Transcriptional lability of brain oxytocin receptor (*Oxtr*) generates diversity in brain OXTR distribution and social behaviors.

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Abstract

Oxytocin receptor (OXTR) modulates social behaviors in a species-specific manner. Remarkable inter- and intraspecies variation in brain OXTR distribution are associated with diversity in social behavior. To investigate potential genetic mechanisms underlying the phylogenetic plasticity in brain *Oxtr* expression and its consequences on social behavior, we constructed BAC transgenic mice harboring the entire prairie vole *Oxtr* locus with surrounding intergenic regulatory elements. Eight independent "volized" (*pvOxtr*) mouse lines were obtained; remarkably, each line displayed a unique pattern of brain expression distinct from mice and prairie voles. Four *pvOxtr* lines were selected for further investigation. Despite robust differences in brain expression, *Oxtr* expression in mammary gland was conserved across lines, suggesting that *Oxtr* expression in brain, but not mammary gland, is highly sensitive to local chromosomal landscape at integration sites. Moreover, different "volized" mouse lines showed differences in partner preference and maternal behaviors. Our results from this cross-species study suggest that species-specific variation in regulatory elements or distribution of transcription factors are not responsible for species-typical brain *Oxtr* expression patterns. Thus, transcriptional hypersensitivity to

surrounding sequence of brain *Oxtr* may be a key mechanism to generate diversity in brain OXTR distribution and social behaviors. This inherent "evolvability" of brain *Oxtr* expression constitutes a novel transcriptional mechanism to generate variability in neuropeptide receptor distribution which, through natural selection, can generate diversity in adaptive social behaviors while preserving peripheral expression. The "volized" *Oxtr* mouse lines are useful for further understanding OXTR regulation and key neural circuits/networks mediating variability in social behaviors.

Significance Statement

Unlike the essential physiological phenomenon including feeding, sex, parturition and lactation, which are conserved across mammalian species, there is extraordinary diversity of social behaviors between and within species. Comparative studies across species suggest that variation in brain oxytocin receptor expression may mediate diversity in social behavior. Subtle variation in human oxytocin receptor sequence have been related to psychiatric phenotypes. Our studies suggest that the oxytocin receptor gene is hypersensitive to sequence variation at its molecular address which leads to variability in brain expression pattern and social behavior. This research provides new insights into the evolvability of genes producing diversity in social behaviors, which allows efficient adaptation of animals to variable environment and potentially provides insight into psychiatric outcomes related to social behavior.

Introduction

Brain oxytocin receptors regulate a wide range of social behaviors including social recognition, maternal care, social bonding, empathy related behaviors, and aggression(1-3). In contrast to sex steroid receptors, which have highly conserved brain expression patterns across species, OXTR shows remarkable inter- and intra-species variation in brain distribution (2, 4). For example, monogamous prairie voles have a brain OXTR distribution in cortex, striatum and amygdala that is distinct from promiscuous montane voles and laboratory rats and mice. Furthermore, there is robust individual variation in brain OXTR expression among prairie voles that is associated with single nucleotide polymorphisms (SNPs), and this variation predicts pair bonding behavior and resilience to early life social neglect(5-8). Different species of primates also differ in brain OXTR distribution, and SNPs in the human OXTR have been linked to variation in social function(9-13). Thus, variation in OXTR distribution in brain likely constitutes a mechanism for the emergence of social traits, potentially including psychiatric endophenotypes.

Species differences in brain gene expression could be mediated by *cis* (via linked

polymorphisms) or *trans* (through diffusible products of distal genes), as a result of adaptation to the environment (14-16). *Cis*-regulatory differences appear to be more commonly responsible for adaptive evolution and interspecific divergence (17). We hypothesized that species-specific *cis* regulatory elements determine the prairie vole pattern of brain *Oxtr* expression and consequently prairie vole-specific social behaviors. Conversely, we hypothesize that species-specific distribution of regulatory proteins, e.g., transcription factors, are not a major contributor to species differences in *Oxtr* expression among rodents. Previously a 5kb fragment containing the 5' flank region of the prairie vole *Oxtr* (pv*Oxtr)* gene was used to make transgenic mice resulting in expression patterns very different from that of prairie vole, which might be due to incompleteness of regulatory elements (18). Since regulatory sequences can reside tens of kilobases from the core promoter, we revisited our hypothesis by creating transgenic mice using a bacterial artificial chromosome (BAC) including 150kb of 5' flanking region, the coding sequence with an added *Cre* sequence, the entire intron and 6kb of 3'flanking region. Our construct covers the entire intergenic region of the pv*Oxtr*, therefore, it presumably would preserve to a large degree the sequence of regulatory element affecting brain region-specific expression. If our hypothesis was correct, we predicted that the mice would express *pvOxtr* in the prairie vole like pattern that would be similar across integration site. In contrast, if species-specific trans regulatory element is the key determinant for the species differences in expression, we expected to see the *pvOxtr* expression in a mouse-typical pattern, based on that mouse*Oxtr* and pv*Oxtr* are highly homologous (>97%). To our surprise, each "volized" (pv*Oxtr*) mouse line, i.e each distinct integration site, displayed a unique pattern of expression in brain distinct from that of either wildtype mice or prairie voles. In contrast, *pvOxtr* expression was conserved across lines in mammary gland. Moreover, our different "volized" mouse lines showed differences in partner preference and maternal behaviors. Our results suggest that transcriptional regulation of *Oxtr* gene in brain is highly sensitive to variation in distal sequences and/or local chromosomal landscape at its molecular address. This lability may impart evolvability in the neuromodulatory system and contribute to species diversity and individual variation in *Oxtr* expression patterns in brain, and be key to the origin of novel social behavioral repertoires.

Results

Creation of the pv*Oxtr-P2A-Cre* BAC transgenic mice

BAC vectors can accommodate the complete structure of genes of interest, including long range cis-regulatory elements required for correct tissue-specific or temporal expression.

They are also more resistant to insertion position effects than smaller transgenes. Reproducible expression from BAC vectors has been achieved for about 500 genes (19). To create pv*Oxtr-P2A-Cre* BAC transgenic mouse line, we obtained BAC clone P236-F17 from a prairie vole BAC library (CHORI-232) (20). This BAC clone carries the entire *pvOxtr* locus with the full intergenic sequence upstream of 5' of *pvOxtr* (150kb) and downstream of 3' of *pvOxtr* (6kb) (Fig1 D). Moreover, *Rad18* gene localized upstream of 5' of *pvOxtr* in this BAC clone is deficient of the first 11 exons (which contain start codon). The *Cav3* gene localized downstream of *pvOxtr* in this BAC clone is its 1st exon including the start codon. Therefore, there will be no interference of the exogenous expression of these two genes flanking *pvOxtr,* and the topological structure around *pvOxtr* locus is preserved to the maximum extent. A P2A-NLS-Cre cassette was inserted in-frame just before the *Oxtr* stop codon to ensure co-expression of *Cre* and *Oxtr* (Fig1 E). Transgenic mice were generated by pronuclear injection using C57BL/6J zygotes. Proper integration of the reporter into the BAC clone was confirmed using gene specific PCR assays and sequencing (Fig.1 E,F and data not shown). Totally we got 11 founder mice carrying the transgenes, which was verified by PCR with multiple primer sets(Fig1 E,G). One founder line was sterile, and two founder lines did not show germ-line transmission. 8 founder lines successfully produced offspring and stably transmitted the transgene to at least 3 generations.

Each Koi line showed a unique expression pattern of transgene in brain, yet conserved expression in mammary gland.

We systemically evaluated 8 founder lines by crossing them with ROSA-26-NLS-*LacZ* mice, a Cre reporter mouse line expressing nuclear-localized beta-galactosidase (21, 22). Since Lac-Z is only expressed in the nucleus of double positive (CRE+, LacZ+) cells but not in the neuronal processes, we could easily assess the expression pattern of our transgene. To our surprise, each Koi founder line displayed a unique pattern of expression in brain distinct from that of wildtype mice, prairie voles, and each other (Fig2A-O, and supplementary Fig1). Almost all Koi lines showed the expression of transgene in olfactory bulb, lateral septum and ventromedial nucleus of the hypothalamus, though with different intensities, suggesting that these regions comprise a stable core *Oxtr* expression network, as these are also regions of overlapping OXTR binding in mice and voles (23, 24). Several Koi lines expressed the transgene in some brain regions specific for voles, including nucleus accumbens (NAc) (Fig2A,B,D Supplementary Fig1A,G,J), prefrontal cortex(PFC)(Fig2A,B,C Supplementary Fig1J), lateral amygdala (Fig2K,L,M

Supplementary Fig1C,I,L), central amygdala (Fig2K,L, N,V Supplementary Fig1I,L), and deep layers of cingulate cortex (Fig2A,B,C Supplementary Fig1C,L), which suggested that cis regulatory elements in the BAC are capable of mediating the expression of *Oxtr* in the reward and reinforcement circuitry of brain, albeit depending on the sequence/structure of distal sequences >150 kb upstream or >22 kb downstream of the transcription start site. None of the Koi lines exactly "mirrored" the vole-specific expression patten. Six of the Koi lines expressed the transgene in broad areas of thalamus (Fig2K,L,N and Supplementary Fig1C,I,L), reminiscent of the strong expression of V1a Vasopressin receptor (*Avpr1a*) in the thalamus(4). Intriguingly, the expression of OXTR in mammary glands showed similar pattern among all the Koi lines and *mOxtr-Ires-Cre* knock-in line (Fig2P-T and Supplementary Fig2).

Transgenes that are introduced by pronuclear injection typically integrate into a single site of the genome as tandem concatamers (25). And increased BAC transgene copy numbers often correlate with increased BAC gene expression. We examined the relationship between transgene copy number and LacZ signal using a widely used protocol of quantitative genomic PCR (26). As expected, the copy number of WT, heterozygous and homozygous *mOxtr-Ires-Cre* knock-in mice was 0, 1 and 2 respectively (Figure2 Z). The copy number of transgene in Koi lines are: Koi-1(12), Koi-2(2), Koi-3(2), Koi-4(1), Koi-5(2), Koi-6(4), Koi-7(9), Koi-8(3). Koi-6 and Koi-7 showed the most limited expression of reporter gene, though the copy number of transgene are 4 and 9 respectively. Koi-4 showed strong expression of reporter gene in NAc, septum and thalamus, though there was only 1 copy of transgene. Therefore, integration site rather than copy number is the determinant of the BAC transgene expression in our Koi lines.

To detect the expression of OXTR in adult Koi lines without the interference of endogenous mOXTR signal, Koi lines were crossed with $Oxtr^{-/-}$ to generate mice in which transgenic pvOXTR was expressed while endogenous mOXTR was absent. We focused on 4 Koi lines: Koi-1, Koi-2, Koi-3 and Koi-4 based on the strong expression of reporter gene in behaviorally relevant neuronal populations. OXTR autoradiography demonstrated that the *pvOxtr* gene product is functionally expressed (Figure 3) and revealed that OXTR binding of Koi-3 line was consistent with the CRE induced reporter gene expression (Fig2 C, H, M and Figure 3 J-L). Especially, the OXTR binding in PFC of Koi-3 was very similar to that of prairie vole. In contrast, Koi-4 showed low OXTR binding in NAc when compared with the high Lac-Z staining (Fig2 D, Figure 3 M), consistent with transient *pvOxtr* expression during developmental, a pattern previously reported in rat NAc(27). Cre-dependent reporter gene product in constitutive *Cre*-recombinase

expressing lines reflect the cumulative expression history of the transgene throughout development. Koi-2, the line which displayed the expression of reporter genes in the broadest regions, did not show strong signal in autoradiography, again suggesting developmental changes in transgene expression. It is interesting that Layer IV of cortex in Koi-4 showed strong OXTR binding (Figure 3 N and O), which is highly likely to originate from the axon projections from the thalamus (Fig2 N). Recently Kiyoshi et al., showed that OXTR binding is likely on proximal dendrites or axon of *Oxtr* expressing cell bodies in prairie voles (24). Consistent with our analysis of reporter gene product, we do not see a clear relationship between OXTR binding copy numbers of transgene. For example, Koi-1 showed low-level OXTR signal (Fig3 D,E,F) with the highest copy number, 12copies, while Koi-4 showed very strong OXTR signal at lateral septum and thalamus (Fig3 N,O), with the lowest copy number, 1copy.

Considering the limited sensitivity of autoradiography, we applied quantitative RT-PCR to further confirm the expression level of *Oxtr* in olfactory bulb, PFC, striatum and thalamus. To allow for the comparation between Koi lines with WT mice, we used primer set which can detect both *mOxtr* and *pvOxtr. Oxtr* mRNA was undetectable in $mOxtr^{-/-}$ mutant mice as expected. Two-way ANOVA revealed that there was a significant main effect of genotype, $F(4, 64)=34.15$, $p<0.0001$. PostHoc Bonferroni test showed that Koi-2 expressed significantly higher *Oxtr* than WT and all the other Koi lines ($p<0.001$ for all). Koi-4 expressed significantly higher OXTR than WT and Koi-1, Koi-3 ($p<0.001$ for all). There was a significant interaction between genotype and brain region, $F(12,64)=26.33$, $p<0.0001$. The relative value of *Oxtr* expression corresponded well to the autoradiography signal in olfactory bulb (Supplementary Fig3), Koi-1 line expressed significantly higher level of *Oxtr* than WT and other Koi lines in olfactory bulb. Koi-4 line expressed significantly higher level of *Oxtr* than WT and other Koi lines in thalamus. The *Oxtr* level in Koi-4 striatum was significantly higher than that of WT and Koi-3 as well (Supplementary Fig3). We also analyzed the *Oxtr* expression with *pvOxtr* specific primer set. As expected, the expression was undetectable in WT mice, and the comparison of *pvOxtr* expression level in 4 brain regions among Koi lines produced similar results (Supplementary Fig3 and Supplementary Fig4).

In summary, although none of our Koi lines mirrored the endogenous OXTR expression in prairie voles, we confirmed the strong expression level of *pvOxtr* in olfactory bulb of Koi-1line, PFC and amygdala of Koi-3 line, striatum and thalamus of Koi-4 lines, and the broad expression of *pvOxtr* in Koi-2 line. As OXTR signaling in some of these regions are similar to that in voles, albeit in separate lines, and postulated to be involved in vole social

behavior(28), we used these lines to test the hypothesis that transcriptional variation in *Oxtr* in brain can contribute to variation in social behaviors.

Different Koi lines showed diverse behaviors in a Partner Preference Test

Partner preference is widely used as a laboratory proxy for pair bonding in prairie voles. We applied a modified version of the partner preference test in our transgenic mice (Fig.4A and B). In a pilot study, we found that Kio-4 on a $mOxt^{+/+}$ background showed a preference for their mate relative to a novel male. We then focused on 4 lines (Koi-1, 2, 3, 4) based on the transgene expression in PFC, BLA or NAc. We bred the transgenic mice with $mOxtr^{-/-}$ mutant line (both on C57/B6 background) to generate offspring expressing transgenic pvOXTR but not endogenous mOXTR. WT C57/B6 mice were also examined. Only female mice were tested. As we did not expect our mice lines to develop a partner preference after a short cohabitation as seen in prairie voles, we employed a protocol, in which 21-days cohousing days were used before PPT. We divided our analysis for 3 phases. In phase 1(habituation phase), the experimental subject was allowed to adapt to the chamber for 5 minutes with both pen-boxes empty. In phase 2(recognition phase), the partner stimulus animal was restricted in one pen-box, and a novel "stranger" stimulus animal was restricted in another pen-box, the experimental subject was allowed to explore the chamber for 10 minutes. In phase 3("preference" phase), the positions of the partner stimulus animal and the "stranger" stimulus animal were switched to avoid position bias, and the experimental subject was allowed to explore the chamber for 20 minutes.

Mixed ANOVA was conducted to examine the effect of genotype and the stimulus on the area stay time during all the phases. In Phase1(Fig.4C), there was no statistically significant interaction between the effects of genotype and position of empty pen-box on stay time, $F(4, 60) = 0.69$, $p = 0.6$. There was no main effect of position on the stay time, $F(1,60)=0.03$, $p=0.87$. Pairwise comparisons corrected by Bonferroni test demonstrated that no group showed significant preference for either of the empty pen-box ($p=0.67$, 0.31,0.56,0.52,0.37 for WT, Koi-1, Koi-2, Koi-3, Koi-4, respectively). There was a significant main effect of genotype, $F(4,60)=3.26$, $p<0.05$. Post Hoc analysis corrected by Bonferroni test revealed that Koi-2 (103.08±2.57s) stayed longer time around empty pen-boxes than Koi-1(91.59±2.77s; $p<0.05$) and Koi-3(91.43±2.64s; $p<0.05$). However, none of the transgenic groups showed significant difference when compared with WT ($p=1$, 0.52,1,1 when Koi-1, Koi-2, Koi-3, Koi-4 was respectively compared with WT).

In Phase 2 (Fig.4D), there was a main effect of stimulus animal on stay time, the time

staying in close proximity to the partner was significantly shorter than the time stay with stranger animal ($F(1,60)=15.38$, $p<0.001$). There was a main effect of genotype on stay time, $F(4,60)=2.62$, $p<0.05$. Post Hoc Bonferroni test revealed that Koi-2(236.91±5.71s) stayed longer time around stimulus animals than Koi-3(210.97±5.93s, $p<0.05$). There was also a statistically significant interaction between the effects of genotype and stimulus animal on area stay time, $F(4, 60) = 2.61$, $p <0.05$. Bonferroni adjusted pairwise comparisons were applied to analyze the simple main effects. Koi-4(stay time with partner: 165.66±15.80s, stay time with stranger: 291.44±17.66s, $p<0.01$) and WT mice (stay time with partner: 192.83±14.37s, stay time with stranger: 256.35±11.05s, $p<0.05$) spent significantly longer time in "stranger" area than in "partner" area, demonstrating a typical novelty preference of mice. There was no significant difference between the stay time with "stranger" and "partner" for Koi-1(stay time with partner: 229.14±15.46s, stay time with stranger: 227.75±17.67s, $p=0.96$), Koi-2(stay time with partner: 227.11±17.35s, stay time with stranger: 246.71±19.06s, $p=0.49$) and Koi-3(stay time with partner: 186.17±12.69s, stay time with stranger: 235.77±15.15s, $p=0.09$) mice.

In Phase3 (Fig.4E), following the initial exploration phase, there was no main effect of stimulus animal on stay time, $F(1,60)=0.04$, $p=0.84$. There was no main effect of genotype on stay time either, $F(4,60)=1.36$, $p=0.26$. There was a statistically significant interaction between the effects of genotype and stimulus animal on area stay time, $F(4, 60) = 4.64$, $p <0.01$. Bonferroni adjusted pairwise comparisons were applied to analyze the simple main effects. Koi-2 mice spent significantly longer time in "stranger" area than in "partner" area (stay time with partner: 382.85±25.48s, stay time with stranger: 509.71±23.49s, $p<0.05$). In contrast, Koi-4 mice spent significantly longer time in "partner" area than in "stranger" area (stay time with partner: 493.64±35.20s, stay time with stranger: 300.85±24.48s, $p<0.01$). There was no significant difference between the stay time with "stranger" and "partner" for WT (stay time with partner: 410.39±37.39s, stay time with stranger: 409.88±30.74s, $p=0.99$), Koi-1(stay time with partner:356.28±24.76s, stay time with stranger: 441.20±41.26s, $p=0.16$) and Koi-3(stay time with partner: 428.51±44.61s, stay time with stranger: 384.66±39.21s, $p=0.45$) mice. Therefore, our different Koi lines demonstrated different preferences in our modified PPT; Koi-4 females preferred to be in close proximity with their partner compared to the novel males.

Different Koi lines showed diverse behaviors in Pups Retrieval Test

Based on the clear and abundant expression of Koi-3 and Koi-4 in the reward-related brain regions revealed by autoradiography (Fig.3J-O), we further tested the parental behaviors

of these two lines (Fig.4F and G). Mixed ANOVA was conducted that examined the effect of genotype and the number of pups retrieved and the latency of pups retrieval. Mauchly's test showed that the sphericity was violated. Therefore, the Greenhouse-Geisser correction was used for the repeated measures ANOVA. There was a significant main effect of genotype, $F(2,48)=29.58$, $p<0.0001$. Koi-4 took a significantly longer time to retrieve pups than WT and Koi-3 ($p<0.0001$ for both). There was also a significant interaction between the effects of genotype and the number of pups retrieved on the retrieval latency, $F(2.40, 57.49) = 4.45$, $p<0.05$. Post Hoc multiple comparation with Bonferroni correction revealed that Koi-4 took a significantly longer time to accomplish the 1st retrieval ($p<0.001$ when compare to WT, and $p<0.0001$ when compare to Koi-3), the 2nd retrieval ($p<0.0001$ when compare to WT and Koi-3), and the 3rd retrieval ($p<0.0001$ when compare to WT and Koi-3). There was no statistically significant difference between WT and Koi-3 ($p=1$, 0.53 and 0.36 for the 1st, 2nd, and 3rd retrieval, respectively).

Crouching time was analyzed as well (Fig.4F and H). There was a statistically significant difference between groups as determined by one-way ANOVA ($F(2,48) = 27.56$, $p<0.0001$). A Bonferroni post hoc test revealed that the crouching time of Koi-4 was statistically significantly longer than WT($p<0.01$) and KOI-3($p<0.0001$). Additionally, the crouching time of KOI-3 was statistically significantly shorter than WT ($p<0.01$). Therefore, two Koi lines with different OXTR expression pattern demonstrated quantitative differences in parental behavior tests.

Discussion

The sex steroid and oxytocin system play important roles in modulating reproductive and related social behaviors in vertebrates. Sexual behavior, i.e., the motivation to mate, is highly conserved across vertebrate species. By contrast, social behaviors, e.g., sociality, mating strategy (monogamy vs polygamy), parental and alloparental behaviors are more nuanced and can vary dramatically across species in the same genus or even between individuals of a single species. It is interesting therefore that the distribution of the steroid receptors is quite conserved among species(29), while that of the OXTR is extremely diverse across and even within species(2, 4). It is even more interesting that although the distribution of OXTR is diverse, its ligand oxytocin is expressed in a highly conserved neuroanatomical pattern in vertebrates(30). Previous work showed that three independent rat lines with puffer fish oxytocin BAC transgene (containing 16 kb of 5' flanking regulatory sequence) expressed the Fugu oxytocin transcript specifically in oxytocin neurons in rat, regardless of the BAC transgene insertion sites (31). Moreover, a similar

transgenic experiment transferring approximately 5 kb of the Fugu oxytocin already resulted in a faithful expression of the fish genes in mouse oxytocin neurons(32). These studies suggest there is a remarkably conserved transcriptional regulatory machinery across species, even between the Fugu and rodent oxytocin genes, despite being separated by 400 million years of evolution. Based on the principle of choosing a proper BAC clone to create BAC transgenic mice library, the most important determinant for correct expression from BAC vectors is inclusion of as much of the 5' and 3' intergenic region(if possible, the entire intergenic region) surrounding a gene of interest within the BAC construct as possible(19, 33, 34). Previously we constructed *Pkcd-BAC-Cre* transgenic mice based on this principle and obtained independent lines with very similar expression patterns (Supplementary Fig. 6). In the current work, the *pvOxtr* BAC clone we chose carries the entire *pvOxtr* locus with the full intergenic sequence upstream of 5' of *pvOxtr* (150kb) and downstream of 3' of *pvOxtr*(6kb). However, the 8 lines we obtained demonstrated remarkably variant expression pattern of pvOXTR in brain, which is different from both prairie vole and mouse. Our data showed that the different pattern is not from the copy number of transgenes. Instead, it is most likely due to the effects from different insertion sites of transgenes. Our results are reminiscent to the observation that virtually every rodent or primate species examined has a unique distribution of OXTR binding in the brain. It is very likely that the distal chromosomal landscape determines subtleties in 3D structure of the BAC transgene, which could modulate the interaction with specific transcription factors in certain brain regions, a phenomenon that does not appear to happen in other transgenes. Single-nucleotide polymorphism in the *Oxtr* intron which explains 74% of the variance in striatal Oxtr expression and social attachment in prairie voles (8). Different *Oxtr*-reporter (either lac-z, EGFP or Venus etc.,) mice created from independent labs showed different expression pattern in brain as well (23, 33, 34), further supporting the ultra-sensitivity and fragility of *Oxtr* gene to subtle changes of cis sequence and local chromosomal architecture. Additionally, SNPs in the human OXTR predict OXTR transcripts in a brain regions-specific manner as well(35). This ultra-sensitivity to genomic variation appears to be tissue specific and mainly appeares in brain, but not in mammary gland, which is responsible for essential lactation in mammals. Based on all of these data, we propose the inherent transcriptional lability of the *Oxtr* in mammal imparts evolvability in expression of the gene in the brain, allowing oxytocin to modulate novel neural circuits, which can then modify various aspects of social behavior, leading to the evolution of diversity in social behaviors. Similar transcriptional lability may impart divergence in vasopressin receptor (*Avpr1a*), a paralogue of Oxtr, whose diverse expression patterns also have been linked to variation in behavior (36-40). By gifting certain neuromodulator receptor genes with

lability, in conjunction with the paracrine actions of their ligands, random mutations easily influences expression pattern of these receptors in brain, making various neural circuits sensitive to the neuromodulator. If the resultant behavior is adaptive, the frequency of the mutation will increase in the population and the social behavioral profile of the population may change. *Oxtr* gene displays a high degree of evolvability (the capacity of an evolving system to generate or facilitate adaptive change) and may be a major generator of social behavioral adaptation and diversity. How exactly variation of gene expression propagates through gene regulatory networks and ultimately results in complex social behavioral phenotypes, still remains to be investigated. Emerging technologies make it possible to study the evolution of gene expression, including new techniques to plumb the effect of chromatin organization and cell- or tissue- specific differences on gene regulation.

The unique expression pattern of *Oxtr* in monogamous prairie vole was mainly observed in PFC, NAc and BLA. We got expression in all of these 3 regions, but in 3 separate lines. Most importantly, the Koi lines with distinct OXTR expression pattern provide us a unique opportunity to test the causal effect of variation of OXTR expression on diverse social behaviors. It is intriguing that Koi-4 line showed preference to their mating partners but not strangers, which is a behavior specific in prairie voles but not in mice. Mice typically show a novelty preference when presented with a novel and a familiar stimulus animal. Multiple studies showed that NAc and PFC play important role in mediating partner preference behavior in prairie voles (41). In Koi-4 line, a robust expression was detected by using Lac-Z reporter mouse method. However, qRT-PCR only detected a modest expression of *pvOxtr* transcripts, and autoradiography barely detected signals in these brain regions. Lines expressing constitutive Cre expression often reflect the cumulative expression history of the gene throughout the organism including both developmental stage and adult stage. Emerging research have shown that species difference exists in developmental patterns of OXTR expression. For example, pre-weaning rats have transient peak OXTR specifically in cingulate cortex (42, 43), while mice have a transient peak of OXTR throughout the entire neocortex(44). Transient OXTR expression may define sensitive periods for oxytocin to shape the brain development of those brain regions in a social stimulus dependent way. OXTR signaling in the NAc during the first two weeks of life appear to shape adult partner preference behavior in prairie voles(5). The brain regions that display the transient appearance of OXTR are not conserved across rodent species, and the transient expression of OXTR may help to shape activity-dependent development and contributes to adult social behaviors in a species-specific manner (45). It is worth studying the OXTR expression pattern during development of our Koi lines in future. Koi-4 line showed distinctive expression in almost the entire thalamus region, which reminds us the

distribution of vasopressin receptor (46). Given the fact that OXTR and AVPR1A genes were derived from a duplication of a single ancestral gene(47), it is not surprising that the similar expression regulation machinery exists. A very recent work showed the important roles of dorsal thalamus in social affiliation in zebrafish (48). The function of thalamus in affiliative behaviors in rodents hasn't yet been investigated. Our preliminary data showed that some neurons in anterior and dorsal thalamus were activated during partner preference task (unpublished data). Oxytocin is released somatodendritically and along axons to diffuse to distal receptor cites (49) . The neuroendocrine action of oxytocin negates the need for oxytocin neurons innervation into OXTR-expressing circuits, and increases the flexibility of the system. The partner preference formation and maintenance need to recruit multi- and cross-modality sensation, social recognition and social memory, selective attention, arousal, and reward system. We propose a model that the sufficient upregulation of the OXTR expression in one or multiple of these systems can tune the network and boost partner preference behavior. However, while the partner preference we observed in our mice is qualitatively similar to what is observed in voles, we do not propose that the mice are monogamous. Rather, we propose that social preferences were biased, and how that may manifest in nature is unknown. The important point though is that simply by redistributing OXTR binding in space and during development, variation in social preference and parental behaviors emerged, which conceivably could impart advantageous behaviors that natural selection could act upon and amplify. Future study will determine how the variation in OXTR distribution in the Koi lines affects neural circuits mediating social behaviors.

In summary, we created mouse lines showing different expression pattern of OXTR in the lab by using a BAC transgenic method. Our work suggested that the evolvability of *Oxtr* gene is a key characteristic of the *Oxtr* gene that allows subtle variation in chromatin landscape, potentially caused by mutations, to alter brain expression patterns leading to the emergence of novel social behaviors, which may allow organisms to adapt to and survive variable environment. Furthermore, the evolvability of *Oxtr* gene is due to its transcriptional sensitivity to genomic variation, including gene polymorphism and local chromatin architecture. Finally, we confirmed the causal effect of OXTR expression on the diversity of social behavioral repertoire. The transcriptional lability of the human OXTR may contribute to endophenotypes relevant to psychiatric disorders(50) Our research on the origins of diversity in social behaviors across species may lead to conceptual understandings relevant for the development of treatments for psychiatric disorders.

Materials and Methods

Vole-Oxtr-P2A-Cre BAC vector construction.

A vole BAC clone P236-F17 containing the *Oxtr* locus was obtained from a prairie vole BAC library (CHORI-232) (20). The NLS-Cre-Frt-Amp-Frt cassette was obtained from Dr. Takuji Iwasato, and was slight modified. P2A sequence(51) was introduced by PCR method with the following primers: fw 5'-ATGGGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCTCTCGAAACTGACAGGAGAACCACC-3'; rv5'-AGACTGGAGTCCGCATAGCCCCCCTCCCCCGCCCCAGGCGCGGTGGGCCAGGCAGGTGGCTCACCTTGACCAAGTTGCTGAAGTTCCTATTCC-3'. Then the P2A-NLS-Cre-Frt-Amp-Frt cassette was inserted in-frame right before the stop codon of *Oxtr* using the Red/ET Recombineering kit (Gene Bridges) after adding homologous arms by PCR with the following primers: fw5'-CACCTTCGTCCTGAGTCGCCGCAGCTCCAGCCAGAGGAGCTGCTCTCAACCATCTTCAGCAATGGGAAGCGGAGCTACTAAC-3'; rv5'-GAGGAGAGGGATACACACCAATAGGCACCTTATACAACTCCACGCACGGCCACCAGGGGCAGACTGGAGTCCGCATAGCC-3'. The ampicillin selection marker was deleted by 706-Flpe induced recombination method (Gene Bridges). A correctly modified BAC clone was verified by using PCR at both the 5' and 3' junctions of the targeted insertion with the following primer pair : fr5'-GCCTTCATCATCGCCATGCTCTT-3' and rv5'-GATGGCTGAGTGACTGGCATCT-3'; The construct was further verified by sequencing using the following 7primers specific either for BAC vector or the p2A-NLS-Cre fragment: 5'- CCTGCAGCCAACTGGAGCTTC-3'; 5'-CTTCCTTGGGCGCATTGACGTC-3'; 5'-TACCTGTTTTGCCGGGTCAG-3；5'-GCCTTCATCATCGCCATGCTCTT-3'；5'-CTGACCCGGCAAAACAGGTA-3'；5'-TCCGGTTATTCAACTTGCACCATGC-3'; 5'-GATGGCTGAGTGACTGGCATCT-3'.

Animals.

Vole-Oxtr-P2A-Cre BAC transgenic mouse lines(Koi lines). Vole-Oxtr-P2A-Cre BAC vector was digested with NotI for linearization and to remove the major components of pTARBAC vector backbone. The Vole-Oxtr-P2A-Cre BAC linearized fragment was purified using CL-4B sepharose (GE Healthcare), and injected into pronuclei of C57BL/6J zygotes. Mice carrying the BAC transgene were identified and confirmed using PCR with 5 pairs of primers: fr5'-TCGACCAGGTTCGTTCACTC-3' and rv5'-

CTGACCCGGCAAAACAGGTA-3'; fr5'- GCCTTCATCATCGCCATGCTCTT -3' and rv5'- TCCGGTTATTCAACTTGCACCATGC -3'; fr5'- GCCTTCATCATCGCCATGCTCTT -3' and rv5'- CTGACCCGGCAAAACAGGTA -3'; fw5'- TACCTGTTTTGCCGGGTCAG -3' and rw5'- GATGGCTGAGTGACTGGCATCT -3'; fr5'-GCCTTCATCATCGCCATGCTCTT-3' and rv5'- GATGGCTGAGTGACTGGCATCT-3'. 8 lines (Koi-1~Koi-8) were confirmed with successful transgene inheritance. These lines were maintained on a congenic C57BL/6J background (at least backcrossed with C57BL/6J for 3 generations) for assessments of BAC DNA copy numbers and transgene expression analysis.

ROSA-26-NLS-LacZ mouse line (21) was obtained from Itohara Lab at RIKEN. ***mOxtr-Ires-Cre*** **knock-in line** (52) and ***mOxtr***$^{-/-}$ **mutant line** (53) were obtained from Nishimori Lab at Tohoku University, and both were backcrossed with C57BL/6J for more than 10 generations. Both Koi lines and *mOxtr-Ires-Cre* knock-in mice were crossed with ROSA-26-NLS-LacZ mice to generate Cre^{+}, LacZ^{+} double positive mice for further gene expression analysis. Koi mice were crossed with Oxtr$^{-/-}$ to generate mice in which transgenic pvOXTR was expressed while endogenous mOXTR was absent, and these mice were used for autoradiography, qRT-PCR and behavioral tests. All mice were generated using continuously housed breeder pairs and P21 as the standard weaning date. Primers used for genotyping were listed in supplementary Table 1. All the animal procedures are approved by Riken and University of Tsukuba Animal Care and Use Committee. Mice were housed under constant temperature and light condition (12 h light and 12 h dark cycle), and received food and water ad libitum.

Determination of transgene copy number

Custom Taqman® MGB probes were synthesized by oligoJp (Thermo Fisher) for detecting the transgenic *Cre* and the mouse *Jun* gene (internal control). The following primer pairs and probes were used: for *Cre* assay, forward primer: (5'-ATGACTGGGCACAACAGACAAT-3'); reverse primer: (5'-CGCTGACAGCCGGAACAC-3'); probe: (5'-FAM- AAACATGCTTCATCGTCGGTC CGG-MGB-3'); for *Jun* assay, forward primer: (5'-GAGTGCTAGCGGAGTCTTAACC-3'); reverse primer: (5'-CTCCAGACGGCAGTGCTT-3'); probe: (5'-VIC-CTGAGCCCTCCTCCCC-MGB-3'). Real-time PCR was performed on Applied Biosystem7500. Transgene copy number was determined through absolute quantification (standard curve method) following the protocol described in (26). Briefly, 2 microliters (20 ng) of genomic DNA samples or copy number standards were analyzed in a 20μL

reaction volume with two primer-probe sets (*Cre, Jun*). The data from transgenic samples were then compared to a standard curve of calibrator samples that are generated by diluting purified BAC DNA (linearized) over a range of known concentrations into wild-type mouse genomic DNA. In addition, no-template controls were included in each experiment. All reactions were performed in triplicate.

Histology

Adult mice(3-4month old) were perfused intracardially with 4% formalin in 0.1M sodium phosphate buffer. Brains were embedded in 2% agarose in 0.1M PB and cut into 100-m-thick sections with a Micro-slicer (Dosaka, Kyoto, Japan). Mammary glands were isolated from virgin female mice(2month old). The brain slices and whole mount mammary glands preparation were stained in X-gal solution (5 mM K3FeCN6, 5mMK4FeCN6,2mMMgCl2, 0.02% NP-40, 0.01% Na-deoxycholate, 1 mg/ml X-gal in 0.1M PB) at 37°C for 6 hours and then were stained with hematoxylin. All experiments were done with positive and negative controls (Cre^+, $LacZ^-$) to monitor the reliability of the X-gal staining.

Receptor Autoradiography

OXTR autoradiography was performed as previously described(24). Briefly, freshly frozen brains were stored at -80˚C. Coronal sections were cut in a cryostat and 20 µm sections were collected and then stored at -80˚C until use in autoradiography. Brain sections were removed from -80˚C storage and air dried, and then fixed for two minutes with 0.1% paraformaldehyde in PBS at room temperature, and rinsed twice in 50 mM Tris buffer, pH 7.4, to remove endogenous OT. They were then incubated in 50 pM 125I-OVTA (2200 Ci/mmol; PerkinElmer; Boston, MA), a selective, radiolabeled OXTR ligand, for one hour. Unbound 125I-OVTA was then washed away with Tris-MgCl2 buffer(50 mM Tris plus 2% MgCl2, pH 7.4) and sections were air dried. Sections were exposed to BioMax MR film (Kodak; Rochester, New York) for five days. Digital images were obtained with a light box and a Cannon camera (Cannon 6D MarkII, Japan). The brightness and contrast of representative images were equally adjusted for all autoradiography images within a panel using Adobe Photoshop.

Quantification of *Oxtr* mRNA expression by qRT-PCR

Real-time quantitative polymerase chain reaction (RT-qPCR) on an Applied Biosystems 7500 Real-Time PCR system was used to quantify *Oxtr* mRNA expression levels in different brain regions of Koi lines. Total RNA of different brain regions was isolated using the RNeasy extraction kit (Qiagen), and cDNA was synthesized after Deoxyribonuclease I (Invitrogen)treatment by using EvoScript Universal cDNA Master(Roche), and then quantitative RT-PCR was performed with FastStart Universal SYBR Green Master (Roche). The relative standard curve method was used to obtain the relative quantities of *Oxtr* expression following the manual of Applied Biosystems. To allow the comparation between Koi lines and WT mice, the following primer pair(which can detect the expression of both *mOxtr* and *pvOxtr*) was used: fr5'-GCCTTTCTTCTTCGTGCAGATG-3' and rv5′-ATGTAGATCCAGGGGTTGCAG-3′ In addition ,to specifically detecting the expression of *pvOxtr*, the primer pair fr5′-GCCTTTCTTCTTCGTGCAGATG-3 and rv5'-AAAGAGGTGGCCCGTGAAC-3′ was used in the 2nd qRT-PCR experiment. GAPDH was used as the endogenous control for both experiments and was amplified by the following primer pair: fr5'-GGGTTCCTATAAATACGGACTGC-3' and rv5'- CCATTTTGTCTACGGGACGA-3'. Samples were analyzed in triplicates. A non-template control was performed to ensure that there was no amplification of genomic DNA.

Partner preference test(PPT)

Subjects were housed with 4 age-matched and same-gender littermates until testing at adulthood (2-5 months old). Ovariectomized female were paired and cohoused with a sexually experienced adult male for 21 days before PPT test. The female was injected with estradiol benzoate (10 μg and 5 μg at 48 h and 24 h before induced mating) and progesterone (500 μg at 4–7 h before induced mating) to ensure high sexual receptivity before mating. Mating was induced on Day2,9 and16. PPT was performed in TimeSSI1 for social interaction test system (O' Hara & Co., Ltd.). Briefly, the experimental subject was placed in a chamber, in which two pen-boxes were located at two diagonally opposite corners. The experimental animal was free to move throughout the chamber and the time spent in close proximity to each pen-box is recorded using an automated mouse tracking system (Fig.4 A, B). In phase 1, the experimental subject was allowed to explore the chamber for 5 minutes with both pen-boxes empty. In phase 2, the partner stimulus animal was restricted in one pen-box, and a novel "stranger" stimulus animal was restricted in another pen-box, the experimental subject was allowed to explore the chamber for 10 minutes. In phase 3, the positions of the partner stimulus animal and the "stranger" stimulus

animal were switched, and the experimental subject was allowed to explore the chamber for 20 minutes.

Parental behavior test

To study the induction of maternal behavior in response to stimulus, we performed pups retrieval test with virgin female mice(2- to 4-month old), following the method described in (54) with some modifications. Briefly, 5 days prior to the behavioral testing, each subject () was isolated and housed in separate home cages in the test room. Nesting material (about 1.5 g of cotton wool) was provided for nest making. The tests were conducted in the dark phase of light/dark cycle (12 hours/12 hours). On the day of the test, each subject was placed in the recording area and allowed 15 minutes for habituation. The stimulus pups (age: P2-P3) were collected from a group of donor mothers immediately before the start of the experiment. A retrieval test began with the placement of 3 stimulus pups on the side farthest form the nest was conducted, and the test was recorded by ARNAN 4 channel security system for 15 minutes. Pup retrieval was defined as picking up the pup and bringing it to the nest. If the subject did not complete the retrieval for 3 pups in 15minutes, the video recording time was extended to 30 minutes. The latency to retrieve each pup (1st, 2nd, 3rd) and the time spent crouching on the pups were time-stamped and calculated manually. A latency of 1800 seconds was assigned if the pups-retrieval wasn't completed in 30minutes. Crouching was defined as the mouse supporting itself in a lactation-position over the pups in the nest. Crouching time on a single pup, two pups, and three pups were summed up as total crouching time. All of the experimenters were blind to the genotype of the subjects.

Statistical analysis

All the statistical analyses were performed by SPSS21(IBM). Mixed ANOVA was used for analyzing PPT data and Pups retrieval data. Mauchly's test of sphericity was used to test whether or not the assumption of sphericity is met in repeated measures. Greenhouse-Geisser correction was applied when the sphericity was violated. Two-way ANOVA was used for analyzing qRT-PCR region specific data across different mouse lines. One-way ANOVA was used for analyzing crouching time in Parental behavioral test. If there was a significant main effect of an independent factor, Post Hoc test was used to do multiple comparisons. If there was a significant interaction between within-subject factor and between-subject factor, Post Hoc pairwise comparisons were applied to analyze the simple

main effects. Bonferroni correction was used for all the Post Hoc tests.

Author Contributions: Q.Z. conceived the project, designed the study, led the teamwork, conducted transgene vector construction and drafted manuscript. Q.Z., L.K. and M.Ts. conducted histological analysis. Q.Z. and M.P. analyzed transgene copy number and gene expression level. Q.Z. and K.I. conducted autoradiography analysis. Q.Z., L.K., M.Ts., M.S. and M.P. performed behavioral test and analyzed the data. Y.N. and M.S. performed ovariectomy surgery. M.Ta. provided the data of *Pkcd-cre* lines in the supplementary part. L.K., M.Ts., M.P., T.S., M.S. contributed to genotyping and mouse maintenance. S.I. provided advice and facility for transgene vector construction. S.O. provided advice and facility for behavioral experiment. L.Y helped conceive the project and provided mentorship during the whole project. All authors discussed the manuscript.

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Figure Legends

Fig.1 The construction of Koi lines (A-C) An example to show the remarkable species difference of OXTR expression. OXTR receptor autoradiography in dorsal caudate putamen(CP) and nucleus accumbens shell(NAccSh) of rat, mouse, and prairie vole was shown in (A),(B) and (C), respectively. (D) The *pvOxtr* BAC clone contains the entire sequence of *pvOxtr* gene(colored green), the complete intergenic sequence both upstream and downstream of *pvOxtr* gene, the 12th exon of *Rad18* (colored blue)and the 2nd exon of *Cav3*(colored purple). The yellow arrows indicate the direction of transcription. (E) The schematic diagram of the strategy to generate Koi mice. The P2A-NLS-Cre-Frt-Amp-Frt cassette was inserted in-frame right before the stop codon of *Oxtr*. And the ampicillin selection marker was deleted by 706-Flpe. PCR primers for verifying the targeted alleles are shown as red arrows. (F) Two correctly modified vector clones were verified by using PCR with p2 and p8. (G) Koi lines were verified by 5 pairs of primers. From Lane1 to Lane8 are 100bp marker, p3+p7, p2+p6, p2+p7, p4+p8, p2+p8, blank and 1kb marker.

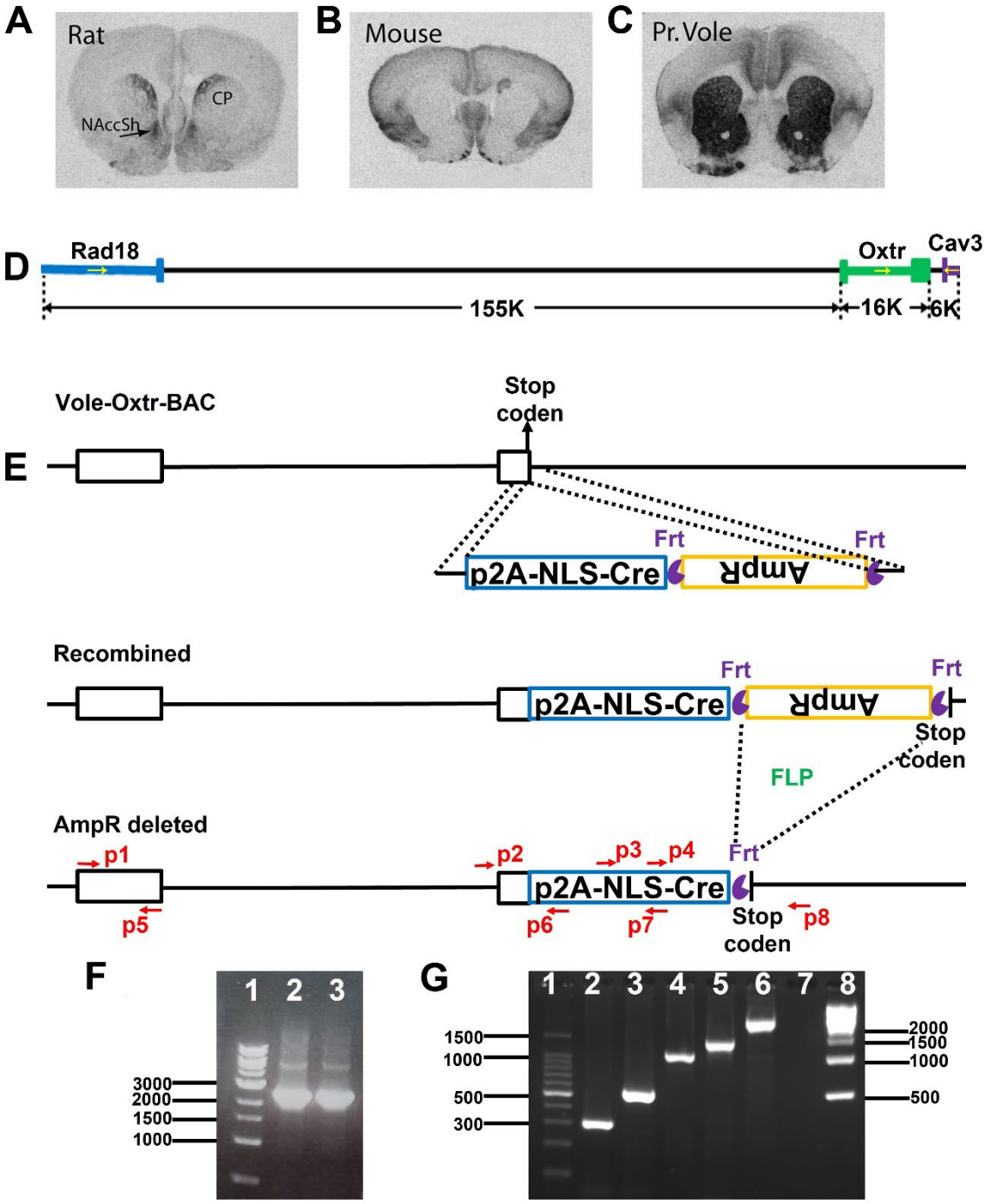
Fig.2 Transgenic CRE mediated Lac-Z reporter gene expression in the brain and mammary gland of Koi lines. X-gal staining of the brains and mammary glands are from the double positive (*Cre*+, *LacZ*+) offspring from Koi-1(A, F, K, P), Koi-2(B, G, L, Q), Koi-3(G, H, M, R), Koi-4 (D, I, N, S) and *mOxtr-Ires-Cre* knock-in line (E, J, O, T). U, V, M, X, Y are the magnified images of the areas within the yellow-border squares from D, N, E, J, O, respectively, to show the distribution of low-density Lac-Z positive cells in these areas. Scale bar=1mm(A-O), 500um(P-T) and 200um(U-Y). (Z) The estimated copy number of transgene of all Koi lines.

Fig.3 The expression of OXTR in the brain of Koi lines. Autoradiographs illustrate the distribution of OXTR in WT(A-C), Koi-1(D-F), Koi-2(G-I), Koi-3(J-L), and Koi-4(M-O). Scale bar=1mm

Fig.4 The social behaviors of Koi lines. (A)The illustration of the procedure of PPT(partner preference test). After co-housing with the mating partner, the experimental mouse is placed in a chamber with an automatic monitoring system, where it can freely explore two pen-boxes localized at two diagonal corners. Phase1(5min): two pen-boxes are empty. Phase2(10min): a stimulating partner mouse is placed into one pen-box and a stimulating stranger mouse is placed in the other one. Phase3(20min): The positions of two stimulating mice are switched(Protocol3). (B)The representative traces of one

experimental mouse during three phases. (C-E) The stay time in the surrounding area of each pen box during three phases was recorded and analyzed. (C)Phase1;(D) Phase 2; (E) Phase 3. WT(n=12), Koi-1(n=14), Koi-2(n=13) Koi-3(n=12) Koi-4(n=14). All the experimental mice are female. (F) The illustration of the procedure of maternal behavior test. (G) Pups retrieval latency and (H)crouching time were recorded and analyzed. WT(n=15), Koi-3(n=18) Koi-4(n=18). The significance of interaction between independent factors: # $p<0.05$; # # $p<0.01$. The significance of main effect or single main effect of genotype: * $p<0.05$; * * $p<0.01$; * * * $p<0.001$; * * * * $p<0.0001$.

Fig.1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FLP
AmpR deleted
p1
p2
p3
p4
Frt
p2A-NLS-Cre
p5
p6
p7
Stop
coden
p8
F
1 2 3
3000
2000
1500
1000
G
1 2 3 4 5 6 7 8
1500
1000
500
300
2000
1500
1000
500

Fig.2

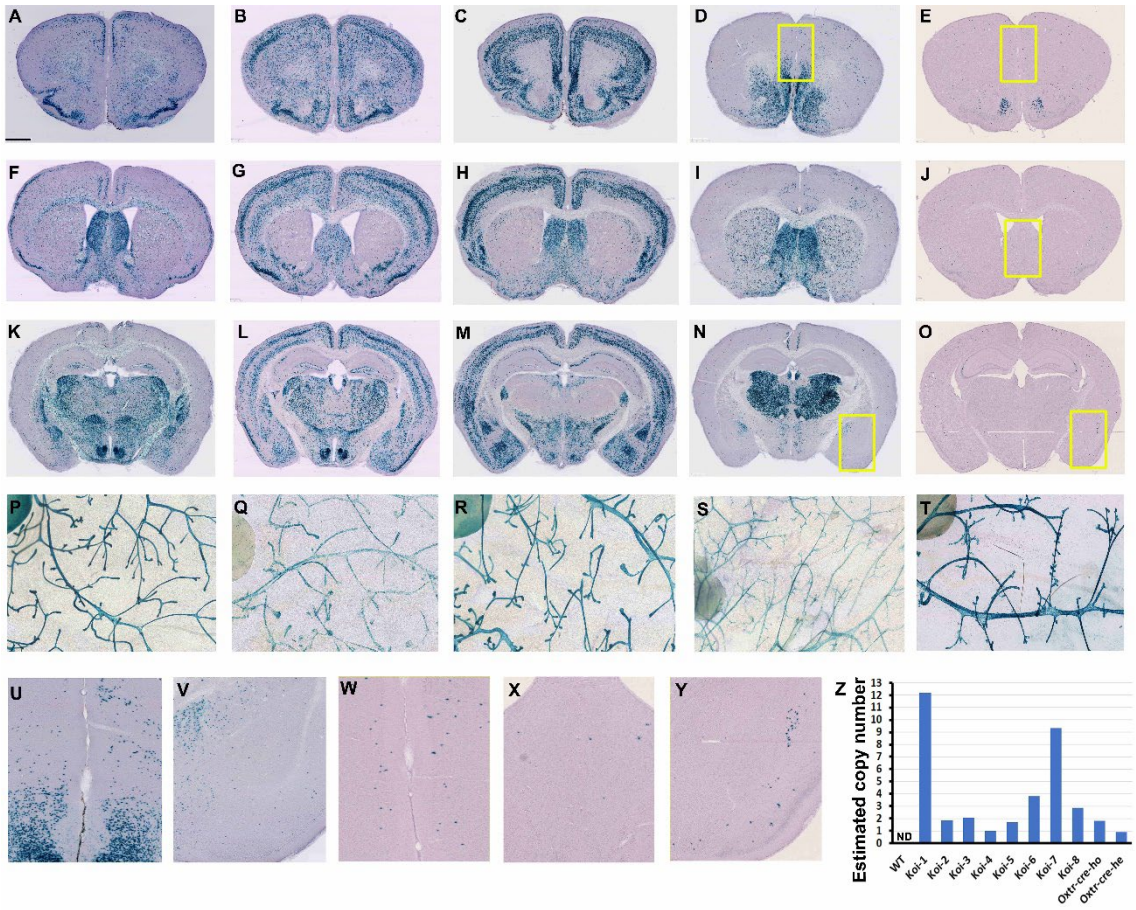

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
Estimated copy number
0
1
2
3
4
5
6
7
8
9
10
11
12
13
ND
WT
Koi-1
Koi-2
Koi-3
Koi-4
Koi-5
Koi-6
Koi-7
Koi-8
Oxtr-cre-ho
Oxtr-cre-he

Fig.3

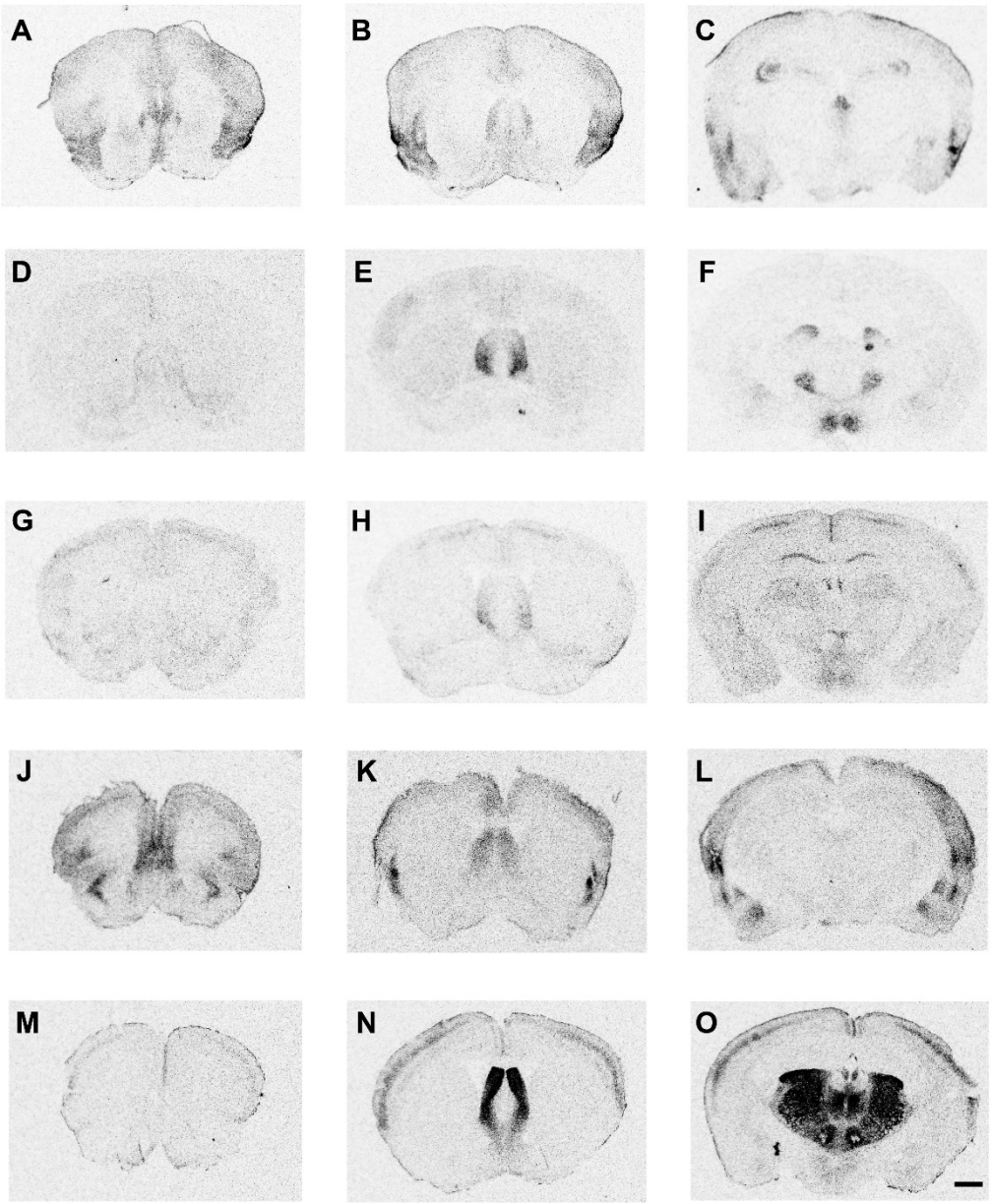
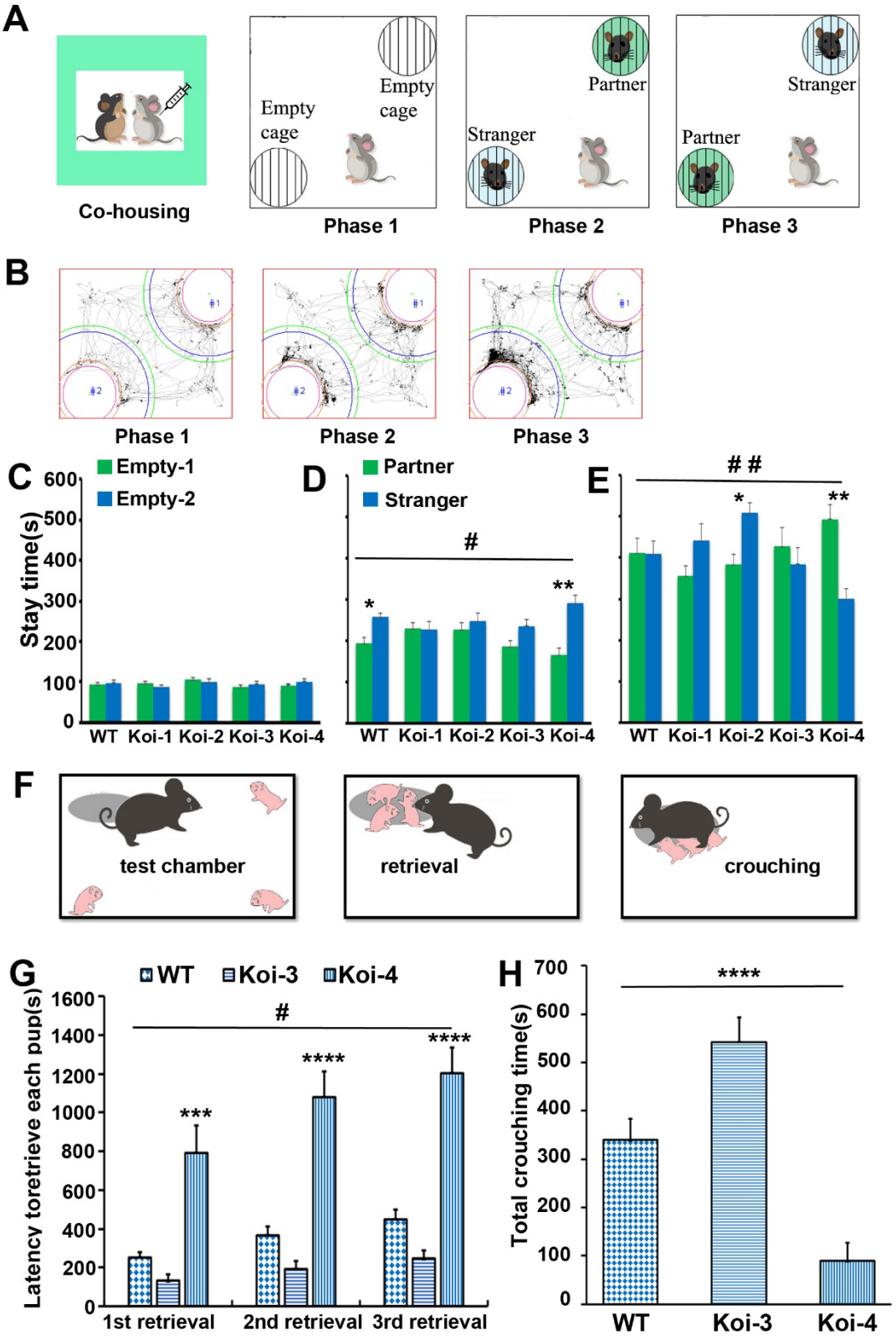
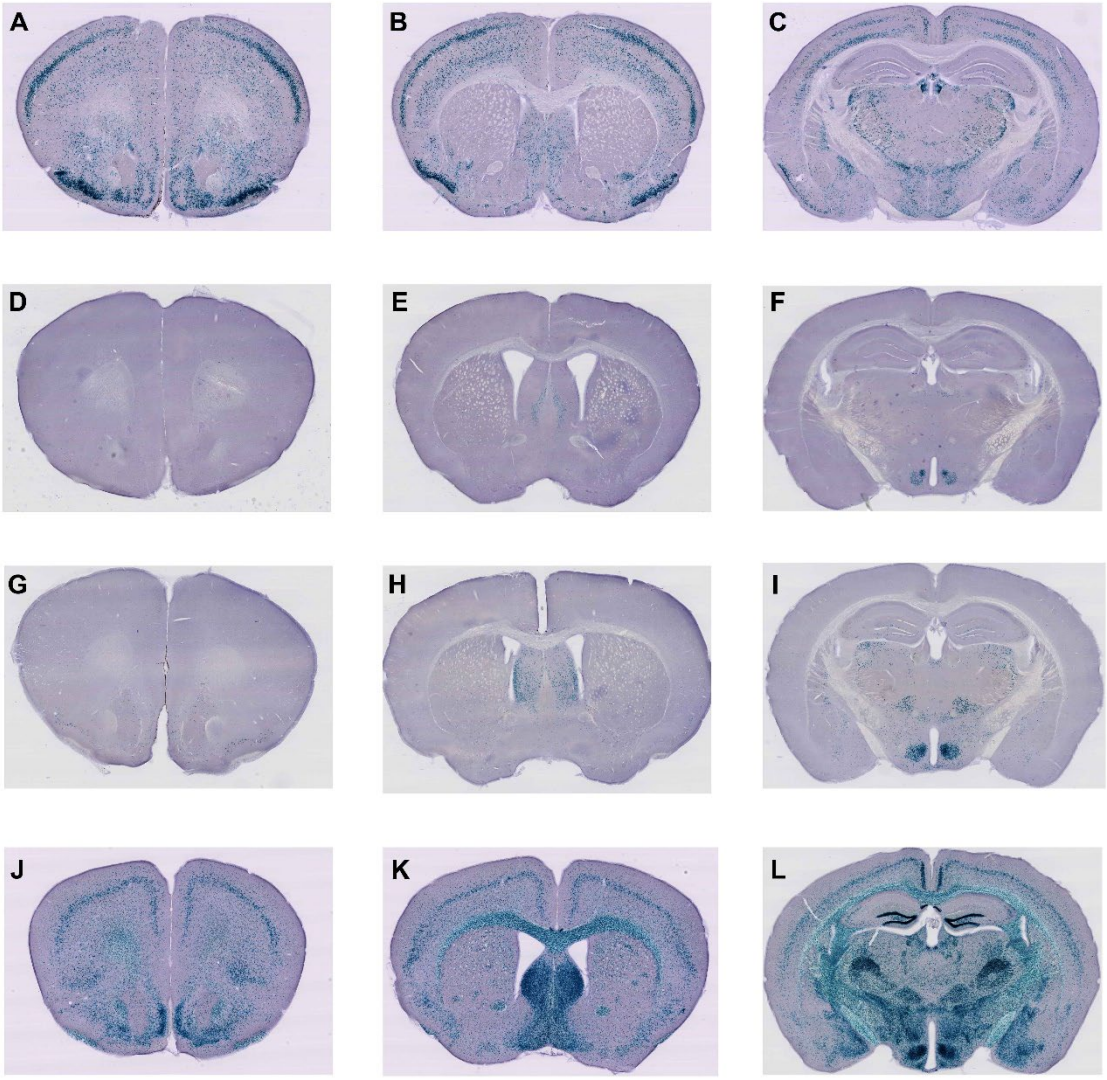


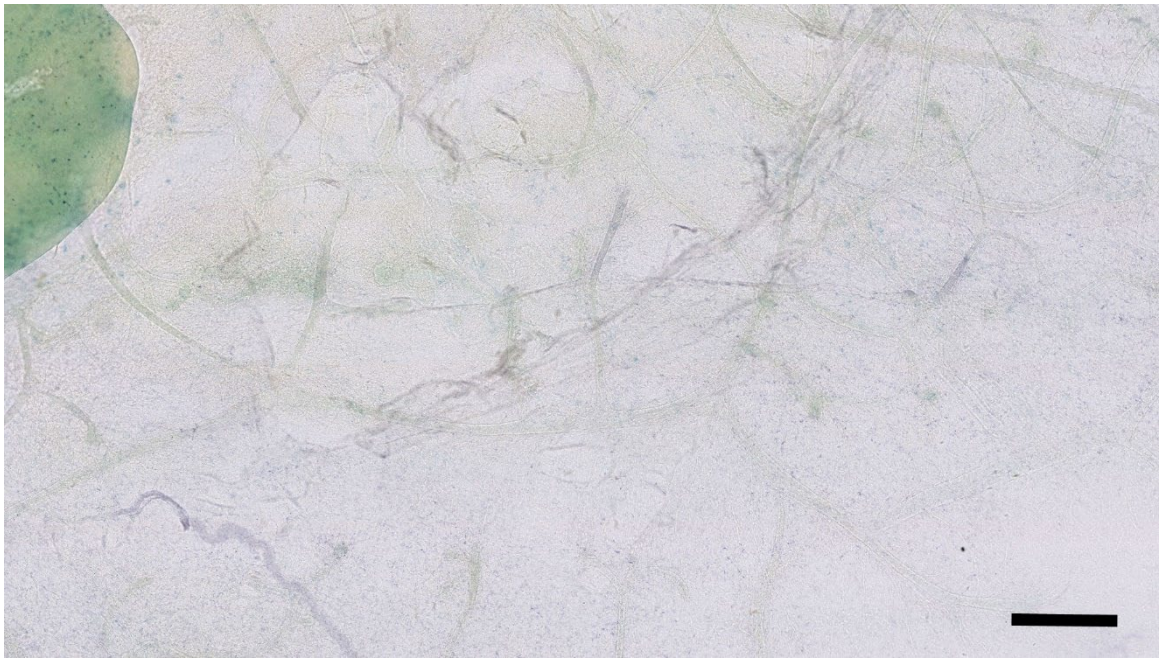
Fig.4


A
Co-housing
Empty cage
Empty cage
Phase 1
Partner
Stranger
Phase 2
Stranger
Partner
Phase 3
B
Phase 1
Phase 2
Phase 3
C
Empty-1
Empty-2
Stay time(s)
600
500
400
300
200
100
0
WT Koi-1 Koi-2 Koi-3 Koi-4
D
Partner
Stranger
#
*
**
WT Koi-1 Koi-2 Koi-3 Koi-4
E
# #
*
**
WT Koi-1 Koi-2 Koi-3 Koi-4
F
test chamber
retrieval
crouching
G
WT
Koi-3
Koi-4
Latency toretrieve each pup(s)
1600
1400
1200
1000
800
600
400
200
0
#
***
****
****
1st retrieval
2nd retrieval
3rd retrieval
H
****
Total crouching time(s)
700
600
500
400
300
200
100
0
WT
Koi-3
Koi-4

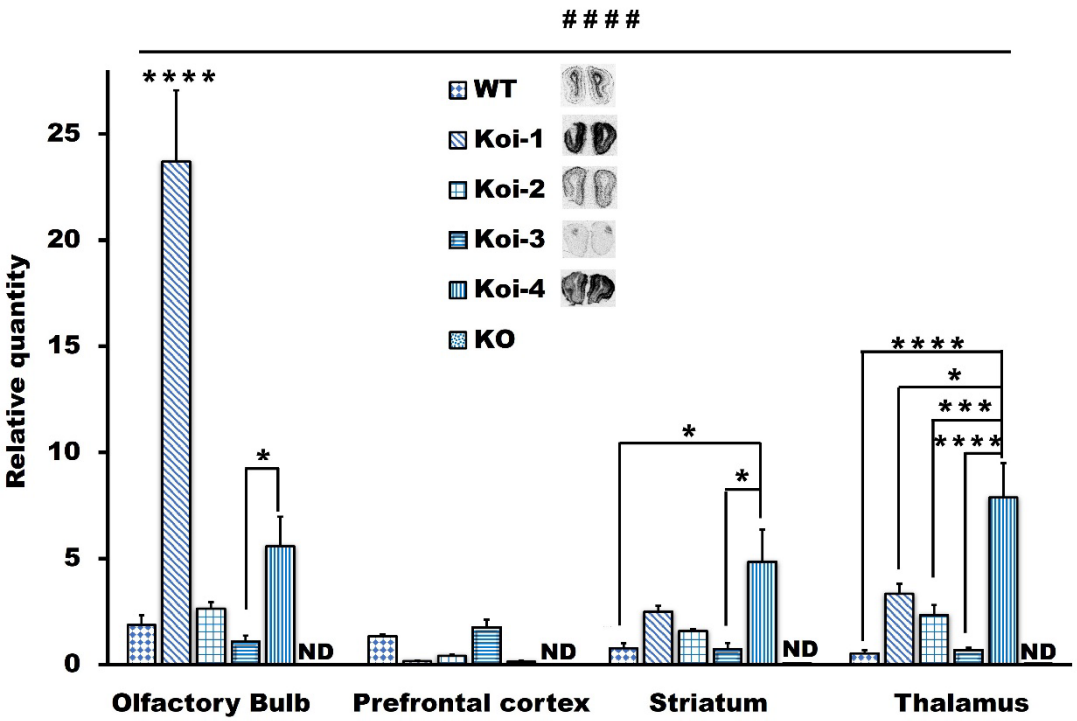
Supplementary Figures



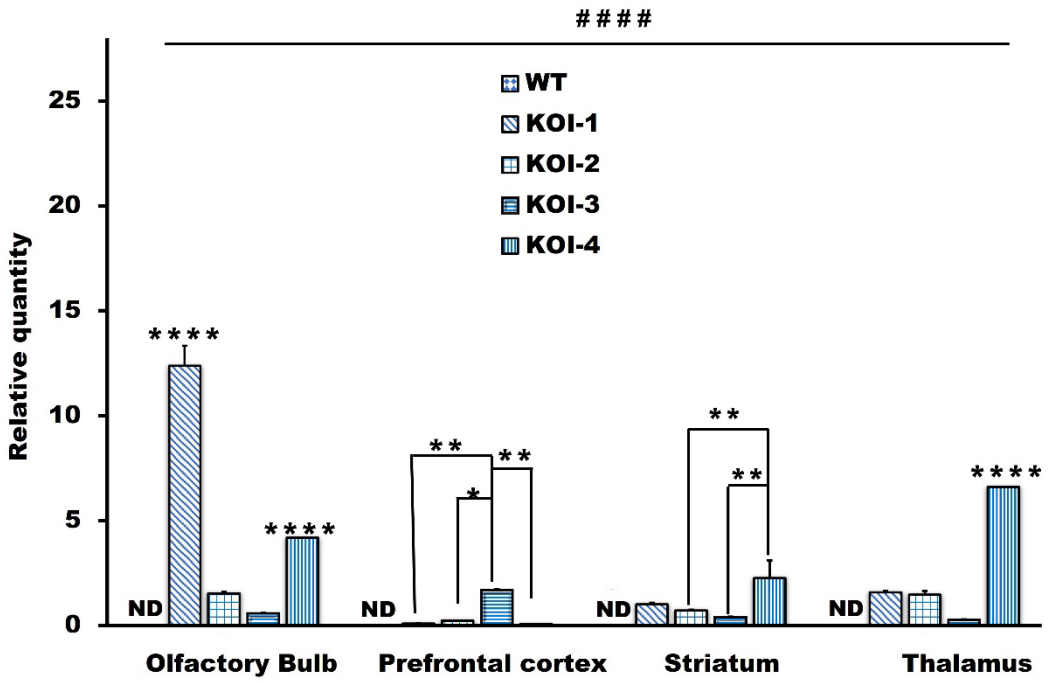
Supplementary Fig.1 Transgenic CRE mediated Lac-Z reporter gene expression in the brain of Koi lines. X-gal staining of the brains are from the double positive (*Cre*+, *LacZ*+) offspring from Koi-5(A-C), Koi-6(D-F), Koi-7(G-I), Koi-8 (J-L). Scale=1mm



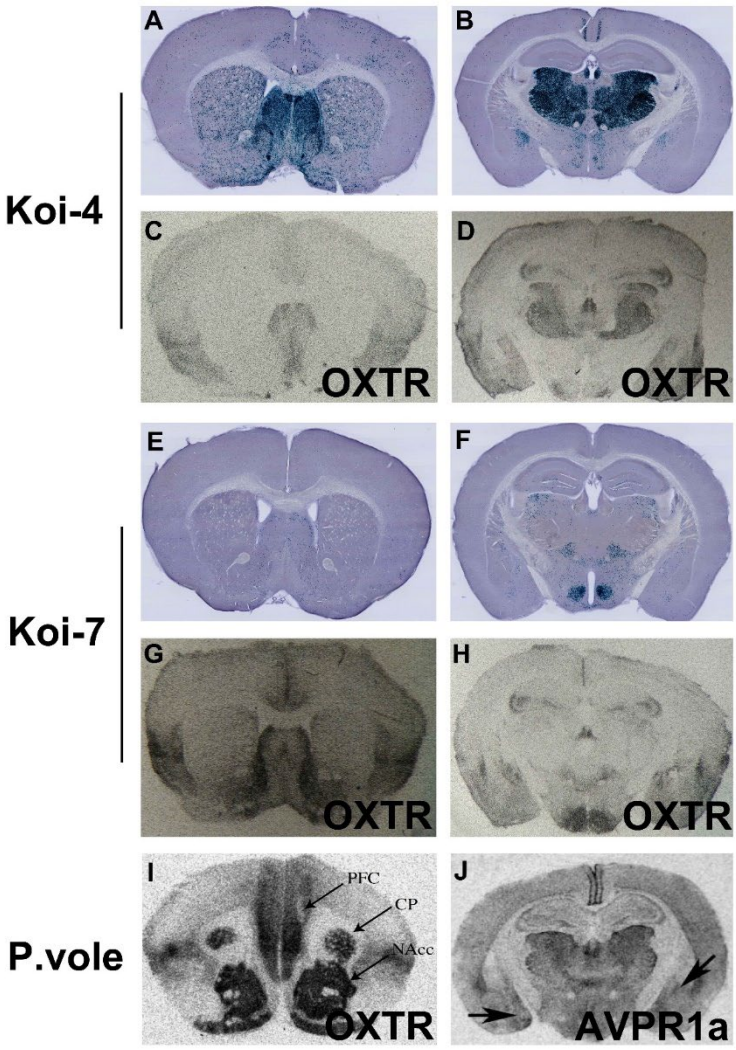
Supplementary Fig.2 Negative control of whole-mount mammary gland staining.
X-gal staining of a mammary gland from (*Cre*-, *LacZ*+) offspring from Koi-4 was shown. The lymph node showed some blue signal, indicating that the lymph node could be stained unspecifically. All the epithelium ducts didn't show any blue signal, verifying the specificity of duct labeling of X-gal staining.



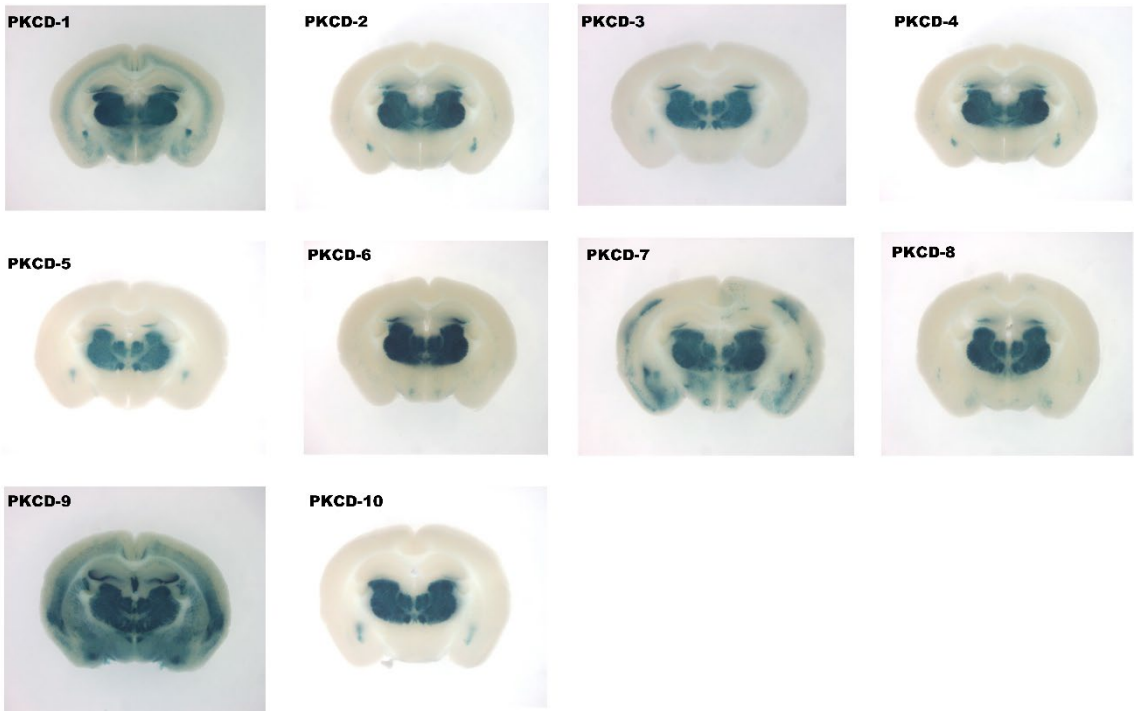
Supplementary Fig.3 Quantitative analysis of *Oxtr* mRNA expression in different brain regions revealed by common primer set recognizing both m*Oxtr* and pv*Oxtr*. The tissue of olfactory bulb, prefrontal cortex, striatum and thalamus from WT, Koi-1, Koi-2, Koi-3, Koi-4 and *mOxtr*$^{-/-}$ mutant mice were analyzed, n=4 for each genotype. Note that the level of *Oxtr* mRNA in *mOxtr*$^{-/-}$ mutant mice was barely detectable. The auoradiograph of OXTR in olfactory bulb from each line were shown along side the legend. There was a significant interaction between position and genotype $F(12,64)=26.33$, $p<0.0001$; Post Hoc Bonferroni test revealed that *Oxtr* expression level in olfactory bulb of Koi-1 was significantly higher than that of other genotype groups($p<0.0001$ for all), and that of Koi-4 was significantly higher than that of Koi-3 $p<0.05$. And the striatum of Koi-4 expressed significantly higher *Oxtr* when comparing with WT and Koi-3($p<0.05$ for both). The *Oxtr* expression level in thalamus of Koi-4 was significantly higher than other genotype groups($p<0.0001$ when comparing with WT and Koi-3, $p<0.001$ when comparing with Koi-2, $p<0.05$ when comparing with Koi-1.) The significance of interaction between genotype and brain region: # # # # $p<0.0001$. The significance of single main effect of genotype: * $p<0.05$; * * $p<0.01$; * * * $p<0.001$; * * * * $p<0.0001$.



Supplementary Fig.4 Quantitative analysis of *Oxtr* mRNA expression in different brain regions revealed by a primer set specifically recognizing pv*Oxtr*. The tissue of olfactory bulb, prefrontal cortex, striatum and thalamus from WT, Koi-1, Koi-2, Koi-3 and Koi-4 mice were analyzed, n=4 for each genotype. Note that the level of *Oxtr* mRNA in WT mice was barely detectable. There was a significant interaction between position and genotype $F(9,48)=89.25$, $p<0.0001$; Post Hoc Bonferroni test revealed that *Oxtr* expression level in the olfactory bulb of Koi-1 was significantly higher than that of other genotype groups($p<0.0001$ for all), and that of Koi-4 was significantly higher than that of Koi-2 and Koi-3 ($p<0.0001$ for both). The *Oxtr* expression level in prefrontal cortex of Koi-3 was significantly higher than that of other groups($p<0.01$ when comparing with Koi-1 and Koi-4; $p<0.05$when compring with Koi-2). And the striatum of Koi-4 expressed significantly higher *Oxtr* when comparing with Koi-2 and Koi-3($p<0.01$ for both). The *Oxtr* expression level in thalamus of Koi-4 was significantly higher than that of other genotype groups($p<0.0001$ for all). The significance of interaction between genotype and brain region: # # # # $p<0.0001$. The significance of single main effect of genotype: * $p<0.05$; * * $p<0.01$; * * * $p<0.001$; * * * * $p<0.0001$.



Supplementary Fig.5 The expression of transgene in Koi-4 and Koi-7 lines. (A, B, E, F) Transgenic CRE mediated Lac-Z reporter gene expression in the brain revealed by X-gal staining. (C, D, G, H) OXTR autoradiography showed the expression pattern of OXTR in nucleus accumbens and thalamus. (I) OXTR autoradiography in the nucleus accumbens of P.vole. (J) AVPR1a autoradiography in the thalamus of P.vole.



Supplementary Fig.6 An example of the expression pattern of the transgene

10 independent *Pkcd* BAC transgenic mouse lines showed expression pattern similar in most brain regions. The BAC construction was made following the same rule as that was used to construct Koi lines. That is, it spans upstream of 5' of *Pkcd*(93kb) and downstream of 3' of *Pkcd* (43kb), which contains the entire intergenic sequence and the upstream *Tkt* gene and downstream *Gm7644* gene.