Current Topics

Translational Research in Neurodevelopmental Disorders: Development of Etiology-Based Animal Models

Cd38 Gene Knockout Juvenile Mice: A Model of Oxytocin Signal Defects in Autism

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Oxytocin (OXT) in the hypothalamus is the biological basis of social recognition, trust, and bonding. We showed that CD38, a leukaemia cell marker, plays an important role in the hypothalamus in the process of OXT release in adult mice. Disruption of *Cd38* (*Cd38*$^{-/-}$) produced impairment of maternal behavior and male social recognition in mice, similar to the behavior 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 vocalization (USV) calls was lower in *Cd38*$^{-/-}$ than *Cd38*$^{+/+}$ pups. These phenotypes seemed to be caused by the high plasma OXT levels during development from neonates 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 differentiating plasma OXT. Contribution by breastfeeding was an important exogenous source for regulating plasma OXT before weaning by the presence of OXT in milk and the dam's mammary glands. The dissimilarity of *Cd38*$^{-/-}$ infant behaviour to *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 behavior, and mice with OXT signalling deficiency, including *Cd38*$^{-/-}$, *Oxt*$^{-/-}$ and *Oxtr*$^{-/-}$ mice are good animal models for developmental disorders, such as autism.

Key words CD38; ADP-ribosyl cyclase; oxytocin; oxytocin release; social recognition; maternal nurturing

1. INRODUCTION

Oxytocin (OXT), a nonapeptide involved in reproduction, is synthesized in the paraventricular nucleus and supraoptic nucleus of the hypothalamus, and flows down along neuronal axons to the posterior pituitary.[1—4] It is secreted into the general circulation from the nerve endings at the pituitary and into the brain from dendrites. It is well known that OXT is linked to social behavior.[5—9] 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 behavior.[3,4—8,14—21] In addition, some studies have shown that increased levels of OXT in the early postnatal period may affect behavior 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*$^{-/-}$) have shown profound social amnesia[14,18,22—26]: Social amnesia can be fully rescued by injection of OXT into the medial amygdale in *Oxt*$^{-/-}$ mice; Impairment of social behavior is clearly observed in pups. These observations suggest that OXT plays important roles in social behavior by OXTR stimulation during brain development throughout the juvenile to adult stages. Adult mice with a null mutation in CD38, a type II transmembrane protein with ADP-ribosyl cyclase activity, showed deficiency in social behavior due to the abnormality of central OXT secretion.[27,28] In addition, decreased formation of cyclic ADP-ribose (cADPR) results in dysfunction of Ca^{2+}-induced Ca^{2+}-release for OXT secretion in hypothalamic OXT neurons.[29] Here, we describe the relationship between social behavior and OXT levels in *Cd38* knockout (*Cd38*$^{-/-}$) mice, as an animal model of autism.

2. AUTISTIC PHENOTYPES IN CD38 NULL MUTANT MICE

Both *Cd38*$^{+/+}$ and *Cd38*$^{-/-}$ mice grew equally 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.

Nishimori *et al.* reported that mice lacking *Oxt* are viable and fertile.[23] OXT-deficient females have no obvious defects in fertility or reproduction (gestation and parturition). However, all offspring die shortly after birth because of the dam's inability to nurse. 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 reproductive behavior, 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 OXT/OXTR for milk ejection, and that CD38 is not directly involved in reproduction (Table 1).

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Table 1. Comparison of Phenotypes in Three Knockout Mice with Defects in Oxytocin Signalling

	$Oxt^{-/-}$ a)	$Oxtr^{-/-}$ b)	$Cd38^{-/-}$ c)
Fertility	+	+	+
Uterine labor	+	+	+
Lactation	−	−	+
Nurturing	−	−	+/−
Growth	−	−	+
Social memory	−	−	−
Locomotor activity	n.d.	High	High
Dam's anxiety	n.d.	+	−
Male aggression	n.d.	+	−

a) Winslow *et al.*, 2000; Ferguson *et al.*, 2001. *b*) Takayanagi *et al.*, 2005. *c*) Jin *et al.*, 2007; Liu *et al.*, 2008. n.d., not determined. +/−, partially impaired under stressful condition such as moth-pup isolation.

We have examined isolation-induced locomotor behavior and ultrasonic vocalisation (USV) of infant male mice[27,28] 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.[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. Higher locomotor activity in $Cd38^{-/-}$ pups was observed that persisted up to 18 min in our tests.[28]

Both $Cd38^{-/-}$ and $Cd38^{+/+}$ pups emitted calls on isolation: the frequency was around 70 kHz and duration was around 60 ms in both genotypes. The calls per 2-min session were less frequent in $Cd38^{-/-}$ infants than wild-type controls, with an average reduction of 38%. These results agreed well with previous observations in $Oxt^{-/-}$ and $Oxtr^{-/-}$ mice.[14,23,24,26] However, the degree of disruption of infant behavior seemed to be much milder in the case of $Cd38^{-/-}$, in comparison with the two OXT-related knockout mouse strains (Table 1). This suggests that $Cd38^{-/-}$ pups retain the ability to interact socially with others to a greater extent than $Oxt^{-/-}$ and $Oxtr^{-/-}$ pups.

Female $Cd38^{-/-}$ mice displayed disrupted maternal behavior after separation of dam and pups.[27] When the pups were temporarily placed outside the nest, normal dams retrieved the pups precisely and very quickly to the same small arena of the nest.[27] $Cd38^{-/-}$ dams took a long time to begin retrieval. They often dropped them during retrieval as if they did not remember the way to the nest, and this resulted in the pups being scattered in different places. However, $Cd38^{-/-}$ dams fed the pups sufficiently for them to grow to the same weight as the controls. These results indicated abnormalities in maternal nurturing behavior in $Cd38^{-/-}$ postpartum mice under 'stressful' conditions, such as separation. This 'neglect-like' behavior partially resembles observations in human cases, in that nurturing is imperfect.

Pregnant $Oxt^{-/-}$ dams clean their offspring after delivery and keep them in the nest.[23] However, milk was not observed in the stomachs of offspring born to $Oxt^{-/-}$ females. Dams failed to elicit milk release. As they have milk in their mammary glands, intraperitoneal injection of OXT produced milk ejection.

$Oxtr^{-/-}$ dams build nests and spend the majority of this period crouching over their pups.[24] However, pups of $Oxtr^{-/-}$ females are found scattered around the cage. $Oxtr^{-/-}$ dams showed a significantly longer latency to begin retrieval and required a longer time to completely retrieve all the pups. The impairment of retrieval observed in $Oxtr^{-/-}$ dams was similar to that in $Cd38^{-/-}$ females (Table 1).

Wild-type ICR 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, $Cd38^{+/+}$ males do not need to investigate further, but instantaneously recognize the paired female. In contrast, $Cd38^{-/-}$ males showed sustained high levels of investigation at each encounter with the same female and the same level of investigation. This 'stalker-like' behavior was due to males' amnesia of conspecifics, because $Cd38^{-/-}$ mice did not have deficits in either olfactory-guided foraging or habituation to a nonsocial olfactory stimulus, and because the CD38 mutants are able to learn the shock experience in the passive avoidance test.

3. PLASMA OXYTOCIN LEVELS

We measured plasma and cerebrospinal fluid (CSF) OXT levels and found that $Cd38^{-/-}$ mice had reduced plasma OXT levels, but elevated levels in the hypothalamus and pituitary tissues.[27,29] These observations indicate that, although OXT is produced and packaged into vesicles in the hypothalamic neurons and posterior pituitary nerve endings in $Cd38^{-/-}$ mice, OXT is not released into the brain and bloodstream. Therefore, OXT does not function sufficiently. Indeed, the behavioral phenotype of $Cd38^{-/-}$ mice could be normalized even by a single subcutaneous OXT injection, because OXT is transported into the brain through the blood-brain barrier, as reported by Jin *et al.*[27] We also used a genetic approach by infusion of a lenti-virus carrying the human CD38 gene into the third ventricle of knockout mice. This procedure resulted in normalization of the plasma and CSF OXT level and thereby of normalized social memory. This indicates that the mechanisms underlying social behavior require CD38-dependent OXT secretion (Fig. 1).

We measured the plasma OXT levels in 5 different phases of development (from 1 week to 2 months) in both genotypes. Surprisingly, the plasma OXT concentration in $Cd38^{-/-}$ pups at 1—3 weeks of age was not decreased, but similar to that in $Cd38^{+/+}$ mice of the same age.[28] However, 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]

4. 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, and the endogenous brain cADPR content is higher in the developing brain.[30,31] ADP-ribosyl cyclase activity was measured in the hypothalamus and posterior pituitary in *Cd38* wild-type and knockout mice during the first two months of life. In the brain regions examined, ADP-ribosyl cyclase activity was higher in the 1-

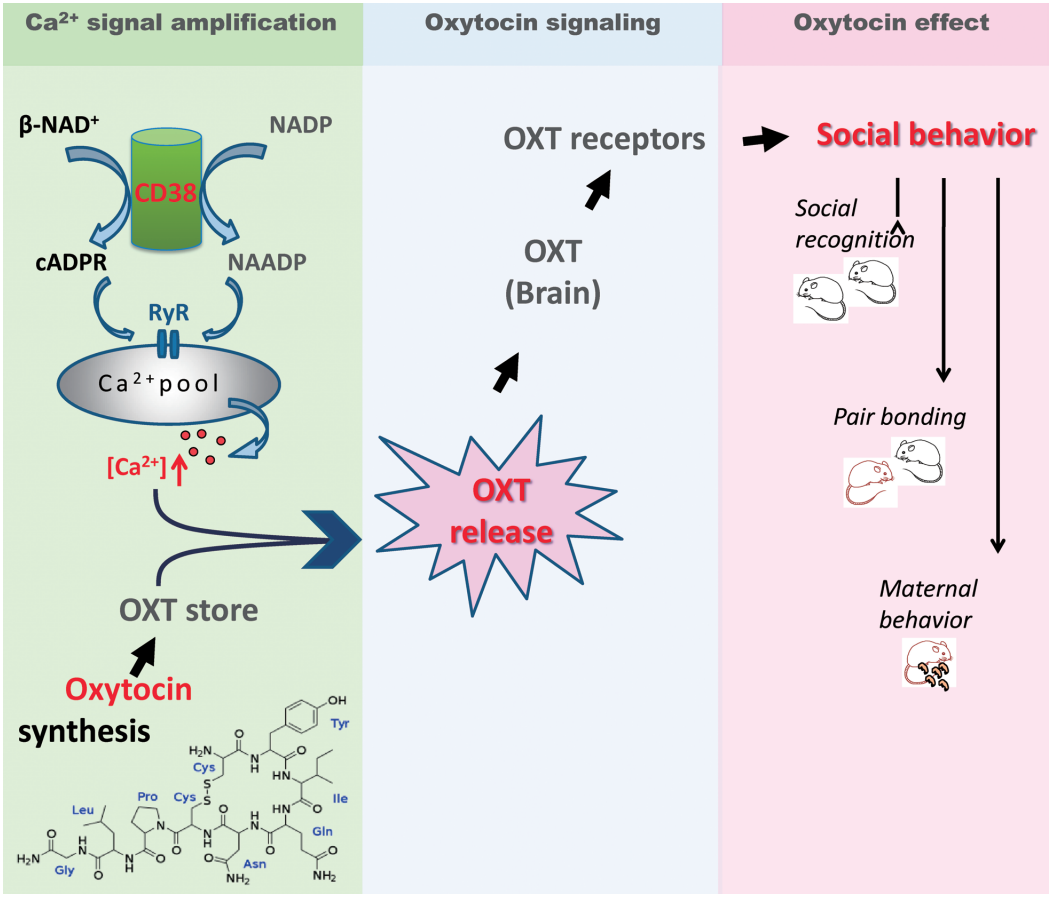

Fig. 1. Regulatory Mechanisms Affecting Oxytocin Activity in the Context of Social Behavior
Regulation at the level of central OXT secretion is due to calcium amplification by CD38 and cADPR.

week-old *Cd38*$^{+/+}$ mice than in age-matched knockout mice, but the difference was not significant. From the second week of life, *Cd38*$^{+/+}$ mice showed significantly higher levels of ADP-ribosyl cyclase activity in the hypothalamus and pituitary in comparison with *Cd38*$^{-/-}$ mice.[27,28] In the hypothalamus, ADP-ribosyl cyclase activity increased with age in *Cd38*$^{+/+}$ mice, but only a slight decrease was observed in *Cd38*$^{-/-}$ mice. ADP-ribosyl cyclase activity was lower in the pituitary than in the hypothalamus in both *Cd38*$^{+/+}$ and *Cd38*$^{-/-}$ mice. Neither *Cd38*$^{+/+}$ nor *Cd38*$^{-/-}$ mice showed significant changes in ADP-ribosyl cyclase activity during development.

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

5. IMPLICATIONS FOR DEVELOPMENTAL DISORDERS

A series of recent studies suggested that OXT is related to autism.[8,9,32—40] 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.[32,40] Infusion or nasal administration of OXT reduces repetitive behaviors or social interaction in adults with Asperger's disorders or autism.[33,40] Tanoue and Oda reported that children in their control group breast-fed significantly longer than autistic infants,[41] and Fries *et al.* provided evidence that OXT is critical for regulating social behavior during early experience in infants.[42] Although the exact mechanism is unclear, based on our data, we propose that lack of adequate exogenous OXT during breastfeeding would affect the normal development of the brain in genetically susceptible infants, thereby increasing the risk of autism. OXT treatment as a refill method has begun in several hospitals world wide, including Kanazawa University Hospital. Very recently, we observed improvement in communication and eye contact, and social behavior in an autistic male with lower intelligence, which lasts for over 2 years. To our knowledge, this is the case with the longest OXT treatment.[40] Interestingly, when the patient stopped to take OXT for several weeks, his social interaction becomes poor, and immediately after reuse, behavior is improved.

6. CONCLUSION

Our studies on *Cd38*$^{-/-}$ mice showed breastfeeding is not only important for infant survival but also for infant brain development. Taken together with plenty of previous integrated studies[3—8,43—47] and reports on *Cd38*$^{-/-}$ mice as a model animal in developmental disorders, 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 behavior. If this system is impaired, autism spectrum disorders could be developed, as described on CD38 single nucleotide polymorphisms of CD38 by Munesue *et al.*[40]

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