https://doi.org/10.1016/j.neuroscience.2016.07.005

Repeated treatment with oxytocin promotes hippocampal cell proliferation, dendritic maturation and affects socio-emotional behavior

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Abstract

Rewarding social behaviors including positive social interaction and sexual behaviors are shown to regulate adult neurogenesis, but the underlying biological mechanisms remain elusive. Oxytocin, a neurohypophysial hormone secreted after exposure to social interaction or sexual behaviors, has a profound role in the formation of social bonding and regulation of emotional distress. While the acute effect of oxytocin was usually studied, relatively scarce evidence showed the behavioral consequence of repeated oxytocin treatment. The purpose of the current study is to investigate the effect of repeated oxytocin treatment on hippocampal cell proliferation, dendritic maturation of new born neurons and social/emotional behaviors. Adult male Sprague-Dawley rats received treatment with either vehicle or oxytocin (1 mg/kg) daily for two weeks. Behavioral tests revealed that oxytocin increased social behaviors and reduced the anxiety- and depressive- like behaviors. Cell proliferation, differentiation and the dendritic complexity of new born neurons in the hippocampus were promoted by oxytocin treatment. Depression- and anxiety-like behaviors were induced by repeated treatment of corticosterone (40 mg/kg) for two weeks while oxytocin treatment reversed the behavioral disturbances. Suppression of cell proliferation caused by corticosterone was reverted by oxytocin treatment in which cell proliferation, cell differentiation, and dendritic complexity increased. The present findings reveal that oxytocin not only enhances cell proliferation, but also promotes the development of the new neurons which is associated with the induction of positive emotional and social behaviors. The results also suggest that oxytocin may be a potential therapeutic agent for treatment of emotional and social dysfunction.

Keywords: Oxytocin; hippocampal cell proliferation; neurogenesis; dendritic complexity; depression-like behaviors; anxiety-like behaviors.

Introduction

The neurohypophysial hormone oxytocin (OXT) is released both centrally and peripherally under rewarding social stimulation such as social interaction with desirable individuals, sexual activity, and suckling (McNeilly and Ducker, 1972). After being synthesized in magnocellular neurons in the hypothalamic supraoptic and paraventricular nuclei, oxytocin is released into the bloodstream via neuronal connections within hypothalamic and limbic regions (Neumann and Landgraf, 2012). Oxytocin is also released from dendrites and perikarya as a neuromodulator and reaches the oxytocin receptors (OXTR) through diffusion via extracellular fluid and ligand binding (Landgraf and Neumann, 2004). As suggested by the regulators of oxytocin secretion, oxytocin is closely associated with social and emotional behaviors. For instance, administration of oxytocin was shown to improve social attachment, show anxiolytic effect on human subjects and may protect an individual from the negative consequence of stress. which implicates the potential therapeutic value of oxytocin in emotion-related disorders(Landgraf and Neumann, 2004; Leuner et al., 2012) . Despite the established roles of oxytocin in regulation of social and emotional behavior, the biological mechanism of its influence remains obscure.

Adult neurogenesis, which describes the production of new functional neurons in the adult CNS, is a complex dynamic process. Internal and external environmental factors can affect the regulation of neurogenesis at different stages including proliferation, migration, differentiation and integration into the existing neural circuitry (Ming and Song, 2005; Song et al., 2012). Neurogenesis can be found predominantly in two distinct regions in the central nervous system (CNS), namely, the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (Song et al., 2012). Cell proliferation, the first step in the neurogenesis process, refers to one complete cell division cycle that can be detected using BrdU which is a marker for DNA synthesis. Once new cells have been born, they differentiate into mature neuronal phenotype and make synaptic connections in the existing circuitry (Malberg, 2004). External factors such as physical exercise and stress have shown to increase and decrease cell proliferation respectively; therefore affecting neurogenesis (Song et al., 2012). Different lines of evidence demonstrate the functional significance of neurogenesis including

learning, sexual behaviors and social behaviors (Leuner et al., 2006; Deng et al., 2010). Since neurogenesis has an impact on different emotional and cognitive behaviors, it is expected that the understanding of neurogenesis at different stages of the neurogenesis process would shed light on pathophysiology of emotional and cognitive disorders, and may provide insight on developing novel clinical treatment (Winner et al., 2011).

As both neurogenesis and oxytocin are shown to regulate social and emotional behaviors, it is possible that they may have intricate interaction to regulate the socio-emotional behaviors. One of the evidence to support the relationship between neurogenesis and oxytocin is that postnatal neuronal growth was observed in the vasopressin and oxytocin-containing nucleus of the pig hypothalamus (Rankin et al., 2003). Recently it was revealed that acute and sub chronic (7 day) treatment of oxytocin increases ventral hippocampal cell proliferation, and the neurogenesis-stimulating effect could be found under stressful situation (Leuner et al., 2012). Meanwhile another *in vitro* study showed that oxytocin treatment promotes neuronal differentiation of adipocyte-derived stem cells (Jafarzadeh et al., 2014). To further explore the effect of oxytocin on cell proliferation and the display of emotional and social behavior, the present study tested 1) the effect of repeated exposure to oxytocin on cell proliferation and dendritic maturation of new neurons; 2) the effect of prolonged exposure to oxytocin on social interaction and depression/anxiety-like behaviors and 3) the potential therapeutic effect of oxytocin in a corticosterone-induced depression- and anxiety-like behavior animal model (Gregus et al., 2005; Brummelte et al., 2006). Since oxytocin signaling may play key roles in the underlying mechanisms of pro-neurogenic effect of socially rewarding behaviors, the molecular mechanisms triggered to promote neurogenesis should be further investigated.

Experimental procedures

Experimental design

Young adult male Sprague-Dawley (SD) rats (31 rats in total, 7-8 weeks of age, 200 to 220 g) were housed in pairs in polycarbonate cages. They were fed *ad libitum* and the room was maintained on a 12-hour alternating light-dark cycle and at 23-25°C. The housing and behavioral procedures were approved by the Animal Subject Ethnics Sub-Committee of the Hong Kong Polytechnic University.

Experiment 1: To study the effect of repeated oxytocin treatment on emotional behaviors and cell proliferation, SD rats (n= 6 rats/group) were injected intra-peritoneal with oxytocin (1 mg/kg/day; Bachem Americas) or equal volume of vehicle (normal saline). Treatment was given around 17:00 every day for 14 consecutive days. The dose of oxytocin used has shown to stimulate neurogenesis in the dentate gyrus in previous studies (Leuner et al., 2012). At day 12-14, 50 mg/kg/day of BrdU was intra-peritoneal injected to label proliferative cells. Behavioral tests were performed at day 14 and 15, which was followed by perfusion at day 16.

Experiment 2: Animals were divided into three groups (n= 6-7 rats/group): 1. Control group (n = 6) with intra-peritoneal injection of vehicle (normal saline) and subcutaneous injection of propylene glycol which was the vehicle used to dilute the corticosterone in groups 2 and 3; 2. Corticosterone treatment group (n = 7) with subcutaneous injection of corticosterone (40 mg/kg) and 3. Oxt+Cort group (n = 6) which received both corticosterone (40 mg/kg) subcutaneously and intra-peritoneal injection of oxytocin (1 mg/kg). The co-administration of oxytocin and corticosterone was performed around 17:00 every day for 14 days. In previous studies, repeated administration of corticosterone at 40 mg/kg showed to reliably induce depressive-like behavior in rats (Kalynchuk et al., 2004; Gregus et al., 2005; Brummelte et al., 2006). In addition, a dose of 40 mg/kg corticosterone has been reported to diminished cell proliferation in the dentate gyrus and subventricular zone (Cameron and Gould, 1994; Leuner et al., 2012; So et al., 2012). Behavioral analysis was carried out at day 14 and 15, followed by transcardial perfusion at day 16.

Behavioral Tests

Forced Swimming Test (FST)

Swimming sessions were conducted in individual transparent cylinders (40 cm height x 30 cm diameter) filled with water at a depth of 30 cm at room temperature. The cylinders were deep enough to avoid that the rats touch the bottom of the cylinder to support themselves. Two swimming sessions were conducted: an initial 15-min pretest for habituation, followed 24 hours later by a 10-min test. Test sessions were video recorded from a front view for scoring later by an observer blinded to the treatment. The behaviors scored in the FST included: (1) time spent immobile – floating in the water without struggling, minimal movement to keep from drowning, with only the necessary movement to keep the head above the water; (2) time spent swimming – making active motions, such as moving around in the cylinder, more than necessary to merely keep the head above water and less movements than those shown when climbing/struggling; and (3) time spent climbing/struggling – showing vigorous active movements with the forelimbs and hind limbs breaking the surface of the water in an clear attempt to get out of the cylinder. The depression like behavior is exhibited when rats spent more time immobile during the test (Gregus et al., 2005; Lau et al., 2011b). After each session, the rats were removed from the cylinders, dried with cloth towels and returned to their home cages.

Open Field Test (OFT)

An arena (72 cm length x 72 cm wide x 40 cm deep; lighting of 550 lux) was used for the OFT to evaluate anxiety in rats when exposed to an unfamiliar environment. A video camera was mounted above the open field adjusted to cover the whole test area. The test was video recorded for scoring later by an observer blinded to the treatment. In the test, each of the animals was put into the arena for 10 minutes and was allowed to explore the arena freely.

For the analysis, the arena was divided into 16 equal squares on screen and the numbers of squares crossed by the rat's neck were counted to measure the locomotor activity. The measurements recorded included: (1) peripheral locomotor – the time spent walking close to the walls of the test field (thigmotaxis); (2) central locomotor activity – time spent in the central 36 cm x 36 cm area of the field. Increased time spent in the

central area or decreased time of latency to enter the central area are indications of anxiety-like behavior (Prut and Belzung, 2003; Gregus et al., 2005; Airan et al., 2007; Gamberini et al., 2015).

Social Interaction Test (SIT)

The test was conducted similar to previous studies (Becker et al., 1999; Gregus et al., 2005). Briefly, an open-field arena of 72 cm length x 72 cm wide x 40 cm deep, with an open top was used. A camera was mounted above the open field arena and adjusted to cover the whole test arena. One day before the test, the animals were familiarized with the arena through a 10-minute exposure. In the test, pairs of unfamiliar rats under the same treatment and with a weight difference no larger than 20 g were introduced at the same time into opposite side corners of the arena. The test was video recorded for later analysis by an observer blinded to the treatment. The total number of social interactions was scored and the interactions were categorized into: (1) non-aggressive behavior – sniffing, following, crawling, social play, grooming; (2) aggressive/defensive behavior – kicking, boxing, wrestling, biting. An indication of increase anxiety is reflected in decreased social interaction between the rats.

Animal Perfusion

On day 16, rats were administered with a lethal dose of anesthetic drug (sodium pentobarbital, 200 mg/kg, intra-peritoneal injection) and transcardially perfused with 4% paraformaldehyde. Before perfusion, 1 ml blood was collected to measure the corticosterone plasma level. Brains were post-fixed overnight at 2-8°C. After post-fixation, brains were transferred to 30% sucrose solution in 0.1M phosphate-buffered saline (PBS) for cryoprotection and stored at 2-8°C (Hillerer et al., 2014). The brain tissue was sectioned into 40µm thick-coronal sections in 1-in-12 series by a cryostat (Shandon Cryotome E, Thermo Electron Corporation). The sections were stored in cryoprotectant at -20°C until immunostaining was performed.

Immunohistochemistry

Immunoperoxidase staining

Immunostaining of DCX positive cells was performed according to previously published reports (Lau et al., 2011a). In brief, affixed brain sections were rehydrated with 0.1M PBS and subjected to antigen retrieval at 80°C for 20 minutes in sodium citrate buffer (pH 6.0). After being rinsed in PBS in three charges, the sections were incubated with rabbit anti-DCX antibody (1:300, Cell Signaling Technology) at room temperature overnight. Then the sections were washed thrice with PBS and incubated in biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories) for 2 hours at room temperature. Staining signal was visualized with Avidin-biotin complex solution (Vector Laboratories) and diaminabenzidine (Dako). DCX-positive cells were stained in brown.

The immunostaining of BrdU cells was conducted similarly as the immunostaining protocol for DCX cells, but the antigen retrieval steps were modified as follows. Antigen retrieval was carried out by inclubating the slides at 80°C for 25 min in preheated sodium citrate buffer. After that, the slides were incubated in 2N HCl at 40°C for 30 min to allow DNA denaturation. Neutralization of the acid was carried out by incubating the slides with 0.1M borax buffer for 15 min at room temperature. After neutralization, the slides were washed with PBS before inculcation with the primary antibody. The primary and secondary antibodies used included 1:300 mouse anti-BrdU antibody (Roche) and 1:200 goat anti-mouse antibody (Dako) respectively (Lau et al., 2011a).

Immunofluorescence

Immunofluorescence staining was applied to detect the presence of BrdU and DCX positive cells. BrdU serves as a marker of proliferative cells while DCX is expressed in immature neurons. The co-immunostaining was performed based on the protocols reported in previous studies (Meshi et al., 2006; Ziv et al., 2006; Lau et al., 2011a). In order to allow anti-BrdU antibody to access the incorporated BrdU, the affixed brain sections were rehydrated in PBS first and incubated in 2M HCl for 30 min at 37°C. Following rehydratation, the sections were incubated in both rat anti-BrdU antibody (1:100, Abcam,) and rabbit anti-DCX antibody (1:300, Cell Signaling Technology) overnight at room temperature. Subsequently, goat anti-rat and goat anti-rabbit antibodies (Alexa Fluor 568 and 488 respectively, 1:200, Life Technologies) were

applied on the sections and incubated for 2 hours. The fluorescent signal was observed and analyzed under a fluorescence microscope Nikon series Eclipse H600L.

Cell counting

Slides were coded prior quantification of cells and analyzed by an experimenter blinded to the treatment. The stereology method used was the fractionator method since the number of cells can be estimated more precisely. This method is more efficient statistically and no estimation of the global volume of the brain region of interest is needed. In the fractionator method, the number of cells is counted within all unbiased counting spaces and multiplied by the reciprocal value of the sampling probability (Schmitz and Hof, 2005; Altunkaynak et al., 2011). Therefore, the estimated number of cells was reported as an absolute number.

Quantification of proliferative cells and immature neurons expressing DCX marker: To measure the number of hippocampal cells that showed proliferation and the number of cells expressing the immature neuron marker DCX, the total number of BrdU-positive cells and DCX positive cells in dorsal area of the dentate gyrus (DG) was counted on every 12^{th} section of the brains using the StereoInvestigator system (version 11, MBF Bioscience) which consisted of a camera interfaced with a Nikon series Eclipse H600L microscope coupled to a motorized stage. Only the DCX expressing cells with tertiary dendrites or above order were counted DCX positive cells. Labeled cells were identified using a 40x objective, the optical fractionator probe and dissector height of 25 µm Six sections per rat were used to count the cells in the dorsal hippocampal area of the dentate gyrus. The number of BrdU-positive cells and DCX-positive cells was presented as mean ± SEM.

Phenotypic analysis: Brain sections co-expressed with anti-BrdU and DCX were observed at 40x magnification using an epifluorescent microscope Nikon series H600L. To determine neuronal differentiation, the number of DCX-positive cells out of 50 randomly selected BrdU-positive cells was counted. When a BrdU positive cell was observed, screening for DCX positive signal was performed by switching the filter on the microscope to detect the fluorescence signal (Meshi et al., 2006). The results were expressed as 'Differentiation Index', which was the proportion of DCX-positive cells over the 50 BrdU+ cells (Plümpe et al., 2006; Hattiangady and Shetty, 2010; Song et al., 2012).

Plasma corticosterone level

The concentration of corticosterone in plasma was measured in the animals from experiment 2. The corticosterone level was determined using a corticosterone ELISA kit following the instructions of the manufacturer (Enzo Life Sciences).

Sholl Analysis

In order to measure the spatial distribution and complexity of the dendritic tree, Sholl analysis by tracing the selected neurons under 20x magnification using NeurphologyJ plugin for ImageJ software was carried out (Gutierrez and Davies, 2007; Meijering, 2010). Ten DXC-positive cells per animal were randomly chosen to have 60 neurons per treatment group (Ramirez-Rodriguez et al., 2011). The criteria followed to select the cells for Sholl analysis included: cells exhibiting tertiary or higher arborization, relatively untruncated dendrites and perpendicularly oriented (Meijering, 2010; Lau et al., 2011a). Series of concentric circles were created around the soma, which was identified on the image in the software. The starting radius, step size and ending radius were set as 10 micrometer, 10 micrometer and 200 micrometers respectively. The number of times that the dendrites intersected the traced circles, known as the intersection number, was counted and the distance from the soma to each intersection was measured. The Sholl analysis was carried out by an experimenter blinded to the treatment. A high intersection number is an indication of a more complex dendritic branching (Lau et al., 2011a; Xiao et al., 2015).

Data analysis

Data was analyzed by SPSS software. Student's t-test was used to compare difference between control and oxytocin-treated groups. To compare the three treatment groups in experiment 2, one-way ANOVA was use and followed by Tukey post-hoc test. When the assumptions of normality and homogeneity of variance were not met, non-parametric tests were used in experiment 2 namely Kruscal-Wallis test followed by Mann-Whitney U post-hoc test were carried out. Statistical significance was defined as $p < 0.05$. All data were illustrated by mean ± SEM.

Results

Experiment 1: Oxytocin reduced depression and anxiety-like behavior, and promoted cell proliferation

In the statistical analysis of the behavioral tests, the assumptions of normal distribution and homogeneity of variance were met and t-student was carried out without any adjustment except for the locomotion analysis in the OFT in which the homogeneity of variance was not met; therefore, adjustment was required in the analysis. FST showed a statistically significant difference between treatment (mean = 113.16 sec. ± SEM = 15.18) and control group (mean = 192.33 sec. ± SEM 24.43) in the time spent in floating ($t(10) = 2.752$, $p = 0.020$, Fig 1A). The climbing behavior between the control treatment (mean = 105.50 sec. ± SEM = 31.71) and oxytocin group (mean = 128.33 sec. ± SEM = 30.48) did not showed any statistically significant difference ($t(10) = -0.519$, $p = 0.615$, Fig 1B). Furthermore, the swimming behavior in the control (mean = 302.16 sec. ± SEM = 18.07) and treatment group (mean = 358.50 sec. ± SEM = 22.22) did not show any statistically significant difference ($t(10) = -1.966$, $p = 0.078$, Fig 1C). When compared to the control group, less time was spent on floating in treatment group, which indicates a decrease in depression-like behavior. Statistically significant difference in number of positive social behavior ($t(10) = -2.956$, $p = 0.014$, Fig 1D) was found between treatment (mean = 24.9 ± SEM = 3.71) and control group (mean = 11.9 ± SEM = 2.3). When compared to control group, treatment group showed a higher frequency of positive social behavior. No aggressive social behavior was observed in all animals. Result from OFT indicated statistically significant difference in time spent in center of the OFT arena ($t(10) = -2.403$, $p = 0.037$, Fig 1E) between the treatment (mean = 22.50 ± SEM = 3.07) and control group (mean = 13.83 ± SEM = 1.88). When compared to control group, treatment group spent significantly longer time in the center, which showed the anxiolytic effect of oxytocin. Similarly, statistically significant difference was observed in the distance traveled in the OFT ($t(7.352) = -4.451$, $p = 0.003$, Fig 1F). The animals in the OXT treated group traveled more distance (mean = 27.97 ± SEM 2.81) than the animals in the control group (mean = 13.98 ± SEM 1.40) which also demonstrate the anxiolytic effect caused by oxytocin.

In the statistical analysis of the cell proliferation and cell differentiation data, the assumptions of normal distribution and homogeneity of variance were met for the BrdU data and BrdU-immunoreactive cells with DCX expression, but homogeneity of variance for the DCX data was not met. Therefore, the t-student test for the DCX data was carried

out adjusting for equal variances not assumed. Significant difference was found between control and experimental group (mean = 6470.33, ± SEM = 832.22 and mean = 10496.33 ± SEM = 1348.58, respectively) in terms of the number of BrdU-positive cells ($t(10) = -2.541$, $p = 0.029$, Fig 2A-C) and the number of DCX positive cells (mean = 7438.75 ± SEM = 1100.70 and mean = 11030.75, ± SEM = 323.57, respectively; $t(3.51) = -3.131$, $p = 0.042$, Fig 2A-C), which indicated that oxytocin treatment promoted cell proliferation of proliferative cells in the hippocampus and the production of new neuroblasts. However, there was no significant difference ($t(10) = 0.056$, $p = -2.160$, Fig 2D-F) in the proportion of BrdU-immunoreactive cells with DCX expression of the treatment group (mean = 0.6367 ± SEM = 0.02) and control group(mean = 0.6517 ± SEM = 0.03).

Dendritic complexity of immature neurons was analyzed by Sholl analysis. There was a significant difference between the two groups in the number of intersections at various distances from soma (Fig 3A-C). The number of intersections of oxytocin group was significantly higher than that of control group. This indicated that oxytocin treatment significantly promotes the dendritic maturation of new neurons.

Experiment 2: Oxytocin reversed the behavioral disturbance and suppression of cell proliferation induced by hypercorticosteronemia

In the statistical analysis, the assumptions of normal distribution and homogeneity of variance were checked. When the assumptions were not met, Kruskal-Wallis followed by Mann-Whitney post hoc test was carried out and the results of the non-parametric statistical analysis were expressed accordingly. Animals from the corticosterone group and the Oxt-Cort treated group did not show any sickness behavior that might be related to the drugs administered. In addition, the mortality rate in all treatment groups was zero. Behavioral tests showed that the antidepressant and anxiolytic effect of oxytocin could reverse the depression and anxiety caused by high dose corticosterone treatment. In FST, the time spent floating showed statistically significant difference among the three groups evaluated ($F(2,16) = 10.645$, $p = 0.001$, Fig 4A). The total floating time of rats with corticosterone treatment was significantly higher than the control ($p = 0.002$) and the group received both corticosterone and oxytocin treatment ($p = 0.007$). The climbing

behavior showed no statistically significant difference among the three groups ($X^2(2)$ = 2.559 , p = 0.278, Fig 4B). Statistically significant difference was observed in the swimming behavior among all the groups (F(2,16) = 6.161 , p = 0.008, Fig 4C). The control and corticosterone groups showed statistically significant difference (p = 0.016). No significant difference was observed between the control and Oxt+Cort group. Similarly, the total number of positive social interaction in SIT of corticosterone group was shown to be significantly lower than the control (U < 0.001, p = 0.004;), and the co-treatment group showed a higher number of positive social interaction compared to the control group (U = 1.50, p = 0.009; $X^2(2)$ = 12.767, p = 0.002, Fig 4D). The results showed that oxytocin reversed the induced depression- and anxiety-like behaviors. In OFT, animals received both oxytocin and corticosterone treatment showed a significantly longer time spent in the center of the arena than the control and corticosterone-treated rats (U = 1.00, p = 0.032; U = 12.00, p = 0.788, respectively) ($X^2(2)$ = 7.192, p = 0.027, Fig 4E). However, no significant difference was found between the control and corticosterone group. Furthermore, no statistically significant difference was observed in the locomotion behavior among the groups in the OFT (F(2,16) = 0.376 , p = 0.693, Fig 4F). Regarding the concentration of corticosterone in plasma, the control group showed high levels of corticosterone when compared with the co-treatment and corticosterone group (detection limit of 26.99 pg/ml, intra-assay variability: low, 8.0 %CV; medium, 8.4 %CV; high 6.6 %CV; inter-assay variability: low, 13.1 %CV; medium 8.2 %CV; high 7.8 %CV; Enzo, catalog No. ADI-900-097). Statistical significant difference was observed among all the groups (F(2,13) = 37.502, p < 0.001, Fig 4G). The control group (mean = 395.88 ± SEM = 54.84) and the Oxt-Cort group (mean = 5.32 ± SEM = 1.40) showed statistically significant difference (p < 0.001) as well as the Cort group (mean = 3.34 ± SEM = 0.34) when compared with the control group (p < 0.001).

Statistically significant difference was observed in the number of DCX positive cells among all the groups ($X^2(2)$ = 9.609, p = 0.008).Corticosterone treatment significantly reduced the number of DCX-positive cells in the hippocampus (U < 0.001, p = 0.004, Fig 5A) while the rats co-treated with oxytocin and corticosterone has a significantly higher number of DCX-positive cells (U = 1.50, p = 0.009, Fig 5A). Sholl analysis of the DCX

positive cells in corticosterone-treated rats showed a significantly lower number of intersections from both the control and oxytocin groups at various distances from soma (Fig 5B-H). These findings revealed that oxytocin could prevent the suppression of hippocampal cell proliferation caused by corticosterone in the hypercortisolemia treatment model.

Discussion

Taking into account that the half-life of oxytocin is 2 minutes in blood and 20 minutes in the cerebrospinal fluid in rats and the administration regimen for 14 consecutive days used in the present study, the observations made in the oxytocin treatment reflected the repeated effects rather than acute effects of oxytocin (Ludwig and Leng, 2006; Leuner et al., 2012). The present study revealed that repeated exposure to oxytocin could significantly enhance cell proliferation, promote dendritic maturation of new neurons and reduce depression- and anxiety-like behaviors. Furthermore, oxytocin could prevent the disruption in cell proliferation and behaviors caused by high dose corticosterone treatment.

As expected and reported in previous studies, suppression of the hypothalamo-pituitary-adrenal (HPA) axis was observed in experiment 2 after repeated exogenous administration of corticosterone which leads to low concentration of corticosterone in plasma. The negative feedback observed after treatment is due to the prolonged exposure to high concentration glucocorticoid that exceeds the concentration required for physiological homeostasis (Young et al., 1995; Andrews et al., 2012).

A significant correlation was also found between the dendritic complexity of DCX-positive cells and social interaction. These findings, collectively, suggest the antidepressant and anxiolytic effect of exogenous oxytocin and the involvement of the neurohypophysial hormone in the regulation of emotional behaviors.

The stimulatory effect of oxytocin on hippocampal cell proliferation was shown by a study conducted by Leuner *et al*. (Leuner et al., 2012), in which cell proliferation in the ventral hippocampus was stimulated after acute or repeated (7 days) administration of oxytocin. The present study provides further information by using different experimental design from Leuner's study. First, since the animals in the previous study were sacrificed three weeks after the treatment, it is possible that the BrdU labeling of cells reflect a mixed effect of oxytocin on both proliferation and survival. In the present study, we sacrificed the rats immediately after the behavioral test, which may better reflect the effect of oxytocin on cell proliferation. Second, as differentiation and maturation of new neurons is another important stage in neurogenesis, the dendritic morphology of new

neurons (indicated by DCX staining) was studied in the present study. Third, the oxytocin treatment was maintained for a prolonged period (two weeks), which will reflect the effect of oxytocin in a repeated treatment manner.

Interestingly, the present study shows that repeated oxytocin treatment increases cell proliferation in the dorsal hippocampus, which is different from the previous finding (Leuner et al., 2012) and the difference may be due to the prolonged treatment period. Furthermore, it is shown that OXT has a stimulating effect on dendritic development of new neurons. With elaborated dendrites, the number of possible synaptic connection within the molecular layer of the dentate gyrus can be increased, which are essential for the integration of the new neurons into the existing hippocampal neural circuit (Lau et al., 2013). The effect of oxytocin on cell proliferation was also shown in corticosterone treated animals which displayed suppressed cell proliferation and dendritic growth of immature neurons. Stress, which is a pivotal trigger in depression, has shown to cause reduction of dendritic length and distal dendritic branching density in immature granule cells in the hippocampus (Sousa et al., 2000; Bessa et al., 2008). In previous studies, it was suggested that synaptic loss as a consequence of dendritic degeneration could be the cause of behavioral deficits observed in stressed animals (Sousa et al., 2000). Furthermore, it has been shown that the effect of stress on dendritic length and complexity can be reverted by antidepressants such as fluoxetine and imipramine (Lussier et al., 2013). Therefore, the effect of oxytocin on dendritic complexity shows antidepressant like effect also at dendritic developmental level when reverting the dendritic degeneration induced by corticosterone. Taken together, these results suggest that OXT stimulates cell proliferation with higher dendritic complexity and may have a positive influence on integration, the last stage of neurogenesis. With the effect to increase the number of new cells and promote dendritic growth, oxytocin may regulate emotional and social behaviors, and exert the therapeutic effect on animals with emotional disturbance. However, as the net increase of new neurons will be affected by the survival rate, future study of oxytocin on neuronal survival would provide further understanding on its neurogenic effect.

Since hippocampus plays important roles in emotion, hippocampal neurogenesis was hypothesized to be involved in emotional regulation and mood disorders (Ruan et al.,

2014). Animal studies showed that rats with impaired adult hippocampal neurogenesis had increased depression-like behavior, elevated anhedonia level (Snyder et al., 2011), and increased anxiety-like behaviors (Revest et al., 2009). Impaired neurogenesis was also found to decelerate the mice's recovery of circulating glucocorticoid level after exposing to stress (Snyder et al., 2011). Furthermore, several animal studies have shown that therapeutic effect of antidepressants was related to neurogenesis (Santarelli et al., 2003). To elucidate the effect of oxytocin on emotional behaviors in both normal and stressed animals, we used FST, OFT and SIT in this study. The role of oxytocin in the regulation of social behavior, anxiety and the HPA axis has been well established (Parker et al., 2010; Ring et al., 2010). In addition, dysregulation of oxytocin has been associated with emotional distress and impairment in social interaction which are frequently observed characteristics of depression (Parker et al., 2010; Yan et al., 2014). The results of present study showed that repeated oxytocin treatment exerted effect on emotions by reducing depressive-like and anxiety-like behaviors. These findings were in line with previous findings on the antidepressant and anxiolytic effect of oxytocin. For instance, an animal study showed that acute and repeated treatments (10 days) of oxytocin at 0.25-1 mg/kg/day were effective in reducing depression-like behaviors in normal animals (Arletti and Bertolini, 1987). In another study, central (0,3 μg) and systemic (30 mg/kg) administration oxytocin significantly reduced the immobility time in mice in the tail suspension test showing improvement in depressive-like behavior (Ring et al., 2010). Furthermore, acute i.p. administration of oxytocin (1 μg/kg) showed antidepressant activity in rats (Nowakowska et al., 2002). Another animal study showed attenuated high anxiety-related behaviors of female rats after 6 days of oxytocin treatment (Slattery and Neumann, 2010a). Our results were in line with these studies, and suggested that oxytocin has antidepressant and anxiolytic effect by increasing cell proliferation.

However, some studies carried out on the effect of oxytocin on depressive-like behavior show conflicting results. In a study carried out by Yan et al., 2014, acute central administration of oxytocin improved depressive-like behavior while acute peripheral administration of oxytocin (1, 2 and 4 mg) did not have any effect on the depression-like behavior in the FST (Yan et al., 2014). Similar results were found in the study carried out

by Slattery and Neumann, 2016 in rats. They found that neither acute (1µg) nor chronic (10 ng/h, 6 days) central administration of OXT showed any effect on the forced swimming test meaning that the depressive state was not attenuated by OXT exposure (Slattery and Neumann, 2010a). As shown in the studies mentioned above, OXT has shown to display antidepressant properties in several preclinical studies while some other studies have shown no alteration in depressive-like behavior in the forced swimming test either in acute or chronic administration as well as when administered centrally or peripherally. The discrepancy in the studies opens an invitation to explore further the regulation of the oxytocinergic system in depression-like behavior since no consistency in the role of OXT has been found in animal studies (Rotzinger et al., 2010; Slattery and Neumann, 2010a, 2010b). In addition, the mechanistic pathways activated by oxytocin at central and peripheral level should be explored more in detail to provide more conclusive evidence of the role of oxytocin and its anti-depressant properties (Slattery and Neumann, 2010b).

Emotion was found to be an important regulator of social behavior (Blair et al., 2004), we tested whether positive social behavior is induced by repeated oxytocin treatment. The purposes of SIT are twofold: assessing anxiety in a social situation and assessing positive/negative social behaviors. The results showed that oxytocin enhanced social interaction, which is in-line with a previous study (Witt et al., 1992), in which the social contact of rats with oxytocin infusion was doubled. Interestingly, strong correlation between dendritic complexity and social interaction was found in the present study. In other words, the dendritic maturation is associated with the display of pro-social behaviors and thus the maturation of new neurons may exert effect on social interaction. Another study on female prairie voles showed that decrease in social interaction could lead to decreased neurogenesis (Lieberwirth et al., 2012). Taken together, although causal relationship between cell proliferation, dendritic maturation and social interaction could not be established in the present study, it is possible that neurogenesis may have a reciprocal interaction with social interaction, which is shown in reproductive behaviors and neurogenesis (Mak et al., 2007; Lau et al., 2011b). Furthermore, OXT may alter social interaction indirectly by altering the emotional state of animals, as emotion mediates social behaviors.

The finding of the present study would provide evidence to support the use of oxytocin in improving positive social behavior, decreasing anxiety-like behavior and increasing cell proliferation. Therefore, oxytocin may have therapeutic value in psychiatric illnesses (Matsuzaki et al., 2012). On the other hand, as rewarding social interaction and sexual stimulus could increase the release of oxytocin and promote neurogenesis, oxytocin may have a role in mediating the neurogenic effect of rewarding social stimulation. If this is the case, it may be able to explain why pro-neurogenic stimuli are usually rewarding to the individual. The rewarding effect of OXT has been previously studied using low doses of MDMA (ecstasy) in which increased plasma levels of OXT in rodents were observed. The results suggest that OXT could also play a role in the rewarding effects of MDMA which supports the action of OXT in reward and positive hedonic states (Slattery and Neumann, 2010b). Chronic peripheral administration of oxytocin has led to positive hedonic state. Nevertheless, no positive hedonic state was observed with intracerebral OXT administration. The lack of consistency in the effects of OXT in animal studies requires more thoroughly investigation to elucidate the mechanism of action of OXT. In this sense, Eliava et al. (2016), studied the OXT signaling pathway of OXT releasing neurons and identified a new population of OXT neurons modulating different biological processes (Eliava et al., 2016). Furthermore, Jurek et al. (2015) investigated the regulatory signaling cascade of OXT in stress response. They found that gene transcription of Crf gene was regulated by OXT via the translocation of CRTC3 (Jurek et al., 2015). Despite the effort to elucidate the signaling pathways involved in the effects caused by OXT at central and peripheral level, more studies are needed to construct a clearer picture of the biological role of OXT and its potential benefit in the treatment of depression.

The current finding might provide an alternate explanation for therapeutic value of social interaction in human. Studies showed that positive social stimuli and social experience increased the level of circulating oxytocin (Uvnäs-Moberg, 1998; Kikusui et al., 2006; Heinrichs and Domes, 2008). The stimulation in neurogenesis by oxytocin may benefit patients with mood or social disorders by reducing negative emotion and promoting social interaction or cognition, which are common problems to be encountered in treatment of mood disorder patients (Chen et al., 2015). The potential involvement and

application value of neurogenesis in rehabilitation of clients with psychiatric illnesses has gained increasing attentions (Eisch et al., 2008). Further investigation is needed to explore the causal relationship between neurogenesis, social interaction and emotion, while thorough understanding of the relationships would inform the clinical practice on effective psychiatric treatment for patients with mood disorders.

Figure Legends

Figure 1: Oxytocin decreased depression-like and anxiety-like behavior. (A): Oxytocin significantly reduced the time spent on floating in the forced swimming test. (B): Oxytocin group shows no difference in climbing behavior in the forced swimming test. (C) Oxytocin group shows no difference in the time spent swimming in the forced swimming test. (D) Oxytocin group shows significantly higher number of social interaction than control group. (E): Anxiety-like behavior is reduced by oxytocin, which is indicated by the significant increase in time spent in the central arena in the open field test and increased locomotion (F). Values expressed in mean ± SEM, *:$p<0.05$ when compared to control group. Student's *t*-test.

Figure 2: Oxytocin enhances cell proliferation, but not cell differentiation in hippocampus. A: Rats with OXT treatment had significantly higher number of BrdU-positive and DCX-positive cells in the dentate gyrus. Photomicrographs of control (B) and oxytocin-treated (C) animals showed that the number of DCX-positive cells in the dentate gyrus is increased by oxytocin. (D): No effect of Oxytocin treatment was found on the differentiation of newly proliferative cells. (E): Immunofluorescent photomicrographs showing the co-labeling of cells with BrdU (red) and DCX (green). (F): A BrdU-positive cell did not co-label with DCX in the dentate gyrus. Values expressed in mean ± SEM, *:$p<0.05$ when compared to control group. Student's *t*-test. Scale bar: 100μm.

Figure 3: Oxytocin promotes dendritic growth of immature neurons. (A) & (B): Photomicrographs and corresponding tracing of DCX-positive new neurons traced by ImageJ from control (A) and oxytocin-treated (B) rats. (C): Sholl analysis of DCX-positive cells of rats from control and oxytocin group. Oxytocin significantly promotes dendritic arborization at the distance 90-180 μm from the soma. Values expressed in mean ± SEM, *:$p<0.05$ when compared to control group. Student's *t*-test.

Figure 4: Corticosterone treatment induced depression- and anxiety-like behavior while co-treatment with oxytocin reversed the behavioral deficit and suppression of HPA axis after administration of corticosterone treatments. (A): Corticosterone-treated animals showed significantly longer duration of floating, which was prevented by co-treatment with oxytocin. (B): No difference is seen in the climbing behavior among the treatments in the forced swimming test. (C): Corticosterone treated group spent less time swimming which was reverted by the co-treatment with oxytocin. (D): Number of positive social interaction in corticosterone-treated animals was significantly lower than the control and co-treatment groups. (E): Co-treatment group shows significantly longer time spent in the central arena of OFT, but not significant difference was found between the control and corticosterone-treatment groups. (F) No statistically significant difference was observed in the locomotion behavior in the OFT. (G) Low concentration of corticosterone in plasma was observed in the corticosterone group and co-treatment

when compared to the control group which is due to suppression of the HPA axis by exogenous administration of corticosterone. Values expressed in Mean ± SEM, *: $p<0.05$ when compared to the other two groups; **: $p<0.01$ when compared to the other two groups. One-way ANOVA with Tukey post-hoc test; Kruskal-Wallis with Mann_Whitney U post-hoc test.

Figure 5: Oxytocin reversed the suppression of cell proliferation and dendritic maturation of new neurons induced by corticosterone. (A): Corticosterone treatment reduced the number of DCX-positive cells with tertiary dendrites in the hippocampus, while co-treatment with oxytocin could reverse the change. (B): Sholl analysis showed that the dendritic complexity of immature neurons is significantly lower in corticosterone group when compared to the control and co-treatment groups. (C-H): representative photomicrographs of DCX-positive cells in control (C & D), corticosterone (E & F) and co-treatment (G & H) group animals. (C, E, G): 100X magnification; (D, F, H): 400X magnification. Scale bar: 100μm. a: $p<0.05$ between corticosterone (Cort) and control group; *: $p<0.05$ when Cort group is compared to Ctrl and OXT groups; #: $p<0.05$ when comparing Cort group with OXT group; One-way ANOVA with Tukey post-hoc test; Kruskal-Wallis with Mann_Whitney U post-hoc test.

Acknowledgements

The study was supported by the Large Equipment Fund, The Hong Kong Polytechnic University (1.51.27.DD6B), and ECS, University Grant Council, H.K. (0112876) to Lau BW.

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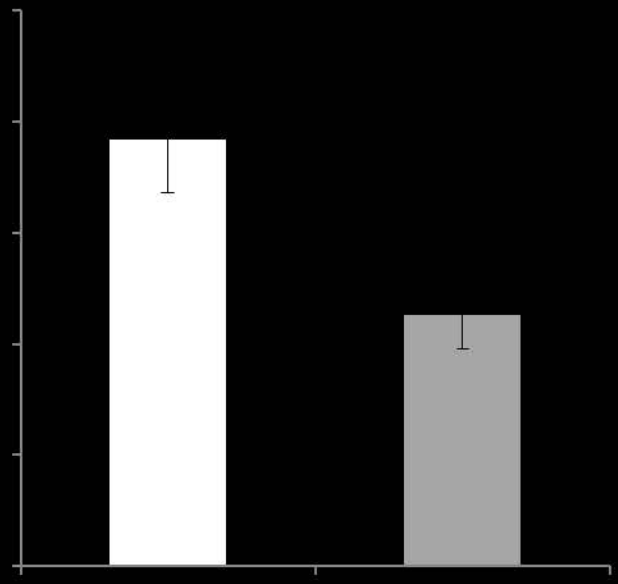

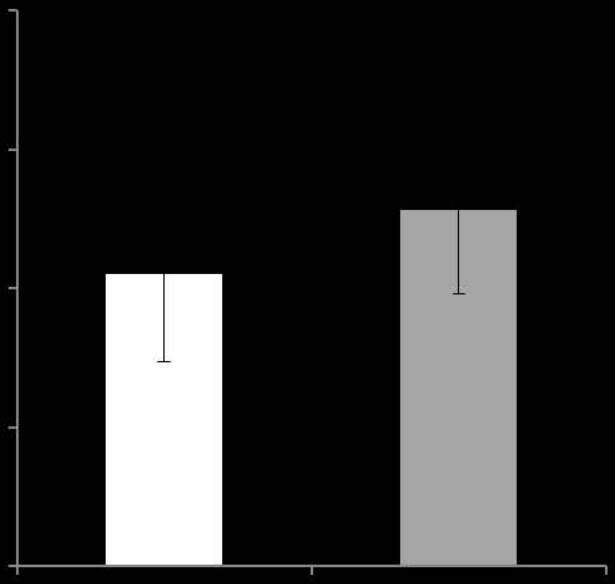

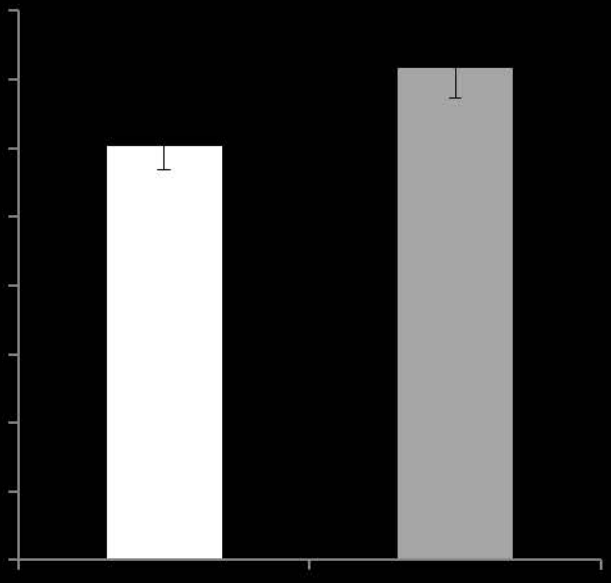

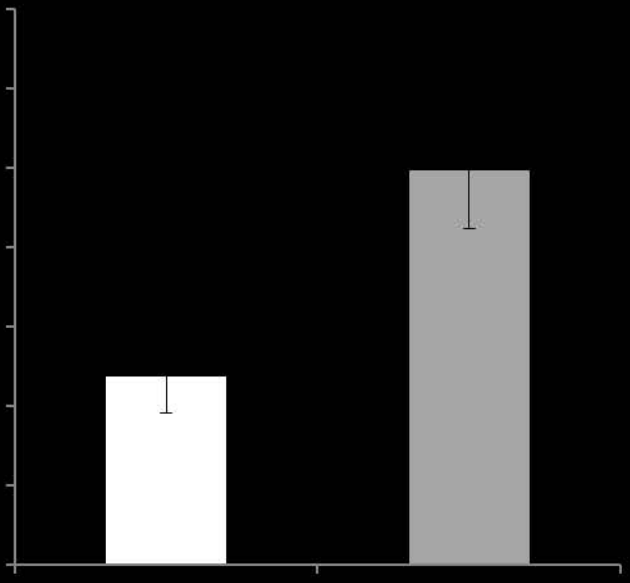

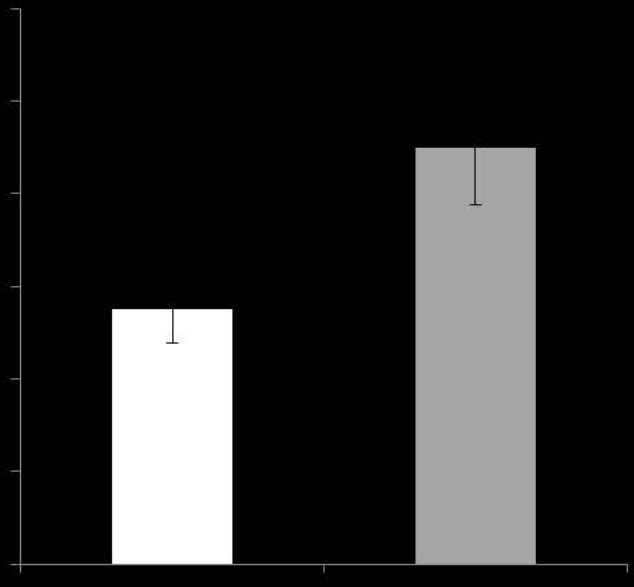

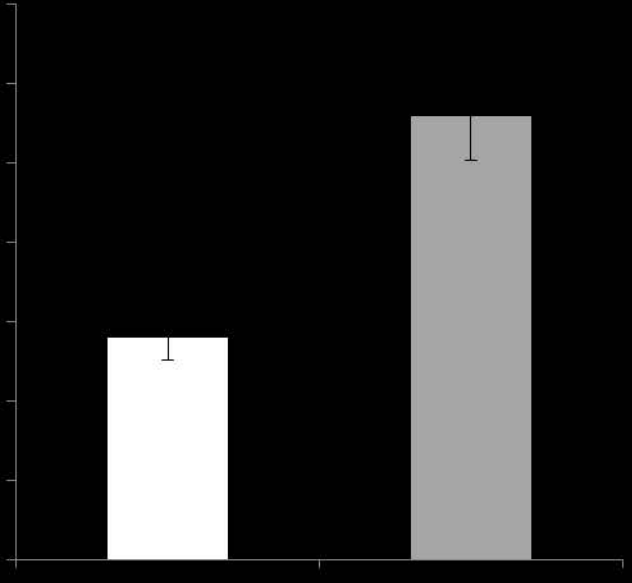

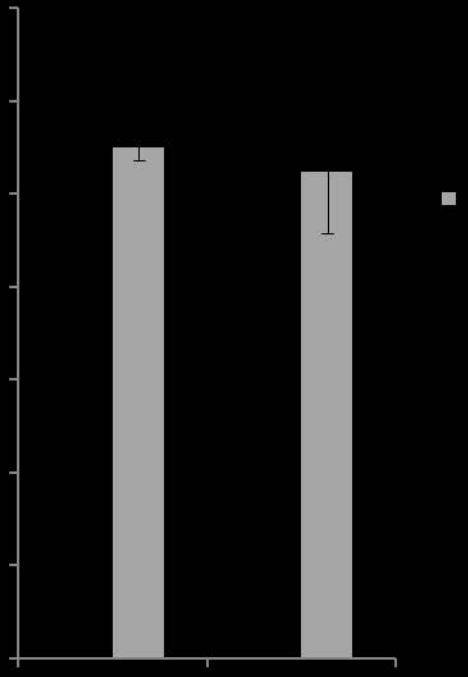

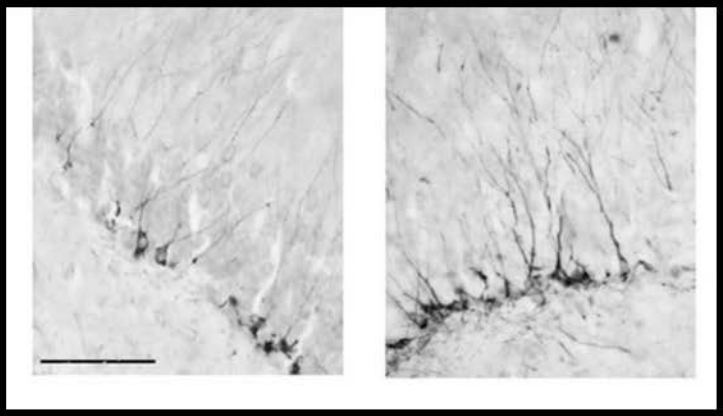

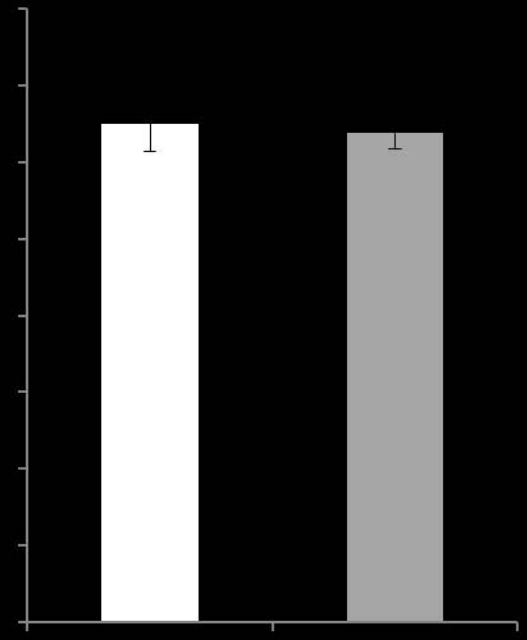

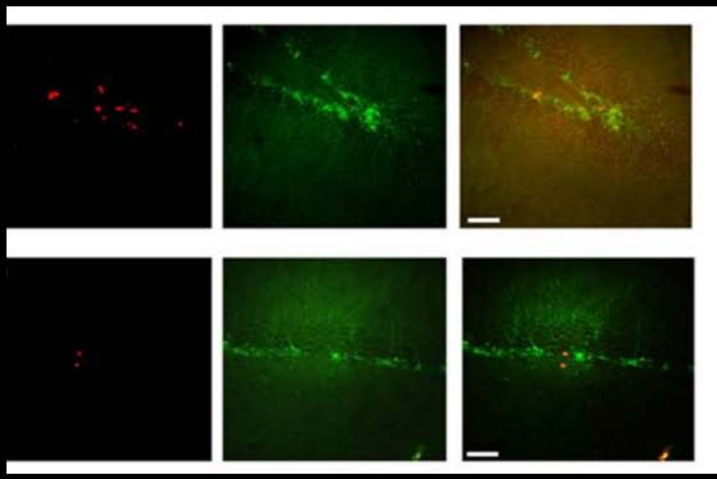

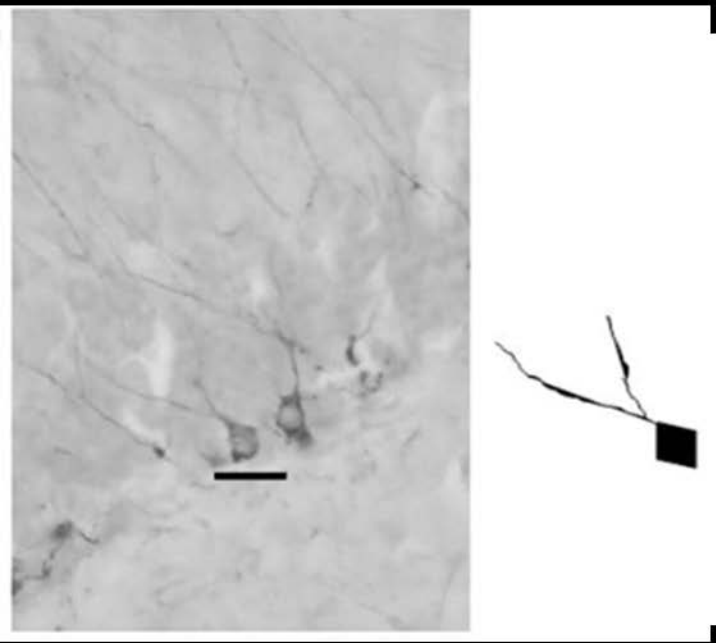

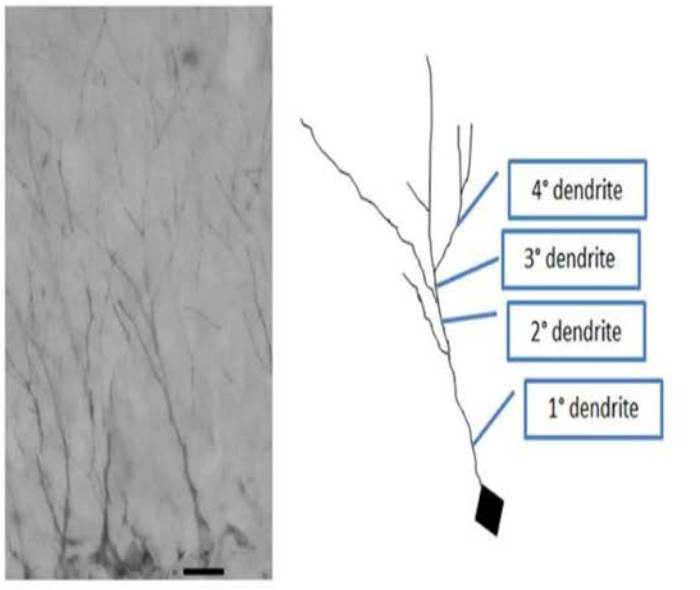
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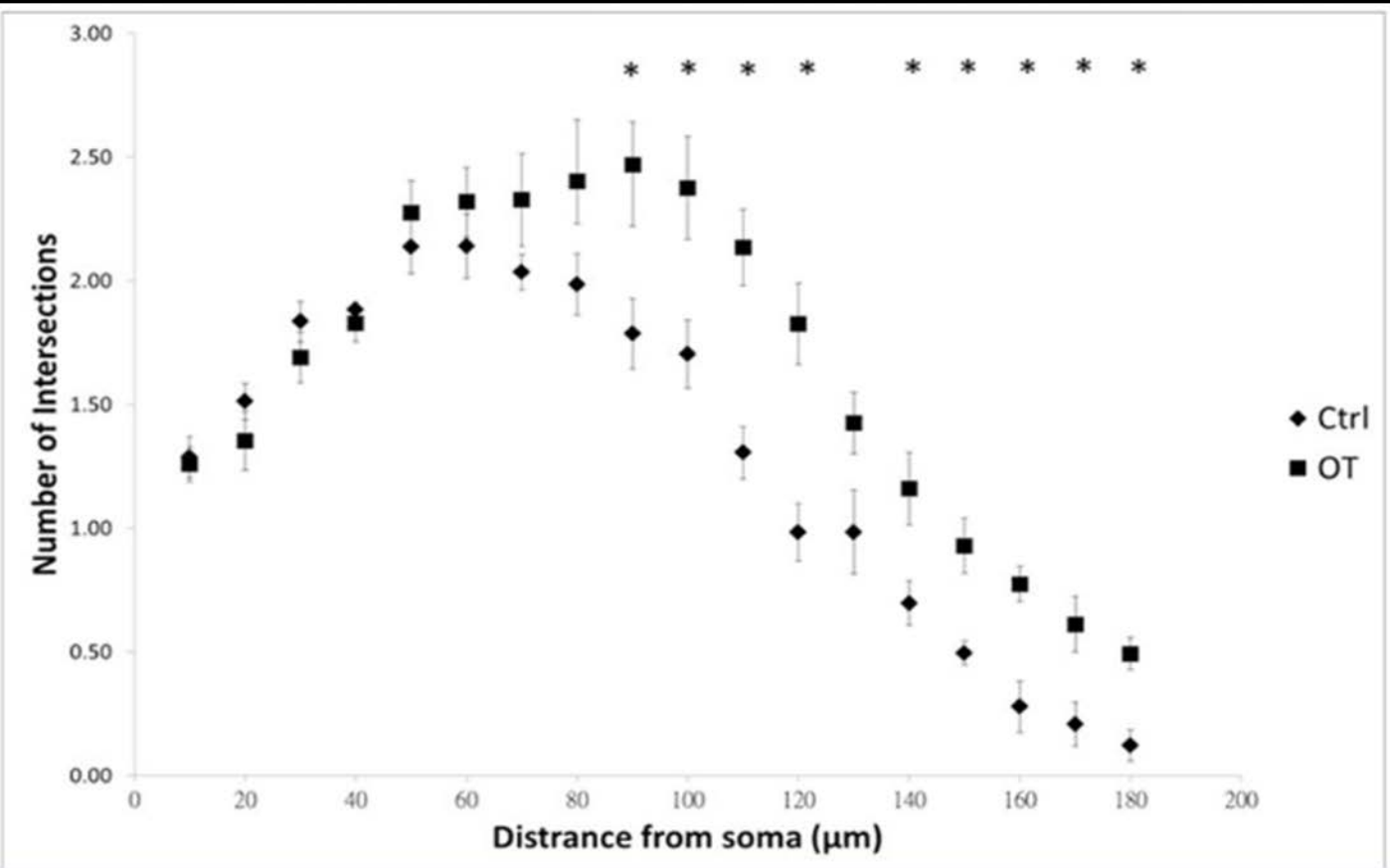
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