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CHAPTER 8

New aspects of oxytocin receptor function revealed by knockout mice: sociosexual behaviour and control of energy balance

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Abstract: To further define the function of the oxytocin receptor (OXTR) in vivo, we generated mice deficient in the Oxtr gene (*Oxtr*−/−). *Oxtr*−/− mice had no obvious deficits in fertility or sexual behaviour, but displayed several aberrations in social behaviours, including male aggression, and mother–offspring interaction. In addition, they showed novel physiological defects including obesity, and dysfunction in body temperature control when exposed to cold. We review here our new findings with *Oxtr*−/− mice, and introduce newly generated Oxtr-Venus knockin mice as a potential tool for examining molecular physiology of Oxtr-neurons.

Keywords: maternal behaviours; social discrimination; aggressive behaviours; thermo regulation; localization of oxytocin receptor expressing neurons

Oxytocin and oxytocin receptor

Oxytocin synthesis

Although occupying less than 1% of the whole brain volume in the case of the human, the hypothalamus is an important control centre that regulates metabolism, body temperature, heart beat rate, blood pressure, water and food intake and behaviour. These controls are generated in response to alterations in the outer and internal environment of the body and performed in part via the endocrine system and through the autonomic nervous system in response to physical and chemical signals. The neuropeptide oxytocin (OXT), with only nine amino acids (a.a.), was first identified as one of the prototypical hypothalamic hormones that is released into the bloodstream from the posterior pituitary. It is now known that OXT released within the brain acts as a neuromodulator/neurotransmitter through its actions on its G-protein coupled receptor (GPCR) (Landgraf and Neumann, 2004). OXT, which is synthesized as a 125 a.a. precursor

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and includes a neurophsyin, is synthesized primarily in magnocellular neurons located at the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. After processing by a protease and formation of an intramolecular disulfide bond, OXT is transported to the posterior lobe of the pituitary and secreted into the blood in response to various physiological stimuli, including vaginocervical and nipple stimulation. In addition to this hypothalamic source, OXT is also synthesized in various peripheral tissues and organs, including the uterine epithelium, ovary, testis, vascular endothelium cell and heart.

Oxytocin receptor

The receptor for oxytocin (OXTR) is a member of the GPCR family and is 388 a.a. in length (Kimura et al., 1992). The cytoplasmic domain (the C-terminal intracellular domain) is known to functionally couple with q or 11 subtype α subunit (G α q/11) of the trimeric G-protein complex. Activation of OXTR by the binding of OXT to its outer membrane domain sequentially activates G-protein α subunit, phopholipase C and PKC, and finally activates numerous cellular proteins and accelerates the outflow of Ca^{++} from the endoplasmic reticulum, leading to several downstream cellular responses.

OXTR is known to be expressed in mammary gland (Kimura et al., 1998), uterine myometrium (Helmer et al., 1998), adipose precursor cell (unpublished data), cardiac muscle of heart (Gutkowska et al., 1997) and vascular endothelium layer (Thibonnier et al., 1999). The function of OXTR in the contraction of uterine myometrium during parturition, and in the contraction of the smooth muscle layer of mammary alveoli during milk ejection after stimulation by suckling is well known.

More recent studies have demonstrated that centrally released OXT, presumably synthesized in the hypothalamus, acts as a neuromodulator or neurotransmitter through OXTR expressed in specific brain regions, where it affects behaviours, memory and other physiological functions of the central nervous system. For example, a role for OXT in the induction of maternal behaviours has been demonstrated in several animal models in which the administration of OXT facilitates maternal behaviour (Pedersen and Prange, 1979; Williams and Griffith 1992; Nelson and Panksepp, 1998). The receptors for OXT expressed in the central nervous system and in peripheral tissues are considered to be the same molecule.

Generation of oxytocin deficient (*Oxt*−/−) mice

To address the reproductive function of OXT, especially with regard to its role in parturition and milk ejection, we generated OXT gene deficient mice (Nishimori et al., 1996).

Defect in reproductive function in mice deficient in oxytocin gene

Although the resultant *Oxt*−/− mice showed no apparent aberration in physical appearance, growth and reproductive ability, and survived as well as the wild-type mice, newborn pups from *Oxt*−/− dams died shortly after birth due to a disruption in nursing. The histology of the mammary glands of the null postpartum mice showed the accumulation of milk, suggesting that a defect in milk ejection was the most likely cause of the neonatal lethality in their pups (Nishimori et al., 1996). In addition, postpartum intraperitoneal injections of OXT given to the *Oxt*−/− females every few hours enabled sufficient milk ejection to keep several offspring from each litter alive as long as the injections continued (Nishimori et al., 1996).

The indispensable function of OXT for milk ejection was clearly proven by the generation of OXT KO mice. However, contrary to expectations, no defect in parturition by the OXT KO mice was observed; null mice showed quite normal delivery of the newborn pups on the due date. In addition, no gross deficits in maternal nurturing behaviour were detected. These unexpected results raised questions concerning the OXT system. (1) Does the OXTR have a ligand(s) other than OXT that could compensate for the loss in OXT?

(2) Is the OXTR, but not OXT, necessary for normal parturition?

Abnormal social behaviours of Oxt−/− *mice*

While *Oxt*−/− displayed grossly normal maternal behaviour, male mutant mice were found to exhibit profound deficits in social recognition, or social amnesia (Ferguson et al., 2000). Interestingly, mutant mice displayed normal cognitive abilities in other tests, such as the Morris Water Maze, and normal olfactory learning for non-social odours. This observation suggests that OXT plays a critical role in the processing of social stimuli, specifically. The deficits in social recognition could be rescued by a central infusion of OXT directly into the amygdala, but not the olfactory bulb (Ferguson et al., 2001).

Generation of oxytocin receptor deficient (*Oxtr*−/−) mice

The lack of the expected phenotype on parturition and maternal behaviour in the *Oxt*−/− mice was particularly puzzling and suggested that alternative ligands or other compensatory effects could have been influencing OXTR activation in the OXT mutant mice. We therefore decided to further define the role of the OXTR system by creating OXTR KO (*Oxtr*−/−) mice (Takayanagi et al., 2005) in collaboration with Dr. Kimura, who first cloned mouse oxytocin receptor gene (Kubota et al., 1996).

Defective phenotype of Oxtr−/− *mice in reproductive function*

Oxtr−/− females and males copulate normally, and their fertilization efficiency was also normal. In addition, and surprisingly, the parturition of *Oxtr*−/− pregnant mice was quite normal without any delay compared to that of the WT animals.

On the other hand, as in the case of the phenotype of *Oxt*−/− postpartum mice, *Oxtr*−/− postpartum mice showed a defect in milk let-down, and all neonatal pups born from the *Oxtr* null mother mice died shortly after birth, presumably because of the lack of feeding and resultant dehydration in the pups. Those observations clearly indicated that the OXTR was not necessary for parturition in mice, and that its ligand, OXT, was also not necessary.

We suspected that some substitutive mechanism existed in mice to maintain the normal parturition machinery even when the OXT system was not functioning. To test this hypothesis, we generated mice doubly deficient in the genes for *Oxtr* and prostaglandinF2 α receptor (Fp, Sugimoto et al., 1997), an important myometrium contractile factor. This "double KO" mice still showed almost normal parturition after the administration of progesterone antagonist just before term (manuscript in preparation).

Pharmacological analysis of the role of oxytocin receptor on contractile reaction with vasopressin in myometrial smooth muscle

Using *Oxtr*−/− mice, we pharmacologically analysed the OXTR in mouse uterus, and confirmed that not only OXT, but also arginine vasopressin (AVP) could signal through the OXTR resulting in the induction of contractile movement of myometrial smooth muscle. In contrast, vasopressin receptor Avpr(V1a) is not expressed in mouse uterine myometrium and no signals through Avpr(V1a) are generated to induce contraction in mouse uterine myometrium in vitro.

In the myometrium of non-pregnant mice, both AVP and OXT induced contractions. The effect of OXT was the most potent, while the maximum contractions induced by these two peptides were almost of the same magnitude. Pharmacological characterization of the mouse OXTR, using the *Oxtr*−/− mice as an experimental control, showed that not only OXT but also AVP induced the dose-dependent contraction of uterine myometrium prepared from both non-pregnant and pregnant (Fig. 1A, B) wild-type females. Further experiments showed that both AVP- and OXT-induced contractions were strongly inhibited by an OXTR antagonist, CL-12-42 ($d(CH_2)_5[Tyr(Me)^2,Thr^4,$-$Tyr\text{-}NH_2^9]OVT$; a generous gift from Dr. Maurice Manning, Medical College of Ohio), but weakly

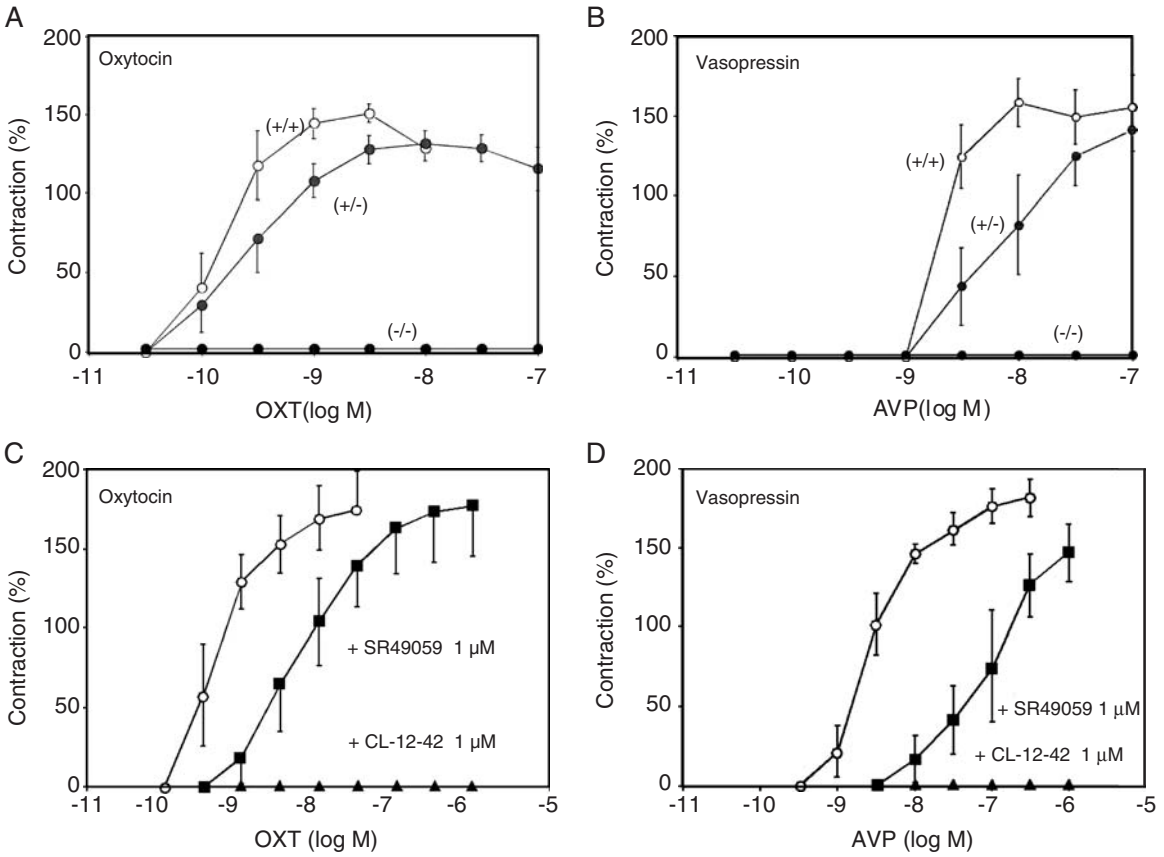

Fig. 1. Cumulative dose–response curve for oxytocin and vasopressin in pregnant mouse myometrium (Adapted with permission from Kawamata et al., 2003). Contractile profiles of myometrium from *Oxtr*−/− pregnant female in response to increased dose of oxytocin (A), or of vasopressin (B) are shown. Contractile profiles of myometrium from WT female in response to increased dose of oxytocin (C), or of vasopressin (D), in the presence of oxytocin receptor antagonist CL-12-42 and V1aRreceptor antagonist SR49059, are shown.

inhibited by a vasopressin Avpr(V1a) antagonist, SR49059; kindly provided by Sanofi Recherche Co. Ltd., France). Similar results were obtained in contraction experiments (Fig. 1C, D) (Kawamata et al., 2003) using myometrium from pregnant WT mice. These results suggested that, in the mouse myometrium, not only OXT- but also AVP-induced contraction was mediated by the activation of OXTRs but not by that of Avpr(V1a) vasopressin receptors. We confirmed the absence of expression of Avpr(V1a) mRNA in the myometrium of WT mice by an RT-PCR procedure (Kawamata et al., 2003). As for human non-pregnant myometrium, in contrast to mouse, we confirmed the inhibition of AVP-induced contraction by SR49059. Our finding, confirmed with experiments using myometrium from *Oxtr*−/− mice, suggests that there are significant differences in the physiological characteristics of OXTRs in contractile responses to AVP and OXT between human and mouse uteri.

Abnormal social behaviours of Oxtr−/− *mice*

Preliminary observations in our *Oxtr*−/− mouse colony suggest an increased frequency of male mice injured in the cages of littermates from *Oxtr*+/− (hetero) parents. We therefore carried out quantitative experiments to measure male aggressiveness using a resident–intruder aggression test.

Elevated aggressive behaviour in Oxtr−/− *male mice*

To test the aggressiveness of the *Oxtr*−/− male mice, we applied a resident–intruder aggression test. Principally, a group-housed C57BL6J male mouse as the intruder (stimulus) was put into the homecage of an individually housed 10-week-old resident male (experimental), and attack duration, frequency and latency to first attack were recorded (see details in Takayanagi et al., 2005). These experiments confirmed increased aggressive behaviour of male *Oxtr*−/− mice (Fig. 2).

Although in our preliminary observation we had not detected a significant elevation in aggressive behaviour in OXT ligand KO mice, we carried out the resident–intruder aggression test to determine the level of aggressive behaviour of *Oxt*−/− male mice as well as that of their wild-type littermates. In contrast to *Oxtr*−/− male mice, *Oxt*−/− male mice did not show any elevation in aggressive behaviour, in comparison with their hetero (*Oxt*+/−) or wild-type littermates. Following this discrepancy between peptide and receptor mutant mice, we hypothesized two explanations: (1) an alternative ligand is activating the OXTR in the *Oxt*−/− knockout or (2) developmental exposure of OXT in the *Oxt*−/− from maternal sources or even siblings.

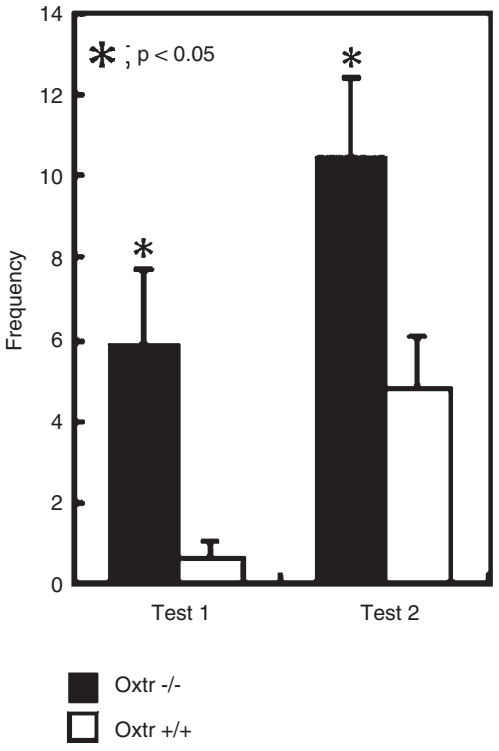

Fig. 2. Aggressive behaviour as measured by the resident–intruder test for *Oxtr*−/− mice (Adapted with permission from Takayanagi et al., 2005). Aggressive behaviour was quantified two times and indicated by attack frequency in the first and second trial (Test 1 and Test 2, 5 min for each test with a 5-min interval. *Oxtr*−/− ($n = 9$) and *Oxt*+/+ ($n = 9$) mice were used.

Supporting the first possibility, Kawamata et al. (2003) reported that AVP, could give an active signal through the OXTR to contract the uterine myometrium in vitro as described in "Pharmacological analysis of the role of oxytocin receptor on contractile reaction with vasopressin in myometrial smooth muscle" of this article. However, AVP apparently failed to suppress aggressive behaviour via the OXTR in *Oxt*−/− mice. We therefore suspected an effect of maternal OXT or OXT synthesized in heterozygous (*Oxt*+/−) or wild-type littermates diffusing through the placental barrier to the null fetus. We normally produced *Oxt*−/− mice by an *Oxt*+/− breeding pair to avoid neonatal lethality of the newborn *Oxt*−/− mice, because of the lack of milk feeding from the null mother mice. This strategy was also good for obtaining all genotypes, such as +/+, +/− and −/−, as littermates, which were ideal for the experiments.

In human, factors whose molecular weight is more than 500 can hardly reach the brain through the blood–brain barrier (BBB), and externally administrated OXT is not considered to be efficiently transported into the brain through this barrier of adult human (Wahl, 2004). However, reports have discussed the possibility of transmission of high molecular hydrophilic materials through the BBB of the fetus, because the foetal barrier may be compromised and the leaked transport of higher molecular weight substances through the BBB of animal embryos may not be negligible (Dziegielewska et al., 1979). In addition, OXT has been reported to be easily transported through placental barrier (Dawood et al., 1979).

Accordingly, we suspected that there was an effect of maternal OXT, diffusing through the placenta and BBB, to the brain of *Oxt*−/− embryos, which might rescue the normal behavioural phenotype including the normally suppressed aggressive behaviours. To test this hypothesis, we generated *Oxt*−/− male mice developed in a maternal environment without possible exposure to OXT by the breeding of *Oxt*−/− parents. Although, in this breeding combination, *Oxt*−/− mothers could not feed their neonatal pups, we cross-fostered them to WT lactating female mice soon after birth to prevent the neonatal lethality of the null pups born from the null breeding pairs. Thus, *Oxt*−/− male mice with the OXTR, but without exposure to OXT during their prenatal development, showed higher aggressive behaviour, similarly to that of *Oxtr*−/− mice (Fig. 3). These results suggested that the aggressive behaviour of *Oxt*−/− male mice, developed in *Oxt*+/− mothers, was suppressed to the normal level by the prenatal exposure of OXTR to maternal plasma OXT, delivered through the premature BBB.

These phenomena suggested the important influence of OXT on prenatal brain development in uteri by which normal social behaviours are established later in adult mice. This may be one of the typical molecular mechanisms by which the maternal milieu regulates foetal development and determines behavioural features in adulthood. In addition, at least in the case of mice, our data showed that appropriate level of exposure of the embryonic brain to OXT was very important for regulating the aggressive response in adulthood.

Further, for some human autistic disorders, a contribution of OXT administered to the pregnant mothers for labour induction has been suggested (Gale et al., 2003). Our observations concerning this suggested mechanism by which aggressive behaviour was established in adult *Oxtr*−/− and

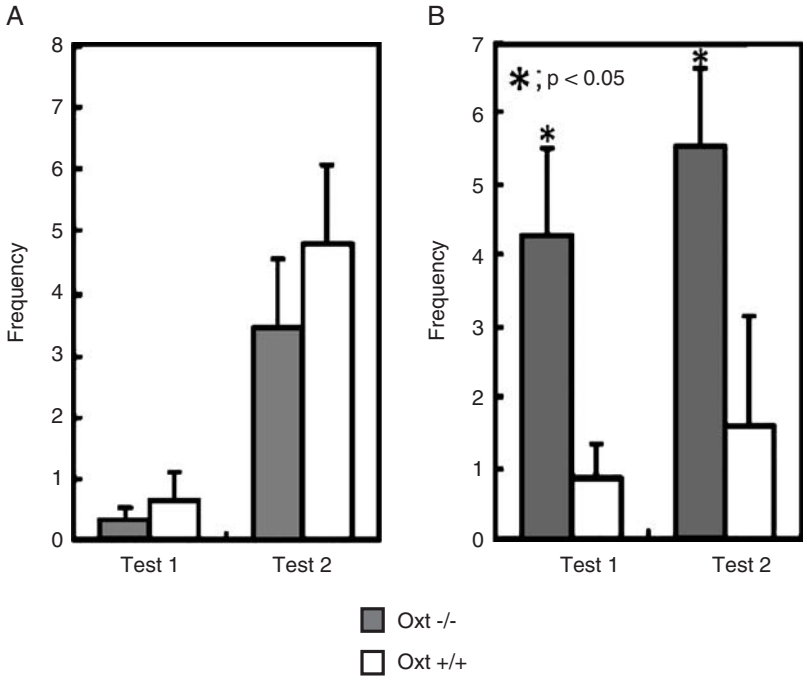

Fig. 3. Aggressive behaviour by the resident–intruder test for *Oxt*−/− mice (Adapted with permission from Takayanagi et al., 2005). Aggressive behaviour was quantified two times and indicated as in Fig. 2. *Oxt*−/− ($n = 11$) and *Oxt*+/+ ($n = 9$) mice from heterozygous intercrosses (A), or *Oxt*−/− ($n = 8$) and *Oxt*+/+ ($n = 7$) mice from intercrosses of homozygous *Oxt*−/− and *Oxt*−/− mice fostered by C57BL/6J females (B), were used. $^{*}p = 0.05$ (Mann–Whitney *U* test). Error bars indicate standard error.

Oxt−/− mice could contribute to understanding some of these disorders. As described above, we first observed increased aggressive behaviour in *Oxtr*−/− male mice. Next, we studied differences in other social behaviours, such as social recognition, maternal behaviours and isolation induced pup ultrasonic vocalizations, because of increasing reports suggesting the importance of neurohypophysial hormones and their receptors in animal social behaviours.

Maternal behaviours of Oxtr−/− *female mice*

We then examined retrieving behaviour and crouching over pups to determine whether aberrations in the maternal behaviours were present in *Oxtr*−/− female mice. Maternal behaviour was assessed in both postpartum (13–18 weeks old) and virgin females (7–9 weeks old) (Fig. 4). Maternal behaviour disruptions were present in both postpartum and virgin *Oxtr*−/− mutant female mice. This observation is in contrast with our previous finding of no gross disruption in maternal behaviour in OXT ligand KO mice. In these experiments, unaffected maternal behaviours were observed in *Oxt*−/− female mice, even those derived from breeding pairs of *Oxt*−/− parents followed by adoption of the newborn *Oxt*−/− mice to the wild-type foster mothers. These observations suggest that the establishment of maternal behaviours in mice requires the presence of OXTR, regardless of the existence of Oxt gene. It is possible, in this case, that other ligands may provide sufficient OXTR activation in the *Oxt*−/− to mediate the development maternal responsiveness.

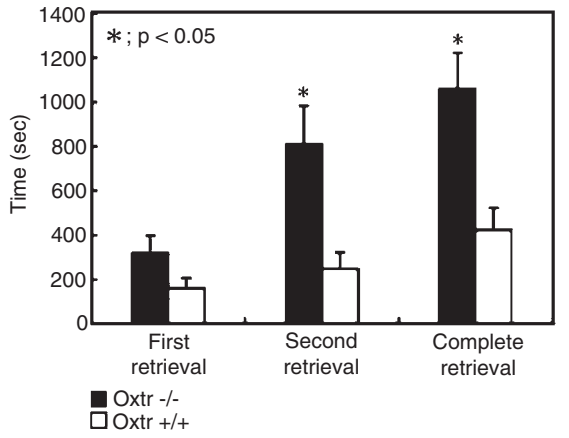

Fig. 4. Maternal nurturing behaviour in female *Oxtr*−/− mice. (Adapted with permission from Takayanagi et al., 2005). Pup retrieval behaviours were tested with *Oxtr*−/− ($n = 9$) or *Oxtr*+/+ ($n = 10$) females from heterozygous intercrosses. Failure to retrieve was assigned as 30 min, the length of the observation period. $^{*}p = 0.05$ and $^{**}p = 0.01$ (Mann–Whitney *U* test). Error bars, standard error.

Social recognition

As mentioned previously, *Oxt*−/− male mice displayed a social amnesia phenotype (Ferguson et al., 2000), strongly suggesting a relationship between the of OXT-receptor system and the control of social behaviour such as social discrimination. A social discrimination test (Landgraf et al., 2003) was also done for *Oxtr*−/− male mice (4–7 months old), singly housed in their own homecage into which group-housed ovariectomized mice were introduced (Ferguson et al., 2000; Takayanagi et al., 2005). We also confirmed that *Oxtr*−/− mice had a defect in social recognition ability as did *Oxt*−/− male mice.

Mother–offspring interaction

Ultrasonic vocalizations (USV) in rodents are signals that play an important communicative role in mother–offspring interaction, and USV of an infant and even those of adult rodents are known to be altered in tone and frequency in response to their emotional situation, suggesting that USV could be used as a quantitative measure of the emotional state such as fear and anxiety in animal models. To understand the effect of a deficit of OXTR on mother–offspring interaction, we examined the isolation-induced USV of *Oxtr*−/− male infants at postnatal day (P)7 when they were separated from their mother. *Oxtr*−/− pups displayed significantly fewer calls than their wild-type littermates (Fig. 5), as *Oxt*−/− males also did (Winslow et al., 2000), while displaying significantly higher levels of locomotor activity during

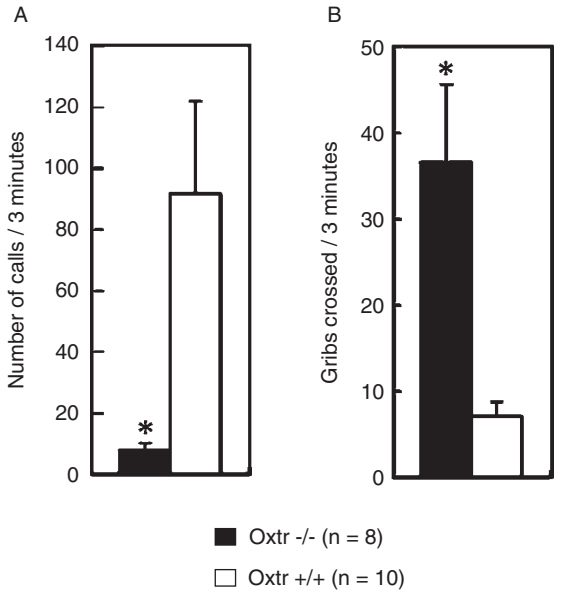

Fig. 5. Infant ultrasonic vocalization (Adapted with permission from Takayanagi et al., 2005). (A) Ultrasonic vocalization of 7-day-old *Oxtr*−/− male pups from Oxtr hetero breeding pairs was tested as described (Takayanagi et al., 2005). The parents were removed from the home cage 20 min before testing. Vocalizations were recorded by using an ultrasonic detector and analysed. (B) Locomotor activity of the same pups.

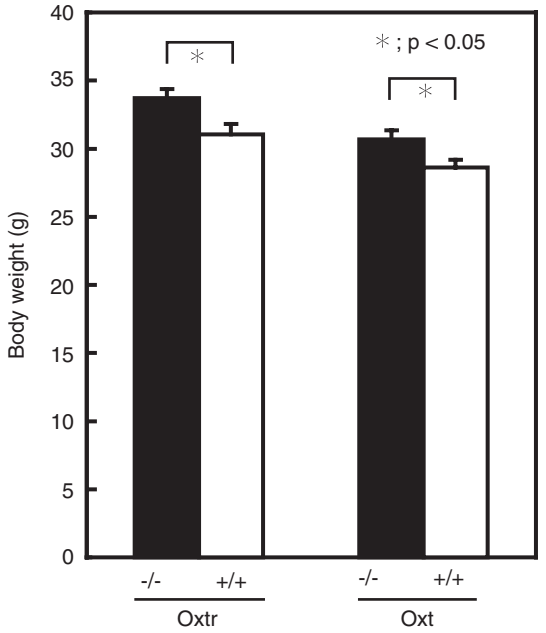

Fig. 6. Body weight of WT ($n = 13$) and *Oxt*−/− ($n = 18$) male mice. $^{*}p < 0.05$ compared with WT mice (20-week-old).

the test. These results from *Oxtr*−/− male pups, showing less USV and more locomotor activity, suggested that the pups without OXTR were more resistant against social isolation and might shift their behaviour towards more exploratory activity (Takayanagi et al., 2008).

Function of oxytocin in energy balance and control of body temperature homoeostasis

The *Oxtr*−/− mice that we generated showed several abnormal phenotypes other than defects in reproductive function and sociosexual behaviours. For example, the *Oxtr*−/− male mice showed slight obesity with aging, and especially 12 weeks after birth or later, they showed a remarkable increase of body weight together with an increase of white adipose tissue (WAT) (Fig. 6) (Takayanagi et al., 2008).

However, they did not show a difference in food intake as compared with the wild-type mice. On the other hand, especially their visceral fat weight increased and enlargement of adipocytes with the accumulation of lipid was observed in both WAT and brown adipose tissue (BAT). In addition, BAT, known as a major tissue generating heat in rodents, showed cells apparently accumulating excessive lipids inside. Because several types of gene-KO mice with abnormal brown adipocytes show aberrations in the generation of heat (Cannon and Nedergaard, 2004), we hypothesized that *Oxtr*−/− mice would also show deficits in heat generation when exposed to cold (5°C). To test this hypothesis, we measured rectal temperature periodically for 2 h during exposure to cold. This test revealed that rectal temperature was significantly decreased in *Oxtr*−/− mice in comparison to that of WT mice (Fig. 7). These tendencies of slight obesity in adult male mice and a rapid decrease of body temperature after exposure to cold were also observed with *Oxt*−/− mice (Kasahara et al., 2008).

We carried out additional experiments to study the mechanism that caused the obesity and aberration in body temperature control observed

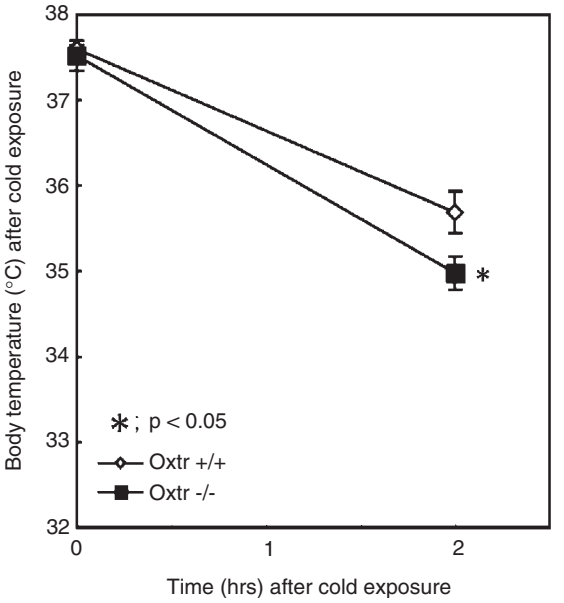

Fig. 7. Body temperature shifts of wild-type (WT, *Oxtr*+/+) and *Oxtr*−/− mice before and after cold exposure. The rectal temperature in 10–12-week-old male WT ($n = 9$) and *Oxtr*−/− ($n = 10$) mice is shown. The initial time point (point 0) shows the measured temperature after 3 h fasting and just before cold exposure (at 5°C). Data shown were mean ± SEM. *Significant difference between WT and *Oxtr*−/− mice.

in both *Oxt*−/− and *Oxtr*−/− mice. Noradrenalin (NA) released from sympathetic nerve terminals regulates heat generation in BAT through adrenergic receptors. We analysed the expression of adrenergic receptors in the BAT of *Oxtr*−/− mice. Adrenergic receptors (AR) are classified into several subtypes, including β3-AR, which accelerates heat generation and lipid oxidation, and α2A-AR, which suppresses lipid catabolism. Quantitative RT-PCR for both ARs mRNA in the BAT of *Oxtr*−/− male mice showed increased expression of α2A-AR and remarkable suppression in the expression of β3-AR indicating a declination in the balance of energy expenditure might have occurred in the *Oxtr*−/− mice to save energy (Kasahara et al., manuscript in preparation). In addition to reports that receptor activity for OXT (Boland and Goren, 1987) or OXTR mRNA was detected (Gould and Zingg, 2003) in adipose tissue, we observed OXTR mRNA mainly expressed in adipocyte precursor cells (unpublished data) but the expression level of the receptor mRNA in mature adipocyte was quite low. All of these findings suggest that the lack of OXTR in adipose precursor cells and the resultant dysfunction in mature adipose tissue may not be the major cause of obesity in *Oxtr*−/− (and even in *Oxt*−/−) mice. On the other hand, the hypothalamus, the center of neurons expressing OXT, is also the center that controls the body temperature. We detected co-localization of OXT and c-fos protein by immunostaining in a portion of neurons in the hypothalamus (PVN) after exposure of WT mice to 5°C for 2 h (Kasahara et al., 2008). These observations strongly suggested that the OXT–OXTR system in the central nervous system contributes to temperature homoeostasis and presumably to the resultant energy balance in the body. With the hypothesis that the imbalance in energy uptake versus intake, presumably caused by the deficit of OXT or OXTR genes in the mice, might give rise to obesity and aberrations in body temperature control after cold exposure, we are further analysing the mechanism causing these phenotypes.

Generation of Oxtr-Venus knockin mice to locate "Oxtr-Neurons"

Although the cloning of the OXTR was achieved more than a decade ago, an effective and reliable anti-OXTR antibody to locate the neurons that express OXTR, to identify the subtype of the neurons, or to delineate circuits including OXTR-neurons has not been produced. Recently, to obtain tools to identify the regions where several important genes are expressed in the brain, the GENSAT project (URL; http://www.ncbi.nlm.nih.gov/projects/gensat) generated several transgenic mice lines expressing EGFP under the control of those genes. The GENSAT project was based on the generation of transgenic mice using BAC clone-derived genomic DNA to control the expression of the marker EGFP gene. This project is of great interest for researchers because in each of the bacterial artificial chromosomes (BAC) transgenic vectors, each of the endogenous protein coding sequences have been replaced by sequences

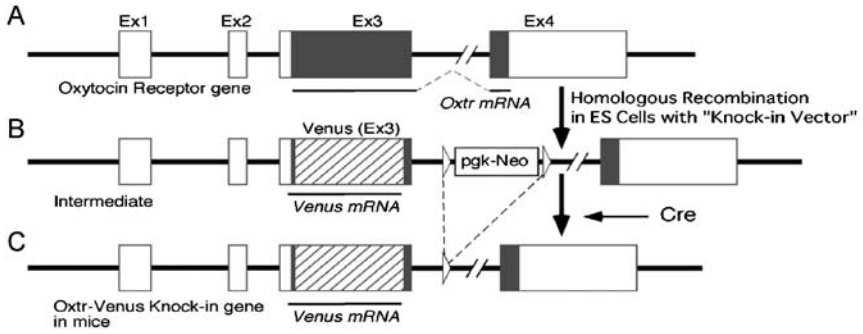

Fig. 8. Strategy for generation of *Oxtr-Venus* knockin mice. (A) Gross intron–exon structure of Oxtr gene in a wild-type mouse. (B) Primary structure of mouse Oxtr gene locus after generation of floxed type Oxtr-Venus knockin mouse using ES cell technology. (C) Final structure of Oxtr gene locus of Oxtr-Venus knockin mouse after deletion of LoxP-Neo-LoxP cassette by crossing with CAG-Cre mouse.

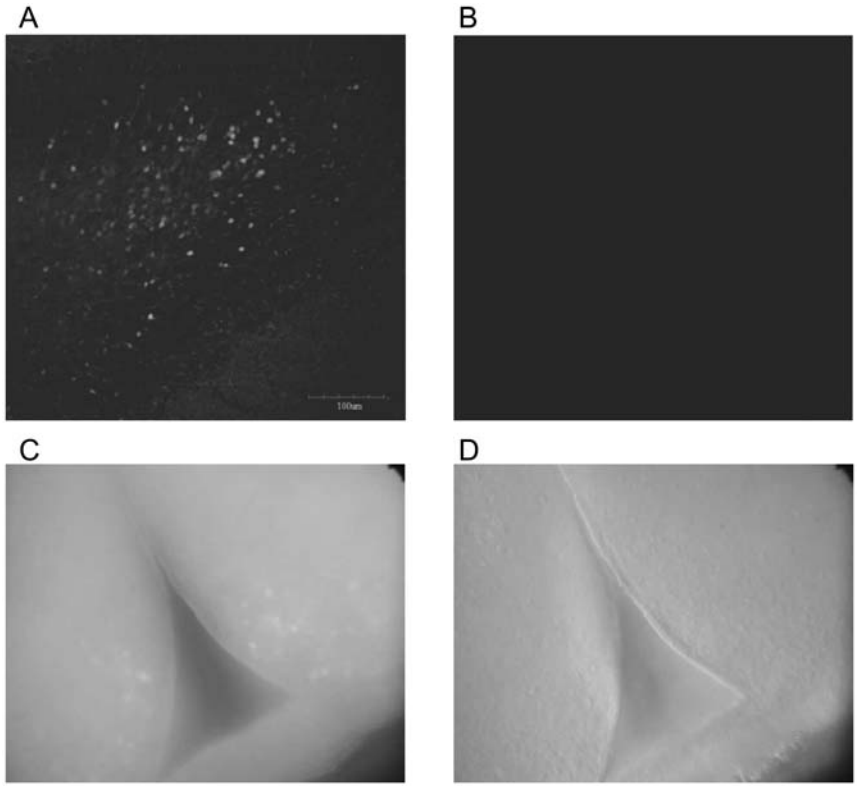

Fig. 9. Venus fluorescence of *Oxtr-Venus* knockin mice. (A, B) Brain sample of *Oxtr-Venus* knockin mice (P40), or control WT mouse (P40) was observed by confocul laser scanning microscope after fixation by PFA perfusion, preparation of sections by cryostat and staining with Anti-EGFP Ab. The original fluorescence of the Venus protein was enhanced by staining with FITC-conjugated secondary antibody. Pictures show neurons emitting fluorescence at the olfactory bulb. (C) Slice of brain (live organ culture) sample prepared from Oxtr-Venus knockin mice (P40) observed by fluorescence microscope. At the arcuate region, many neurons showed clear fluorescence suggesting that it is suitable for further electrophysiological analysis. (D) The same slice under a visible field.

encoding the EGFP reporter gene making them easily detectable and measurable. However, using this strategy one must carefully consider whether the distribution of the EGFP marker is equal to that of the endogenous gene products, because the expression of multiply inserted trans-genes may be affected by the circumstances of the chromosomal locus where they are randomly integrated.

Recently, we independently generated *Oxtr-Venus* knockin mice, whose OXTR coding region in the chromosome was substituted by the Venus (enhanced YFP, Nagai et al., 2002)-coding sequence, using homologous recombination. The aim of generating *Oxtr-Venus* knockin mice is to obtain a tool to characterize Oxtr-producing cells, and to identify live Oxtr-neurons for various subsequent analyses such as electrophysiological experiments, with minimal interference that might be caused by artificial manipulation of the gene responsible for the intrinsic expression of OXTR.

The *Oxtr-Venus* knockin vector (Fig. 8) was composed of the OXTR gene sequence, whose exon 3 was substituted with the Venus structural gene sequence, and was introduced into embryonic stem (ES) cells using a conventional technique. With the resultant *Oxtr-Venus* knockin mice, we detected Venus fluorescence in the lateral septum (LS), cortical amygdaloidal nucleus, medial amygdaloidal nucleus, arcuate nucleus, olfactory nucleus and so on (Fig. 9) (Yoshida et al., submitted). The *Oxtr-Venus* knockin mouse may be an ideal experimental tool for further analysis such as electrophysiology that require accurate information concerning the distribution of the Oxtr-neurons and identification of their subtype.

Abbreviations

a.a.	amino acids
AVP	arginine vasopressin
AVPR(V1a)	vasopressin receptor V1a
BBB	blood brain barrier
ES	embryonic stem
GPCR	G-protein coupled receptor
OXT	oxytocin
OXTR	oxytocin receptor
USV	ultra sonic vocalization
WT	wild type

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