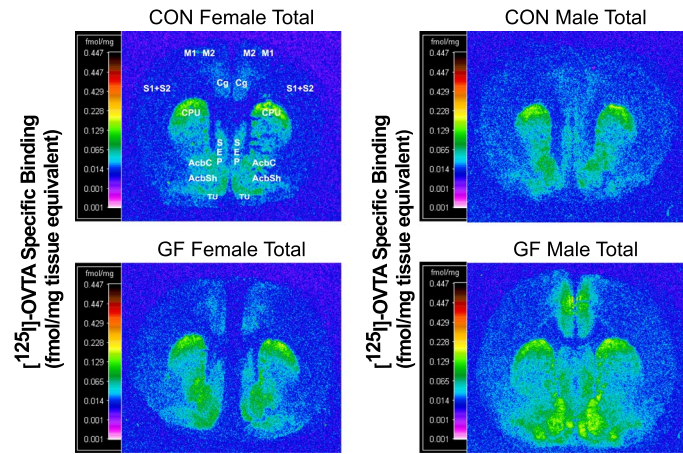


several forebrain regions over the first 150 days from birth with profound transient increases of OTR levels detected in specific olfactory nuclei (AOM, AOV, AOL), the SEP and the AcbC at PND 22, which declines significantly in adulthood. Similar pattern of ontogenic variation was reported by Newmaster 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

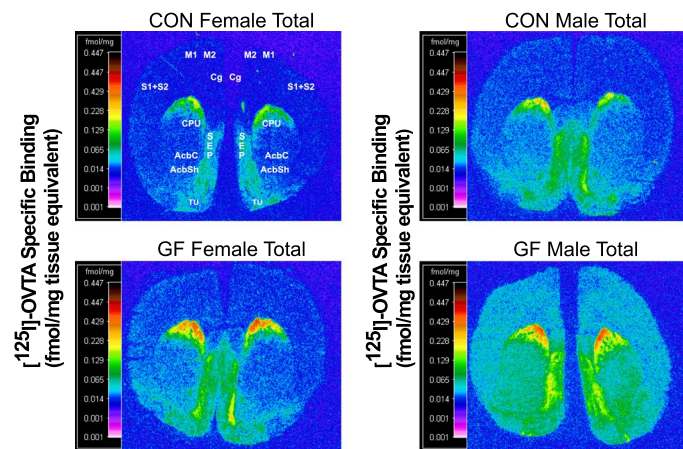
developmental changes in OTR on behavioral development, albeit during a sensitive developmental period. Interestingly, the lack of significant interactions between "age and sex," "age \times microbiota status," "age \times microbiota status \times sex," and "age \times microbiota status \times sex \times region" may signify that the ontogenic patterns of variation of OTR, at least at those three 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 reflect the low statistical power.

Given the vast body of evidence highlighting the sexually dimorphic nature of OT effects on certain behaviors (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 versus 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 analyzed across the three age groups. The lack of brain-specific

PND 8



PND 22



Adult

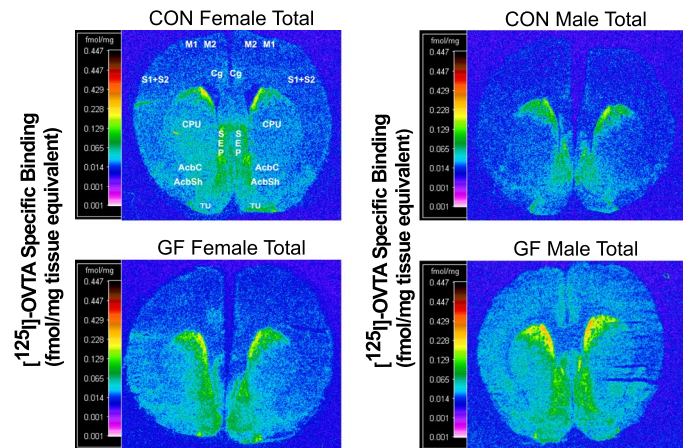


FIGURE 3 Computer-enhanced representative autoradiograms of OTR binding in coronal forebrain sections of male and female GF and CON rats at PND 8, 22, and adult. The represented images are of total $[^{125}\text{I}]\text{-OVTA}$ binding at the level of the CPU and SEP (Bregma 1.20 mm) at PND 8, 22, and adult. $[^{125}\text{I}]\text{-OVTA}$ (50 pM) was used for total binding. Regions analyzed from this bregma have been labeled in CON females of all three developmental stages. The color bar illustrates a pseudo-color interpretation of black and white film images in fmol/mg tissue equivalent. AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cg, cingulate cortex; CON, conventional; CPU, caudate putamen; GF, germ-free; M1 + M2, motor cortex one and two; S1 + S2, somatosensory cortex one and two; SEP, septum; Tu, tubercle

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 & Kramer, 2005) although some studies have revealed higher or lower OTR levels in specific brain regions of male rodents versus female (Dumais et al., 2013; Mitre

et al., 2017; Newmaster et al., 2020). Species, strain, age, and brain region differences where OTR density was analyzed 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 versus males in

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.

* $p < .05$ versus Male CON; [#] $p < .05$ versus Female CON.

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 & Walther, 1999; Young et al., 1998). Whether these sexual dimorphic “trends” contribute to the profound sexual dimorphic behavioral responses of OT remains to be determined.

Given the emerging evidence demonstrating an essential contribution of the gut microbiome to neurobehavioral 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” ($p < .069$) was detected across all regions, age groups, and genders, a significant “sex \times 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 versus 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 analyzed. 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 behavior and performance (Dinan & Cryan, 2016; Warner, 2019) and expands it to the central OTR system. In

support of our findings, Erdman and Poutahidis (2014) reported that a *L. 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 up-regulation 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, Georgiou, et al., 2014). Interestingly, this central oxytocinergic dysregulation has been shown to be concomitant with the emergence of social deficit and emotional impairment, behaviors which were reversed by administration of the OT or OT analog (Lee et al., 2007; Zanos, Georgiou, et al., 2014), pointing toward a causal relationship between central oxytocinergic dysregulation and socio-emotional impairment. Therefore, we can hypothesize 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 behavior (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 & 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,

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
LOMOVO	CON	Female	0.175	0.038	48.182	0.098	0.252
		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

(Continues)

TABLE 3 (Continued)

Brain Reg * GF status * Sex							
Brain Reg	Microbiota status	Sex	Mean	Std. error	df	95% confidence interval	
						Lower bound	Upper bound
CG	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 SUP	CON	Female	0.101	0.022	39.379	0.057	0.146
		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 DEEP	CON	Female	0.087	0.023	42.969	0.041	0.132
		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 DEEP	CON	Female	0.103	0.027	55.537	0.049	0.157
		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
SEPTUM	CON	Female	0.541	0.048	48.408	0.443	0.638
		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: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; AOL, lateral anterior olfactory; AOM, medial anterior olfactory; AOV, ventral anterior olfactory; Cg, cingulate cortex; CON, conventional; CPu, Caudate putamen; GF, germ-free; LOMOVO, lateral, medial, & ventral olfactory; M1 and M2 SUP and Deep, superficial and deep primary motor cortex one and two; M2, motor cortex; PrL, prelimbic cortex; S1 and S2 SUP and DEEP, superficial and deep primary somatosensory cortex; SEP, septum; Tu, tubercle.

* $p < .05$; **** $p < .0001$ versus male CON; # $p < .05$; ### $p < .001$ versus female GF.

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 & 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 female 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 behavior remains to be elucidated but given the key role of oxytocin on social behavior this is likely. Interestingly, GF rodents exhibit deficits in social behavior (Desbonnet et al., 2014; Warner, 2019) and altered anxiety-like behavior (Crumeyroille-Arias et al., 2014; Neufeld et al., 2011) and exhibit increased repetitive stereotypic behaviors 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 behavioral consequence of this sex difference of microbiota effect on central OTR to determine its role in the etiology 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 & 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 remains 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 signaling 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 four-way (sex \times age \times microbiota status \times region) as well as some three-way and two-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 behavior, 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 & 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 behavior.

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 toward the causation of neuropsychiatric disorders later on in life. Here, we demonstrate for the first time that gut microbiome colonization 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 neurobehavioral functioning as well as neurodevelopmental disorders such as autism.

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CONFLICT OF INTEREST

No conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Felix Effah: carried out the experiments, acquisition of data and data analysis, preparation of figures and tables, wrote the manuscript. **Nivea Karla de Gusmao Taveiros Silva:** Contributed to the data analysis, preparation of figures and tables, and writing of the manuscript. **Rosana Camarini:** contributed to the study design, data analysis and interpretation, and written part of the manuscript. **Fatima Joly:** Contributed by breeding the GF rats that were utilized in this study and contributed with the study design. **Sylvie Rabot:** Contributed by breeding the GF rats that were utilized in this study, contributed with the data interpretation and writing of the manuscript. **Vincent Bombail:** Correspondent author who planned study design, contributed to the experiments, 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.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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