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著者	Higashida Haruhiro, Lopatina Olga A., Yoshihara T., Pichugina Yu A., Soumarokov Andrei A., Munesue Toshio, Minabe Yoshio, Kikuchi Mitsuru, Ono Yasuki, Korshunova N., Salmina Alla B.
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Oxytocin Signal and Social Behaviour: Comparison among Adult and Infant Oxytocin, Oxytocin Receptor and CD38 Gene Knockout Mice

**H. Higashida^{a,b,c*}, O. Lopatina^{a,c,d}, T. Yoshihara^b, Y. A. Pichugina^{a,e},
A. A. Soumarokov^e, T. Munesua^{b,f}, Y. Minabe^{b,f}, M. Kikuchi^f, Y. Ono^f,
N. Korshunova^a and A. B. Salmina^d**

^aDepartment of Biophysical Genetics, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan,

^bResearch Center for Child Mental Development, Kanazawa University, Kanazawa 920-8640, Japan,

^cCore Research for Evolutional Science and Technology, Tokyo 102-0075, Japan,

^dDepartment of Biochemistry, Medical, Pharmaceutical and Toxicological Chemistry, Krasnoyarsk State Medical University, Krasnoyarsk 660022, Russia,

^eDepartment of Psychiatry, Krasnoyarsk State Medical University, Krasnoyarsk 660022, Russia,

^fDepartment of Psychaitry and Neurobiology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan,

Running title: CD38 knockout mice display abnormalities in social behavior

*Correspondence should be addressed to: Haruhiro Higashida, Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan. Tel/Fax: 81-76-265-2455. E-mail, Haruhiro@med.kanazawa-u.ac.jp

Abstract

Oxytocin (OXT) in the hypothalamus is the biological basis of social recognition, trust, love and bonding. Previously, we showed that CD38, a proliferation marker in leukaemia cells, plays an important role in the hypothalamus in the process of OXT release in adult mice. Disruption of *Cd38* (*Cd38*^{-/-}) elicited impairment of maternal behaviour and male social recognition in adult mice, similar to the behaviour observed in *Oxt* and OXT receptor (*Oxtr*) gene knockout (*Oxt*^{-/-} and *Oxtr*^{-/-}, respectively) mice. Locomotor activity induced by separation from the dam was higher and the number of ultrasonic vocalisation (USV) calls was lower in *Cd38*^{-/-} than *Cd38*^{+/+} pups. However, these behavioural changes were much milder than those observed in *Oxt*^{-/-} and *Oxtr*^{-/-} mice, indicating less impairment of social behaviour in *Cd38*^{-/-} pups. These phenotypes seemed to be caused by the high plasma OXT levels during development from the neonatal period to 3-week-old juvenile mice. ADP-ribosyl cyclase activity was markedly lower in the knockout mice from birth, suggesting that weaning for mice is a critical time window of plasma OXT differentiation. Breastfeeding was an important exogenous source of plasma OXT regulation before weaning due to the presence of OXT in milk and the dam's mammary glands. The dissimilarity between *Cd38*^{-/-} infant behaviour and those of *Oxt*^{-/-} or *Oxtr*^{-/-} mice can be explained partly by this exogenous source of OXT. These results suggest that secretion of OXT into the brain in a CD38-dependent manner may play an important role in the development of social behaviour.

Key words: Oxytocin, oxytocin receptor, social recognition, maternal nurturing, CD38, ADP-ribosyl cyclase

Introduction

Oxytocin (OXT), a nonapeptide involved in reproduction, is synthesised in the paraventricular nucleus and supraoptic nucleus of the hypothalamus, and travels down neuronal axons to the posterior pituitary (1-4). OXT is secreted into the general circulation from the nerve endings of the neurohypophysis and into the brain from dendrites. It is well known that OXT is linked to complex social behaviour (5-9; Fig. 1A). In humans, intranasal OXT may promote trust (10), gaze (11) or face recognition (12) and infusion of OXT can increase generosity (13). In rodents, OXT is highly involved in social interaction, social recognition, pair bonding and maternal behaviour (14-21). In addition, animal studies have shown that increased levels of OXT in the early postnatal period may affect behaviour and last into adulthood (8, 15, 22), and that subcutaneous administration of low doses of OXT facilitates social recognition (21). Two types of mice with OXT (*Oxt*) or OXT receptor (*Oxtr*) gene knockout (*Oxt*^{-/-} or *Oxtr*^{-/-}) show profound social amnesia (14, 18, 22-26; Fig. 1B). Social amnesia can be fully rescued by injection of OXT into the medial amygdale in *Oxt*^{-/-} mice. Impairment of social behaviour is clearly observed even in pups. These observations suggest that OXT plays an important role in social behaviour by stimulation of OXTR during brain development throughout the juvenile to adult stages.

Recently, we reported that CD38 is required for regulation of social behaviour by OXT in mice (27, 28). The transmembrane glycoprotein, CD38, detected in leucocytes is expressed on many neuronal cells, though its function in the brain remains unclear (29, 30). CD38 possesses ADP-ribosyl cyclase

activity, which produces cyclic ADP-ribose (cADPR) from an abundant substrate in the brain, β -NAD⁺ (29). cADPR is a potential intracellular second messenger and a cofactor, together with Ca²⁺, for Ca²⁺ mobilisation from ryanodine-sensitive Ca²⁺ pools (30, 31). Adult mice with a null mutation in CD38 showed deficiencies in social behaviour, such as mate recognition and memory in males and pup retrieval in females, due to the abnormality of central and peripheral OXT secretion (27, 28). We also showed that decreased formation of cyclic ADP-ribose (cADPR) results in dysfunction of Ca²⁺-induced Ca²⁺-release for OXT secretion in hypothalamic OXT neurons (27). Here, we present an overview of the relationship between social behaviour and OXT levels in CD38 knockout mice, and compare the results among three different genotypes: *Oxt*^{-/-}, *Oxtr*^{-/-} and *Cd38*^{-/-}.

Growth of knockout mice

Both *Cd38*^{+/+} and *Cd38*^{-/-} mice grew well and gained weight (27, 28). There were no significant sex-related differences in body weight, indicating that these mice fed well from the infant stage through the dam's milk and by solid food after weaning to the adult stage. *Cd38*^{-/-} mice showed no deficits in lactation/milk ejection (Table 1).

Nishimori *et al.* reported that mice lacking *Oxt* are both viable and fertile (23). OXT-deficient females have no obvious defects in fertility or reproduction, including gestation and parturition. However, all offspring die shortly after birth because of the dam's inability to nurse. However, postpartum injection of OXT into the *Oxt*-deficient dams restores milk ejection and rescues the offspring (23).

Similarly, *Oxtr*^{-/-} mice are viable and have no obvious defects in fertility or reproductive behaviour, although dams exhibit normal parturition followed by defects in lactation and maternal nurturing (24, 25). These results indicate that the OXT/OXTR/CD38 signalling pathway is not essential for normal parturition in mice, but milk ejection requires OXT/OXTR (Table 1).

Stress-induced behaviour in infant mice

We have examined isolation-induced locomotor behaviour and ultrasonic vocalisation (USV) of infant male mice (24, 26) on postnatal day 7. *Cd38*^{-/-} males showed significantly higher levels of locomotor activity during the first 3 min after separation from the dam, when they were examined individually in the grid-crossing test in an observation chamber (28). Next, the pups' activity was measured simultaneously with 4 pups together for a much longer period, in which there was no physical contact but some type of interaction, probably with USV as discussed below. Higher locomotor activity in *Cd38*^{-/-} pups was observed that persisted up to 18 min in the test, although the difference was not marked compared to the other mice (28).

Both *Cd38*^{-/-} and *Cd38*^{+/+} pups emitted calls on isolation. The properties of USV were similar in that the frequency was around 70 KHz and duration was around 60 ms in both genotypes. However, the calls per 2-min session were less frequent in *Cd38*^{-/-} infants than wild-type controls, with an average reduction of 38% (Table 2). These results agreed well with previous observations in *Oxt*^{-/-} and *Oxtr*^{-/-} mice (14, 23, 24, 26). However, the degree of disruption of infant behaviour seemed to be much milder in the case of *Cd38*^{-/-}, in comparison with

the two OXT-related knockout mouse strains (Table 2). These observations suggested that *Cd38*^{-/-} pups retain the ability to interact socially with others to a greater extent than *Oxt*^{-/-} and *Oxtr*^{-/-} pups.

Maternal nurturing behaviour in female mice under stress

Female *Cd38*^{-/-} mice displayed disrupted maternal behaviour after separation of dam and pups (27). We observed the dams' behaviour with their newborn pups in home cages with the pups temporarily placed outside the nest. Normal dams retrieved 5 pups tested precisely and very quickly (with the average latency of 43 ± 3 s) to the same small area of the nest (27), while *Cd38*^{-/-} dams took a significantly longer time to begin retrieval (the average latency of 76 ± 8 s; *p* <0.01), moved around continuously and sniffed as if their interest was drawn to many things other than their pups. They often dropped the pups during retrieval as if they did not remember the way to the nest, and this resulted in the pups becoming scattered in different places. However, *Cd38*^{-/-} dams fed the pups sufficiently for them to grow to the same weight as the controls, as mentioned above. These results indicated clear abnormalities in maternal nurturing behaviour in *Cd38*^{-/-} postpartum mice under stressful conditions, such as separation. This imperfect and neglect-like nurturing behaviour partially resembles observations in human cases, because a child's or pup's basic needs, *i.e.*, staying in a warm and comfortable house or nest, are not adequately met (32, 33).

Pregnant *Oxt*^{-/-} dams clean their offspring after delivery and keep them in the nest (23). The dams quickly retrieve offspring that are moved outside the

nest. However, milk was not observed in the stomachs of offspring born to *Oxt*^{-/-} females. Dams encouraged latching on behaviour by newborn offspring to the nipples, but failed to elicit milk release. As they have milk in their mammary glands, intraperitoneal injection of Oxt produced milk ejection.

Oxtr^{-/-} dams, however, build nests and spend the majority of this period crouching over their pups (24). However, pups of *Oxtr*^{-/-} females are often found scattered around the cage. The dams' responses to pups placed in different corners of the cage were monitored. *Oxtr*^{-/-} dams showed a significantly longer latency to begin retrieval and required a longer time for complete retrieval of all the pups. The impairment of retrieval observed in *Oxtr*^{-/-} dams was similar to that in *Cd38*^{-/-} females.

Social amnesia in male mice

Normal mice that experienced repeated pairings with the same female showed a significant decline in the time spent investigating the female upon subsequent presentations of the same animal (14, 22). *Cd38*^{+/+} young adults showed this phenotype, which was due not to loss of interest but to retained memory of the paired female. Therefore, they did not need to investigate further, but instantaneously recognised the paired mate. In contrast, *Cd38*^{-/-} males showed sustained high levels of investigation at each encounter with the same female and the same level of investigation. This behaviour was due to males' amnesia of conspecifics (14).

As *Cd38*^{-/-} mice did not have deficits in either olfactory-guided foraging or habituation to a nonsocial olfactory stimulus, as determined from the preference

ratio of consumption of isovaleric acid solution (which was a noxious or offensive odour) in their drinking water, the impairment of social memory did not depend on deficits in main olfactory bulb function (27). Although the function of CD38 in social behaviour could be very specific to the particular neural circuitry involved, the data presented to date do not exclude a more general cognitive dysfunction. Therefore, we examined whether the CD38 mutants are able to learn the shock experience in the passive avoidance test. The results indicated that they can indeed learn. We concluded that *Cd38*^{-/-} males with persistent interest during repeated presentations fail to develop social memory. The abnormality found in *Cd38*^{-/-} resembles a memory deficit found in *Oxt*^{-/-} and *Oxtr*^{-/-} mice (14, 24) (Table 2).

Plasma OXT levels in adult mice

To determine the link between CD38 and OXT, we measured plasma and cerebrospinal fluid OXT levels (Fig. 2, A, B) and found that, in comparison with wild-type, *Cd38*^{-/-} mice had reduced plasma OXT levels, but elevated levels in the hypothalamus and pituitary (27). These observations indicated that although OXT is produced and packaged into vesicles in the hypothalamic neurons and posterior pituitary nerve endings in *Cd38*^{-/-} mice, it is not released into the brain and bloodstream. Therefore, OXT does not function. Indeed, the behavioural phenotype of *Cd38*^{-/-} mice could be normalised even by a single subcutaneous OXT injection, because OXT is transported into the brain, probably through the blood – brain barrier, as reported by Jin *et al.* (27; Fig. 2, C, D). We also used a genetic approach by infusion of a virus carrying the human CD38 gene into the

third ventricle of knockout mice. This procedure resulted in normalisation of the plasma and CSF OXT levels and thereby of normalised social memory, indicating that the mechanisms underlying social behaviour require CD38-dependent OXT secretion.

Plasma OXT levels in infant mice

As mentioned above, the degree of disruption of infant behaviour seemed to be much milder in the case of *Cd38^{-/-}*, and this speculation prompted us to measure plasma OXT levels to examine whether the OXT level in pups is decreased as adults. We measured the plasma OXT levels in 5 different phases of development (from 1 week to 2 months) in both genotypes (Fig. 3A). Surprisingly, the plasma OXT concentration in *Cd38^{-/-}* pups at 1-3 weeks of age was not decreased, but was similar to that in *Cd38^{+/+}* mice of the same age. However, as expected, at 2 months of age (young adult) following weaning, we found a significantly lower plasma concentration of OXT in *Cd38^{-/-}* than in *Cd38^{+/+}* mice (28).

Critical period of plasma OXT levels in juvenile to adult stages

The above decrease in OXT concentration after weaning during development observed only in *Cd38^{-/-}* mice suggests an important critical period to distinguish different plasma OXT switching from the juvenile stage to the adult stage. As breast milk is the only food source in lactating pups, we speculated that OXT is inevitably taken in from the breast milk. To confirm this, we performed quantitative analysis to determine whether OXT is present in the mammary

glands of lactating dams and milk in pups. Milk curd was found in the stomachs of the offspring born to *Cd38*^{-/-} females (Fig. 4), which was quite different from that in those born to *Oxt*^{-/-} and *Oxtr*^{-/-} females (23, 24). OXT was abundant in the mammary gland tissue and breast milk in lactating dams of both genotypes, with no significant difference between *Cd38*^{+/+} and *Cd38*^{-/-} animals (28). In the three stages of growth and development, the plasma OXT seems to be controlled from different sources. (1) Foetal stage: The foetus can obtain OXT from the placenta, which is linked with the mother, because OXT is transported to the foetus through the blood-placental-barrier (34). (2) Infant stage (breastfeeding stage): the dams provides OXT to the pups from breast milk until weaning. As milk is the only food source for pups during lactation, breastfeeding is the sole exogenous origin of plasma OXT. (3) Adult stage (weaning stage): during this period, the plasma OXT is derived entirely from its own synthesis and secretion in the absence of exogenous supply. OXT levels remained high in wild-type mice during all developmental stages. In contrast, OXT levels decreased significantly after weaning in CD38 knockout mice. These observations can explain why the *Cd38*^{-/-} mice begin to show different pathological phenotypes at each stage.

ADP-ribosyl cyclase activity

In the central nervous system, ADP-ribosyl cyclase activity corresponding to CD38 was detected as early as embryonic day 15 in mouse development (35), and the endogenous brain cADPR content is higher in the developing brain and declines in adults (35). ADP-ribosyl cyclase activity was measured in the hypothalamus (Fig, 3B) and posterior pituitary (data not shown) in CD38

wild-type and knockout mice during the first two months of life.

In the hypothalamus, ADP-ribosyl cyclase activity in 1-week-old mice was 6% that in 2-month-old *Cd38^{+/+}* mice. Its activity in *Cd38^{+/+}* mice was significantly higher than that in age-matched knockout mice. From the second week of life, *Cd38^{+/+}* mice showed significantly higher levels of ADP-ribosyl cyclase activity in the hypothalamus, and the difference in comparison with *Cd38^{-/-}* mice increased markedly (27, 28). In *Cd38^{+/+}* mice, ADP-ribosyl cyclase activity in the hypothalamus decreased slightly with age.

ADP-ribosyl cyclase activity in the pituitary was lower than that in the hypothalamus in both *Cd38^{+/+}* and *Cd38^{-/-}* mice (data not shown). *Cd38^{-/-}* mice showed little or no increase in ADP-ribosyl cyclase activity in the pituitary during development.

The role of CD38 in regulation of OXT secretion through cADPR-mediated intracellular calcium signalling has been demonstrated in adult mice. Here, we found lower and similar levels of ADP-ribosyl cyclase activity were found in the hypothalamus and pituitary of both 1-week-old *Cd38^{+/+}* and *Cd38^{-/-}* pups. Based on these observations, we speculated that the level of ADP-ribosyl cyclase activity is also relatively low at the foetal stage, suggesting that maintenance of plasma OXT relies mainly on the exogenous source from the placenta and breast milk. As CD38 knockout pups suddenly lose their exogenous OXT supply after weaning and the intrinsic activity of ADP-ribosyl cyclase remains at a low level, the resulting relative shortage of endogenous OXT release during development of the adult stage thus affects the social recognition behaviour in adult animals.

Implications for developmental disorders

A series of recent studies suggested that OXT may be related to autism (3-8, 36-39). It has been reported that plasma OXT levels in autistic children are lower than those in age-matched normal controls, although the precise deviation is very small (35). Infusion of OXT reduces repetitive behaviours in adults with autistic and Asperger's disorders (37). However, these studies focused largely on autism in older children and adults. There have been only a few studies in infants during breastfeeding and the early postnatal period; Tanoue and Oda reported that children in their control group breast-fed significantly longer than autistic infants (40), and Fries *et al.* provided evidence that OXT is critical for regulating social behaviour during early experience in infants (41). Although the precise mechanism is unclear, our data in mice suggest that lack of adequate exogenous OXT during the infant period would affect the normal development of the brain in genetically susceptible infants, thereby increasing the risk of autism.

OXT treatment as a compensatory method has been proposed (42), and its use has begun in several hospitals, including Kanazawa and Krasnoyarsk State Medical University Hospitals (43). In some cases, we have observed improvements in social behaviour, such as increased eye contact and positive communication (43), as suggested based on previous human studies reported previously (10-13, 44, 45).

Recently, Ebstein *et al.* suggested some interesting links between ASD and CD38 in humans (46). Genetic polymorphisms in the vasopressin-OXT pathway, notably the arginine vasopressin receptor 1a, the OXTR, neurophysin I

and II, and CD38 contribute to deficits in socialisation skills in ASD patients. They presented the first evidence that CD38 expression in lymphoblastoid cells derived from subjects diagnosed with autism can be correlated with social skill phenotype. Very recently, Ebstein's group and our group found associations between two single nucleotide polymorphisms in CD38 and ASD subjects (43, 47). Further clinical trials of OXT treatment using such biomarkers in CD38 in patients with ASD are warranted.

Conclusion

Although the mechanism of by which milk OXT is transported into the brain through the blood-brain-barrier is unclear, it is undeniable that breastfeeding is not only important for infant survival but also for infant brain development. Taken together with the observations of previous integrated studies (3-8, 20, 30, 48-51), we concluded that different sources of OXT seem to impact brain development at different stages of growth, and thus maintenance of high OXT level is secured by these two sources for development and social behaviour.

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Table 1
Comparison of phenotypes in three knockout mouse strains

	<i>Oxt</i> ^{-/-} *	<i>Oxtr</i> ^{-/-} **	<i>Cd38</i> ^{-/-} ***
Fertility	+	+	+
Uterine labour	+	+	+
Nurturing			
Milk ejection	-	-	+
Lactating action	+	+	+
Pup retrieval	+	+/-	+/-
Crouching over	+	+	+
Growth	-	-	+
Social memory	-	-	-
Locomotor activity	n.d.	High	High
Mam's anxiety	n.d.	+	-
Male aggression	n.d.	+	-

* 22, 26; **24; ***27 and 28.

+, not impaired; -, impaired; +/-, partially impaired under stressful condition; n.d., not determined.

Table 2

Comparison of infant ultrasonic vocalisation and locomotor activity in three knockout mouse strains

	Knockout strain		
	<i>Oxt</i> ^{-/-} *	<i>Oxtr</i> ^{-/-} **	<i>Cd38</i> ^{-/-} ***
Call	-80%	-95%	-38%
Locomotor	n.d.	+500%	+161%

* 22, 26; **24; ***27 and 28. n.d., not determined.

Figure legends

Figure 1. Regulatory mechanisms affecting oxytocin activity in the context of social behavior. (A) Regulation at the level of central and peripheral OXT secretion and action. (B) Disruption of components of the CD38/OT/OTR system affects social memory. (C) Treatment strategy to improve OXT-mediated regulation of social behavior.

Figure 2. Oxytocin and vasopressin levels in cerebrospinal fluid (CSF). (A) Cerebrospinal OXT concentrations in wild type ($Cd38^{+/+}$), knockout ($Cd38^{-/-}$) and $Cd38^{-/-}$ mice re-expressing hCD38 by injection of lentiviral vector. (B) AVP concentrations in CSF. Cerebrospinal OXT levels after subcutaneous administration of OXT (100 ng/ml) or saline (0.2 ml) in $Cd38^{+/+}$ (C) and $Cd38^{-/-}$ (D) mice. Means \pm s.e.m. n = 3-6. *, $p < 0.01$ from $Cd38^{+/+}$ and $Cd38^{-/-}$ mice re-expressing hCD38.

Figure 3. Plasma concentrations of oxytocin (A) and ADP-ribosyl cyclase activity in the hypothalamus (B) during development in $Cd38^{-/-}$ (circle) and $Cd38^{+/+}$ (triangle) mice. Bar indicates the weaning period. s.e.m. are within symbols. n = 5-11. *, $p < 0.01$ from $Cd38^{-/-}$.

Figure 4. Oxytocin levels in breast milk (A) and mammary gland (B) in $Cd38^{+/+}$

and *Cd38*^{-/-} lactating mice. n = 4-6. No significant difference.

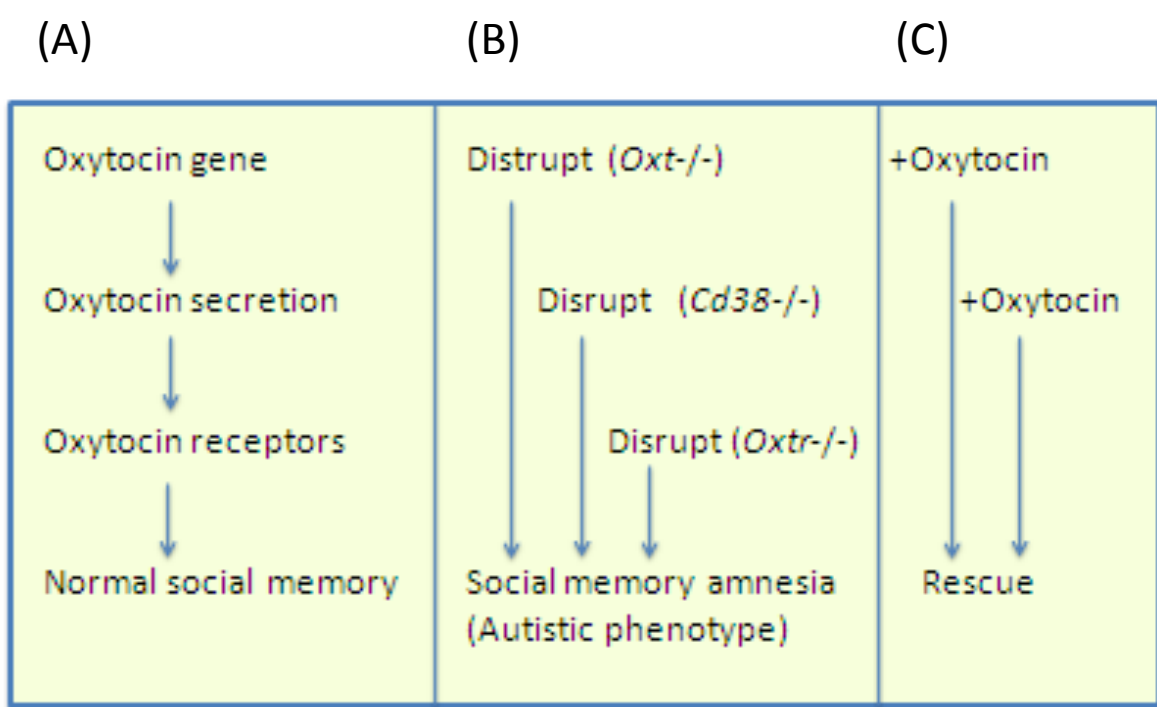


Fig. 1

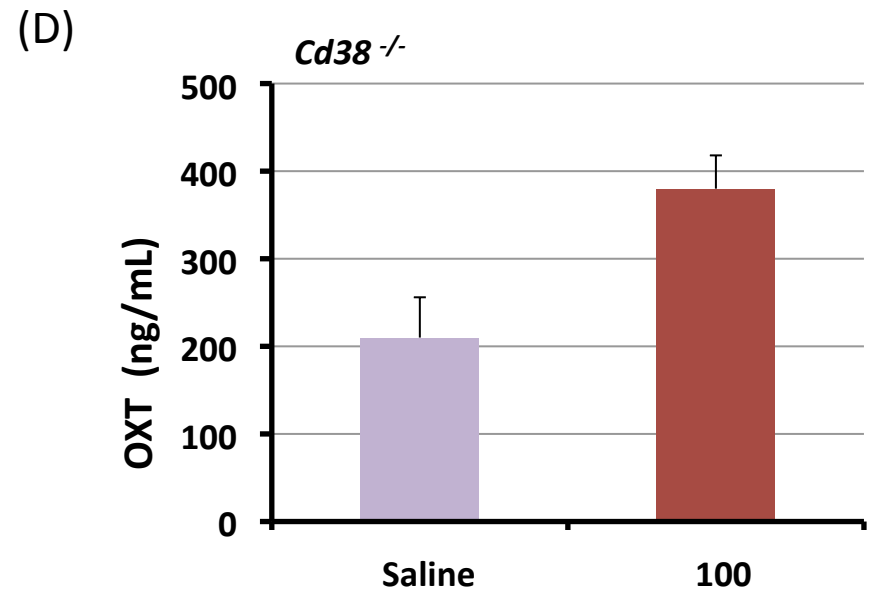
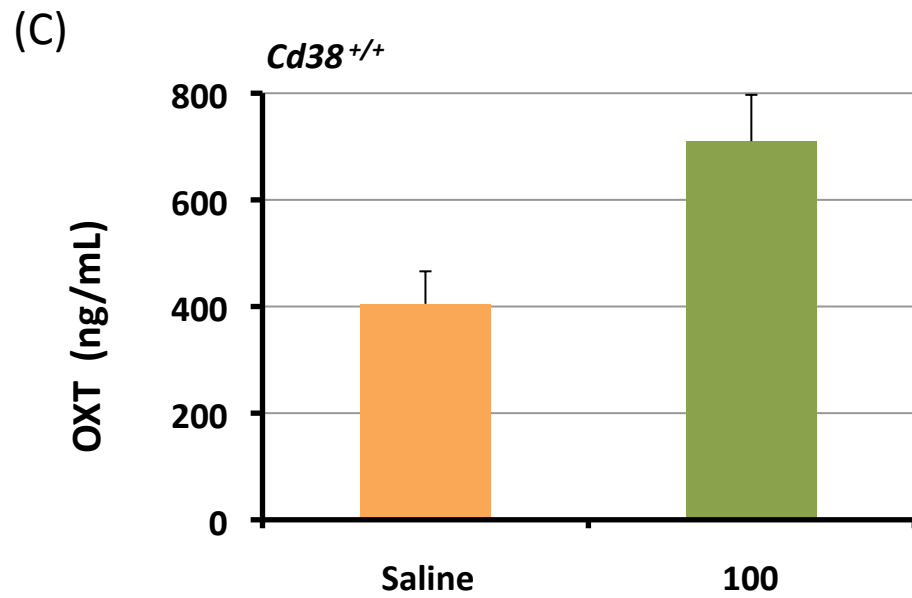
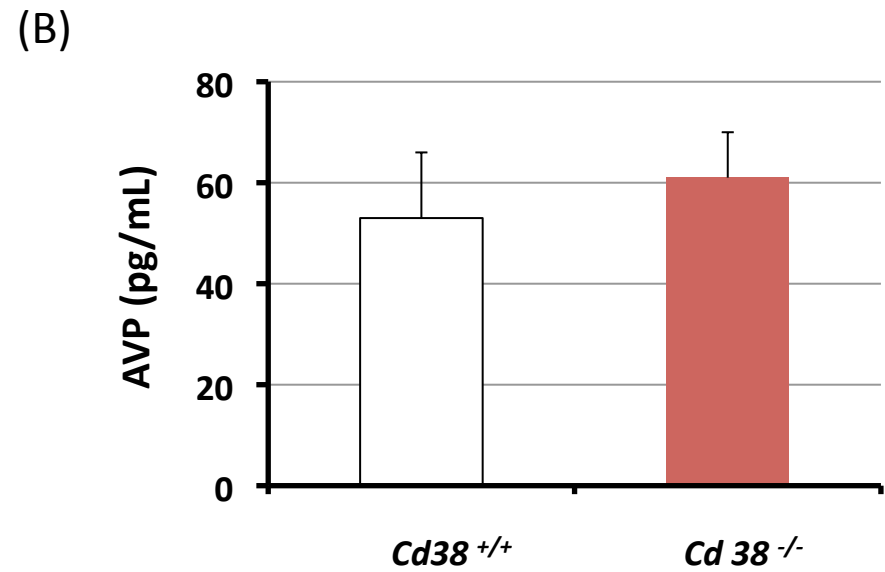
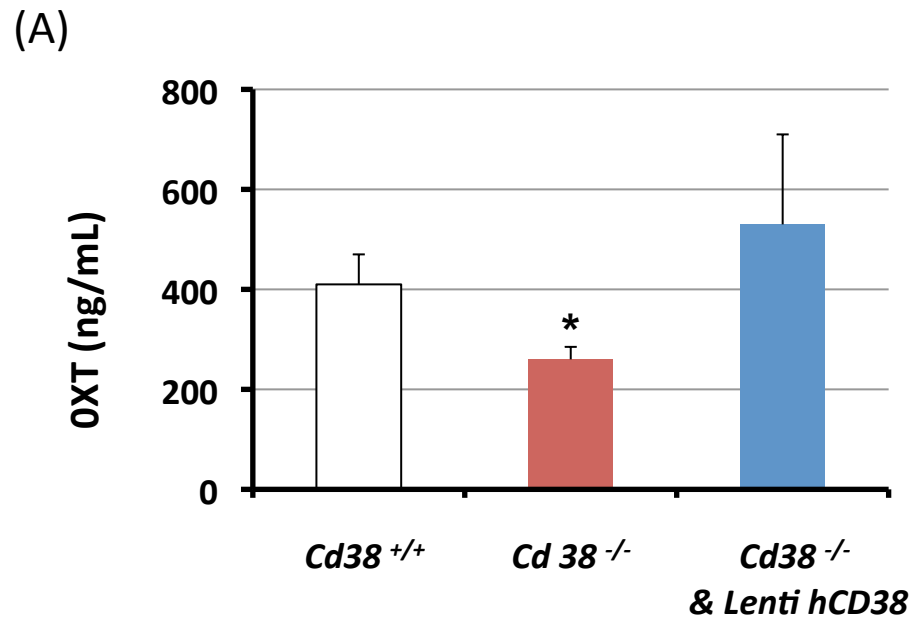


Fig. 2

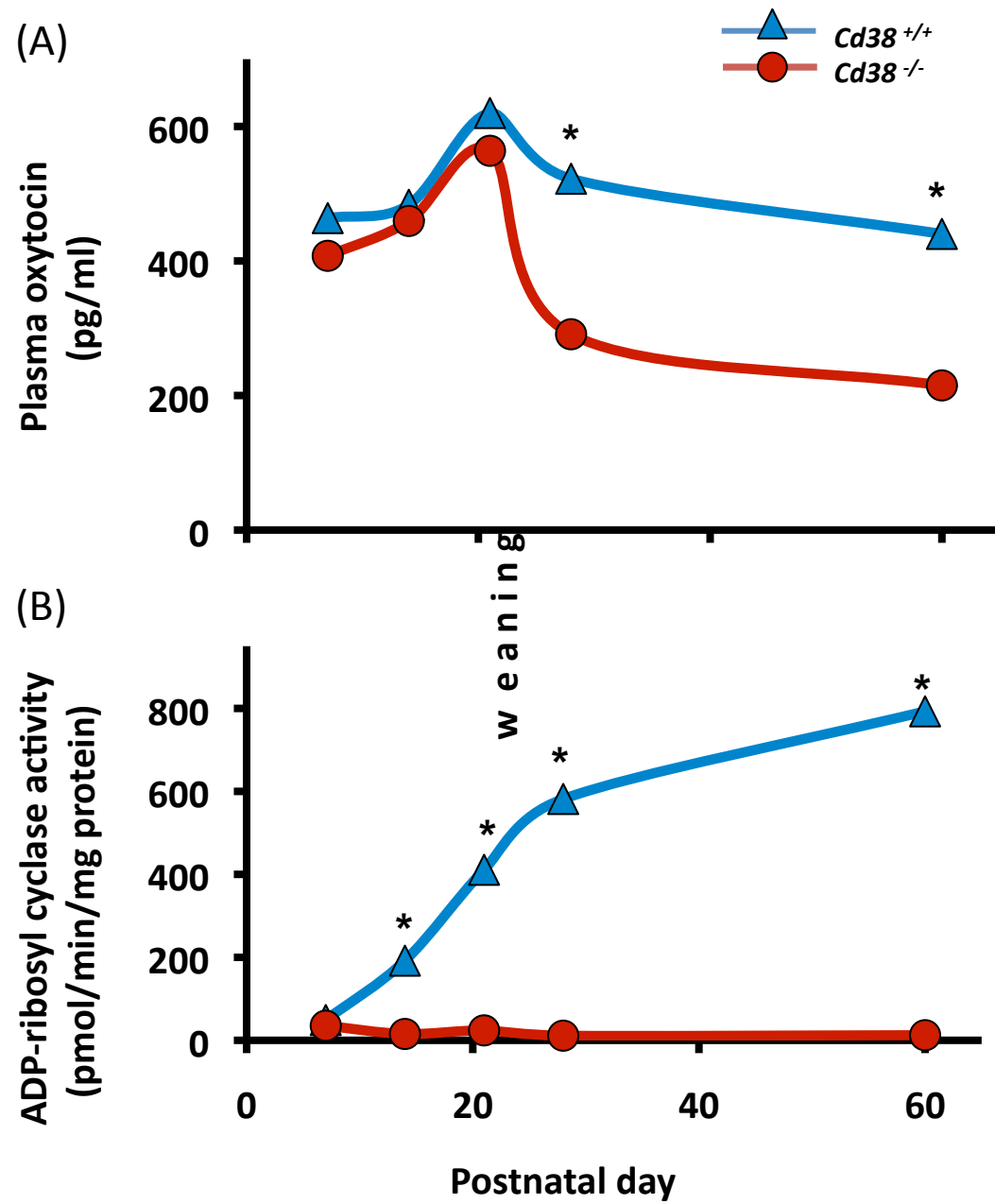


Fig. 3

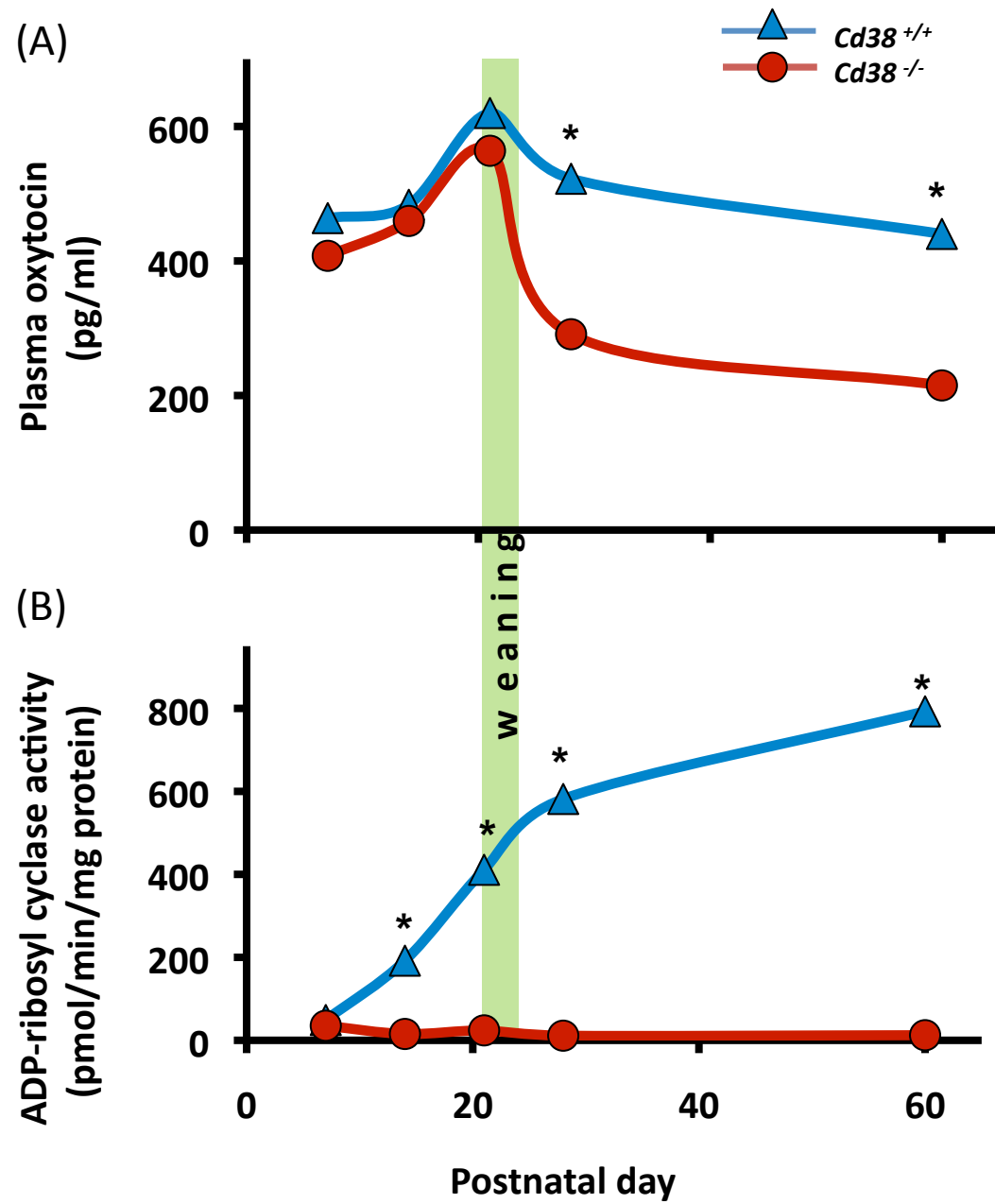


Fig. 3

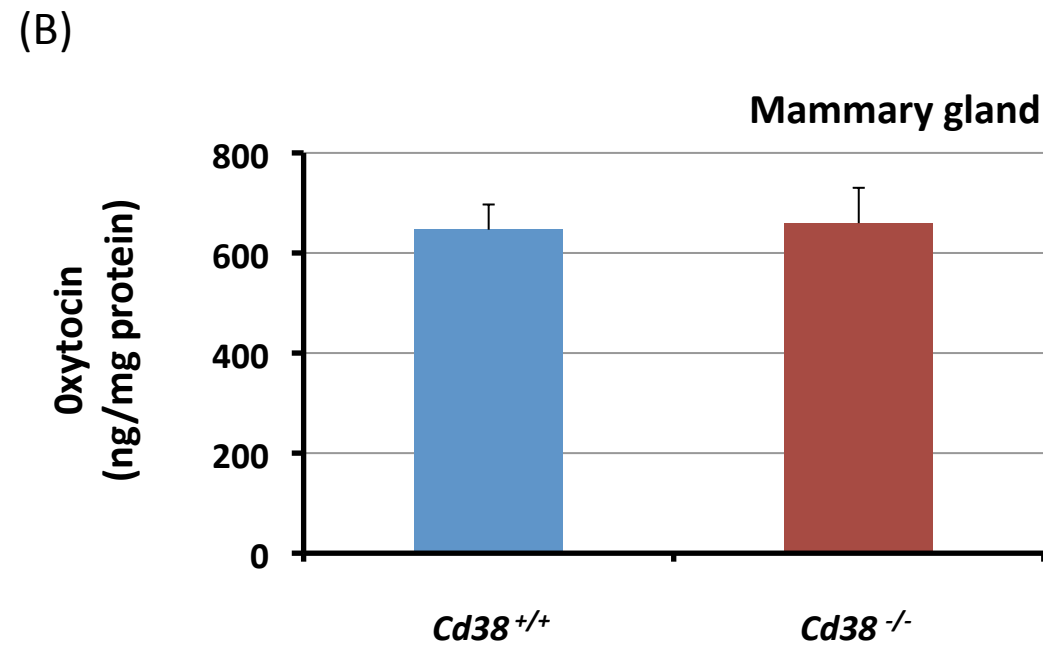
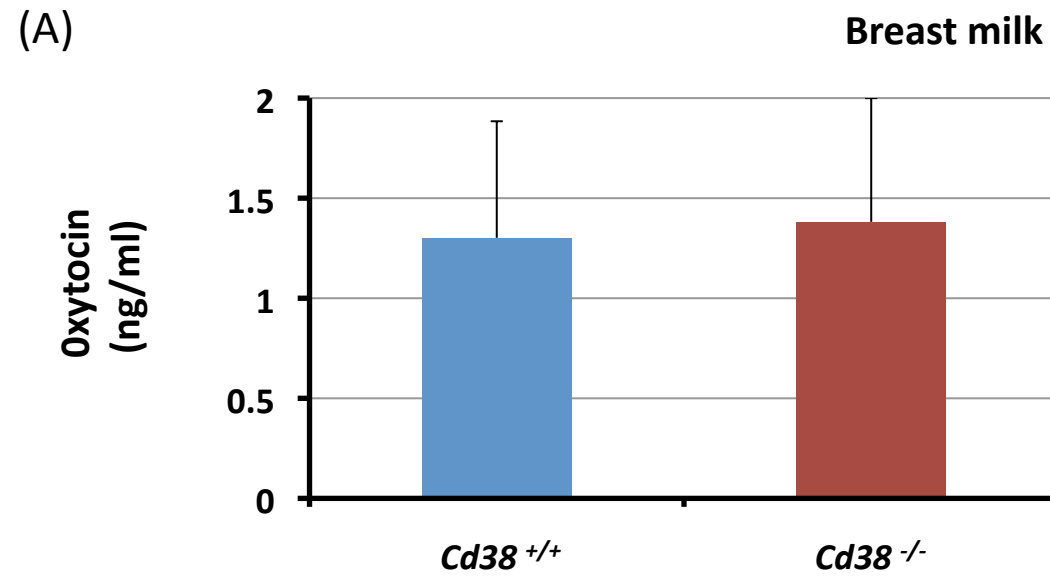


Fig. 4