Vascular RAGE transports oxytocin into the brain to elicit its maternal bonding behaviour in mice

Yasuhiko Yamamoto1, Mingkun Liang2, Seiichi Munesue1, Kisaburo Deguchi3, Ai Harashima1, Kazumi Furuhara2, Teruko Yuhi2, Jing Zhong2, Shirin Akther2, Hisanori Goto1, Yuya Eguchi1, Yasuko Kitao4, Osamu Hori4, Yoshitake Shiraishi5, Noriyuki Ozaki5, Yu Shimizu1,6, Tomoya Kamide4,6, Akifumi Yoshikawa4,6, Yasuhiko Hayashi6, Mitsutoshi Nakada6, Olga Lopatina2,7, Maria Gerasimenko2, Yulia Komleva7, Natalia Malinovskaya7, Alla B. Salmina2,7, Masahide Asano6, Katsuhiro Nishimori9, Steven E. Shoelson10, Hiroshi Yamamoto1,11 & Haruhiro Higashida2,7

Oxytocin sets the stage for childbirth by initiating uterine contractions, lactation and maternal bonding behaviours. Mice lacking secreted oxytocin (Oxt−/−, Cd38−/−) or its receptor (Oxtr−/−) fail to nurture. Normal maternal behaviour is restored by peripheral oxytocin replacement in Oxt−/− and Cd38−/−, but not Oxtr−/− mice, implying that circulating oxytocin crosses the blood-brain barrier. Exogenous oxytocin also has behavioural effects in humans. However, circulating polypeptides are typically excluded from the brain. We show that oxytocin is transported into the brain by receptor for advanced glycation end-products (RAGE) on brain capillary endothelial cells. The increases in oxytocin in the brain which follow exogenous administration are lost in Ager−/− male mice lacking RAGE, and behaviours characteristic to abnormalities in oxytocin signalling are recapitulated in Ager−/− mice, including deficits in maternal bonding and hyperactivity. Our findings show that RAGE-mediated transport is critical to the behavioural actions of oxytocin associated with parenting and social bonding.
Oxytocin mediates both physiological and psychosocial events surrounding mammalian birth, including uterine contractions, initiation of lactation, and maternal bonding. Oxytocin produced by oxytocinergic neurons in hypothalamic paraventricular and supraoptic nuclei (PVN, SON) has both central and peripheral actions, the latter through posterior pituitary release into the circulation. Systemic loss of either oxytocin or oxytocin receptors (Oxt−/− and Oxt−r−) disrupts maternal nurturing behaviours in mice. This is restored by administration of exogenous oxytocin in Oxt−/− but not Oxt−r− mice, suggesting that peripheral oxytocin has central effects. Mice lacking CD38, a cyclic ADP ribose synthetase and hydrolase necessary for secretion of oxytocin, also display this characteristic behavioural phenotype, which is also reversed following administration of exogenous oxytocin. Exogenous oxytocin in humans also appears to have behavioural effects, particularly in such social deficit-related psychiatric disorders as autism and schizophrenia. Numerous clinical trials are determining the psychopharmacological effects of peripherally administered oxytocin in a variety of conditions. The potential for peripherally administered oxytocin to act centrally is supported by measures of it in the brain. Oxytocin concentrations are maximal in human or primate cerebrospinal fluid and mammalian hippocampus and amygdala within 10–60 min of peripheral administration. Because many peripheral peptides and proteins do not pass freely into the central nervous system, we hypothesised a specialised uptake mechanism for oxytocin to cross the blood-brain barrier (BBB) and gain access to the CNS.

RAGE, a member of the immunoglobulin superfamily of pattern recognition receptors, is not known to be related to oxytocin or oxytocin signalling. Full-length, membrane bound RAGE (mRAGE) is present on many cell types. Endogenous soluble RAGE (sRAGE), the product of an alternatively spliced mRNA, is found in the circulation as it lacks a membrane-spanning domain. A second soluble form, ectodomain-shed RAGE (esRAGE), is a proteolytic product of mRAGE. The three RAGE forms bind various ligands with similar affinities, including multiple advanced glycation end-products (AGEs) and amyloid β peptides. Here, our findings show that the expression of RAGE on capillary endothelial cells of the blood-brain barrier is both necessary and sufficient for the transport of oxytocin into the brain. The increases in oxytocin in the brain which follow exogenous administration are lost in RAGE knockout male mice (Ager−/− mice lacking the mouse receptor for AGEs gene). Behaviours characteristic to abnormalities in oxytocin signalling are recapitulated in Ager−/− mice, including deficits in maternal bonding and hyperactivity, and both transport and behavioural deficits are restored in Ager−/− mice following transgenic, endothelial cell expression of RAGE. These findings indicate that RAGE-mediated transport can explain the degree of brain oxytocin recruitment previously reported at the molecular level, and demonstrate that oxytocin is critical to the behavioural actions of oxytocin associated with parenting and social bonding.

Results

Oxytocin-RAGE interactions. Survival of Ager gene null (Ager−/−) pups is often low due to maternal neglect, with even mild maternal stress leading to dramatic reductions in offspring survival. This is clearly a maternal deficit, as newborn Ager−/− pups transferred to wild-type (WT) post-partum mothers are nurtured and survive at near normal frequencies. By contrast, normal offspring of WT mothers do poorly when transferred to post-partum Ager−/− mothers. Offspring neglect in the absence of gross anatomic or histological defects is a behavioural deficit reminiscent of the characteristic disorders seen with loss of oxytocin function, as in Oxt−/−, Oxt−r− or Cd38−/− mice. Survival rates for Ager−/− pups fostered by WT mothers were lower than for WT offspring raised by their WT mothers, and survival rates for WT pups transferred to Ager−/− mothers tend to be higher than for Ager−/− offspring raised by their Ager−/− mothers (Fig. 1a, b). This too is seen in Oxt−/−, Oxt−r−, or Cd38−/− mice and attributable to intraterine and peripartum effects of loss of oxytocin function occurring prior to the exchange of offspring. We therefore hypothesised potential interactions between oxytocin and RAGE signalling pathways.

Multiple methods were used to demonstrate direct binding of oxytocin and RAGE. Surface plasmon resonance methods showed concentration-dependent oxytocin binding to immobilised recombinant esRAGE, with an apparent dissociation constant (Kd) of 179 nM (Fig. 1c). Oxytocin-RAGE binding was further confirmed using plate assays where either oxytocin or esRAGE was immobilised, and esRAGE or oxytocin was in solution, respectively (Fig. 1d, e).

Endogenous oxytocin and soluble RAGE also associate in human circulation. Using gel permeation chromatography to separate serum proteins based on molecular size, oxytocin immunoreactivity co-eluted with soluble RAGE (Supplementary Figure 1a). Larger amounts of free oxytocin eluted later, as the molar amount of oxytocin in blood exceeds that of soluble RAGE. Endogenous oxytocin in human serum (30 pmol/50 ml) also co-eluted with soluble RAGE following isolation by anti-RAGE antibody affinity chromatography (Supplementary Figure 1b). Mass spectrometry was employed for further structural identification. In this case, we used immobilised esRAGE to isolate oxytocin from 50 ml human serum, and repeated the procedure after adding a synthetic oxytocin standard (100 ng) (Supplementary Figure 1c). Analyses of the eluted fractions by liquid chromatography-tandem mass spectrometry (LC-MS/MS) showed that endogenous oxytocin in human serum is structurally indistinguishable from the synthetic oxytocin standard, as they eluted at identical times (Supplementary Figure 1d) and have identical parent ions (M+H+ 1008.2). Mass spectrometry of the eluted material from the immobilised esRAGE did not identify structurally related peptides such as arginine-vasopressin which may cross-react in immunoassays.

Oxytocin binding to soluble RAGE suggests it also binds membrane RAGE, but oxytocin neither stimulates a RAGE-dependent NF-kB reporter nor inhibits reporter induction by AGEs, a ligand that activates the NF-kB reporter (Supplementary Figure 2a). Furthermore, oxytocin did not activate NF-kB-independent Rac1 or Cdc42 signalling, which are also suggested to function downstream of RAGE (Supplementary Figures 2b and 2c). Therefore, while oxytocin binds soluble and presumably membrane RAGE, it neither induces nor inhibits intracellular signals reported for RAGE ligands. Moreover, oxytocin binding to soluble RAGE was weakly or non-specifically inhibited by potential RAGE ligands: S100B, AGEs, amyloid β or high-mobility group B1 (HMGB1) (Supplementary Figures 3a–d).

To further explore potential oxytocin-RAGE binding modes, we subdivided extracellular RAGE into its V, C1 and C2 domains. S100B, AGEs, amyloid β, and HMGB1 all bind the RAGE V domain. Oxytocin binding to soluble RAGE was blocked by addition of recombinant V domain, marginally inhibited by C1 domain, and not blocked by C2 domain (Supplementary Figure 3e), suggesting that oxytocin binds the V domain, but at a distinct site from S100B, AGEs, amyloid β or HMGB1.
Oxytocin transport across the BBB. In vitro model systems are used to predict the ability of compounds to cross into the CNS and exert central effects. We used one such system constructed using primary cultures of monkey brain capillary endothelial cells (EC) coupled with rat pericytes and astrocytes to assess requirements for endothelial RAGE in the transport of oxytocin (Fig. 2a). Endothelial transport of oxytocin was dose-dependent (Fig. 2b) and of a similar rate to transport of centrally acting drugs. RAGE was either left at endogenous levels or reduced in the ECs by shRNA knockdown (Fig. 2c). The integrity of the in vitro primate “BBB” was unaffected by RAGE knockdown, assessed by high transendothelial electrical resistance (TEER) (Fig. 2d). Notably, oxytocin transport was reduced selectively in the blood-to-brain direction following RAGE knockdown (Fig. 2b). Reverse transport was much less efficient (Fig. 2e), and according to calculated apparent permeability (Papp) measures, inconsequential (Fig. 2f).

Immunohistochemical analyses showed RAGE in vascular patterns limited to CD31 (platelet endothelial cell adhesion molecule)-positive endothelial cells, which are the components of the BBB, of the hippocampus and choroid plexus of WT (Fig. 3a, b) but not of Agε−/− mice (Supplementary Figure 4a). We also performed confocal microscopic analyses to observe RAGE expression in the circumventricular organs (CVOs) of the brain, referred to as “windows of the brain”, and found that RAGE expression was clearly observable in the endothelial cells of the neurohypophysis (Supplementary Figure 4b), but barely in those of the sensory CVOs, including the organum vasculosum of the lamina terminalis, subfornical organ, and area postrema. Further analysis revealed co-localisation of RAGE with cavelolin-1, a marker of plasma membrane invaginations (caveolae), in endothelial cells and the choroid plexus in WT mice (Supplementary Figure 4c), suggesting a role of RAGE in the oxytocin transfer (trafficking) through caveolae-associated endocytosis and transcytosis (Supplementary Figure 4d). By contrast, oxytocin receptors, which could theoretically act as oxytocin transporters, were not co-localised with endothelial cells in numerous sections of 5 brain regions of Venus mice expressing a fluorescent oxytocin receptor reporter (Supplementary Figures 4e and f).

RAGE-dependent transport of oxytocin across the BBB. Oxytocin concentrations in blood and CSF were assessed following a subcutaneous injection of oxytocin (30 ng/mouse; approximately 1.1 μg/kg of body weight). Steady-state levels in blood (∼20 pg/ml) increased rapidly, peaking within 10 min after injection and returning to baseline by 1–2 h in both WT and Agε−/− male mice (Supplementary Figure 5a). By contrast, levels of oxytocin in CSF from the cisterna magna of WT mice increased more gradually to maxima at 1–2 h after returning to baseline at 4 h (Fig. 4a). Despite equivalently high serum levels following injection of identical amounts of oxytocin (Supplementary Figure 5a), concentrations in the CSF of Agε−/− mice remained at baseline levels (Fig. 4a).

In separate experiments we cannulated the third ventricles of WT and Agε−/− mice and withdrew CSF for oxytocin measurements. Following subcutaneous injection, oxytocin increased in third ventricle CSF of WT but not Agε−/− male mice (Fig. 4b and Supplementary Figure 5b). Microperfusion methods showed that oxytocin also increased in relevant regions of the brain, including the amygdala and PVN of the hypothalamus, of WT but not Agε−/− male mice following subcutaneous, intravenous or intranasal administration of...
oxytocin but not saline carrier (Fig. 4c, d and Supplementary Figure 5c). The direct CSF measures (Fig. 4a, b) and microperfusion results (Fig. 4c, d) confirm that oxytocin in the blood is transported across the BBB as suggested by Landgraf and his colleagues\textsuperscript{16,17,20} and to our knowledge, show for the first time that endothelial RAGE is required for oxytocin transport into the CNS.

Because this is such an important point, we further investigated oxytocin transport following selective transgenic (Tg) expression of RAGE in endothelial cells of both WT and Ager\textsuperscript{−/−} mice using an Flk-1 promoter\textsuperscript{26,41}. Protein expression in both lines was equivalent to the amount of endogenous endothelial RAGE in WT mice (WT = 1, WT + Tg=2, KO + Tg=1). CSF concentrations were measured following subcutaneous injections of oxytocin. Tg expression in Ager\textsuperscript{−/−} mice increased oxytocin in CSF to the WT concentration, whereas Tg expression in WT mice minimally affected CSF oxytocin concentrations (Fig. 4e and Supplementary Figure 5d). Stable isotope-labelled oxytocin was used to more accurately assess transport into the CSF. Following subcutaneous injection, [\textsuperscript{13C,15N}]oxytocin was undetectable in CSF of Ager\textsuperscript{−/−} male mice, whereas amounts in the CSF of WT or KO + Tg mice were equivalent (Fig. 4f and Supplementary Figure 5e). Although oxytocin has been found to produce sex-specific effects\textsuperscript{48–50}, RAGE-mediated oxytocin transport was also observed in female WT-mice (Fig. 4g). These results demonstrated that RAGE is both necessary and sufficient for oxytocin transport into the brain in both male and female mice.

Endothelial RAGE is upregulated in brain capillaries after bilateral common carotid artery occlusion (BCCAO)\textsuperscript{23}. Using this method we found a ~2-fold increase in capillary RAGE (Fig. 5a, b). We predicted this would also promote oxytocin transport. As a control, we showed this procedure does not cause
endothelial RAGE in Ager<sup>−/−</sup> mice would reverse the behavioural phenotypes. There was a substantial rescue of the Ager<sup>−/−</sup> phenotype, as 63.4% of the transgenic Ager<sup>−/−</sup> (KO + Tg) offspring survived, compared to only 10.5% survival for the non-transgenic Ager<sup>−/−</sup> (KO) offspring (P < 0.001) (Fig. 7a). Litters contained both transgenic Ager<sup>−/−</sup> and non-transgenic Ager<sup>−/−</sup> offspring, and all offspring were raised by their respective birth mothers. In addition, offspring survival rate was lower in endothelial RAGE-deficient (EC-KO) mothers (P < 0.001; Fig. 7b).

Social behaviour in RAGE knockout mice. Clinical studies have linked autism and Asperger’s disorders and attention-deficit/hyperactivity disorders to oxytocin deficiency<sup>7,8,34</sup>, and the potential use of exogenous oxytocin to treat these conditions is being assessed in clinical trials<sup>10,11,54–57</sup>. Ager<sup>−/−</sup> male mice are also known to be hyperactive, with greater speeds and distances travelled during both light-dark transition and open field tests<sup>58</sup>. These have been suggested to be potential mouse equivalents of human anxiety properties. We therefore reasoned that the hyperactivity seen in Ager<sup>−/−</sup> male mice might also be related to a deficiency in transport of peripheral oxytocin into the CNS, and administered exogenous oxytocin to WT and Ager<sup>−/−</sup> male mice. Normal activity levels of WT mice were unaffected following administration of either subcutaneous or intraventricular oxytocin (Fig. 7c, d). By contrast, the elevated distances travelled and average movement speeds of Ager<sup>−/−</sup> mice in light-dark transition tests were normalised after intraventricular but not subcutaneous administration of oxytocin. Concordantly, intraventricular but not nasal administration of oxytocin normalised distances travelled by Ager<sup>−/−</sup> mice in open field tests (Fig. 7e). These findings provide psychopharmacological support for the importance of vascular RAGE-dependent transport of peripheral oxytocin into the brain, which appears to be necessary for the development and maintenance of normal social behaviours.

Discussion

Consistent with effects on maternal behaviour, mouse RAGE gene ablation (Ager<sup>−/−</sup>) mice leads to maternal neglect and dramatically decreased offspring survival. This phenotype is reminiscent of mice lacking oxytocin (Oxt<sup>−/−</sup>) or with deficiencies in oxytocin secretion (Cd38<sup>−/−</sup>) or action (Oxtr<sup>−/−</sup>)<sup>35,38,39</sup>. Notably, OT release from the hypothalamus was not affected by the RAGE-deficiency (Supplementary Figure 6). Supporting a primary role for oxytocin transport by endothelial RAGE, selective transgenic expression of RAGE in the endothelium of Ager<sup>−/−</sup> mice rescued the maternal behaviour deficit. The potential for RAGE to serve as an oxytocin transporter is further supported by results from our biochemical binding studies, the in vitro BBB model system, where unidirectional blood to brain transport was seen, and by measurements of oxytocin in various regions of the CNS of WT and Ager<sup>−/−</sup> male mice before and after peripheral administration of oxytocin. Oxytocin concentrations were consistently elevated in the third ventricle and cisterna as well as amygdala and PVN of WT but not Ager<sup>−/−</sup> mice. c-Fos activation in the mPOA and BNST is a classical maternal behavioural response<sup>8,51</sup>, which was also recapitulated by exogenous oxytocin administration in WT but not Ager<sup>−/−</sup> mice. These observations of peripheral oxytocin effectiveness indicate that hypothalamic oxytocin in Ager<sup>−/−</sup> mice was not sufficient to induce maternal behaviour, suggesting the importance of central release of oxytocin stimulated by circulating oxytocin<sup>59</sup>.

The affinity of oxytocin binding to the oxytocin receptor has often been estimated to be K<sub>D</sub> ~100 nM, although a high affinity form with K<sub>D</sub> ~1 nM has also been reported<sup>60</sup>. Therefore our estimated K<sub>D</sub> ~180 nM for binding between RAGE and oxytocin...
seems biologically reasonable. Under normal physiological conditions the primary effects of oxytocin are peripheral, and the endothelial RAGE transport system would not be engaged. However, during childbirth when both peripheral and central actions of oxytocin are needed, blood concentrations increase dramatically, which may saturate peripheral receptor occupancy and promote transport across the BBB. Levels of oxytocin in the periphery are lower than those in the CSF, in humans16,61,62, providing a concentration gradient for what appears to be a transport mechanism: this point of view depends on the methods

![Fig. 4 Transport of oxytocin into the brain. a Oxytocin concentrations in CSF from the cisterna magna after subcutaneous injection of 30 ng oxytocin in WT (Ager+/+) or Ager−/− male mice (n = 3-15/data point, *p < 0.05). b Oxytocin concentrations in the CSF of the third ventricles before and 90 min after subcutaneous administration of oxytocin (30 ng) (n = 7-9). c Oxytocin concentrations in microperfusates of the amygdala before and after intranasal (20 ng) oxytocin. Closed circles, oxytocin in WT mice; open triangles, oxytocin in Ager−/− mice; inverted open triangles, saline in WT mice; open squares, saline in Ager−/− mice (n = 4-13; *p < 0.05). d Oxytocin concentrations in microperfusates of the paraventricular nuclei (PVN) of WT mice before and 60 min after intravenous (iv), nasal (in) or subcutaneous (sc) administration of oxytocin (n = 6-8). e, f Transgenic (Tg) mice expressed human RAGE selectively in endothelial cells either on WT or Ager−/− (KO) backgrounds. Oxytocin concentrations in the CSF were measured 60 min after subcutaneous injections of oxytocin (e; n = 4-14) or [13C,15N]oxytocin (f; n = 9-10). g Oxytocin concentrations in the CSF of female mice were measured 60 min after subcutaneous injections of oxytocin (OT) (n = 4-10). n.d., not detected; ns, not significant. Values are mean ± SEM]
of oxytocin measurements in blood, because a recent report has shown much higher blood OT levels than previously thought using a robust nanoLC-MS platform63. Endothelial RAGE signalling is not required for the central actions of oxytocin, much like the short form of the leptin receptor64. It remains unclear how oxytocin is transported by RAGE on the capillary endothelium. It is most likely that a vesicular trafficking system, involving endocytosis and transcytosis, a transcellular transportation across endothelial cells, is involved in oxytocin transportation mediated by RAGE carrier proteins65 (Supplementary Figure 4d).

Anterograde transport of oxytocin produced in the PVN and SON of the hypothalamus leads to its accumulation in axon terminals of the posterior pituitary, where it is released into the systemic circulation. Surges in the release and circulating concentrations of oxytocin occurring during childbirth activate oxytocin receptors in the reproductive organs1,4, including the uterus where oxytocin stimulates cervical dilation and uterine contractions and the mammary glands to stimulate lactation. Peripheral oxytocin may also act centrally to initiate maternal bonding behaviours5,8. Pharmacological studies clearly show that exogenous oxytocin has behavioural effects5,8,12,24,66, which further demonstrates that peripheral oxytocin can act centrally17,18. However, this has been a point of much debate and dissension13,15,55,67, as polypeptides typically do not cross the BBB without specific transport mechanisms20,45, and mechanisms for oxytocin transport were unknown. Findings presented here show that membrane-associated RAGE on endothelial cells and potentially elsewhere in the CNS binds to and transports peripheral oxytocin into the brain, as recently shown16–19,66.

It has been reported that intranasally administered oxytocin may be transported to the olfactory nerve via olfactory sensory neurons located in the mucous layer, or may reach the trigeminal nerve via trigeminal ganglion cell fibres, which are also located close to the surface of the nasal cavity67,68. Unfortunately, current experiments do not provide any evidence for such transport mechanisms. However, capillaries are dense in the mucus membrane, and thus the oxytocin can easily enter into the bloodstream17,68,69. Once nasally applied oxytocin is conveyed by the blood stream, RAGE in the cerebral micro-vessels will transport oxytocin into the brain. RAGE transport seems to be responsible for the brain uptake of oxytocin nasally delivered oxytocin as treatment for human ASD patients or administration of mae- does of pitocin during labour, which can potentially affect maternal and foetal/newborn behaviour. Instead of direct nasal transport bypassing the BBB, one of the main mechanism may be uptake from the blood via RAGE, because rapid increases in plasma oxytocin concentrations have been reported16; on the other hand, one study reported only a small increase in men70.

![Fig. 5 Oxytocin transport after transient brain ischaemia.](https://example.com/figure5)

- **a** Transient brain ischaemia was induced by 15 min of bilateral common carotid arteries occlusion (BCCAO) as described in Methods. RAGE and CD31 expression in vascular cells of the hippocampus were assessed 24 h after ischaemic insults; nuclei are stained with DAPI (Bar = 100 μm).
- **b** Quantitation of RAGE induction in CD31-positive endothelial cells (n = 5).
- **c** Fluorescein dye was used to check nonspecific vascular leakage and BBB damage following BCCAO (n = 3–6). ns, not significant.
- **d** Oxytocin concentrations in CSF from the cisterna magna of BCCAO or sham-operated WT (RAGE+/+) or Ager−/− male mice (n = 4–9). Values are mean ± SEM.
It has been reported that approximately 0.002% of the peripherally applied amount of oxytocin (5 μg) reaches the CNS at 10 min after subcutaneous or intraperitoneal administration in rats\textsuperscript{71}, while the bioavailability is approximately 2% in rats, as measured by LC/MS after administration of 500 μg oxytocin in rats\textsuperscript{72}. Rault et al. reported a bioavailability of 0.001% of i.n. application of 50 μg oxytocin in female pigs\textsuperscript{18}. With an intra-carotid artery bolus injection of \textsuperscript{125}I-labelled or \textsuperscript{3}H-labelled oxytocin, it was estimated that 1–2% of the oxytocin peptide accumulated on the BBB in rats\textsuperscript{20}. In our study, the estimated availability of oxytocin in the CNF was 0.2%, after subcutaneous administration of 30 ng oxytocin in mice, which was a much lower amount of oxytocin than used in previous studies. The findings on oxytocin reaching the CNS were inconsistent among studies; this may be due to differences in treatment protocols, species, and detection methods. Further studies are required to determine the exact bioavailability.

The neural circuitry responsible for the peripartum acquisition of maternal bonding behaviours in rodents and mammals more generally is increasingly understood. Classical mapping studies revealed roles for the mPOA and adjacent vBNST\textsuperscript{51–53,73,74}. Lesions in the mPOA identified important roles for dopaminergic neurons projecting to the ventral tegmental area (VTA) involved in reward and reinforcement learning\textsuperscript{51,53}. Serotonergic signalling is also critical for the acquisition of maternal behaviour, as demonstrated by Pet1\textsuperscript{−/−} mice with diminished serotonin synthesis in the CNS\textsuperscript{75}. The loss of maternal bonding by Pet1\textsuperscript{−/−} mice has been attributed to deficits in the mPOA and BNST, which are innervated by serotonergic neurons. Oxytocin receptors expressed in the mPOA and BNST are also critical for normal maternal behaviour, which raises important questions about sites of oxytocin expression and mechanisms of oxytocin supply. Our findings suggest that peripheral oxytocin is transported by RAGE into the CNS to activate oxytocin receptors directly.

Finally, there is precedence for RAGE to serve as a peptide transporter across the BBB. For example, RAGE has been shown to transport amyloid β peptide across the BBB, which promotes Alzheimer disease-like symptoms in susceptible mice. RAGE has
also been implicated in the pathogenesis of diabetes complications. Since both of these are deleterious, pathological effects, RAGE is being targeted for inhibition in drug discovery efforts. By contrast, our findings demonstrate that RAGE is required for normal physiological functioning of oxytocin, which may also be inhibited by these drug discovery strategies. Parental bonding and nurturing and numerous other socially interactive behaviours are promoted by oxytocin and may either be missing in its absence or replaced by aggressive or anti-social behaviours.

Mass spectrometry of the eluted material from the immobilised esRAGE in human serum did not identify arginine-vasopressin; additionally, RAGE did not transport it in an in vitro blood–brain barrier (BBB) assay system (Supplementary Figure 7). In conclusion, the contribution of peripheral oxytocin to centrally-mediated behaviours has long been predicted and may now be understood and studied more precisely in view of the RAGE-mediated transport mechanism reported here. This transport mechanism is not gender-specific, but equally function in male and female mice (Fig. 4g).

Methods

Chemicals. Oxytocin was obtained from the Peptide Institute (Osaka, Japan). Liquid chromatography mass spectrometer (LC-MS)-grade water, acetonitrile (ACN), formic acid, trifluoroacetic acid (TFA), and trichloroacetic acid (TCA) were purchased from Wako Chemicals (Tokyo, Japan). Stably-labelled oxytocin [13C5,15N2]-Pro7 and [13C6,15N1]-Leu8 (Scrum Co. Ltd., Tokyo, Japan), as described previously. Liquid chromatography mass spectrometer (LC-MS)-grade water, acetonitrile (ACN), formic acid, trifluoroacetic acid (TFA), and trichloroacetic acid (TCA) were purchased from Wako Chemicals (Tokyo, Japan). Stably-labelled oxytocin [13C5,15N2]-Pro7 and [13C6,15N1]-Leu8 (Scrum Co. Ltd., Tokyo, Japan), as described previously.

Animals. RAGE knockout (Ager−/−), deletion of the mouse gene of the receptor for AGE, Ager−41 and wild type (WT, RAGE+/+) mice (C57BL/6J) were produced by crossbreeding heterozygous mutant mice. Endothelial RAGE-transgenic (Tg) mice were crossed with Ager−/− mice to yield endothelial RAGE-Tg Ager−/− mice (KO + Tg). Both male and female mice were maintained under standard cage conditions (24 °C; 12-h light/dark cycle, lights on at 8:45 a.m.) with sawdust bedding and food and water ad libitum. Breeding pairs were maintained in separate cages. Offspring were weaned at 21 days of age and housed in same-sex sibling pairs. For monitoring offspring survival, pregnant WT and Ager−/− mice were transferred to a new environment (clean cages) 1 day before delivery. The offspring were either kept with biological mothers or caged with postpartum mothers of the alternative genotype. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Chromatography. Polypeptides were separated using a Superdex 75 pg HiLoad 26/600 column (GE Healthcare Japan, Tokyo) eluted at 1.0 ml/min with 5 mM ammonium acetate pH 7.8. Human serum (12 ml) preincubated with 48 ng oxytocin was separated and an enzyme immunosorbent assay (EIA) was used to identify OT in eluting fractions. Anti-RAGE monoclonal antibody (1.0 mg) or purified esRAGE (0.5 mg) were coupled to NHS-activated HiTrap (GE Healthcare). Sera samples (50 ml) from healthy consenting adults were applied to the HiTrap anti-RAGE column equilibrated with 50 mM Tris–HCl (pH 7.4) and 0.15 M NaCl. Alternatively, the low molecular weight (<3000, Amicon UltraproBEK) fraction of human serum (50 ml) was applied to the HiTrap-esRAGE column previously equilibrated with phosphate-buffered saline (PBS). After washing the bound proteins were eluted with 100 mM glycine–HCl buffer (pH 2.5) for HiTrap-anti-RAGE or 10 mM Tris–HCl (pH 7.4) and 2.0 M NaCl for HiTrap-esRAGE and the eluates were analysed by EIA or LC-MS/MS and Western blotting.

Surface plasmon resonance assay. Isolated human esRAGE was immobilised to BIAcore CM5 research grade sensor chips with the amine coupling kit to a density of ~5000 response units (RU). Oxytocin binding kinetics to the immobilised
esRAGE was analysed using a BIAcore 2000 system (GE Healthcare Japan) and mobile phase 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA and 0.005% (V/V) surfactant P 20 at 25 °C and 20 μl/min. The sensor chips were regenerated with 10 mM NaOH and 0.5% SDS (W/V).

Plate binding and competition assay. Oxytocin binding to RAGE and competition by RAGE ligands were assayed with oxytocin-coated 96-well plates, human esRAGE, and horseradish peroxidase (HRP)-conjugated anti-human RAGE antibody (100 μg/ml) indicated concentration was immobilized in 96-well microtitre plates, 1 μg/ml esRAGE was added, and bound esRAGE was detected immunochemically using covalently coupled (RAGE antibody) horseradish peroxidase-catalysed oxidation of tetramethylbenzidine. RAGE ligands were S100B (Sigma-Aldrich, St. Louis, MO, USA), AGE-BSA (glyceraldehyde-derived AGE-BSA)27,32, amiody β 1-42 (Sigma-Aldrich), and high-mobility group box 1 (HMGB1, Sigma-Aldrich)22.

Luciferase assay. O6 rat glioma cells expressing RAGE and stably transduced with NK:β F-promoter-driven luciferase construct27,28 were incubated for 24 h in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 0.1% foetal bovine serum (FBS) prior to 4 h stimulation with ST, S100B or AGE-BSA (glyceraldehyde-derived AGE-BSA)27,32. The luciferase activities were assayed with a Luciferase Assay System (Promega, Madison, WI, USA).

Mass spectrometry. High MW proteins in diluted mouse plasma were precipitated with 2% TCA: supernatants obtained after centrifugation were analysed by LC-MS/MS. Mouse CSF samples diluted 1:5 with PBS (25 μl) were analysed directly. Human plasma samples (50 μl) spiked with 100 ng of oxytocin and 75 ng of S100B (Pierce BioScience, Rockford, IL, USA) for 1 h at concentrations used in animal experiments. The eluting 1.0 ml fractions (Fig. 1) were analysed by LC-MS/MS. The 25 μl samples were separated using a ZORBAX 300SB-C8 column (2.1 × 150 mm, 5 μm; CA, USA) eluted with a linear gradient of 0.1% (v/v) formic acid and acetonitrile (0–30%) at 0.3 ml/min and 50 °C. Eluted proteins were detected in the multiple reaction monitoring (MRM) mode of the MS/MS system (Shimazu, UFLC, Kyoto, Japan; AB Sciex 4000 QTRAP, Framingham, MA, USA); positive ion electrospray (ESI) mode (capillary voltage 5 kV); desolvation voltage (75 V); curtain gas (12 l/h, 65 ml/min); temperature 80 °C. After CSF collections, blood samples were obtained in the perifusion site. The CSF samples were thawed and diluted 1:20 in assay buffer77. CSF samples were assayed without protein extraction due to the low protein concentration in the CSF. The plasma samples (100 μl) were thawed on ice and assayed without protein extraction77, when an additional extraction step may have resulted in lower than actual oxytocin levels. Each sample volume was so small, the CSF was assayed without extraction, though unextracted samples result in binding of complexes of oxytocin stuck to interacting proteins. The oxytocin assay had two linear ranges, which covers a lower concentration range from a few to 30 pg/ml, and a higher concentration range between 50 and 1000 pg/ml. The inter-assay and intra-assay coefficients of variation were less than 15%.

Oxytocin release from the hypothalamus. WT and Ager−/− male mice were anaesthetised with pentobarbitial sodium (50 mg/kg). The entire bilateral hypothalami were obtained and placed separately in different wells of a 12-well plate with 0.4 ml normal Locke’s solution (pH 7.25) containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 2.2 mM CaCl2, 10 mM glucose, 10 mM HEPES, and 0.01% BSA, in a 35 °C water bath. The incubation medium was replaced 10 times every 3 min, as described80. Beginning at the 11th replacement, the temperature was shifted to 38.5 °C. From the 13th–15th replacements, cyclic ADP-ribose (a final concentration of 100 μM, Sigma) was added into the Locke’s medium at 38.5 °C. A healing dummy was used to provide mechanical anchor screws and biocompatible dental cement. All surgical procedures were completed within 30 min. A healing dummy was used to provide mechanical stability during the implantation and the healing period of 2 weeks80. The microperfusion probe (4 mm length of the coaxial tube, 2.5 mm in diameter) consisting of a 20 G fluorinated ethylene propylene guide cannula was replaced before sampling with an inflow/outflow tubing on the day of the experiments80. This tubing was connected to two glass Hamilton microsyringes placed in syringe pumps (Eicom, Osaka, Japan). Microperfusion was pumped into the probe at a flow rate of 0.1 μl/min, and the samples were withdrawn at the faster rate of 1 μl/min. The tubing was conducted for 2 h. Both microprobes were perfused without sampling for 60 min before the first 30-min microperufases from the amygdala, PVN, and prefrontal cortex were collected. The microperufases were mixed under sterile conditions and consisted of 154 mM NaCl, 2.2 mM CaCl2, 5.6 mM KCl, 2.3 mM NaHCO3, and 0.15% BSA (pH 7.4)80. Beginning immediately after the nasal application, 4 additional microperufases were taken at 30-min intervals. After the termination of the experiments, the brains were removed and snap-frozen to obtain 40-μm cryo-cut stained brain slices later for the histological verification of the perfusion site.

Enzyme immunoassay for oxytocin. Oxytocin immunoreactivity levels in serum and CSF were quantified by using an oxytocin ELISA kit (Biosource, Life Sciences, NY, USA, formerly Assays Designs, MI, USA), following the manufacturer’s manual. The CSF samples (5 μl) were thawed and diluted 1:20 in assay buffer77. CSF samples were assayed without protein extraction due to the low protein concentration in the CSF. The plasma samples (100 μl) were thawed on ice and assayed without protein extraction77, when an additional extraction step may have resulted in lower than actual oxytocin levels. Each sample volume was so small, the CSF was assayed without extraction, though unextracted samples result in binding of complexes of oxytocin stuck to interacting proteins. The oxytocin assay had two linear ranges, which covers a lower concentration range from a few to 30 pg/ml, and a higher concentration range between 50 and 1000 pg/ml. The inter-assay and intra-assay coefficients of variation were less than 15%.
A recovery test was performed following subcutaneous injection of oxytocin (100 ng/ml × 0.3 ml) or intravenous application of oxytocin (100 ng/ml) for 10 min at 2 µl/min, through implanted canulae, as described above.

Open field test. The open field test is meant to assess anxious behaviour. It uses a wooden box (60 × 60 × 20 cm) covered with polypropylene and having an outline of lines (30 cm). Animals are in the box for 10 min and recorded movements into the centre are digitally recorded and analysed using ANY-maze software. This test is based on the idea that mice naturally prefer to be near protective walls rather than being exposed to potential dangers in open spaces. Test chambers are cleaned after each trial.

Cerebral ischaemia model. WT and Ager−/− male mice (8 to 12-weeks-old and weighing 20–30 g) were used for the experiments. The animals were anaesthetised with 2.0% halothane and maintained with 0.5% halothane through a facemask. Brain ischaemia was induced via bilateral occlusion of the common carotid arteries (BCCAO) for 15 min using microvascular clips as described previously. Laser-Doppler flowmetry was used to measure cerebral cortical microperfusion (3 mm lateral to bregma). In our experimental model, the mice that exhibited <15% of the baseline control microperfusion during the first minute of occlusion were used in subsequent experiments. Rectal temperature was maintained at 36.5–37.5 °C by using a heat lamp and a blanket until the mice were completely alert. The control animals underwent a sham-operation that was identical with the exception of the occlusion. To quantify accumulative BBB leakages, 200 µl of sodium fluorescein (Sigma-Aldrich) at a concentration of 6 mg/ml in PBS was injected via the venous occlusion. The mice were perfused with a 4% PFA solution overnight at 4 °C. Brain regions were fixed in a 4% PFA solution overnight at 4 °C. Brain regions were cut into 24 large blocks. The blocks were sliced on a microtome into 20-µm-thick sections. The sections were pre-incubated in blocking solution (3% bovine serum albumin and 0.3% Triton X-100 in PBS) for 1 h, then incubated with an anti-c-Fos antibody (sc-52, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the blocking solution for 12 h at 4 °C. After three washes with washing buffer, the sections were incubated with goat anti-rabbit IgG antibody coupled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) in the blocking solution for 1 h at room temperature. The images were obtained by using an Olympus IX71 inverted microscope equipped with a cooled CCD camera (Cool SNAP HQ2; Roper Scientific, Tucson, AZ, USA). The number of c-Fos immuno-positive nuclei in each brain section were recorded and analysed using Metamorph software (Molecular Devices, Downingtown, PA, USA).

Immunoelectron microscopy. The immunogold staining method was applied to the amygdala as previously described. Briefly, after 10 min of oxytocin (100 ng/ml × 0.3 ml) injection, wild type (WT, Ager+/−) and Ager−/− mice were perfused with a mixture containing 2% paraformaldehyde and 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2). The tissue blocks were then fixed by immersion for 4 h at 4 °C in the same solution and washed for 1 h with 0.1 M phosphate buffer (pH 7.2). After washing, the tissue blocks were dehydrated and embedded in LR-White resin (London Resin Co.). Ultrathin sections were mounted on nickel grids. The sections were washed with PBS, incubated in a blocking solution of 1% bovine serum albumin (BSA) and 0.05% sodium azide (NaN₃) in PBS for 15 min, and then exposed with anti-oxytocin polyclonal antibody (1:5000, Chemicon International, Inc. USA) overnight at 4 °C. After washing twice with PBS, the sections were incubated with 5 nm gold-conjugated goat anti-rabbit secondary antibody (1:100, Sigma, USA) in a solution containing 0.1% BSA in PBS for 4 h at room temperature. The sections were washed with PBS and then with distilled water. After washing, the sections were stained with uranyl acetate and analysed under a transmission electron microscope (JEM, Tokyo Japan) by using an 80-kV accelerating voltage.

Statistical analysis. P values were calculated by using two-tailed Student’s t-test for pair wise comparisons, and one-way or two-way analysis of variance (ANOVA) followed by Bonferroni’s or Tukey’s test for multiple comparisons, unless otherwise stated. The Kaplan–Meier survival analysis was performed to compare survival curves between the different groups of mice. A P-value of < 0.05 was considered statistically significant. Data are expressed as mean ± SEM. Statistical data with F values are shown in Supplementary Table 1.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings in this study are available from the corresponding author upon reasonable request. The source data of each figure are presented as a Supplementary Data 1.

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Author contributions

Y.Y., K.D., O.H., H.Y. and H.H. designed the experiments. Y.Y., S.M., A.H., H.G., Y.E., Y. Shir., N.O. and Y.Shim. performed biochemical and cell culture experiments and production of genetically engineered mice. M.L., K.F., T.Y., J.Z., S.A., O.L, M.G., Y.K., N.M. and A.B.S. carried out endocrinological and behavioural experiments. K.D. and A.H. analysed oxytocin in a LC-MS/MS machine. Y.K. and O.H. performed confocal microscopical analyses. Y.S., T.K., A.Y., Y.H. and M.N. carried out mouse carotid artery occlusion experiments. M.A. made mouse lines with germ cell technology. K.N. provided brain specimens of oxytocin receptor reporter mice. Y.Y., S.E.S. and H.H. interpreted the results and wrote the paper with comments from K.D., O.H. and H.Y.

Additional information

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