Oxytocin inhibits corticosterone-induced apoptosis in primary hippocampal neurons

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Abbreviations: ANOVA, one-way analysis of variance; CORT, corticosterone; DIV, day in vitro; DMSO, dimethyl sulfoxide; GR, glucocorticoid receptor; OT, oxytocin; OTR, oxytocin receptor; TBST, tris-buffered saline with Tween 20; TUNEL, TdT-mediated dUTP nick end labeling assay.

ABSTRACT

Stress is an adaptive and coordinated response to endogenous or exogenous stressors that pose an unpleasant and aversive threat to an individual's homeostasis and wellbeing. Glucocorticoids, corticosterone (CORT) in rodents and cortisol in humans, are adrenal steroids which are released in response to stressful stimuli. Although they help individuals to cope with stress, their overexposure in animals has been implicated in hippocampal dysfunction and neuronal loss. Oxytocin (OT) plays an active role in adaptive stress-related responses and protects hippocampal synaptic plasticity and memory during stress. In this study, we showed that OT inhibits CORT-induced apoptosis in primary mouse hippocampal neurons. OT receptors (OTR) were expressed in primary mouse hippocampal neurons and glial cells. CORT induced apoptosis in hippocampal neurons but had no effect on apoptosis in glial cells. OT inhibited CORT-induced apoptosis in primary hippocampal neurons. OT was unable to protect primary hippocampal neurons prepared from OTR KO mice from CORT-induced apoptosis. These results indicate that OT has inhibitory effects on CORT-induced neuronal death in primary hippocampal neurons via acting on OTR. The findings suggest a therapeutic potential of OT in the treatment of stress-related disorders.

Key words: oxytocin; stress; corticosterone; apoptosis

Stress is an adaptive and coordinated response to an internal or external challenge that is perceived as unpleasant or threatening to homeostasis, wellbeing or survival of an individual. The physiological stress response comprises the rapid activation of the sympatho-adrenal axis and the release of catecholamines from the adrenal medulla and the delayed activation of the hypothalamic–pituitary–adrenal (HPA) axis and the release of glucocorticoids, corticosterone (CORT) in rodents and cortisol in humans from the adrenal cortex (Lucassen et al., 2014). All these aim to use energy resources more efficiently by shifting attention on the most urgent and important elements to prepare individuals to be able to cope with stress while less urgent vegetative functions, such as digestion and absorption, are temporarily suppressed (Joëls et al., 2012).

Exposure to strong and long-term physical and psychological stress leads to the hyperactivity of the HPA axis and elevated glucocorticoid levels (Dettmer et al., 2012). Glucocorticoid receptors (GR) are expressed in many brain structures, particularly in the hippocampus. This makes the hippocampus vulnerable to elevated glucocorticoid levels as seen in a stress response (de Kloet et al., 2005; Swaab et al., 2005; Lucassen et al., 2010). Previous studies show that stress induces atrophy and loss of neurons in the adult hippocampus (Watanabe et al., 1992; Stein-Behrens et al., 1994; McEwen, 1999). Negative correlations were observed between glucocorticoid levels and hippocampal size and cognitive function (Lupien et al., 1998). In depressed adolescent patients, smaller hippocampal volumes were observed compared to healthy controls (MacMaster and Kusumakar, 2004). Administration of a GR agonist induces apoptosis in the dentate gyrus and CA3 pyramidal cell layers (Sousa et al., 1999; Almeida et al., 2000). Hippocampal damage could result from the hypersecretion of glucocorticoids amounting to neuronal death or the failure of adult neurogenesis in the dentate gyrus (Tae et al., 2011). We hypothesized that high corticosteroid levels as seen in stress and depressed patients might cause hippocampal volume reduction, hippocampal damage and impaired cognitive function by

inducing apoptosis of hippocampal neurons.

Oxytocin (OT), a neuropeptide produced mainly in the paraventricular and the supraoptic nuclei of the hypothalamus, is essential in parturition and lactation (Swanson and Sawchenko, 1983). In the brain, OT plays an important role in regulation of emotional, parental, affiliative and sexual behaviors. Previous studies showed that OT mediates antistress and antidepressant-like effects in mice and rats (Uvnas-Moberg and Petersson, 2005; Matsuzaki et al., 2012). Plasma OT levels increase during stress responses and decrease stress in humans (Taylor et al., 2006). In addition, centrally administered OT inhibited stress-induced CORT release in rats (Windle et al., 1997). OT receptors (OTRs) are strongly expressed in mouse hippocampus and amygdala (Tomizawa et al., 2003; Freeman and Young, 2016). OT maintains hippocampal synaptic plasticity and memory during stress (Lee et al., 2015). OT also stimulates adult neurogenesis in rats subjected to glucocorticoid administration or cold water swim stress (Leuner et al., 2012). OT may exert anti-stress effects by protecting hippocampal neurons from the damaging effects of glucocorticoids. In the present study, we demonstrated that OT inhibits CORT-induced apoptosis in primary hippocampal neurons.

MATERIALS AND METHODS

Animals

C57BL6 pregnant female mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Japan). OTR knockout (KO) mice were backcrossed to achieve a C57BL/6 genetic background for more than 6 generations (Matsushita et al., 2012). Mice were housed at 25 °C with 12-h light/dark cycles in the Department of Animal Resources of Okayama University. Water and standard rodent chow were available ad libitum. All experimental procedures were approved by the Animal Ethics Committee of Okayama University (OKU-2015522 and OKU-2016285).

Primary hippocampal neuron cultures

Primary cultures of hippocampal neurons were prepared as described previously by Gitler et al. (2004). Newborn pups (postnatal day 0-2) were decapitated and their hippocampi dissected under a light microscope and aseptic conditions. For OTR-KO mice, littermates produced by crossing heterozygous mice were genotyped on postnatal day 0, and newborn pups homozygous for OTR were used to make primary cultures. Cells were harvested from a homogenized pool of hippocampi and were plated at a density of 60,000 to 80,000 cells/well on poly-D-lysine-coated 4-well plates (Thermo Fisher Scientific, USA). Cultures were maintained in neurobasal–A medium enriched with GlutaMAX (Thermo Fisher Scientific, USA) and B27 supplement (Thermo Fisher Scientific, USA) in a humidified incubator in an atmosphere of 5% CO_2 at 37°C. On fourth day in vitro (4 DIV), 0.5 ml of fresh growth medium was added to each well, and on the following day 2.5 µl of 1 mM cytosine arabinoside (Sigma-Aldrich, USA) were added to each well to prevent the growth of glial cells. For the glial cell culture, minimum essential media (MEM), supplemented with L-glutamine, fetal bovine serum, penicillin/streptomycin, (Thermo Fisher Scientific, USA) and 45% glucose (Sigma-Aldrich, USA), was used. The cells were cultured for a total of 7 days before proceeding to further experiments.

Drug Application

OT (O4375) and CORT (27840) were purchased from Sigma-Aldrich. The OT stock 100 μM solution was prepared with deionized water and the CORT stock 100 mM solution was prepared with dimethyl sulfoxide (DMSO) before use. Seven DIV primary hippocampal neurons were used in experiments for drug treatment. The neuronal cultures were exposed to CORT with or without OT for 24 h. Control cultures were treated with DMSO, at a final concentration of less than 0.5%.

Immunoblot analysis

Western blotting was performed as described previously (Tomizawa et al., 2003). Hippocampal neurons were lysed with N-PER neuronal protein extraction reagent (Thermo Fisher Scientific) containing protease inhibitor (Roche Applied Science, Germany) and phosphatase inhibitor (Roche Applied Science, Germany) and centrifuged at 10000 g for 10 min at 4°C. The supernatant was assayed for total protein concentrations using Pierce™ BCA assay kit (Thermo Fisher Scientific). HeLa cells were prepared by sonication in boiled 1% sodium dodecyl sulfate buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% skim milk in 1 × Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature. Then the membranes were washed 3 times with 0.1% skim milk in 1 × TBST for 5 min and incubated overnight at 4°C with the appropriate antibodies: anti-OTR (abcam, UK) (Gong et al., 2016; Kaneko et al., 2016) at 1:2000; and anti-β-actin (Sigma-Aldrich, USA) at 1:4000. After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (anti-rabbit IgG for anti-OTR or anti-mouse IgG for anti-β-actin, both at 1:10,000 dilution) (Sigma-Aldrich, USA) for 1 h at room temperature. Western blot bands were detected by enhanced chemiluminescence technique using ECL prime detection kit (GE Healthcare, USA).

Immunocytochemistry

Primary hippocampal neuronal cultures were fixed with 4% paraformaldehyde in 1 × phosphate-buffered saline for 10 min, permeabilized with 0.1% Triton X-100 in 1 × phosphate-buffered saline for 10 min, and blocked with 10% goat serum prepared in 0.1% Tween 20 in 1 × phosphate-buffered saline for 30 min at room temperature. Rabbit monoclonal antibody against OTR (anti-OTR; abcam, UK) and mouse polyclonal antibody against microtubule associated protein 2 (anti-MAP2; abcam, UK) were used as primary antibodies. Subsequent steps were performed using secondary antibodies labelled with fluorescent dyes: goat anti-rabbit IgG Alexa Fluor 488 conjugate (Invitrogen, USA) and goat anti-mouse IgG Alexa Fluor 555 conjugate antibodies (Invitrogen, USA). For nuclear staining, Hoechst was added at 1:1000.

In situ detection and measurement of apoptotic cells by TdT-mediated dUTP nick end labeling assay (TUNEL)

For in situ detection of apoptotic cells, the in situ cell death detection kit (Roche Applied Science, Germany) was used as previously described (Musumeci et al., 2011). The assay makes use of the enzymatic action of TdT, which adds dUTP labeled with TMR red to the ends of DNA fragments. The cultured cells were rinsed once with 1× PBS, fixed with 4% paraformaldehyde in PBS for 1 h at room temperature and permeabilized using freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate. Then the fixed cells were incubated with TUNEL reaction mixture in a humidified atmosphere at 37°C for 1 h in the dark. For negative control samples, TdT was omitted from the reaction. After rinsing the cells with PBS, Hoechst (1:1000) was added to the wells for 5 min for nuclear staining. TUNEL positive cells were counted manually, and the percentage of positive cells was calculated for each sample.

Statistical analysis

To make each batch of primary cultures of hippocampal neurons, a pool of dissociated cells was collected from hippocampi dissected from 2-3 littermate pups. On average, one pup yields 2 four-well plates with a cell density of 60000-80000 cells/well. The total number of culture plates required for all experiments was 24, and we used 15 pups to make the plates. In this study, data are shown as the mean + S.E.M, and 'n' denotes the number of wells used for each condition. We have tested whether our experimental data meet the criteria for one-way analysis of variance (ANOVA), by Shapiro-Wilk test for normality and Levene's test for equal variance. To test the effects of different concentrations of CORT on primary hippocampal neurons and glial cells, a one-way ANOVA followed by Tukey–Kramer post hoc test or Bonferroni test was used to compare multiple conditions. To test the protective effects of OT against CORT on primary neurons, Welch's ANOVA followed by Games-Howell test was used. *P* values less than 0.05 were considered significant.

RESULTS

Expression of OTR in primary mouse hippocampal neuronal cultures

Immunofluorescent analysis of 7 DIV primary mouse hippocampal neurons revealed that OTR was strongly expressed and localized in the soma of mature cultured hippocampal neurons (Fig. 1A). OTR expression was also observed in primary dendrites of neurons and glial cells but not as strong as its expression in the soma. Western blot analysis showed that both primary hippocampal neurons and human cervical carcinoma cell line HeLa expressed OTR at similar levels (Fig. 1B).

CORT induced apoptosis in primary hippocampal neurons but not in glial cells

Previous studies showed that 100 μM CORT induces apoptosis in mouse hippocampal neurons (Xu et al., 2011; Nakatani et al., 2014). To examine the effect of CORT in primary mouse hippocampal neurons in this study, neurons and glial cells were treated with vehicle or 10, 50, 100, or 500 μM CORT for 24 h, and then the extent of apoptosis was measured by TUNEL assay. A one-way ANOVA analysis yielded significant differences among the groups [F (4, 10) = 20.88, P < 0.01, one-way ANOVA; Fig. 2A]. CORT induced apoptosis in primary hippocampal neurons in a dose dependent manner; compared to the control group, a significant increase in TUNEL-positive cells started at a dose of 50 μM CORT, and the number became higher with 100 μM CORT and the highest with 500 μM CORT [P < 0.05 for 50 μM CORT and P < 0.01 for 100 and 500 μM CORT, post hoc Tukey–Kramer; Fig. 2A]. However, glial cells were resistant to the apoptosis-inducing effect of CORT. Only at a very high CORT concentration (500 μM) were a significant number of apoptotic cells observed in glial cultures [P < 0.01, Bonferroni; Fig. 2A].

OT attenuated CORT-induced apoptosis in primary hippocampal neurons

To explore the effects of OT on CORT-induced apoptosis, primary hippocampal neurons were incubated for 24 h in 100 μM CORT with or without 1 μM OT. A significant effect of OT on

CORT-induced apoptosis was observed [F (2, 11.72) = 18.74, P < 0.01, Welch's ANOVA; Fig. 2C]. The number of TUNEL-positive cells was significantly higher in CORT-treated neurons [P < 0.05, Games-Howell; Fig. 2C] than in those treated with vehicle, whereas co-treatment with OT caused a dramatic decrease in the number of apoptotic cells [P < 0.05, Games-Howell; Fig. 2C]. From these findings, it can be concluded that OT protects hippocampal neurons from the deleterious effects of CORT.

OT failed to rescue primary mouse hippocampal neurons prepared from OTR-KO mice from CORT-induced apoptosis

To determine whether the effects of OT on CORT-induced apoptosis are mediated by OTR expressed in hippocampal neurons, we used primary cultures prepared from OTR-KO pups. OTR-KO hippocampal neurons were treated with vehicle, 100 μM CORT (CORT) or 100 μM CORT + 1 μM OT (CORT+OT) for 24 h. Statistical analysis revealed significant differences between groups [F (2, 8.65) = 9.23, P < 0.01, Welch's ANOVA; Fig. 3B]. Quantification of TUNEL-positive cells revealed that 100 μM CORT induced significant apoptosis in the OTR-KO neurons [P < 0.05, Games-Howell; Fig. 3B] while cotreatment with OT failed to protect primary neurons from CORT-induced apoptosis in the absence of OTR. These results confirmed that OT exerted its action against CORT via acting on OTRs expressed in hippocampal neurons.

DISCUSSION

Major depressive disorder is among the leading causes of disability worldwide (Vos et al., 2015). One of the major causal or exacerbating factors of depression is long term stress or psychological trauma (Liu and Alloy, 2010). Dysregulation of the HPA axis activation is common in depressed patients, and elevated plasma glucocorticoids and corticotrophin releasing hormone levels were frequently reported (Varghese and Brown, 2001). CORT has been shown to involve in hippocampal dysfunction and damage (Sousa et al., 1999; Almeida et al., 2000; Zhu et al., 2006), whereas OT has been shown to mediate antistress and antidepressant-like effects in both animals and humans (Uvnas-Moberg and Petersson, 2005; Matsuzaki et al., 2012). The present study attempted to explore the possible neuroprotective effect of OT against CORT-induced neuronal damage in hippocampal neurons.

We used primary cultures of hippocampal neurons prepared from early postnatal mice as an in-vitro cellular model to explore the neuroprotective function of OT in the brain. Hippocampal neurons retain their morphological and functional characteristics even when they are grown in primary cultures. To verify the suitability of the primary cultures in our experiments, we examined the expression of OTR in these cultures. Leonzino et al. (2016) reported that OTR expression was detected in cultured hippocampal neurons already at DIV1 and increased over time. Immunocytochemistry of OTR showed that they are mainly expressed in neurons, but surrounding glial cells also have some degree of expression. Regarding subcellular localization, OTRs are mainly located on the soma of neurons, but in some cases, their expression extends to primary dendrites. Cultured hippocampal neurons also express abundant GRs and mineralocorticoid receptors (Crochemore et al., 2005). Early expression of OTR and GR suggests that there might be an interplay between OT and glucocorticoids in development of the brain during pre- and post-natal periods, possibly by OT protecting developing neurons from the strains and stresses imposed by the process of labour and the outside world.

CORT has been shown to cause hippocampal damage in a number of ways; altering dendritic tree of hippocampal neurons (Woolley et al., 1990; Watanabe et al., 1992; Magariños et al., 1996), apoptosis of hippocampal neurons (Zhu et al., 2006; Liu et al., 2011) and inhibition of adult neurogenesis in dentate gyrus (Yu et al., 2004). Our group focused on CORT-induced apoptosis in hippocampal neurons. Our findings highlighted two salient points: firstly, high concentrations of CORT were required to induce neuronal death in mouse hippocampal neurons, and secondly, glial cells in cultures were refractory to CORT-induced apoptosis. CORT induced apoptosis in primary cultures of hippocampal neurons in a dose dependent manner. Significant apoptosis started to be seen with 50 μM CORT, and the number of apoptotic cells increased with increase in CORT concentration. Our findings are similar to what was observed in other groups. Nakatani et al. (2014) reported that high exposure of CORT (100 μM for 72 hours) was required to induce significant cytotoxicity in primary mouse hippocampal cultures. Xu et al. (2011) also reported that CORT administration at a concentration greater than 50 μM for 24 h induced significant cell death in mouse hippocampal cell line HT-22. In contrast, 1 μM CORT was enough to cause significant decrease in neuronal viability in primary rat hippocampal neurons (Liu et al., 2011). Our findings and others suggest that there is a species difference in susceptibility of hippocampal neurons to the damaging effects of CORT. However, even at high CORT stimulation, glial cells were resistant to CORT-triggered apoptosis. Yu et al. (2011) also reported that, unlike hippocampal neurons, astrocytes are resistant to glucocorticoid-induced apoptosis. They also reasoned that astrocytes might have lesser production of reactive oxygen species as well as a greater capacity to buffer their cytotoxic actions (Yu et al., 2011). The differential action of CORT on neurons and glial cells is interesting, and will require further research to understand why glial cells are less prone to the deleterious effects of CORT.

Given that CORT-induced hippocampal damage has been implicated in depression, aging and prolonged glucocorticoid therapy, finding a molecular agent that can protect hippocampal neurons

from the adverse effects of CORT could have significant clinical benefits. In this regard, we tested the potential protective role of OT in CORT-induced apoptosis, as OT is released in response to stressful stimuli and has been shown to have antidepressant- and antianxiety-like effects in animal studies (Arletti and Bertolini, 1987; Matsushita et al., 2010). In our study, OT counteracted the action of CORT and protects the hippocampal neurons from apoptosis. To confirm whether anti-apoptotic effects of OT were mediated via OTR, we tested the effect of CORT and OT using primary hippocampal cultures derived from OTR KO mice. As OT failed to rescue hippocampal neurons from CORT-induced apoptosis in the absence of OTR, it was concluded that OT acts via OTR to protect them.

There are some limitations in interpretation of our results. Firstly, we used in-vitro cultures of hippocampal neurons to test our hypothesis. They are different in a number of ways from mature neurons in adult brain. They do not have a structural and humoral support of a network of glial cells. They do not have extensive connections with other functionally distinct neurons as in an adult brain. Their transcriptome might be different from adult neurons. Secondly, CORT levels that can amount to significant cytotoxicity to hippocampal neurons in our study are relatively high compared to levels that can be achieved in a stress paradigm in vivo. Peak plasma CORT levels in mice that can be achieved by acute or chronic stress were reported around 1000 ng/mL (i.e. 2.9 μM) (Gong et al., 2015). It seems that mouse hippocampal neurons in primary cultures were resistant to CORT, and we reasoned that high resistance of hippocampal neurons to CORT might be partly related to difference in expression of GR either in terms of the number or the isotype, as well as in glucocorticoid metabolism. Varga et al. (2013) reported that in the hypothalamus, hippocampus, and prefrontal cortex in rat pups, the expression and protein levels of GR and mineralocorticoid receptors were decreased compared to adult animals, while those of 11beta-hydroxysteroid dehydrogenase 2, an enzyme which converts CORT and cortisol into inactive metabolites, were increased. Whether this is also true in mice should be answered with future

experiments.

Although our results cannot be directly interpreted into what would be observed in vivo, our findings highlighted the possible mechanism of OT acting as an antagonist against a stress hormone, CORT, in young and developing hippocampal neurons. As primary hippocampal neurons are retrieved from newborn mice, they can be viewed as young, and immature neurons developing to form a mature neuronal network in the face of challenges such as the process of labour, adaptation to external world, and in our case, artificial environment. This time in life coincides with stress non-responsive period during which the HPA axis is less responsive to stressful stimuli (Schapiro, 1968). Although the underlying mechanisms were not very clear, this might reduce the exposure of developing neurons from the toxic effects of glucocorticoids. The process of labour also results in a dramatic increase in plasma OT level in mother, which might cause a parallel increase of plasma OT in fetuses. In humans, fetal plasma OT levels were significantly higher after vaginal delivery than after elective cesarean section (Kuwabara et al., 1987; Marchini et al., 1988). OT in fetal plasma also seems to come from the fetal pituitary, as evidenced by significantly higher plasma levels in the umbilical artery compared to maternal levels (de Geest et al., 1985). Current and previous findings suggest the role of OT as a neuroprotective agent in the developing brain.

Previous studies have linked perturbed OT signaling to several neurodevelopmental and psychiatric disorders, and many tried to evaluate its potential application in such patients. Intranasal OT has been shown to have positive effects in patients with post-traumatic stress disorder (Olff et al., 2015; Frijling et al., 2016; van Zuiden et al., 2017) and major depressive disorder (Scantamburlo et al., 2015; Domes et al., 2016). Despite these developments in appraising OT as a therapeutic agent in stress-related disorders, the therapeutic value of OT in the context of psychotherapy remains limited, and needs both basic science and translational research for further evaluation.

In conclusion, OT has inhibitory effects on CORT-induced neuronal death in primary cultured hippocampal neurons, and these effects are mediated via acting on OTR. The findings suggest that OT could have a physiological role in the development of brain as well as a pharmacological value in treating stress-related disorders.

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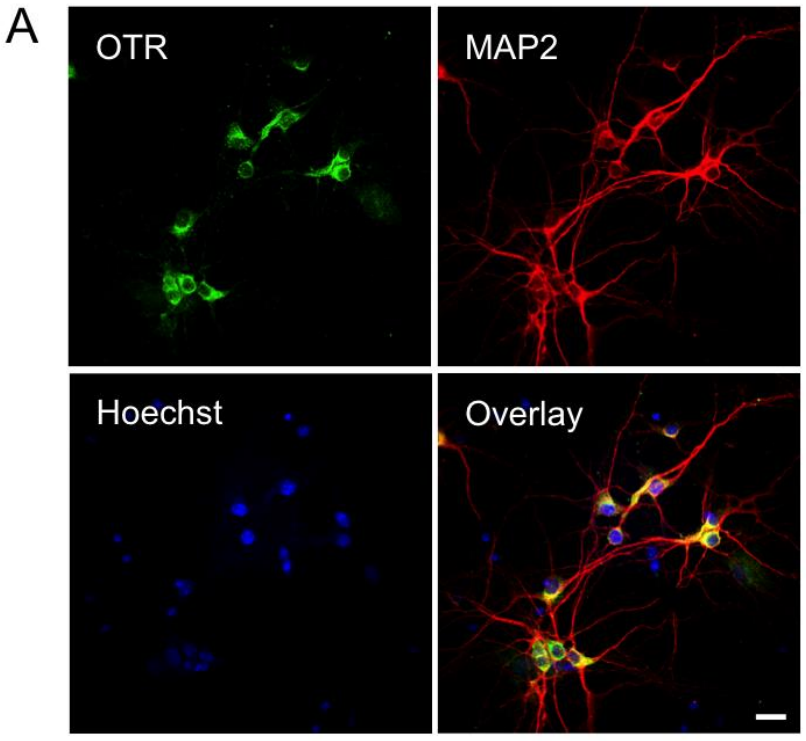
FIGURE LEGENDS

Figure 1. The expression of OTR in primary mouse hippocampal neuronal cultures. (A) Primary hippocampal neurons were stained for OTR (green), MAP2 (red) and Hoechst (blue). Immunofluorescent analysis of 7 DIV primary mouse hippocampal neurons revealed that OTR is localized in the cell bodies of mature cultured hippocampal neurons. Scale bar, 20 μm. (B) Western blot analysis showed that both primary hippocampal neurons and human cervical carcinoma cell line HeLa expressed OTR.

Figure 2. OT protects primary mouse hippocampal neurons from CORT-induced apoptosis. (A) Neurons and glial cells were treated with vehicle, 10, 50, 100, or 500 μM CORT for 24 h. The degree of apoptosis was measured by TUNEL assay. Data represent mean + SEM. * $P < 0.05$ and ** $P < 0.01$ vs. control group; $n = 3$. A one-way ANOVA followed by Tukey–Kramer post hoc test for neurons or Bonferroni test for glial cells was used to compare each condition. (B) Representative images of cultured neurons treated with vehicle (Control), 100 μM CORT (CORT) and 100 μM CORT + 1 μM OT (CORT+OT). Scale bar, 50 μm. (C) Quantification of TUNEL-positive cells with CORT treatment. Data represent mean + SEM. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. CORT group; $n = 8$. Welch's ANOVA followed by Games-Howell test was used to compare each condition.

Figure 3. OT failed to protect primary mouse hippocampal neurons from OTR-KO mice against CORT-induced apoptosis. (A) Representative images of OTR-KO hippocampal neurons treated with vehicle (Control), 100 μM CORT (CORT) and 100 μM CORT + 1 μM OT (CORT+OT) for 24 h. Scale bar, 50 μm. (B) Quantification of TUNEL-positive cells with CORT and OT treatment. Data represent mean + SEM. * $P < 0.05$ vs. control group; $n = 6$-7. Welch's ANOVA followed by Games-Howell test was used to compare each condition.

Fig. 1



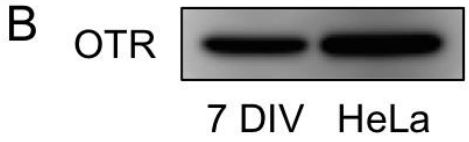


Fig. 2

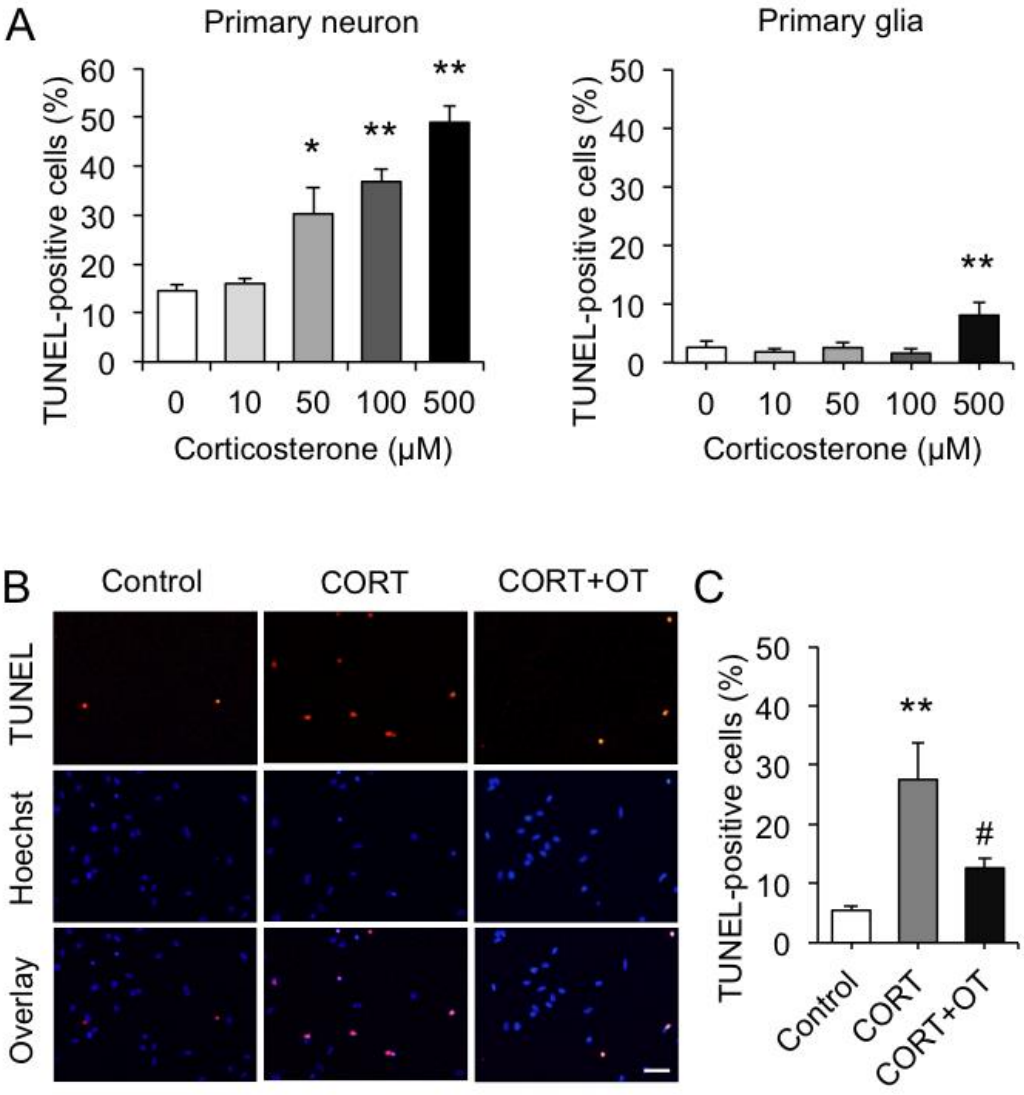
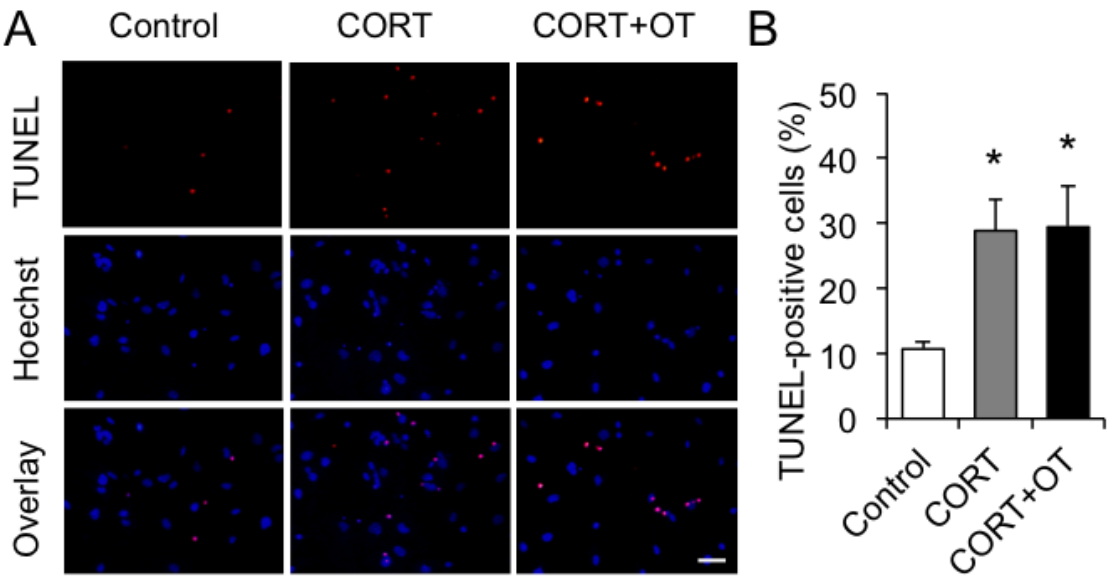

A
Primary neuron
TUNEL-positive cells (%)
0
10
20
30
40
50
60
*
**
**
0
10
50
100
500
Corticosterone (μM)
Primary glia
TUNEL-positive cells (%)
0
10
20
30
40
50
**
0
10
50
100
500
Corticosterone (μM)
B
Control
CORT
CORT+OT
TUNEL
Hoechst
Overlay
C
TUNEL-positive cells (%)
0
10
20
30
40
50
**
#
Control
CORT
CORT+OT

Fig. 3


A
Control
CORT
CORT+OT
TUNEL
Hoechst
Overlay
B
TUNEL-positive cells (%)
50
40
30
20
10
0
*
*
Control
CORT
CORT+OT