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Research Report

RGS2 mediates the anxiolytic effect of oxytocin

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ABSTRACT

The neuropeptide oxytocin (OT) has been shown to exert multiple functions in both males and females, and to play a key role in the regulation of emotionality in the central nervous system (CNS). OT has an anxiolytic effect in the CNS of rodents and humans. However, the molecular mechanisms of this effect are unclear. Here we show that OT induced the expression of regulator of G-protein signaling 2 (RGS2), a regulatory factor for anxiety, in the central amygdala (CeA) of female mice. Bath application of OT increased RGS2 levels in slices of the amygdala of virgin mice. RGS2 levels in the CeA were higher in lactating mice than in virgin mice. In contrast, RGS2 levels in mice that had given birth did not increase when the pups were removed. Acute restraint stress for 4 h induced RGS2 expression within the CeA, and local administration of an OT receptor antagonist inhibited this expression. Behavioral experiments revealed that transient restraint stress had an anxiolytic effect in wild-type females, and RGS2 levels in the CeA correlated with the anxiolytic behavior. By contrast, in the OT receptor-deficient mice, restraint stress neither increased RGS2 levels in the CeA nor had an anxiolytic effect. These results suggest that OT displays an anxiolytic effect through the induction of RGS2 expression in the CNS.

1. Introduction

OT is the classical reproductive hormone in female mammals, promoting uterine contractions during labor and milk ejection during lactation (Gainer and Wray, 1994; Neumann, 2001). OT also acts as a neurotransmitter/neuromodulator to regulate a range of central nervous system functions in both males and females, including emotional (Neumann, 2008), parental (Numan and Insel, 2003), affiliative (Insel and Shapiro, 1992), and sexual (Argiolas and Gessa, 1991) behaviors, as well as spatial and social cognition (Bielsky and Young, 2004; Tomizawa et al., 2003). Moreover, OT is an important regulator of anxiety (Bale et al., 2001; Blume et al., 2008; Neumann et al., 2000a) and of stress-coping circuitries (Ebner et al., 2005; Huber et al., 2005). For instance, OT released in the hypothalamus mediates mating-induced anxiolysis in rats (Waldherr and Neumann, 2007). Psycho-social or physical stressors like forced swimming and restraint stress evoke the release of OT in various areas

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known to be involved in the modulation of stress mechanisms, including the amygdala and the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei (Ebner et al., 2000; Ebner et al., 2005; Wigger and Neumann, 2002). In humans, moreover, intranasal OT promotes trust, and reduces the level of anxiety, possibly at the level of the amygdala (Heinrichs et al., 2003; Kirsch et al., 2005; Kosfeld et al., 2005; Labuschagne et al., 2010; Slattery and Neumann, 2010).

The amygdala is a region of the brain particularly relevant to the processing of behavioral and neuroendocrinal stress responses (Gray, 1996), especially with respect to the oxytocinergic system (Bale et al, 2001; Neumann et al, 2000a). Within the amygdala, specifically in the medial and central (CeA) subnuclei, a substantial number of oxytocinergic fibers (Sofroniew, 1983) and OT receptors have been detected (Barberis and Tribollet, 1996; Gimpl and Fahrenholz, 2001), suggesting that locally released OT is a potential mediator of the complex stress response. Indeed, local blockade of OT receptors within the amygdala resulted not only in altered emotionality (Bale et al, 2001; Neumann, 2002) but also in a dis-inhibition of the hypothalamo-pituitary-adrenal (HPA) axis of rats (Neumann et al, 2000b).

There is no doubt that OT in its activated state is an endogenous neuromodulator with an anxiolytic effect in response to stress. However, the precise mechanism of OT receptor-mediated effects and the involvement of subsequent intracellular signaling cascades, for example in the anxiolytic effects of OT, are only beginning to be elucidated (Blume et al., 2008). There is an OT receptor, which is a G-protein-coupled receptor (GPCR) that couples to a complex intracellular signaling pathway (van den Burg and Neumann, 2011). The binding of OT with the receptor activates the MAP kinase cascade both *in vitro* (Tomizawa et al., 2003) and *in vivo* (Blume et al, 2008) and induces the phosphorylation of cAMP response element-binding protein (CREB), a transcription factor critical to neuronal development, synaptic plasticity and memory formation (Han et al., 2007; Lonze et al., 2002; Tomizawa et al., 2003). These results suggest the anxiolytic effect of OT to occur through the regulation of gene expression.

Regulator of G-protein signaling (RGS) proteins are key modulators of G protein-coupled receptor signaling by virtue of their ability to accelerate the intrinsic GTP hydrolysis activity of G subunits (Hollinger and Hepler, 2002; Hollinger and Hepler, 2004). RGS2 was one of the first mammalian RGS proteins to be identified, (Siderovski et al., 1996) and stimulates GTPase activity through interaction with Gqα *in vitro* (Heximer et al., 1997; Hollinger and Hepler, 2002). Previous studies have shown that RGS2 modulates anxiety in both mice and humans (Cui et al., 2008; Flint, 2003; Oliveira-dos-Santos et al., 2000; Smoller et al., 2008; Yalcin et al., 2004). For instance, the use of transgenic and knockout mice as well as quantitative trait locus (QTL) techniques in the laboratory has led to the identification of candidate genes related to fear- and anxiety-related behaviors (Norrholm and Ressler, 2009). In mice, a QTL on chromosome 1 is associated with anxiety-related phenotypes (Flint, 2003); the principal quantitative trait gene for this linkage signal has been identified as *RGS2* (Yalcin et al., 2004). In addition, *RGS2* gene polymorphisms have been associated with panic disorder (Leygraf et al., 2006) and completed suicides (Cui et al., 2008).

The aim of the present study was to clarify the molecular mechanism behind the local anxiolytic effect of OT in the amygdala. We first identified RGS2 as a gene whose expression is up-regulated by OT and investigated whether RGS2 expression was increased in OT-applied amygdala slices and lactating mice. Moreover, we examined whether acute restraint stress induced OT secretion and RGS2 expression in CeA of female mice, resulting in an anxiolytic effect. Finally, a deficiency and blockade of the OT receptor was found to abrogate these effects of acute restraint stress.

2. Results

2.1. *Up-regulated genes in primary cultured neurons treated with oxytocin*

We first examined up-regulated genes in primary cultured neurons treated with OT by a microarray analysis. Five genes were up-regulated more than two-fold compared with control neurons (Table 1). The greatest increase was shown by the RGS2 gene (Table 1). Moreover, we examined up-regulated genes in the central amygdala (CeA) of lactating mice at postpartum 7 days by a microarray analysis. Only RGS2 was up-regulated more than two-fold among the five genes in the CeA of lactating mice compared with virgin mice (data not shown).

2.2. *Induction of RGS2 expression in the CeA by OT and during motherhood*

To investigate whether OT induced RGS2 expression in the CeA, amygdala slices were incubated with OT or PBS, and the RGS2 level in the CeA was examined by Western blotting. OT induced RGS2 expression in the CeA of virgin mice 60 and 120 min after the addition of OT (Fig. 1A). It was next examined whether the RGS2 level increased in the CeA during motherhood. The RGS2 level at 1 day postpartum was the same as that in virgin mice (Fig. 1B). However, the protein level was significantly increased at 3, 7 and 10 days postpartum (Fig. 1B). Interestingly, RGS2 levels were not increased in females when the pups were removed after labor (Fig. 1B). These results suggest that OT or other suckling- or birth-related factors such as prolactin may induce RGS2 expression in the CeA of female mice. Therefore, we next examined

Table 1 – Up-regulated genes in primary cultured neurons treated with oxytocin. Relative gene expression in OT-treated neurons compared with control neurons.

Gene Name	Fold change
Mus musculus regulator of G-protein signaling 2 (Rgs2), mRNA	4.70
PROTOCADHERIN PRECURSOR PCDH	3.28
Mus musculus brain derived neurotrophic factor (Bdnf), mRNA	3.02
Mus musculus host cell factor C1 (Hcfc1), mRNA	2.45
Mus musculus protein kinase C, epsilon (Prkce), mRNA	2.36

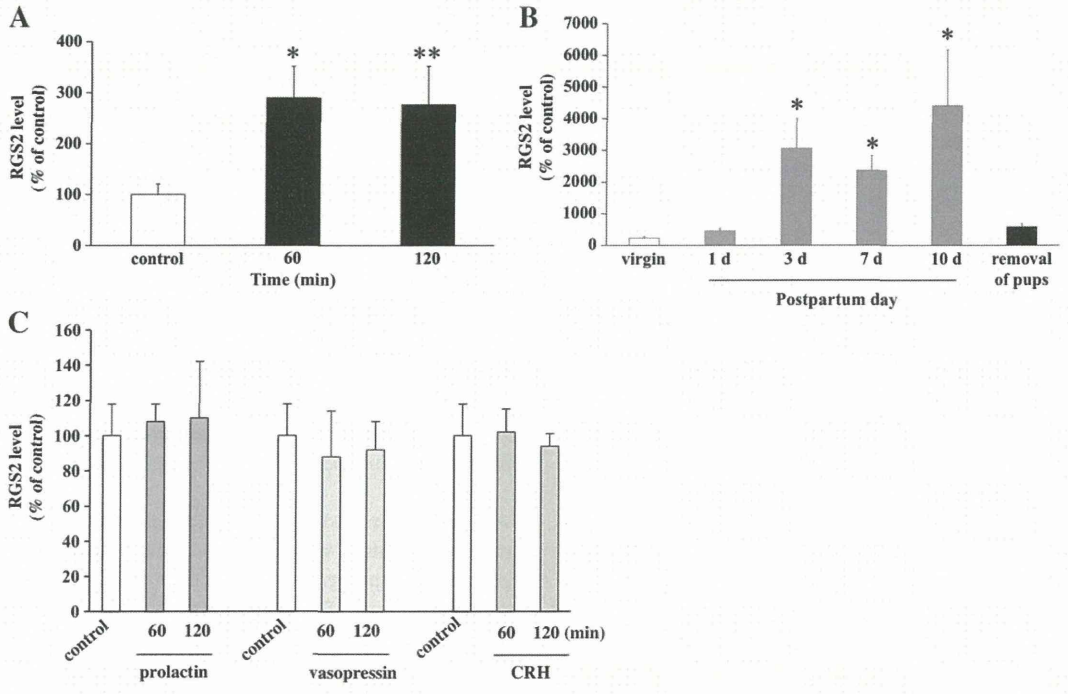


Fig. 1 – Induction of RGS2 expression in the CeA by OT treatment (A) and during motherhood (B) but not by prolactin, vasopressin or CRH (C). (A) Amygdala slices of female mice were incubated with OT for 60 and 120 min. As a control, the slices were incubated with ACSF. The RGS2 level in the CeA was examined by Western blotting. n=4–5 each. * P<0.01, ** P<0.05 *v.s.* control. (B) The CeA of lactating mice or of lactating mice whose pups were removed immediately after birth was taken out on the days indicated after birth, and RGS2 levels were compared with those in age-matched virgin mice (virgin). n=4–5 each. * P<0.001 *v.s.* age-matched virgin mice (virgin). (C) Amygdala slices of female mice were incubated with prolactin, vasopressin and CRH for 60 and 120 min. As a control, the slices were incubated with ACSF. The RGS2 level in the CeA was examined by Western blotting. n=5 each.

whether prolactin, vasopressin and corticotropin-releasing hormone (CRH) induced RGS2 expression in CeA slices. Incubation with the hormones had no effect on RGS2 levels (Fig. 1C).

2.3. *Induction of RGS2 level by restraint stress*

Stress exposure such as a 10-min forced swimming session causes a significant increase in OT release in the amygdala (Ebner et al., 2005). We next examined the effect of restraint stress on the RGS2 protein level in the CeA of female mice. The mice were restricted for 4 h per day. Restraint stress for 3 and 7 days significantly induced production of the protein (Fig. 2A) and the expression of the mRNAs (Fig. 2B) in the CeA.

To investigate whether OT mediated the restraint stress-induced RGS2 expression in the CeA, the effect of the OTR antagonist on the protein and mRNA levels was examined. The antagonist had no effect on normal RGS2 levels in the CeA (Fig. 2C), but inhibited the restraint stress-induced RGS2 expression (Fig. 2C). As a result, the protein level was decreased to that in control mice.

2.4. *Anxiolytic effect of short-term restraint stress*

It was next examined whether the repeated restraint stress protocol, shown to trigger RGS2 expression in the CeA, alters anxiety-related behavior in female mice. Females were subjected to restraint stress (4 h/day) for 2 days and anxiety-related behavior was tested in the elevated plus-maze 24 h later. Anxiolytic behavior was evaluated based on the time spent in the open arms. Mice exposed twice to restraint stress spent significantly more time in the open arms (control, 22.5±2.7%; Stress (+), 29.4±3.6%) (Fig. 3A), suggesting that stress exposure reduced anxiety in female mice. In mice exposed to restraint stress, the number of entries in the closed arms was the same as that of control mice (control, 12.5±2.3; Stress (+), 13.3±3.9%, P>0.05), suggesting the locomotor activity of the mice exposed to restraint stress to be the same as that of control mice.

We next examined whether the RGS2 level in the CeA was correlated with anxiety-related behavior (i.e. % time spent in the open arms). Females subjected to restraint stress for 2 days were tested in an elevated plus-maze and the RGS2 level in the CeA was examined. The RGS2 level correlated with anxiolytic behavior (Pearson's rank correlation, R squared=0.0537, 0.39, P=0.0067). (Fig. 3B).

2.5. *No anxiolytic effect of restrain stress in OTR KO mice*

To investigate whether OT mediates the anxiolytic effect of restraint stress, OTR KO mice were twice subjected to restraint stress (4 h/day) for 2 consecutive days. On day 3, 24 h after the

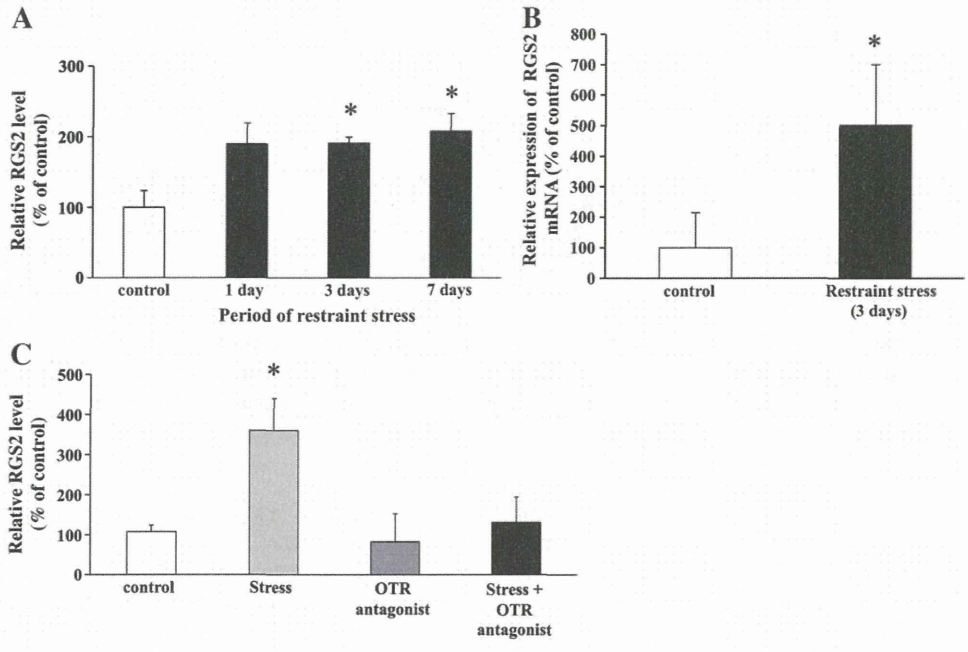


Fig. 2 – Effect of restraint stress on RGS2 protein and mRNA levels in the CeA of female mice. (A) Female mice were subjected to restraint stress (4 h per day) for the indicated period. The amygdala was removed 24 h after the last restrain stress. n=6–7 each. (B) Effect of restraint stress on the expression of RGS2 mRNA. The mice were subjected to restraint stress (4 h per day) for 3 days (3 d). The amygdala was removed 24 h after the last stress. n=8 each. (C) Effect of an OTR antagonist on the restraint stress-induced RGS2 expression. The antagonist (5 ng) was injected into the bilateral amygdala every 24 h for 5 days. Mice were subjected to restraint stress (4 h/day) 24 h after the first injection of the antagonist for 4 successive days. Twenty-four hours after the last restraint stress, the bilateral amygdalas were removed. As controls, mice were injected with vehicle (PBS). n=7–10 each. * P<0.05 *v.s.* control.

last restraint exposure, an increase in anxiety-related behavior was found in stressed OTR KO mice compared with the un-stressed KO mice (Fig. 4A). RGS2 levels did not differ between stressed and un-stressed OTR KO females (Fig. 4B).

3. Discussion

The present study provided the following four important findings (Table 2). First, OT induced RGS2 expression in the CeA of female mice. Second, repeated restraint stress also induced RGS2 expression in the CeA. Third, RGS2 levels in the CeA correlated with anxiolysis. Fourth, restraint stress neither increased RGS2 levels in the CeA nor had an anxiolytic effect in OT receptor-deficient or OT receptor antagonist-injected mice.

Psycho-social or physical stressors like restraint stress and forced swimming evoke the release of OT in various areas of the CNS including the amygdala (Ebner et al., 2000; Ebner et al., 2005; Wigger and Neumann, 2002). The release of OT is believed to protect against stress-evoked anxiety. The present study also showed that short-term restraint stress had an

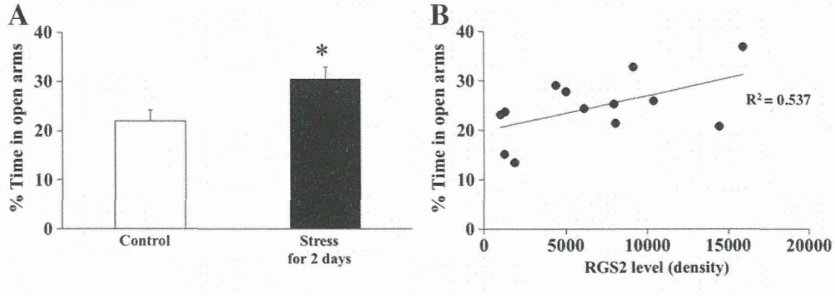


Fig. 3 – Anxiolytic effect of short-term restraint stress. (A) Effect of restraint stress for 2 days on time spent in the open arms on day 3. n=10 each. * P<0.05 *v.s.* control. (B) Comparison of the Spearman rank correlation between RGS2 levels in the CeA of females and time spent in the open arms. Mice were subjected to restraint stress (4 h/day) for 2 days and then tested on an elevated plus maze on day 3. After the test, mice were sacrificed and the CeA was used for Western blotting of RGS2.

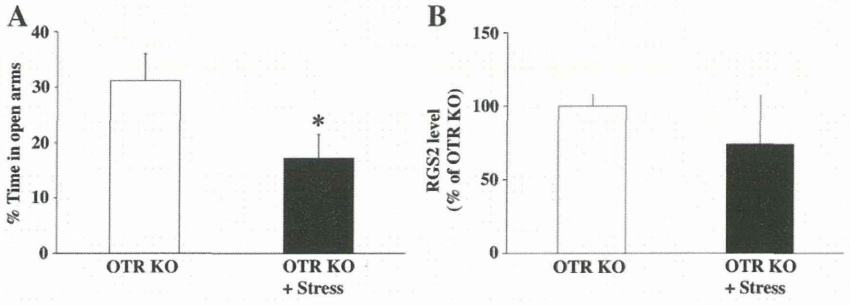


Fig. 4 – Effect of restraint stress on the anxiety of OTR KO mice (A) and RGS2 levels in the CeA (B). (A) OTR KO females were subjected to restraint stress (4 h/day) for 2 days and then tested on an elevated plus maze 24 h after the last stress. n=5 each. * $P<0.05$ *v.s.* OTR KO mice with no restraint stress (OTR KO). (B) OTR KO females were subjected to restraint stress (4 h/day) for 2 days. Twenty-four hours after the last stress, the CeA was removed and the RGS2 level was examined by Western blotting.

anxiolytic effect in wild-type females but not in OT receptor-deficient or OT receptor antagonist-injected mice. These results suggest that OT is an important modulator of anxiety and of stress-coping circuitries (Fig. 5).

Anxiety disorders represent one of the most common psychiatric conditions in the World and epidemiological studies have indicated that as many as 29% of people will, at some point in their lives, suffer from anxiety disorders (Hawgood and De Leo, 2008). As a means of gaining a better understanding of the risk factors for developing anxiety disorders as well as to tailor individualized treatment options for anxiety, there has recently been a significant effort to investigate the genetic bases for anxiety disorders (Norrholm and Ressler, 2009). RGS2 was identified as a modulator of anxiety in both mice and humans (Cui et al., 2008; Leygraf et al., 2006; Yalcin et al., 2004). RGS2 represents a potential target for pharmacotherapy and compounds, which induce its expression may provide a potential treatment for anxiety disorders. However, no drugs, hormones or cytokines, which induce RGS2 expression in the CNS, especially in the CeA, have yet been identified. In the present study, we showed that OT induced RGS2 expression in the CeA of mice. Although a number of studies have shown a potential anxiolytic effect of OT, the mechanism involved has been unclear (Slattery and Neumann, 2010). Induction of RGS2 expression may be one mechanism behind the anxiolytic effect of OT and OT may be of therapeutic benefit in subsets of patients with anxiety disorders.

In the present study, we showed that OT functions in female mice. The question of whether OT also has an anxiolytic effect through the induction of RGS2 expression in males arises. OT receptors and OT-binding sites are distributed throughout the amygdala, with a particularly high density in its central part in males (Kremarik et al, 1993; Yoshimura et al, 1993). Force swim stress triggers the release of OT in the CeA of male rats (Ebner et al., 2005). Moreover, sexual activity and mating with a receptive female reduce the level of anxiety and increase risk-taking behavior in male rats and the effect is inhibited by the administration of OTR antagonists (Waldherr and Neumann, 2007). These results suggest that OT may induce RGS2 expression in males. Further study is needed to examine the effect of OT in males as a potential therapeutic for the treatment of anxiety disorders.

The present study focused on RGS2 among OT-induced genes. However, brain-derived neurotrophic factor (BDNF) was also an up-regulated gene in primary cultured neurons treated with OT by microarray analysis (Table 1). In rodents, chronic stress decreases the expression of BDNF, which can lead to neuronal atrophy in the hippocampus and other brain structures, while direct hippocampal infusion of BDNF has anxiolytic and antidepressant effects (Berry et al., in press). Blood BDNF levels are decreased in subjects diagnosed with major depressives, antidepressants can revert this neurobiological change (Berry et al., in press; Duman and Monteggia, 2006). These results suggest that OT may also have anxiolytic effects through the induction of BDNF expression. To investigate whether RGS2 is a principal target of the anxiolytic effects of OT, further study is needed to show whether blockade of upregulation of RGS2 in the CeA such as by siRNA techniques enhance stress-induced anxiety.

In conclusion, OT induces RGS2 expression in the CeA of female mice, resulting in an anxiolytic effect.

Table 2 – A summary of the results.

	Wild-type		OTR KO	
	Nursing	Restraint stress	Restrain stress + OTR antagonist	Restraint stress
RGS2	↑	↑	–	–

↑, up-regulation; –, unchanged.

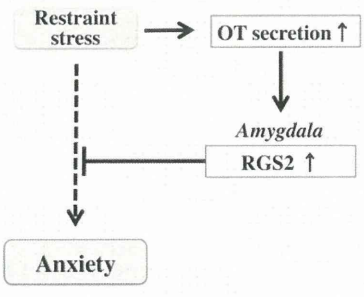


Fig. 5 – Scheme of molecular mechanism on anxiolytic effect of oxytocin.

4. Experimental procedures

4.1. Subjects

Female C57BL6 mice aged 10 to 12 weeks were used for all experiments except those with OT receptor (OTR)-deficient mice.

OTR-deficient mice (OTR-KO) were purchased from Deltagen (San Mateo, CA, USA) (Matsushita et al., 2010). The wild-type (WT) female littermates were used as controls. Originally in an equal mix of the C57BL/6 and 129X1/SvJ strains (RW4 embryonic stem cell line), they have been repetitively backcrossed for 4 generations with C57BL/6 mice (from Deltagen). The females aged 10 to 12 weeks were used for all experiments. The WT females used as controls were littermates.

Animals were housed at 25 °C with 12-h light/dark cycles and free access to water and standard rodent chow in the Department of Animal Resources of Okayama University. To investigate the effect of nursing, pups of female mice were removed immediately after giving birth and their brains were removed 5 days after labor. All procedures were approved by the Animal Ethics Committee of Okayama University (OKU-2010164).

4.2. Application of oxytocin, vasopressin, prolactin and corticotropin-releasing hormone (CRH) in amygdala slices

The brains of virgin mice were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) bubbled with a gaseous mixture of 95% O_2 and 5% CO_2. The composition of the ACSF was as follows: NaCl, 124 mM; KCl, 4.4 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.3 mM; NaH_2PO_4, 1 mM; $NaHCO_3$, 26 mM; and glucose, 10 mM. The amygdala region was dissected, and 400-μm transverse slices were prepared using a microtome (VT1200S, Leica Microsystems GmbH, Wetzlar, Germany). The amygdala slices were incubated in ACSF at 30 °C for 1 h before the application of OT. The slices were incubated with 0.1 μM OT (Sigma-Aldrich, St. Louis, MO, USA), 0.1 μM vasopressin (Sigma-Aldrich), 100 ng/ml prolactin (Cedarlane labs, Burlington, Ontario, Canada) or 0.1 μM CRH (Sigma-Aldrich) in a tube for specified periods before protein isolation for Western blotting.

4.3. Microarray analysis

The microarray analysis was performed using a mouse whole genome oligo DNA chip (Agilent Technologies, Santa Clara, CA, USA) as described previously (Fujimura et al, 2011). Briefly, mRNAs (200 ng) from primary cultured neurons treated with 0.1 μM OT or PBS for 2 h and CeA of lactating mice or virgin mice were pooled into separate master total RNA mixes, and labeled with Cy-3 or Cy-5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA. For detection of genes with significant differential expression between the control group and mice treated with OT, each image was processed using Agilent Feature Extraction ver.8.5.1.1.

4.4. Western blot analysis

Western blotting was carried out at high stringency, essentially as previously described (Tomizawa et al., 2003). Briefly, the CeA was punched out from amygdala slices. Homogenates of CeA were prepared by sonicating the tissue in boiled 1% SDS buffer. The homogenates (50 μg) were separated by electrophoresis through a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). Blots were probed with primary antibodies against RGS2 (1:500 dilution, anti-RGS2 antibody, SC-1020 [Santa Cruz Biotech., Santa Cruz, CA]) and Actin (AC-40, Sigma-Aldrich), and secondary antibodies before the bands were visualized using a commercial ECL detection kit (GE Healthcare). The band densities were normalized to Actin for each sample. The quantitative analysis was performed using analysis software (Quantity One [Bio-Rad, Hercules, CA]).

4.5. Restraint stress

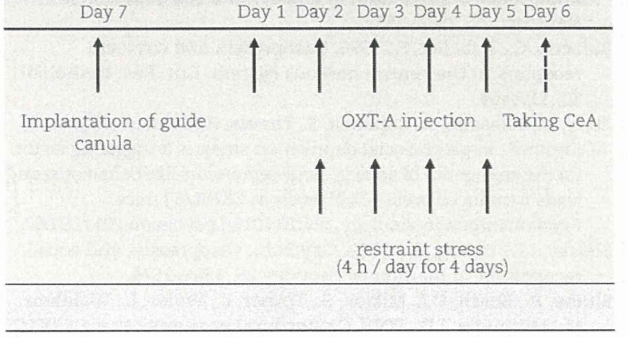
Polypropylene tubes (50-ml conical tubes [Iwaki, Tokyo, Japan]), with holes for proper ventilation, were used to induce restraint stress during the experimental period. Mice were restrained in the tubes for 4 h per day during the dark cycle (1800–2200 h) for the periods indicated.

4.6. Injection of an OT antagonist into the central amygdala

Seven days before the OT injection, mice were placed in a stereotactic device and implanted with an 8-mm long 26-gauge stainless steel cannula just above the left and right CeA (1 mm posterior, 2.5 mm lateral, 1.5 mm ventral from the bregma; Paxinos and Watson). A selective OT receptor (OTR) antagonist, desGly-NH2(9),d(CH2)5[Tyr(Me)2,Thr4] OVT (Sigma-Aldrich), was infused (5 ng/ 0.5 μl) using a 11-mm long 31-gauge needle every 24 h for 5 days (between 9:00 and 10:00). As controls, mice were injected with vehicle (PBS).

Mice were subjected to restraint stress (4 h/day) every 24 h for 4 successive days. The first stress was applied 24 h after the first injection of the OTR antagonist. The time schedule of the experiments is shown in Table 3.

Table 3 – Time schedule of the injection of OT antagonist and restraint stress in mice.



4.7. *Behavioral testing*

Anxiety-related behavior was tested in the elevated plus-maze (EPM), a plus-shaped apparatus elevated above the floor with two dark (7 lux) enclosed arms and two open (30 lux) arms (Lister, 1987). Each mouse was placed in the center of the test apparatus, facing a closed arm, to begin and behavior was monitored with a video camera suspended from the ceiling directly above the center of the maze. Data collection was performed with a digital tracking system (LimeLight ver. 2.0, Actimetrics) interfaced with a Pentium-class personal computer. The time spent in each arm and number of closed and open arm entries were scored for a 5-min test period. Anxiolytic activity was indicated by increased exploration of the open arms of the plus-maze, i.e. by an increased percentage of entries into or percentage of time spent on the open arms of the maze. The total number of entries into closed arms was used as a measure of overall motor activity.

4.8. *Statistics*

Data are shown as the mean ± S.E.M. Student's *t*-test or Mann-Whitney's *U*-test was used to identify significant differences between two conditions and a one-way ANOVA or a two-way ANOVA followed by Tukey-Kramer's *post-hoc* analysis was used to compare multiple conditions. Correlations between each term were analyzed using Pearson's rank correlation coefficient. P values less than 0.05 were considered to be significant.

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Identification of a splicing variant that regulates type 2 diabetes risk factor CDKAL1 level by a coding-independent mechanism in human

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Single-nucleotide polymorphisms (SNPs) in *CDKAL1* have been associated with the development of type 2 diabetes (T2D). CDKAL1 catalyzes 2-methylthio modification of adenosine at position 37 of tRNA^{Lys}(UUU). A deficit of this modification causes aberrant protein synthesis, and is associated with impairment of insulin secretion in both mouse model and human. However, it is unknown whether the T2D-associated SNPs in *CDKAL1* are associated with downregulation of CDKAL1 by regulating the gene expression. Here, we report a specific splicing variant of *CDKAL1* termed *CDKAL1-v1* that is markedly lower in individuals carrying risk SNPs of *CDKAL1*. Interestingly, *CDKAL1-v1* is a non-coding transcript, which regulates the CDKAL1 level by competitive binding to a *CDKAL1*-targeting miRNA. By direct editing of the genome, we further show that the nucleotides around the SNP regions are critical for the alternative splicing of *CDKAL1-v1*. These findings reveal that the T2D-associated SNPs in *CDKAL1* reduce *CDKAL1-v1* levels by impairing splicing, which in turn increases miRNA-mediated suppression of CDKAL1. Our results suggest that *CDKAL1-v1*-mediated suppression of *CDKAL1* might underlie the pathogenesis of T2D in individuals carrying the risk SNPs.

INTRODUCTION

Recent advances in genome-wide association studies have successfully revealed a number of loci associated with susceptibility to type 2 diabetes (T2D) (1–4). Among these risk loci, the *Cdk5 Regulator Subunit Associated Protein 1-Like 1* (*CDKAL1*) locus is one of the most reproducible loci in European and Asian populations (5). Individuals carrying T2D-associated single-nucleotide polymorphisms (SNPs) in *CDKAL1* show impaired insulin secretion and up to a 2-fold increase in the risk of T2D (1). We showed that Cdkal1 is a mammalian methylthiotransferase that catalyzes the 2-methylthio (ms^2) modification of N^6-threonyl-carbamoyladenosine (t^6A) to produce 2-methylthio-N^6-threonyl-carbamoyladenosine (ms^2t^6A) at position 37 of tRNA^{Lys}(UUU) (6). The ms^2-modification of tRNA^{Lys}(UUU) is critical for the accurate decoding of the lysine codons AAA and AAG by stabilizing the codon–anticodon interaction (7). The precise decoding of the lysine codon by ms^2-modification was particularly important for proinsulin synthesis, because a lack of Cdkal1 significantly compromised the proper translation and processing of proinsulin (7). As a result, the Cdkal1 knockout mice showed impaired insulin secretion and glucose metabolism. Furthermore, the downregulation of 2-methylthio modification level was also associated with impaired insulin secretion and T2D risk in human (8). Taken together, these results suggest that 2-methylthio modification in tRNA^{Lys}(UUU) by Cdkal1 is critical for precise protein synthesis and the development of T2D.

In contrast to the discovery of molecular function of CDKAL1 and its pathological relevance, there remains fundamental question whether there is a correlation between the T2D-associated SNPs in *CDKAL1* with the regulation of the *CDKAL1* gene. Like most of disease-related SNPs, T2D-associated SNPs in *CDKAL1* are located in deep intronic region (9). These intronic regions are neither conserved across species, nor do these regions contain predictable regulatory elements (1). Because of these obstacles, how the T2D-associated SNPs in *CDKAL1* would affect gene function and lead to the development of T2D remains unknown.

In the present study, we investigated the functional role of T2D-associated SNPs in *CDKAL1* and showed that the SNPs were actively involved in the regulation of cellular CDKAL1 levels through a unique post-transcriptional mechanism.

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