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Post-weaning social isolation regulates social exposure-induced vasopressin release in the paraventricular nucleus

Yu Wang *MD*, Shu-min Deng *MD*, Qun-ying Fu *MD*, Xu Liu *MD*

Department of Neurology, The First Affiliated Hospital, China Medical University, Shenyang, China

Abstract

Background & Objective: Early life stresses, such as social isolation, have lasting effects on the development of emotion and behavior, in which vasopressin plays important roles. This study aimed to assess the possible association of central release of vasopressin with social isolation. *Methods*: The social isolation model was performed in male mice who endured 6-week social isolation after weaning. Vasopressin expression in the paraventricular nucleus of hypothalamus (PVN) was measured with immunohistochemistry. Released vasopressin from PVN was measured with radioimmunoassay. *Results*: Vasopressin immunoreactive cells number decreased in the PVN, medial parvocellular division in social isolation-reared mice, compared to the group-reared counterparts. Social isolation decreased short social exposure-induced vasopressin release from PVN. Isolation-reared mice exhibited anxiogenic profile and difficulty in social recognition.
Conclusions: This study provides new evidence for the important role of vasopressin in the development of emotional and social behaviors.

INTRODUCTION

Early stressful experience, such as social isolation, can cause long-lasting alterations in the development of the brain. The alterations have been manifested as increased vulnerability to neuropsychiatric disorders such as anxiety and depression in adulthood.[1] Social isolation is associated with altered activity of neuroendocrine systems.[2] Central arginine vasopressin (AVP) is thought to play important roles in the regulation of emotional behaviors (anxiety, depression, stress coping) and social behaviors (social attachment, parental care, social recognition, aggression).[3]AVP immunoreactivity is found to correlate with the difference in social behaviors and to vary with social experience. In some social isolation models, for example in maternally separated male rats, there were remarkable decrease of AVP immunoreactivity and mRNA expression in the paraventricular nucleus of the hypothalamus (PVN).[4,5] Rats receiving high levels of licking and grooming as pups had increased AVP-V1a receptor binding in the central nucleus of amygdale in adult, and were less fearful and more maternal than the rats receiving low levels of maternal licking and grooming.[6] In contrast, male mice with V1a receptor knockout exhibited anxiety-like behaviors and social recognition impairment.[7]

AVP is predominantly synthesized in the somata of hypothalamic magnocellular neurons in the supraoptic nucleus (SON) and PVN. It is packed into neurosecretory granules, which are transported through axons into the terminals in neurohypophysis.[8] AVP positive neuron is also found in parvocellular division of PVN from immunostaining.[9] These AVP producing cells in the PVN and SON project to posterior pituitary and release AVP into circulation. Circulating AVP level may thus serve as the indices of activity within the neuropeptidergic cells in the PVN or SON. Children who were reared in extremely aberrant social environments where they were deprived of the usual care-giving had a lower peripheral level of basal AVP than the family-reared children.[10] AVP from the PVN also diffuses to the limbic areas through extracellular fluid where it can potentially mediate behavioral responses via receptors located in these areas.[3] Therefore, central release of AVP in stress condition may be the key process for the subsequent effects.

Neural and behavioral developments of rodents mirror those of human. The developmental timing of isolation is a crucial determinant of its effects on the animal. The period from weaning to early adulthood is often referred to as the adolescence for rodents, and widely used in the investigation of social isolation in mouse and rat models.[11]

Address correspondence to: Dr. Yu Wang, Department of Neurology, The First Affiliated Hospital, China Medical University, Shenyang, 110001, China. Tel: 13940242439, E-mail: 88446486@163.com

The present study was designed to examine the effects of post-weaning social isolation on AVP release and expression in the PVN, as well as related emotional and social behaviors. We hypothesized a causal relationship between stress at adolescence stage and the later developments of emotional and social behaviors at adult stage.

METHODS

Animals

Male C57BL/6 mice born from nulliparous females were used. The animals at 21 days of age were weaned and randomly divided into control group (housed with 2 or 3 siblings) or isolation-reared group (singly housed). To avoid any confounding factor of litters, pups from same dam were evenly divided into the two groups. C57BL/6J strain of mice was chosen as this was a strain frequently used in genetic analyses of brain and behavior.[12] The mice had free access to food and water under a 12 : 12 h light/ dark cycle (lights on 07.30 h) at 22 ± 2°C and 40-70% relative humidity. All animal experiments were approved by Animal Care and Use Committee at China Medical University (Permit number CMU62043013), which complied with National Institute of Health Guide for Care and Use of Laboratory Animals.

Behavioral tests

After 6 weeks of social isolation, the mice were used for anxiety-related behavioral tests (elevated-plus maze test, open-field test, light–dark test) and social behavioral test. There was a two-day interval between two tests. These tests were performed between 8:00am to 16:00pm to minimize the risk of diurnal differences in behaviors. All tests were videotaped and later scored by a blinded observer. Each pair of control and isolation-reared mice received experimental procedures alternately. All behavior testing apparatuses were cleaned thoroughly between subjects. Each test was performed according to previous reports[9,13,14] and described in Appendix 1.

Implantation of microdialysis probes

The measurement of released AVP in the PVN was performed according to the previous reports with a few modifications.[13,15] After the social behavior test, the mice were anaesthetized with avertin and positioned in a stereotaxic frame. A microdialysis probe (CUP11, CMA/microdialysis, Stockholm, Sweden) was implanted with its two tips bilaterally targeting the PVN (coordinate: 0.2 mm caudal, 0.3 mm lateral to the midline, 5.5 mm deep). The probes were flushed and filled with sterile Ringer's solution, and were fixed to the skull with two jeweler's screws and dental cement. Two approximately 5 cm long pieces of polyethylene tubing filled with Ringer's solution were connected to the inlet and outlet of the microdialysis probe and fixed with dental cement. The implantation sites of probes were checked in the brain sections after all experiments were done. Only the data from the mice with correct probes implantation were analysed.

Monitoring of AVP release in the PVN during social recognition test

The mice were given 2 days to recover from the surgery procedure to minimize non-specific stress response. Then the social recognition test was performed and dialysates were collected during the test to measure AVP release. The microdialyasis probes were connected to a syringe mounted onto a microinfusion pump via the polyethylene tubing and were perfused with sterile Ringer's solution (3.0 μl/min, PH 7.4) for 2 h to establish equilibrium between inside and outside of the microdialysis membrane.

The social recognition test and dialysates collection is shown in Figure 1A. At 15 minutes prior to the social recognition test, one dialysate was collected as the basal level. Then the social recognition test was performed and 5 dialysates were collected at every 15 minute intervals. The first dialysis was started immediately after a 5-minute exposure (1st exposure) in the recognition test. The dialysates were collected directly into eppendorf tubes containing 0.1 M HCl, immediately frozen on dry ice, and subsequently stored at -20°C until the quantification of AVP were determined by AVP-specific radioimmunoassay according to the instruction from the company (RK-065-07, Phoenix Pharmaceuticals, Inc).

Social recognition test

The social recognition test was performed according to the previous report.[16] A resident mouse from the isolation-reared group or control group was exposed to an intact male mouse (stranger 1) for a 5-min period (1st exposure). After a 30 min-interval, the stranger 1 mouse and another intact male mouse (stranger 2) were exposed to the resident mouse for the second 5-min period (2nd exposure). The time

spent for the resident mouse to investigate both mice was measured. Mouse or rat tends to show sniffing behavior towards unfamiliar conspecifics compared to the familiar ones. Preference index was calculated by the ratio of time spent in investigating the stranger 2 to the sum of time used in investigating both stranger 1 and 2 mice. The mice that failed to discriminate stranger 1 and stranger 2 had preference index 50%.

Immunohistochemistry

After a 6-week social isolation, the mice (Not used in previous experiment) were anaesthetized with avertin (250 mg/kg body weight) and perfused intracardially heparinized saline (20 mU/mL) followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS). The brains were immediately removed and postfixed in same fixative containing 15% sucrose solution overnight, were followed by cryoprotection with 30% sucrose solution one more day. The coronal sections (30 μm) were cut using a freezing microtome, collected into 0.1 M PBS, and then used for immunohistochemistry to examine the AVP expression. In immunohistochemistry, the anatomically matching sections containing PVN region were incubated with anti-AVP antibody (diluted 1: 1000, Chemicon International Inc., Temecula, CA, USA) for 48 h at 4°C, followed by incubation of horseradish peroxidase labelled 2nd antibody at 4°C overnight. The sections were thoroughly washed with PBS after antibodies incubation. The AVP immunoreactivity was visualized as a brown cytoplasm precipitate by DAB procedure. The AVP immunoreactivity positive neurons in PVN region from 4 anatomically matching sections per mouse were counted bilaterally.

Statistical analysis

Data were expressed as mean ± SEM, and analyzed using Mann-Whitney U test. $p < 0.05$ was considered statistically significant.

RESULTS

Social isolation affected anxiety-related behaviours and social behaviors

Anxiety-related behaviors were examined with elevated-plus maze test, open field test and light-dark test in sequence. Similar to previous reports[17-19], our results from the three tests showed that 6-week social isolation induced severe anxiogenic profiles (Supplementary Figure 1). Social behaviors test

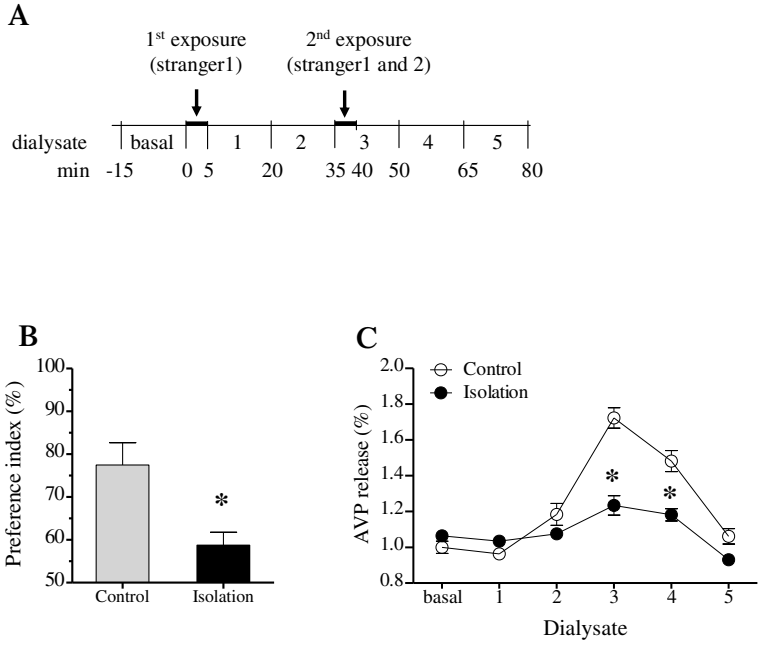

Figure 1. The effects of post-weaning social isolation on social recognition and AVP release in the PVN. Mice (n=8) with six-weeks post-weaning social isolation were used for social recognition test and measurement of AVP release in the PVN. The time course for the social recognition test and 6 dialysate collections were depicted (A). Preference index was calculated (B). The quantification of AVP in the dialysates was measured using radioimmunoassay, and expressed as the percentage of the basal level in the control group (C). $^{*}p<0.05$ *vs.* the control group.

showed that post-weaning social isolation reduced social behaviors (Supplementary Figure 2).

Post-weaning social isolation affected social recognition function

In the social recognition test, the control mice had preference index up to 77 ± 5.21%, showing good social recognition ability. However, in the isolation-reared mice, the preference index significantly decreased to 58 ± 2.99% (Figure 1B). These data demonstrated that the post-weaning social isolation impaired social recognition function.

Post-weaning social isolation affected the release of AVP in the PVN during social recognition test

The local release of AVP in the PVN was checked during social recognition test (Figure 1C). The basal level of vasopressin was 4.16±1.87 pg/ml, which was similar between the control mice and isolation-reared mice. In the control mice, the AVP level after first exposure to a strange mouse was not significantly different with the basal level. However, the second exposure to the familiar mouse and another strange mouse triggered a remarkable increase of AVP level, which was followed by gradual decrease towards the basal

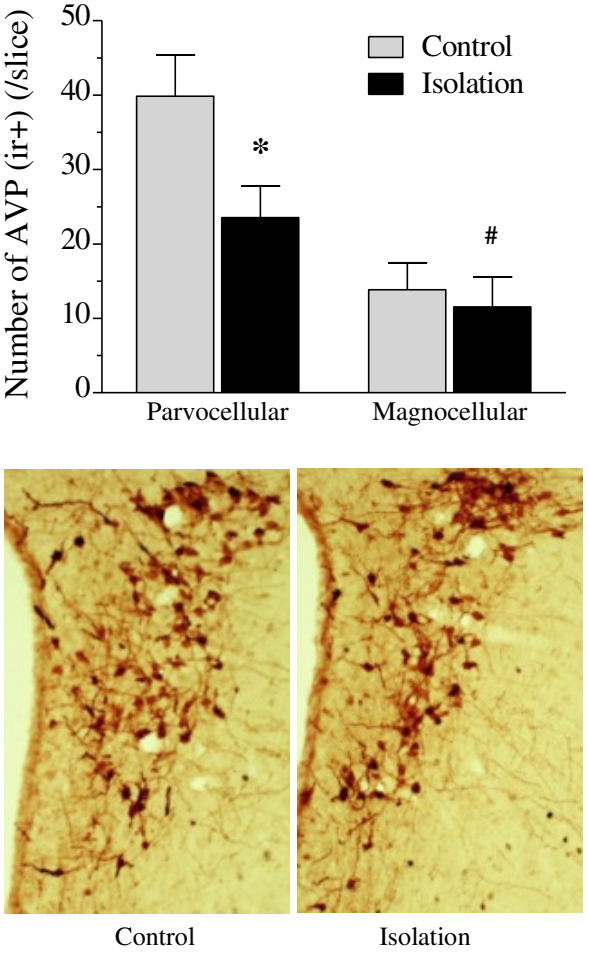

Figure 2. The effect of post-weaning social isolation on AVP expression in the PVN. The mice were sacrificed after a 6-week social isolation. The brains were removed, and cut into 30-μm thick sections. The sections containing PVN were used for immunohistochemistry to detect the AVP immunoreactivity. The AVP immunoreactivity positive (ir+) neurons in the parvocellular and magnocelluar parts of PVN were counted. Representative pictures and quantified data from 6 mice in each group were shown. * $p<0.05$, # $p>0.05$ *vs.* the control group.

level. Like in the control mice, AVP level in the isolation-reared mice increased significantly after the second social exposure, and decreased toward the basal level with time. However, when compared to the control mice, the AVP level in isolation-reared mice was lower, with significant difference during 1st and 2nd 15 min-period following second social exposure ($p<0.05$). There was no significant difference of AVP level in first exposure between the isolation-reared mice and control mice.

Post-weaning social isolation affected tissue expression of AVP in the PVN

The effect of post-weaning social isolation on AVP expression in the PVN was examined using immunohistochemistry (Figure 2). Isolation-reared mice had less number of AVP positive neurons in medial parvocellullar division of PVN than the control mice (39.84±5.54 *vs.* 23.56±4.25, $p<0.05$). There were no differences in AVP positive neuron number in the posterior magnocellular division in the two groups (13.84±3.65 *vs.* 11.56±4.01, $p>0.05$).

DISCUSSION

Our study showed that post-weaning social isolation increased anxiety-related behaviors, decreased social behaviors and recognition ability. Social isolation also reduced AVP positive neurons in the parvocellular division of PVN. To our knowledge, this was the first time that social isolation has been shown to reduce AVP release in the PVN following short social recognition exposure.

A number of reports have shown the adverse effects of post-weaning social isolation on the development of emotional and social behaviors.[9,17-19] Our results from elevated-plus maze test, open field test, dark-light test and social test confirmed this finding.

Normal emotional, social and recognitive developments require physical interactions during the developmental period until the early adulthood. Early physical interactions with peers are essential for the appropriate development of social behavior.[20] Social isolation rearing of male rats during a critical window period (pre- to mid-adolescence) resulted in long-lasting increase in anxiety-like behaviors that were not reversed by resocialization.[11] Retrospective investigation has shown that children with behavioral inhibition have higher prevalence of multiple anxiety disorders, particularly overanxious and phobic disorders.[21] It suggests that behavioral inhibition may be associated with social anxiety in children. Our results showed social isolation induced both anxiety and behavioral inhibition, suggesting a close relationship between them. However, whether this behavioral inhibition is a specific precursor to anxiety needs further investigation.

There is increasing number of studies investigating the mechanisms involving social isolation and behavior. The activity of hypothalamic-pituitary-adrenal (HPA) axis varies in a variety of stressful experiences, including social isolation. The activation of HPA axis consists of the release of corticotropin releasing hormone (CRH) and AVP from the PVN into the anterior pituitary gland. This in turn stimulate the adrenal glands to produce and release glucocorticoids. HPA axis is immature at birth and its early activity can be strongly regulated by early social experiences.[4] We hypothesized that post-weaning social isolation may affect AVP expression and release in PVN. In this study, we observed that AVP immunoreactivity in the PVN decreased significantly after 6 weeks of post-weaning social isolation. This is consistent with the previous reports observed in maternally separated male rats and mice.[4,5] Moreover, the decreased AVP neurons were in the parvocelluar division, not in magnocellular division.

Parvocellular neurosecretory neurons of the PVN have axons projecting to the median eminence, where their neurosecretory nerve terminals release hormones at the primary capillary plexus of the HPA system. Parvocellular AVP has been found to have important linkage with emotion. In an animal model of anxiety using fawn-hooded rats, there was reduced AVP expression in the parvocellular cells of the PVN.[22] Two-week isolation also significantly decreased AVP immunoreactive neurons in medial parvocelluar division, ventral zone in male rats, but not in magnocellualr division.[9] Thus, our result provides further evidence for the importance of AVP expression in PVN, especially parvocellular division. There are several reports that show different results for AVP expression induced by social isolation. In male prairie voles, post-weaning social isolation enhanced, but not decreased AVP mRNA expression in PVN.[17] The difference between peptide and mRNA may result from different mouse subspecies, or translation dysfunction. The data from adult prairie voles also showed different result: social isolation in adults increased the plasma levels of AVP, as well as oxytocin and corticosterone.[23] The difference

between animals of different age indicates that social isolation taking place at adolescence stage may block the development of immature AVP producing system.

Social recognition is critical for establishing and maintaining social structures in animals living together.[12] Social isolation had marked adverse effect on recognition function.[9] Consistent with the previous reports, our findings from behavioral assessment showed that the isolation-reared mice had difficulty in social recognition. AVP is a known molecular mediator of social recognition. Specially, exogenous AVP injection into the intracerebroventricule of adult male rats rapidly improved conspecific recognition and consolidation of olfactory information.[24] AVP receptor knockout or antagonist impaired social recognition, but not general object recognition.[8,24] In this study, short social recognition exposure triggered a rapid and profound AVP release in the PVN of the control mice. Together with the previous reports involving exogenous AVP injection [23] and AVP receptor knockout or antagonist[8,24], our results suggest that the rapid AVP release play an important role in recognition. In post-weaning social isolation-reared mice, the AVP release decreased greatly. Together with low AVP immunoreactivity in PVN, these data indicate that the released AVP may come from local production in PVN, but not from distal transportation.

In summary, a possible causal linkage of post-weaning social isolation and behaviors may be depicted as follows. Post-weaning social isolation damages the development of the AVP-producing system (shown as decreased AVP immunoreactivity), and then leads to the decreased release of AVP upon social exposure. The low release of AVP underlies the recognition deficit, which results in social behavior inhibition and anxiety.

ACKNOWLEDGEMENT

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DISCLOSURE

Conflict of interest: None

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Supplemental experimental procedures

Anxiety-related behavior tests

In the elevated-plus maze test, a maze consisted of two open and two enclosed arms of the same size (25×5 cm) with 15 cm-high transparent walls (60 lux) was used. In this test, the total distance and time spent in the open arms and the frequency of entries into the open arms were recorded over a 5-min period. In the open-field test, the mice were placed in a corner of an open-field apparatus (60×60×40 cm; 60 lux of illumination). The total distance and the time spent in the central area were recorded over a 10-min period. In the light–dark test, an apparatus consisted of a cage (40×40×30 cm), which was divided into two equal chambers by a black partition containing a small opening. One chamber was made of white plastic and illuminated (100 lux), and the other chamber was black and dark. The mice were placed in the dark chamber and allowed to move freely between the two chambers. The total distance and time spent in each chamber were recorded over a 5-min period.

Social behavior test

Social behavior test was performed following anxiety-related behavior tests. Two control mice from different cages or two isolation mice were placed together in a test cage (29×18×12 cm). An infrared video camera and infrared ray light emitting diodes were attached at the top of the cage. The images from each cage were captured at a rate of one frame per second. The behaviors were video-monitored over one day. Social interaction was measured by counting the number of particles in each frame. One particle indicated that the two mice were in contact with each other. Two particles indicated that the two mice were in separation. The percentage of time spent for the direct contact and for the separation was calculated. Total locomotor activity was also measured. The duration of various social behaviors during the first 5 min following the placement of the pairs of mice into the test cage was also hand-scored, including approaching, following, anogenital sniffing, nose-to-nose sniffing, crawling over and under, grooming each other, sleeping together, and fighting.

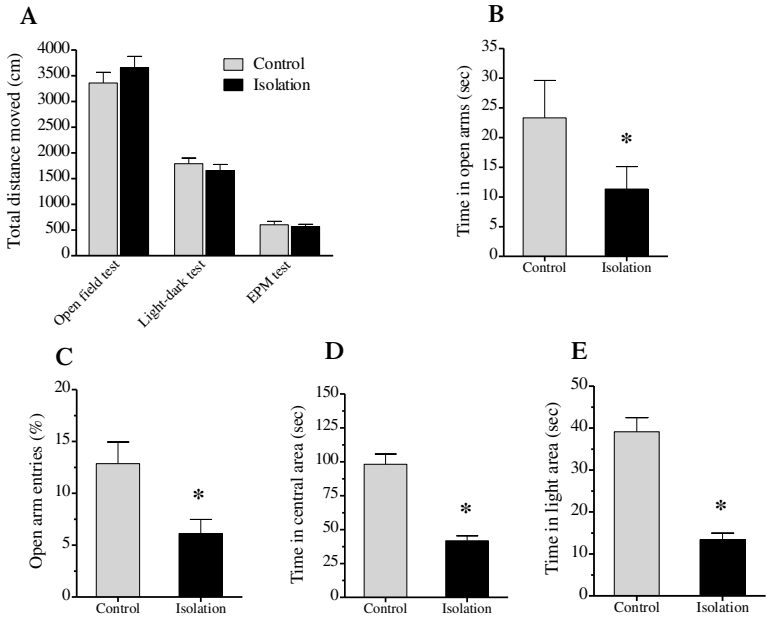
Supplemental experimental results

Social isolation affected anxiety-related behavior and social behaviors

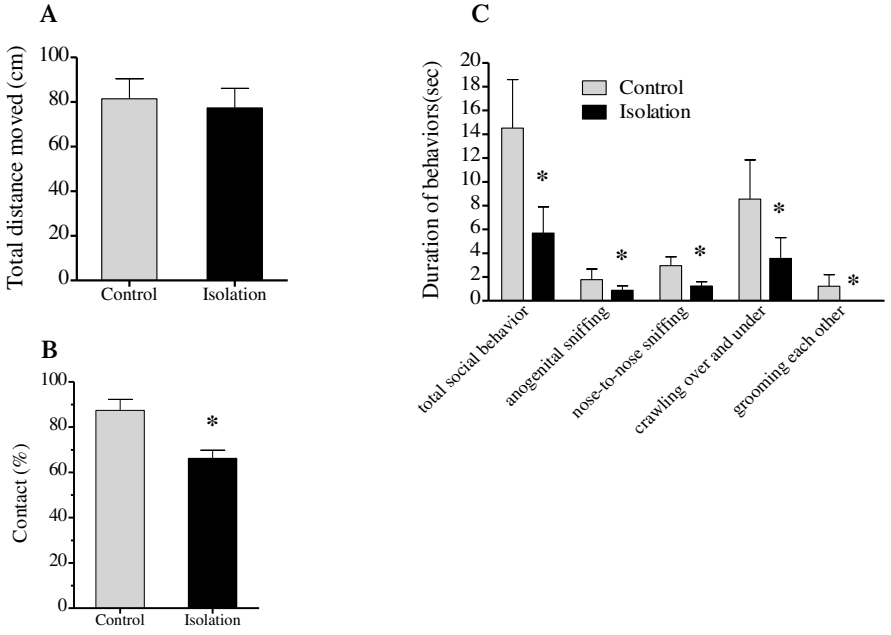
Anxiety-related behaviors were examined with elevated-plus maze test, open field test and light-dark test in sequence. The mouse with a higher level of anxiety tends to stay less in the central area of the open field, in the light area of the light-dark box, in the open arms of the elevated-plus maze, and has fewer entries into the open arm of the elevated-plus maze. In all the three tests, there was no difference in the total distance moved between the isolation mice and control mice (Supplementary Figure S1A), indicating that the isolation rear did not affect the abilities of exploration and locomotion. In the elevated-plus maze test, the isolation mice spent less time in the open arms (Supplementary Figure S1B) and had fewer number of open arm entries (Supplementary Figure S1C) compared to the control mice. Similar results were found in the open field test and the light-dark test. The isolation mice spent less time in the central area and light area in the open field test (Supplementary Figure S1D) and light-dark test (Supplementary Figure S1E), respectively. All these results suggest that the isolation mice are more anxious than the control mice.

Post-weaning social isolation affected social behaviors

Social behaviors between two isolation mice or two control mice were examined. There was no significant difference in the total distance moved between the isolation mice and control mice (Supplementary Figure S2A). The control mice spent more time staying still in contact with each other. In contrast, the isolation mice spent less time in contact with each other (Supplementary Figure S2B). Social behaviors in the first 5 min following the first encounter were further analyzed. Compared to the control mice, the isolation mice spent less time in anogenital sniffing, nose-to-nose sniffing, crawing over and under, and grooming each other (Supplementary Figure S2C). No approaching, following, sleeping together and fighting were observed in the first 5 min (Data not shown). These data suggest that post-weaning social isolation reduces social behaviours.



Supplementary. Figure S1. The effects of post-weaning social isolation on anxiety-related behaviors. After a 6-week social isolation, the mice were used to test anxiety-related behaviors in sequence in a 5 min of elevated-plus maze (EPM) test, 10 min of open field test and 5 min of light-dark test. The total distance moved in the three tests (A), the time spent in the open arms (B) and the percentage of the number of open arm entries (C) in the EPM test, the time spent in the center area of the open field test (D), and the time spent in the light area of the light-dark test (E) were shown (n=16). * $p<0.05$ *vs* the control group.



Supplementary Figure S2. The effects of post-weaning social isolation on social behaviors. After the tests of anxiety-related behaviors, the mice performed the social behavior test. Two isolation mice or two control mice from different cages were placed together in a test cage and their behaviors were video-monitored over 24 h. The percentage of time spent in contact in 24 h (A), the total distance moved (B) and the time spent in different social behaviors in the first 5 min (C) were shown (n=8). * $p<0.05$ *vs* the control group.