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TITLE

Oxytocin induced labor causes region and sex-specific transient oligodendrocyte cell death in neonatal mouse brain

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Brain injury caused by induced labor
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Abstract

Aim

Previous reports showed associations between oxytocin induced labor and mental disorders in offspring. However, those reports are restricted in epidemiological analyses and its mechanism remains unclear. In this study, we hypothesized that induced labor directly causes brain damage in newborns and results in the development of mental disorders. Therefore we aimed to investigate this hypothesis with animal model.

Methods

The animal model of induced labor was established by subcutaneous oxytocin administration to term-pregnant C57BL/6J mice. We investigated the neonatal brain damage with evaluating immediate early gene expression (c-Fos, c-Jun and JunB) by...
quantitative polymerase reaction and TUNEL staining. To investigate the injured brain cell types, we performed double-immunostaining with TUNEL staining and each brain component specific protein, such as Oligo2, NeuN, GFAP and Iba1.

Result

Brain damage during induced labor led to cell death in specific brain regions, which are implicated in mental disorders, in only male offspring at P0. Furthermore, oligodendrocyte precursors were selectively vulnerable compared to the other cell types. This oligodendrocyte-specific impairment during the perinatal period led to an increased numbers of Olig2-positive cells at P5. Expression levels of oxytocin and Oxtr in the fetal brain were not affected by the oxytocin administered to mothers during induced labor.

Conclusion

Oligodendrocyte cell death in specific brain regions, which was unrelated to the oxytocin itself, was caused by induced labor in only male offspring. This may be an
underlying mechanism explaining the human epidemiological data suggesting an association between induced labor and mental disorders.

Keywords

Brain damage, induced labor, mental disorder, oligodendrocyte, oxytocin

Abbreviations

ASD: autism spectrum disorder; BP: bipolar disorder; Cb: cerebellum; Ctx: cortex; DAB: diaminobenzidine; EDTA: ethylenediaminetetraacetic acid; ELISA: enzyme-linked immunosorbent assay; Hb: habenular nucleus; Hippo: hippocampus; HRP: horseradish peroxidase; IEGs: immediate early genes; KO: knockout; Oxt: oxytocin; Oxtr: oxytocin receptor; PB: phosphate buffer; PBS: phosphate buffered saline; PFA: paraformaldehyde; PFC: prefrontal cortex; PVN: paraventricular hypothalamic nucleus; qPCR: quantitative polymerase chain reaction; SZ: schizophrenia; SON: supraoptic nucleus; TUNEL: TdT-mediated dUTP nick end labeling;
Introduction

Induced labor has been reported to decrease the risk of cesarean section, meconium-stained amniotic fluid, and perinatal death in post-term pregnancy\textsuperscript{1-4}. Accordingly, the adoption of induced labor has drastically increased since the early 1990s in clinical practice\textsuperscript{5-8}. The rate of induced labor has dramatically increased from approximately 9\% to 20\% in developed countries between 1990 and 2013\textsuperscript{9,10}. However, induced labor leads to adverse effects, such as hyperactive uterine contraction and uterine rupture\textsuperscript{11-14}. These adverse effects are mainly associated with pregnancy-related complications and the prognosis of both mother and infant for conditions such as cerebral palsy. Other than those investigating infant mortality and cerebral palsy, there are few studies on the influence of induced labor on the offspring after the perinatal period. However, recent epidemiological studies suggest an association between induced or augmented labor with oxytocin and the development of several mental disorders in the offspring\textsuperscript{15-17}. For example, one cohort study tracking over 600,000 live births indicated that induced and augmented labor led to 35\% higher risk for autism spectrum disorder (ASD) in delivered boys than non-induced and non-augmented labor.
Other cohort studies indicate that oxytocin administration to mothers during the perinatal period for the induction or augmentation of labor is associated with a 2.4-fold increase in the risk for bipolar disorder (BP) in offspring, and that augmentation of labor with oxytocin is modestly associated with a 13% higher risk for ASD in male offspring. These epidemiological studies are quite suggestive, but are still controversial, as human populations have diverse genetic and environmental backgrounds. The mechanisms underlying these epidemiological associations between induced labor with oxytocin and mental disorders in the offspring should thus be re-evaluated in experimental animals under properly controlled genetic and/or environmental conditions. The association between induced labor using oxytocin and mental disorders in the offspring may be explained by two possible mechanisms. First, brain damage caused by hyperactive uterine contractions may arise from the overdosing of the uterine contraction drug. Second, a pharmacological side effect of oxytocin itself may impair the offspring brain. Many studies have assessed the relationship between fetal brain damage, such as brain ischemia, and mental disorders, such as ASD, BP, and schizophrenia (SZ). Furthermore, oligodendrocyte-lineage cells are sensitive to
hypoxia during the perinatal stage \(^{24-26}\). In fact, abnormal myelination due to oligodendrocyte impairment has been found in the brains of patients diagnosed with ASD \(^{27}\), BP \(^{28-31}\), and SZ \(^{28,29,32}\). We hypothesized that the oligodendrocyte impairment caused by brain damage during induced labor may explain the increased incidence of mental disorders after the maturation of offspring. Several studies using an animal model have investigated the relationship between fetal brain damage and mental disorders. These studies have used occlusion of the middle cerebral artery or the internal carotid artery. However, these methods do not replicate the clinical conditions present during induced labor in humans, as occlusion of arteries is not involved in the process of induced labor. Therefore, the establishment of experimental animal models for induced labor is necessary to address its potential risks in humans.

In this study, we established a new animal model of induced labor to replicate the human clinical condition. We selected oxytocin as a uterine contraction drug to induce labor, as oxytocin is the most popular of the many uterine contraction drugs and is used all over the world. Using this animal model, we aimed to clarify the potential underlying mechanisms explaining the human epidemiological data.
Methods

Animals

Wild-type C57BL6/J mice and DBA/2 mice were purchased from CLEA Japan (Tokyo, Japan) and Japan SLC (Fukuoka, Japan). Oxytocin receptor knockout (Oxtr KO) mice and Oxtr reporter mice expressing Venus under the control of the Oxtr promoter were backcrossed onto the C57BL6/J genetic background (Oxtr-Venus). Three to five animals were housed in each cage at 23 ± 1ºC under a 12-hour light cycle and were provided with food and water ad libitum. Three- to 5-month-old female mice were mated to obtain pregnant mice. The day on which a copulation plug on the female mouse was observed was denoted as gestational day 0.5 (day 0.5). All animal experiments (anesthesia, sacrifice, surgical operation, etc.) were carried out based on the recommended protocol of Tohoku University, approved after the evaluation of animal experiments committee of Tohoku University

Continuous infusion of oxytocin
On day 18.5, pregnant mice were anesthetized with isoflurane and a micro-osmotic pump (model 1003D; Alzet; CA, USA) was subcutaneously implanted. Before the surgical procedure, pumps were loaded with oxytocin (Bachem; CA, USA) dissolved in phosphate-buffered saline (PBS) at different concentrations to provide 0.6, 6, 18, or 240 µg/day of the drug.

**Plasma extraction and enzyme-linked immunosorbent assay**

Blood samples were collected from the pregnant mouse heart directly 5 hours after pump implantation using ethylenediaminetetraacetic acid. To collect the plasma, we centrifuged total blood at 1,600 g for 15 minutes at 4°C. We then extracted the peptides from plasma using Sep columns containing 200 mg of C18 (Phoenix Pharmaceuticals; CA, USA). We determined oxytocin concentrations using an enzyme-linked immunosorbent assay (ELISA) (Phoenix Pharmaceuticals; CA, USA) according to the manufacturer’s instructions.

**Quantitative polymerase chain reaction**
Total RNA was extracted from brain samples by homogenization in TRIzol reagent (Ambion; Thermo Fisher Scientific; Kanagawa, Japan) following the manufacturer’s instructions. One microgram of RNA was reverse-transcribed to cDNA using the PrimeScript RT reagent kit with genomic DNA eraser (Takara; Shiga, Japan) according to the manufacturer’s instructions. SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara; Shiga, Japan) and Thermal Cycler Dice Real Time System (Takara; Shiga, Japan) were used for real-time PCR to detect gene expression. The primers shown in Supplementary Table 1 were used for cDNA detection.

**Immunohistochemistry**

Mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate-buffer (PB), and the brains were dissected out. Brains were postfixed in 4% PFA/PB for 24 hours and 50-µm-thick coronal sections were obtained using a vibratome (VT1200S; Leica; Tokyo, Japan). The sections were permeabilized with 0.5% Triton X-100 dissolved in PBS, and then blocked with 5% normal horse serum and 0.3% Triton X-100 dissolved in PBS. The
sections were incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: rabbit anti-green fluorescent protein (GFP) (1:250; MBL; Aichi, Japan), rabbit anti-glial fibrillary acidic protein (GFAP) (1:200; Frontier Institute; WA, USA), rabbit anti-Iba1 (1:200; Wako; Tokyo, Japan), mouse anti-NeuN (1:200; Millipore; Darmstadt, Germany), mouse anti-Olig2 (1:500; Millipore; Darmstadt, Germany), and rabbit anti-oxytocin (1:2,500; Millipore; Darmstadt, Germany). The anti-GFP antibody was used to detect Oxtr, with enhanced sensitivity. The sections were rinsed and incubated for 2 hours at 23 ± 2°C with the following secondary antibodies: Alexa 488- or 594-conjugated mouse or rabbit immunoglobulin G (1:1,000; Vector; CA, USA). Horseradish peroxidase-conjugated streptavidin and diaminobenzidine were used for visualization and detection of oxytocin. Images were obtained using a confocal laser microscope (Zeiss LSM780; Tokyo, Japan).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling**

The detailed procedure for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is described in a previous publication. 35. Coronal sections
(25-μm-thick) were prepared using a cryostat (Leica CM1520; Tokyo, Japan) and stored at -80°C until use. TUNEL was performed using the DeadEnd colorimetric TUNEL system (Promega; Tokyo, Japan) according to the manufacturer’s instructions. Alexa 488- or 594-conjugated streptavidin was used for visualization and detection of TUNEL-positive cells.

Statistics

Statistical analyses were performed using Graphpad Prism version 6.0 (GraphPad Software) and R software. Data are expressed as mean plus standard error of the mean. The results were analyzed using paired tests. Multiple comparisons were carried out using the Steel-Dwass or Kruskal-Wallis tests. The Mann-Whitney U tests were used for 2-group comparisons. Values of P <0.05 were considered statistically significant.

Results

Establishment of induced labor model with subcutaneous oxytocin administration
To mimic the conditions present during induced labor in humans, oxytocin was subcutaneously and continuously administered to term-pregnant mice using an implanted osmotic pump to maintain oxytocin concentrations in the blood. Subcutaneous implantation is expected to decrease stress, as it does not affect the perinatal behavior of mice when compared to intravenous administration through a cannula.

The onset of labor occurred faster in groups receiving over 0.6 µg/day of the drug than in the PBS group. However, there were no significant differences in the time until delivery between the different groups receiving oxytocin (0.6 µg/day, 6 µg/day, 18 µg/day, or 240 µg/day) (Figure 1A). Oxtr KO mice were not susceptible to labor induction by oxytocin administration. This indicates that oxytocin has a critical role in the shortening of time until labor. To confirm oxytocin administration, we evaluated the blood concentrations of oxytocin in dams using an ELISA 5 hours after pump implantation. Subcutaneous administration of oxytocin increased the blood concentration of oxytocin in a dose-dependent manner, indicating that the administration was successful (Figure 1B).
We did not observe any statistical differences in the viability of pups after delivery among the PBS, 0.6 µg/day, and 6 µg/day groups. In contrast, almost all of the pups delivered from mothers receiving over 18 µg/day of oxytocin died within 24 hour of delivery (Figure 1C). After delivery, pups in the 6 µg/day group did not have any differences in growth when compared to those in the PBS group (Figure 1D). To determine whether induced labor leads to brain damage in the newborns, we evaluated the RNA expression levels of immediate early genes (IEGs) c-Fos, c-Jun, and JunB, which are known to be associated with the early phase of brain damage. As shown in Figure 1E, the expression levels of IEGs increased in an oxytocin dose-dependent fashion in pup brains 5 hours after pump implantation. However, oxytocin administration to Oxtr KO mothers did not lead to increased expression of IEGs.

Based on these data, we concluded that the appropriate dose of oxytocin to cause brain damage during induced labor not enough to affect the mortality is around 6 µg/day. This 6 µg/day of oxytocin is almost equal to the dose in clinical practice (1mg = 600 U, Japanese Pharmacopoeia). We further analyzed the influence of induced labor with 6 µg/day of oxytocin using this animal model causing brain damage on the
neonatal brain.

**Brain damage in neonatal brain during induced labor leads to region-specific cell death in only male offspring.**

To determine the brain regions impaired during induced labor, we assessed cell death using TUNEL in fetal brains harvested and fixed 24 hours after delivery. As shown in Figure 2, the numbers of TUNEL-positive cells increased in the prefrontal cortex (PFC), habenular nucleus (Hb), and periventricular nucleus (PVN) in the induced labor group, while the numbers of TUNEL-positive cells did not increase in other regions, such as the cerebral cortex, hippocampus, and cerebellum. Notably, increased cell death was not observed in the brains of female mice born in the same litters as the impaired neonatal male mice (Figure 3). These data suggest that cell death due to the brain damage during induced labor is region- and sex-specific.

**Brain damage during induced labor leads to oligodendrocyte-specific cell death.**

To determine the cell type of the TUNEL-positive cells described in Figure 2,
we carried out double-staining for Olig2 and TUNEL. Previous studies indicate that Olig2 is a marker of cell of oligodendrocyte lineage, including mature myelinating oligodendrocytes\textsuperscript{38,39}. As shown in Figure 4, about 20-30\% of Olig2-positive cells were also TUNEL-positive, while about 1\% of NeuN-positive cells were TUNEL-positive (NeuN is a neuronal marker). Similarly, about 1\% of GFAP-positive cells (astrocytes) were TUNEL-positive (data not shown). The total numbers of Iba1-positive cells (microglia) were increased in the induced labor group. This is in good agreement with previous reports, as Iba1-positive cells have phagocytic activity and are important for the removal of dead cells in the brain (data not shown).

We next investigated the influence of the observed perinatal oligodendrocyte cell death on myelination during the maturation of offspring. As shown in Figure 5, the numbers of Olig2-positive cells were significantly increased in the induced labor group at P5 by a compensatory mechanism.

**Maternal oxytocin administration does not directly influence the expression levels of oxytocin and Oxtr in the brains of male offspring.**
Previous studies indicate that oxytocin administered to mothers during induced labor travels through the placental barrier and the immature fetal blood-brain barrier. Accordingly, Wahl et al. have hypothesized that oxytocin administered to mothers leads to the down-regulation of oxytocin and Oxtr expression via negative feedback in the fetal brain, finally resulting in the development of some mental disorders, and especially ASD. Therefore, we assessed the direct pharmacological effect of oxytocin on oxytocin and Oxtr expression in the offspring.

Since some of the pups in the 6 and 240 µg/day groups were already born 6 hours after pump implantation, we investigated Oxtr expression 5 hours after implantation and obtained pup brains following cesarean section. After isolation of RNA from pup brains, we analyzed Oxtr mRNA expression using qPCR. We did not observe any differences in Oxtr expression between pups delivered after oxytocin exposure (including the 240 µg/day group) and those in the non-exposed group (Figure 6A). We also investigated the cytotoxic effects of oxytocin on Oxtr-expressing cells, as there was high exposure to oxytocin during induced labor. To evaluate the effects of Oxtr expression, we mated wild type (+/+) females with Oxtr-Venus homogeneous...
knock-in males (v/v) and measured Venus expression in cells using immunohistochemistry in male offspring (v/+). We investigated whether the TUNEL-positive cells in male offspring were expressing Oxtr or not by utilizing Oxtr-Venus reporter mice. As a result, the TUNEL-positive cells were not specifically merged with those of the Venus-labeled Oxtr-positive cells (Figure 6B).

We next investigated the distribution of oxytocinergic neurons in the PVN and supraoptic nucleus (SON). Since most neurons were still not positioned and characterized at the perinatal stage, we assessed the oxytocinergic neurons in the adult stage. At 3-5 months of age, we assessed the distribution of oxytocinergic neurons using immunohistochemistry of oxytocin itself. The numbers of oxytocinergic neurons were not altered between the oxytocin-exposed and non-exposed groups in both the PVN and SON (Figure 6C).

**Discussion**

To date, several studies have reported that induced labor increases the risk of psychiatric disorders, such as ASD and BP in offspring. However, the underlying mechanisms for these epidemiological findings are completely unknown. To our
knowledge, this is the first study to investigate these potential mechanisms using an experimental animal model. The key findings of our study are as follows: (1) brain damage during oxytocin-induced labor in the perinatal stage leads to region-specific cell death in only male offspring, (2) cells of oligodendrocyte lineage are selectively vulnerable to the brain damage during induced labor, (3) oxytocin itself administered to mothers during induced labor did not influence the expression levels of fetal endogenous oxytocin and Oxtr.

As shown in Figure 2,3, sensitivity to the brain damage during induced labor was region- and sex-specific. To the best of our knowledge, no previous study has reported sex-specific adverse effect of induced labor. The cell death caused by brain damage during induced labor was observed only in the PFC, Hb, and PVN. Interestingly, these regions are known to be associated with the development of several mental disorders. According to Bicks, the PFC plays an important role in the control of social cognition, social motivation, social memory, and social hierarchy in both humans and rodents, and the impairment of this region leads to some of the symptoms observed in patients with ASD and those with SZ. Hb and PVN are implicated in the
development of BP. In particular, several histological and imaging studies have shown that Hb volume is decreased in patients with BP \(^{48,49}\). In addition, glial dysfunction in the Hb leads to depressive behaviors in mice \(^{50}\). It has also been reported that patients with BP have neuron loss in the PVN \(^{51}\). Taken together, our results imply that the region-specific cell death caused by the brain damage during induced labor might be a potential risk factor for the development of some mental disorders. We show that male offspring have higher susceptibility to this region-specific cell death than female offspring. This sex difference of our result may be caused by the brain protective effect of estrogen and estrogen receptor (ER). As Raghava et al. and Brotfain et al. reviewed, the estrogen and ER brain protective effect for brain injury has been shown in both an animal experiment and human clinical observational reports\(^{52,53}\). Based on these previously data, it was known that female had lower mortality rate and better functional outcomes after brain injury. This suggests that brain damage during induced labor may be associated with mental disorders such as ASD, which was known to have sex differences in morbidity.

We determined the subpopulations of dead cells in the PFC, Hb, and PVN.
Interestingly, oligodendrocyte lineage cells were selectively vulnerable to the perinatal brain damage caused by induced labor when compared to other brain cells, such as neurons, astrocytes, and microglia. This phenomenon is consistent with the pathophysiology of periventricular leukomalacia, which is caused by ischemia during the perinatal period in humans. Recent studies indicate that the impairment in myelination caused by oligodendrocyte dysfunction is strongly associated with the pathogenesis of some mental disorders in both humans and rodents. Treatments for ASD, SZ, and BP, such as antipsychotic drugs, antidepressants, and electroconvulsive therapy, affect shared signaling pathways, such as the Akt and glycogen synthase kinase-3 pathways, which are involved in myelination. Therefore, new therapeutic and preventive targets for the protection of oligodendrocytes and myelination during brain development have been under investigation. The numbers of oligodendrocytes were significantly increased at P5 in our study. The increased oligodendrocyte cell numbers may reflect a compensatory mechanism to help replace impaired oligodendrocytes. Although we haven’t investigated the oligodendrocyte functions in developed stages, it has previously been suggested that transient
oligodendrocyte cell loss causes persistent changes in gene expression and microstructures, even though the number of oligodendrocytes or its precursors were compensated\textsuperscript{58}. Furthermore, such oligodendrocyte compensatory changes are also observed in postmortem brains from patients with BP \textsuperscript{22}. The potential risk for the development of mental disorders caused by oligodendrocyte cell death during the perinatal remains unclear, even if the oligodendrocyte impairment is transient and subsequently rescued. As indicated in a review by Kleinhans, several studies show that oligodendrocyte abnormalities including insufficient myelination observed in the earlier stage of development are no longer evident in later childhood or adolescence in patients with ASD \textsuperscript{59}. These data suggest that abnormal oligodendrogenesis caused by oligodendrocyte impairment during the perinatal period might contribute to the abnormal neural circuitry and connectivity. These abnormalities may be present even when morphological abnormalities are no longer apparent following brain maturation. The formation of myelin sheaths is crucial for neural plasticity, homeostasis, and repair of damage. Impairments in these repair processes during brain development might be associated with the pathogenesis of some mental disorders, such as ASD, SZ, and BP.
We next investigated whether our observations were due to direct pharmacological side effects of oxytocin administered to mothers during induced labor. Many previous studies have reported that oxytocin and Oxtr KO mice display autistic behaviors\(^{33,60}\). In addition, reduced expression levels or single nucleotide polymorphisms of oxytocin and Oxtr have been observed in patients with ASD and SZ\(^{61-63}\). Furthermore, a recent study suggests that perinatal exposure to oxytocin or oxytocin receptor antagonists leads to social impairment\(^{64}\). We investigated Wahl’s hypothesis (described above) by determining the numbers of oxytocinergic neurons and Oxtr expression. No significant differences in Oxtr expression or the numbers of oxytocinergic neurons were observed between the oxytocin-exposed and non-exposed groups. Furthermore, the TUNEL-positive cells did not merge with Oxtr-positive cells specifically. Based on these data, we concluded that oxytocin induced labor did not affect the oxytocin/Oxtr system in the neonatal brain in our study. Our results suggest that oxytocin does not induce direct pharmacological effects associated with mental disorders, as long as it is used at an appropriate dose.

Here we established a new experimental animal model for induced labor.
However, caution should be used when applying our experimental animal data to humans, as there are species-specific differences between mice and humans. One important species difference is the glia/neuron ratio. As previously described, the glia/neuron ratio in the whole brain is generally about 1 to 10 in humans, while it is about 1 to 4 in rodents. There is thus a possibility that glial function in humans is different from that in rodents. We therefore plan to establish an induced labor animal model in primates to more closely mimic the human clinical condition.

In conclusion, our findings indicate that oligodendrocyte lineage cell death in specific brain regions is caused by the brain damage during induced labor in only male offspring. This may be an underlying mechanism explaining human epidemiological data on the relationship between induced labor and the development of some mental disorders, such as ASD and BP. We also show that the above-described cell death is caused by the brain damage, and is not due to the pharmacological effects of oxytocin, which are mediated by Oxtr. These results suggest that it is important to lessen the brain damage during induced labor to reduce the potential risks in neural developmental impairment resulting in the mental disorders in the future.
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Authors’ contributions

TH, YH, EK and SM designed and carried out the all experiments, and interpreted the results. TH, YH, TF and KN prepared the manuscript. TH and KH performed the data analysis. MK, AI and ST helped with the interpretation of the data and critical revision
of the manuscript from the clinical viewpoint, SH and KN from the basic viewpoint.

All authors read and approved the final version of the manuscript.

**Disclosure**

The authors declare that no conflicts of interest exist.
References


32. Roussos P, Haroutunian V. Schizophrenia: susceptibility genes and


44. Malek A, Blann E, Mattison DR. Human placental transport of


Figure 1. Establishment of an animal model of induced labor with subcutaneous oxytocin administration

A, Time until labor was remarkably shortened at doses over 0.6 µg/day. Oxtr KO mice were not susceptible to labor induction by oxytocin administration (n = 8-12 dams per group).

B, Subcutaneous administration of oxytocin increases the plasma oxytocin concentration in a dose-dependent fashion at 5 hours after pump implantation. (n = 5-6 dams per group).

C, The viability of pups after delivery following administration of PBS, 0.6 µg/day of oxytocin, or 6 µg/day of oxytocin was not statistically different. However, viability following administration of 18 µg/day or 240 µg/day of oxytocin was extremely low when compared to the other three groups. Viability was defined as the percentage of live pups per litter (n = 8-12 dams per group).

D, Growth of pups in the 6 µg/day group was not different from that of pups in the PBS group (n = 17 pups per group).

E, Increased expression levels of IEGs in an oxytocin dose-dependent fashion was
detected in pup brains 5 hours after pump implantation. This effect was not seen following oxytocin administration to Oxtr KO dams. *c*-Fos and *c*-Jun had dose-dependent increases; JunB had a similar tendency (n = 15-20 pups per group).

* P < 0.05  ** P < 0.01  *** P < 0.001  N.S: not significant
Figure 2. **Transient brain damage during induced labor causes region-specific cell death in male offspring.**

TUNEL-positive cells were increased in the prefrontal cortex, habenular nucleus, and periventricular nucleus in the induced labor group at **24 hours after delivery**. The numbers of TUNEL-positive cells did not increase in other brain regions, such as the cerebral cortex, hippocampus, and cerebellum. The increased cell death was observed only in male offspring (n = 4-5 pups per group).

Scale bar = 100 µm

* P < 0.05  ** P < 0.01  N.S: not significant
Figure 3. **Transient brain damage during induced labor does not increase the cell death in female offspring.**

Increased cell death was not observed in the brains of female mice born in the same litters as the impaired neonatal male mice at **24 hours after delivery.** The numbers of TUNEL-positive cells did not increase not only in the cerebral cortex, hippocampus and cerebellum but also the prefrontal cortex, habenular nucleus, and periventricular nucleus in female offspring. (n = 4-5 pups per group).

Scale bar = 100 µm

N.S: not significant
Figure 4. Transient brain damage during induced labor causes oligodendrocyte-specific cell death.

About 20-30% of Olig2-positive cells in male offspring in the oxytocin-induced labor group were also TUNEL-positive at 24 hours after delivery. About 1% of NeuN-positive cells were also TUNEL-positive (n = 4-5 pups per group).

Arrows and arrowheads indicate double-positive cells for Olig2 and TUNEL.

Scale bar = 100 µm

* P < 0.05   ** P < 0.01   N.S: not significant
Figure 5. **Increased numbers of Olig2-positive cells at P5 after brain damage**

The numbers of Olig2-positive cells (red) significantly increased in the induced labor group in the prefrontal cortex, habenular nucleus, and periventricular nucleus at P5 (mHb: medial habenula, lHb: lateral habenula, n = 8-10 mice per group).

Scale bar = 100 µm

* P < 0.05   ** P < 0.01
Figure 6. **Maternal oxytocin administration did not directly influence oxytocin or Oxtr expression in the brains of male offspring.**

A, There was no difference in Oxtr expression between pups in the non-exposed vs. oxytocin-exposed groups (including the 240 µg/day group, n = 15-20 pups per group).

B, Almost none of the TUNEL-positive cells (red) were Venus-labeled Oxtr-expressing cells (green) in the oxytocin-exposed group in the prefrontal cortex, habenular nucleus, and paraventricular nucleus (PVN).

C, There were no differences in the numbers of OXT immunoreactive oxytocinergic neurons in the PVN and supraoptic nucleus between adult mice in the 6 µg/day group and the PBS group (n = 7 mice per group).

Scale bar = 100 µm

N.S: not significant
Supplementary Table 1. **Sequence of primers used in this study**
Figure 1. Establishment of an animal model of induced labor with subcutaneous oxytocin administration

A, Time until labor was remarkably shortened at doses over 0.6 µg/day. Oxtr KO mice were not susceptible to labor induction by oxytocin administration (n = 8-12 dams per group).

B, Subcutaneous administration of oxytocin increases the plasma oxytocin concentration in a dose-dependent fashion at 5 hours after pump implantation. (n = 5-6 dams per group).

C, The viability of pups after delivery following administration of PBS, 0.6 µg/day of oxytocin, or 6 µg/day of oxytocin was not statistically different. However, viability following administration of 18 µg/day or 240 µg/day of oxytocin was extremely low when compared to the other three groups. Viability was defined as the percentage of live pups per litter (n = 8-12 dams per group).

D, Growth of pups in the 6 µg/day group was not different from that of pups in the PBS group (n = 17 pups per group).

E, Increased expression levels of IEGs in an oxytocin dose-dependent fashion was detected in pup brains 5 hours after pump implantation. This effect was not seen following oxytocin administration to Oxtr KO dams. c-Fos and c-Jun had dose-dependent increases; JunB had a similar tendency (n = 15-20 pups per group).
* P < 0.05  ** P < 0.01  *** P < 0.001  N.S: not significant
Figure 2. Transient brain damage during induced labor causes region-specific cell death in male offspring. TUNEL-positive cells were increased in the prefrontal cortex, habenular nucleus, and periventricular nucleus in the induced labor group at 24 hours after delivery. The numbers of TUNEL-positive cells did not increase in other brain regions, such as the cerebral cortex, hippocampus, and cerebellum. The increased cell death was observed only in male offspring (n = 4-5 pups per group).

Scale bar = 100 µm

* P < 0.05  ** P < 0.01  N.S: not significant
Figure 3. Transient brain damage during induced labor does not increase the cell death in female offspring. Increased cell death was not observed in the brains of female mice born in the same litters as the impaired neonatal male mice at 24 hours after delivery. The numbers of TUNEL-positive cells did not increase not only in the cerebral cortex, hippocampus and cerebellum but also the prefrontal cortex, habenular nucleus, and periventricular nucleus in female offspring. (n = 4-5 pups per group).

Scale bar = 100 µm
N.S: not significant
Figure 4. Transient brain damage during induced labor causes oligodendrocyte-specific cell death. About 20-30% of Olig2-positive cells in male offspring in the oxytocin-induced labor group were also TUNEL-positive at 24 hours after delivery. About 1% of NeuN-positive cells were also TUNEL-positive (n = 4-5 pups per group).

Arrows and arrowheads indicate double-positive cells for Olig2 and TUNEL. Scale bar = 100 µm

* P < 0.05  ** P < 0.01  N.S: not significant
Figure 5. Increased numbers of Olig2-positive cells at P5 after brain damage
The numbers of Olig2-positive cells (red) significantly increased in the induced labor group in the prefrontal cortex, habenular nucleus, and periventricular nucleus at P5 (mHb: medial habenula, lHb: lateral habenula, n = 8-10 mice per group).
Scale bar = 100 µm
* P < 0.05  ** P < 0.01
Figure 6. Maternal oxytocin administration did not directly influence oxytocin or Oxtr expression in the brains of male offspring.

A, There was no difference in Oxtr expression between pups in the non-exposed vs. oxytocin-exposed groups (including the 240 µg/day group, n = 15-20 pups per group).

B, Almost none of the TUNEL-positive cells (red) were Venus-labeled Oxtr-expressing cells (green) in the oxytocin-exposed group in the prefrontal cortex, habenular nucleus, and paraventricular nucleus (PVN).

C, There were no differences in the numbers of OXT immunoreactive oxytocinergic neurons in the PVN and supraoptic nucleus between adult mice in the 6 µg/day group and the PBS group (n = 7 mice per group).

Scale bar = 100 µm
N.S: not significant

179x229mm (300 x 300 DPI)