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Altered ADAR1 in mice affected by social isolation stress-induced cognitive deficits

Adenosine deaminase acting on RNA (ADAR) activity increases in response to inflammation. Social isolation stress is related to neuroinflammation; however, it remains unclear whether ADAR1 is altered in response to social isolation stress-induced cognitive deficits. To investigate our hypothesis that ADAR1 displayed patterns of change in response to social isolation stress, we addressed this issue systemically by isolating Kunming mice for 2, 4 and 8 weeks individually since postnatal 21 days to set up isolation mouse model. Furthermore, we arranged re-socialization group to evaluate the alterations of ADAR1 in the cognitive deficits recovery. The results of behavior tests showed that social isolation stress resulted in cognitive dysfunction, which was recovered by re-socialization in re-gregarious rearing group. Furthermore, the immunohistochemistry and western blot results displayed that both the immunoreactivity and protein expression of ADAR1 in the hippocampus and frontal cortex increased obviously as compared to the same age mice without isolation. The above abnormal alterations of ADAR1 were recovered by re-socialization in re-gregarious rearing group. Our study supports the hypothesis that ADAR1 is altered in mice affected by social isolation stress-induced cognitive deficits.
Altered ADAR1 in the social isolation stress-induced cognitive deficits mice

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Adenosine deaminase acting on RNA (ADAR) activity increases in response to inflammation. Social isolation stress is related to neuroinflammation; however, it remains unclear whether ADAR1 alters in response to social isolation stress-induced cognitive deficits. To investigate our hypothesis that ADAR1 displayed patterns of change in response to social isolation stress, we addressed this issue systemically by isolating Kunming mice for 2, 4 and 8 weeks individually since postnatal 21 days to set up isolation mouse model. Furthermore, we arranged re-socialization group to evaluate the alterations of ADAR1 in the cognitive deficits recovery. The results of behavior tests showed that social isolation stress resulted in cognitive dysfunction, which was recovered by re-socialization in re-gregarious rearing group. Furthermore, the immunohistochemistry and western blot results displayed that both the immunoreactivity and protein expression of ADAR1 in the hippocampus and frontal cortex increased obviously as compared to the same age mice without isolation. The above abnormal alterations of ADAR1 were recovered by re-socialization in re-gregarious rearing group. Our study supports the hypothesis that ADAR1 is altered in the social isolation stress-induced cognitive deficits.

Key words: social isolation; cognitive ability; ADAR1

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Social isolation defined as an objective reduced social contact (Khodaie et al., 2015) is a kind of psychosocial stressor (O’Keefe et al., 2014; Barratt et al., 2011). Reports from both humans (Grant et al., 2009) and rodent models (Fone and Porkess, 2008) suggest that social isolation leads to cognitive dysfunction (Yusufishaq & Rosenkranz, 2013). So far, the mechanisms of social isolation stress-induced cognitive deficits are not clarified completely. Prefrontal cortex and hippocampus are vulnerable to be disrupted by social isolation stress (Buechel et al., 2014). Moreover, the mechanisms on social isolation stress-induced cognitive deficits in detail are listed as follows. Hypothalamic–pituitary–adrenal (HPA) axis activity is impaired (Sandström & Hart, 2005); Loss of neuronal plasticity combined with change of glutamate and serotonin in cortex and hippocampus is evident (Araki et al., 2014); 5-hydroxytryptamine (5-HT) 1A binding in the frontal cortex is decreased (Hellemans et al., 2005); Prefrontal dopaminergic systems are activated transiently (Araki et al., 2014); The sensitivity for dopamine in the pyramidal neurons of prefrontal cortex is reduced (Baarendse et al., 2013); Hippocampal potassium ion channel currents change (Quan et al., 2010); Polysialyalted form-neural cell adhesion molecule is decreased (Pereda-Pérez et al., 2013); loss of NMDA receptors (Strømme Johannesen et al., 2002) and increased AMPA receptor activity are shown in the prefrontal cortex (Araki et al., 2014); the activity of rapamycin pathway in the prefrontal cortex is enhanced (Meffre et al., 2012); Hypo-responsive to psychosocial stress also is related with aging (Buechel et al., 2014) and synaptic changes in the pyramidal neurons of adult prefrontal cortex (Baarendse et al., 2013). The above findings are focused on the alterations of neurotransmitter systems, neuron plasticity, regeneration and the function of HPA axis mainly; most of studies are in post-receptor mechanism. However, little is known about the change of ADAR1, an enzyme involving RNA editing and inflammation, in the brain in response to social isolation stress.

ADAR1 (Gene ID:ADAR) is the RNA editing enzyme belonging to ADAR family, which catalyzes the process of converting adenosine to inosine (A-to-I) in pre-mRNA. A-to-I RNA editing takes place in 5-hydroxytryptamine 2C receptor (5-HT2CR), Glutamate receptor (GluR) B subunit of α-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid (AMPA)
receptor, α-3 subunit of the gamma-aminobutyric acid (GABA) A receptor and KV1.1 potassium channel. ADAR is not only an enzyme involving in the RNA editing of 5-HT2CR, being well known to be implicated in regulation of cognitive ability (Bombail et al., 2014) with distributing in the center nervous system widely, but also is related to inflammation (Yang JH et al; 2003). Neurogenic neuroinflammation may become maladaptive and aggravate the outcomes of stress (Kareliuta K1 et al; 2011). ADAR1 expression level is influenced by inflammatory mediators including tumor necrosis factor, lipopolysaccharide, and endotoxin (Lukasz et al., 2013). However, the reports about the role of brain inflammation in the pathophysiology of neuropsychiatric disorders are limited. So far, no published paper is found on whether ADAR1 expression patterns change in the hippocampus and frontal cortex undergoing social isolation stress-induced cognitive deficits. It is then worthwhile to analyze the change and distribution characters of ADAR1 in the cognitive-related brain areas undergoing social isolation stress to help to better understand the mechanism for social isolation-induced cognitive deficits epigenetically. In this study, we systemically addressed those issues by isolating post-weaning Kunming mice for 2, 4 and 8 weeks. Since adolescence is critical in the development period, during this period many physiological and neurobiological changes occur as an individual transit into adulthood. We also designed re-socialization group in adolescent period. We found that different durations of social isolation stress led to cognitive deficits accompanied with altered ADAR1 expression in the hippocampus and prefrontal cortex. Furthermore, we adapted the isolated mice to gregarious rearing with their littermates at adolescent period naturally, and found that re-socialization recovered cognitive deficits and abnormal changes of ADAR1 induced by social isolation stress. Our study demonstrates that ADAR1 alters in the social isolation stress-induced cognitive deficits mice, which can be recovered by re-socialization in re-gregarious rearing group. These findings give the clue to clarify the mechanisms of social isolation stress-induced cognition deficits epigenetically.

Materials and methods

Animals
Seventy healthy male Kunming (KM) mice were used from Dalian Medical University, Laboratory Center, ID: 0003746. KM mouse is the most commonly used outbred murine in China. Actually, KM mice have been used in neuroscience widely (Cui LB et al., 2013, Yu Y et al., 2015, Peng X et al., 2013). Mice at the age of 21 days old (15 ± 5g) were used in the beginning of the study. They were housed with a 12-h rhythm of day/night cycle, the temperature of 21 ± 1°C and the humidity at (55 ± 5)% . The mice were fed with food and water ad libitum. They were divided into 7 groups randomly (Figure 1A), 10 mice each group. The mice were placed in a plastic cage (Beijing Heli Technology Development Co. Ltd. China. 290 × 178 × 160 mm; 5 mice per cage). The isolated mice were housed individually in a standard sized cage for 2, 4 and 8 weeks designated as SI 2W (isolation of 2 weeks), SI 4W (isolation of 4 weeks) and SI 8W (isolation of 8 weeks). Re-socialization group mice (SI 2WR) returned to the cage with 4 littermates and were reared until adult after 2 weeks isolation. Control groups were the same age mice without isolation stress (C 2W, C 4W and C 8W). All experimental procedures were in accordance with the animal ethics standards and regulations for the Administration of Affairs Concerning Experimental Animals.

Methods

Objection Recognition Test (ORT)

The ORT (Figure 1B) was performed according to the previous procedure (Võikar et al., 2005). This test is used to measure the non-spatial cognitive ability. The test apparatus (XR-XX117, Shanghai Xinruan Technology Co. Ltd. China) was put in the testing room for observing the animal behavior. The mouse was put in a white plastic box (40 × 40 × 35 cm). The cubic objects (A, B and C, 5 × 5 × 5 cm) for recognition were woody blocks. Objects A and B with black color and object C with black and white pattern, were used for recognition in the tests. The objects could not be moved by the mouse in virtue of the objects' heavy weights. The procedure of ORT was designated with two trials including the sample and the test trial. Each mouse was put into an empty box for 5 min in order to make it adapt to the new environment. Then, during the sample trial, object A and object B were placed oppositely with 24 cm from each other. To explore for 5 min, every mouse was placed in the middle between the two objects. Then, the mouse returned back with an interval of 4 h.
During the test trial, object B was replaced by object C. Object C was unfamiliar to the mouse. The mouse explored for 5 min in object A and object C. The behavior of the mouse was recorded. The time for exploring the objects was measured by the trained researcher.

Objection Location Test (OLT)

The OLT procedure was similar with ORT (Võikar et al., 2005). This test is used to measure the spatial cognitive ability. As can be seen in Figure 1C, the cubic objects A and B were the same as objects A and B in the ORT. The acclimation was carried out in the same way as in the ORT. In the sample trial, objects A and B were put in the similar location in the ORT. The mouse explored the objects for 5 min and then returned to the home cage for a 4 h interval. Afterward, in the test trial, object B was moved to the opposite direction toward the object A, then the mouse was left to explore for 5 min. The time of exploration in both the sample and test trial was recorded.

In both ORT and OLT, only the time when the nose of the mouse was closing to the objects within 2 cm was analyzed. After each trial, the objects and the boxes were cleaned in order to avoid olfactory cues by using 75% ethanol. The discrimination index (DI) was calculated as follows: $DI = (Tn - Tf)/(Tf + Tn)$: $Tn$ and $Tf$ mean the time took on exploring familiar and new objects (or locations) in 5 min respectively.

Spontaneous Alternation Test in T maze

Spontaneous alternation test was used to measure work memory. The procedure was according to the paper (Sandstrom & Hart, 2005). The maze (XR-XT111, Shanghai Xinruan Technology Co. Ltd. China) was consisted of three plexiglas arms with the same size, two opposite arms and one central arm, which were connected to make a “T” shape (46 cm × 71 cm). Two sliding doors controlled the access of two opposite arms. At the edge of the center arm, there was a door, which can be closed to restrict the mouse to stay at the dead end of T maze. The mouse had been trained for 4 days. The mouse was measured for 14 trials/session each time. In each session, the mouse was placed at the dead end and the door was closed. The door was opened after 5 s to make the mouse can enter into one of the arms freely. Once the mouse selected one arm, the opposite access was occluded. When the mouse returned back and stayed at the dead end of the center arm, it was kept there for 5 s. After that, the
mouse started the next trial with opening the door again. The alternation times were collected and measured.

Immunohistochemistry Staining

The mouse was injected with 4% chloral hydrate for anesthesia (400 mg/kg, i.p.). They were perfused transcardially by the treatment with 1% and 4% paraformaldehyde respectively. The mice brains were put into 4% paraformaldehyde, and then put in phosphate buffer saline (PBS) with 20% sucrose overnight at 4°C. The slices were 16 μm by cutting on the microtome-cryostat. The selected slices were rinsed in PBS×3, 10 min each time, then the slices were incubated in 1% bovine serum albumin, after that, the ADAR1-Ab (P110) (1:100, Proteintech, USA) covered the slices overnight at 4°C. Then the sections were rinsed in PBS×3, 10 min each time and further were cultivated with biotinylated second antibody (ZSJQ-BIO company, China) at room temperature for 1.5 h. After rinsing the sections, avidin-biotin complex was used to incubate for 2 h at room temperature. Then diaminobenzidine was used to show coloration. Negative control slices were incubated with PBS without the antibody. After that, the sections were staining with 1% thionine (sigma) to mark the neurons.

Western Blot

The brain areas including prefrontal cortex and hippocampus were selected on ice. The tissues were homogenized in 1 ml cold lysis buffer (Keygen Biotech, China) containing 10 μl phosphatase inhibitor, 1 μl protease inhibitors, 5 μl phenylmethane sulfonyl fluoride (PMSF). Shook tubes by hand, centrifugated at 10,000 g for 5 min at 4°C. The supernate was removed to a fresh tube and stored at -80°C. The protein content was assessed by using a BCA protein assay kit (Keygen Biotech, China). 30 mg protein was added in 7.5% sodium dodecyl sulfate-polyacrylamide (SDS) gel, and then, the protein was transferred into nitrocellulose membranes. ADAR1-Ab (1:1000, Proteintech, USA) was assessed by western blot analysis. GADPH-Ab (1:1000, Beyotime Company, China) was used as an internal control for each sample. Horseradish peroxidase-coupled rabbit anti-goat (1:5000, ZSJQ-BIO Company, China) and anti-mouse (1:5000, ZSJQ-BIO Company China) antibodies were used. The grey value of ADAR1 and GADPH protein expressions was detected by BIO-RAD (Hercules, CA) gel
analysis software.

Statistical Analysis

All data were expressed as the mean ± SD and the data were analyzed statistically with Graph-Pad Prism (GraphPad Software Inc.) and SPSS 21.0. T test was used to analyze the variance between social isolation groups and control groups; ANOVA was used to analyze the differences among groups. \( P<0.05 \) was considered statistically significance.

Results

Objection Recognition Test (ORT) and Objection Location Test (OLT)

In the ORT and OLT, the discrimination index (DI) of the mice undergoing 2, 4 and 8 weeks of social isolation (SI) decreased significantly as compared to that of the same age mice without isolation (Figure 1D). These results demonstrated that the mice displayed the decreased spatial and non-spatial cognition ability after undergoing isolation for 2, 4 and 8 weeks, moreover, no obvious difference was found between SI 2WR and the same age mice without isolation (C 4W). These results suggested that social isolation stress induced abnormal spatial and non-spatial cognition abilities, which were recovered by re-socialization in SI 2WR group.

Spontaneous Alternation Times

In spontaneous alternation test, alternation times of SI 2W group were less than those of C 2W group in day 1 and day 4; in day 2, alternation times of SI 4W were more than those of C 4W group; in day 4, alternation times of SI 8W were more than those of C 8W group. The alternation times of SI 2WR re-socialization group showed no obvious changes as compared with those of the same age mice without isolation stress (Figure 2). These results demonstrated that the mice displayed the abnormal work memory abilities after undergoing isolation rearing of 2, 4 and 8 weeks. Re-socialization in SI 2WR group rescued the abnormal work memory ability induced by social isolation stress.

Nissl staining and DAB staining

Thionine is the Nissl staining to mark the neurons. The results (see arrow 1 and 2 in Figure 3B) showed that ADAR1 expressed in a part of neurons. Analyzing the immunohistochemistry results of ADAR1 showed that as compared to the mice without
isolation in the same age, the immunoreactivity of ADAR1 enhanced significantly in hippocampus and frontal cortex in the mice undergoing 2, 4 and 8 weeks social isolation stress (Figure 3B and 3C). Statistical analysis (Figure 3D) revealed the higher number of ADAR1 immunoreactive positive cells in frontal cortex of 2, 4 and 8 weeks isolation mice compared with the same age mice without isolation; similarly, in CA1 and hilus, the higher numbers of ADAR1 immunoreactive positive cells were found in 2 and 8 weeks isolation mice.

These results suggested that social isolation led to increased immunoreactivity of ADAR1. Expectedly, the re-socialization group displayed no difference of that in frontal cortex and CA1 with the same age mice without isolation in control groups, which suggested re-socialization rescued the social isolation stress-induced abnormally enhanced immunoreactivity of ADAR1 in frontal cortex and CA1. Additionally, the number of ADAR1 immunoreactive positive cells in the re-socialization group reduced obviously in hilus as compared with the control mice without isolation, which suggested immunoreactive change of ADAR1 in hilus involved in the recovering mechanism of re-socialization.

**Western Blot**

Western blot results of ADAR1 (p110) were consistent with those of immunohistochemistry staining results mostly. In comparison with the same age mice without isolation, the expression of ADAR1 (p110) in the hippocampus elevated significantly in 2 and 4 weeks isolation rearing mice as well as that in the frontal cortex of 8 weeks isolation rearing mice (see Figure 4). The above results suggested that social isolation caused an increase in ADAR1 (p110) expressions.

In addition, as compared with 4 weeks social isolation rearing mice, the expression of ADAR1 (p110) was lower in the hippocampus. The results support the hypothesis that re-socialization reverses the social isolation stress-induced high expression of ADAR1 (p110). Actually, we were also focused on the alteration of ADAR1 (p150) and ADAR2 expression, however, no obvious results were found based on very weak expression in both the social isolation stress groups and the relative control groups (data not shown).

**Discussion**
This study examined alterations of ADAR1 in the frontal cortex and hippocampus of social isolation stress-induced cognitive deficits mice. Three major findings were summarized here: (1) Different distribution characters of ADAR1 in the frontal cortex and hippocampus represented dynamic changes of ADAR1; (2) Short, middle and long durations of social isolation stress altered distribution characters of ADAR1 in frontal cortex and hippocampus, which suggested the involvement of ADAR1 in response of social isolation stress; (3) Re-socialization recovered the cognitive deficits and alterations of ADAR1 led by social isolation stress.

**Social isolation-induced cognitive deficits**

Our study demonstrated that isolated rearing mice induced spatial and non-spatial cognitive deficits as well as abnormal work memory, which are consistent with previous published papers (Khodaie et al., 2015). Social isolation results in cognitive dysfunction, which is also supported by the view that individual rearing has effects on impairing cognition ability on the following behavior tests (Benner et al., 2014; Green & McCormick, 2013). In the rotating T-maze, 6 weeks isolation leads to reversal-learning impairment in Wistar rats (Quan et al., 2010); C57BL/6J and DBA/2 strains reared in 7 weeks isolation since postnatal day 28 show significantly impaired fear conditioning (Võikar et al., 2005); The learning memory ability decreases in isolation-reared rats by the performance of Morris water maze and attention set shifting. The above studies prove that rearing in social isolation leads to cognition deficits in rodents. Actually, more and more studies demonstrate that social isolation stress causes the abnormal cognition ability in human beings (Shankar et al., 2013). Social engagement including enough social connections and participation in social activities can prevent cognitive decline (Green et al., 2008; Crooks et al., 2008). However, the mechanisms of cognitive deficits created by social isolation stress and the recovery from the re-socialization are not clearly understood until now. Undoubtedly, isolated rearing rodents, a kind of stress model, is a good method to understand physiological, pharmacological characteristics and pathogenesis in human beings undergoing social isolation stress (Yusufishaq et al., 2013).

Currently, the mechanisms about cognition decline brought by rearing in social isolation stress are associated with the hippocampus and frontal cortex related neuron circuits involving
in the senior cognition function. Rearing in social isolation damages the structure of frontal cortex (Djordjevic et al., 2010) and hippocampus (Võikar et al., 2005). The detail mechanisms explored as mentioned before, include abnormal function of neurotransmission (Shao S et al., 2014; Baarendse et al., 2013), synaptic plasticity (Pereda-Pérez et al., 2013), apoptosis (Khodaie et al., 2015), potassium ion channel currents change (Quan MN et al., 2010), the accumulation of oxidative products (Djordjevic et al., 2010), regeneration (Pereda-Pérez et al., 2013) and the dysfunction of HPA axis (Sandstrom & Hart, 2005). These findings are focused on the upstream mechanisms mainly. Until now, little is known about the alterations on distribution characters of ADAR1 in the hippocampus and frontal cortex in response to social isolation stress epigenetically. ADAR1 is one of ADAR family and catalyzes the conversion process of adenosine to inosine (A-to-I) in post transcription level (Buechel et al., 2014; Baarendse et al., 2013). Social isolation alters neuroinflammatory response (Karelina K, er al., 2009). Additionally, ADARs involve in inflammation (Yang JH et al; 2003). Based on the current knowledge, we hypothesized that ADAR1 altered in the brain of social isolation stress-induced cognitive dysfunction mice. Our findings about increased expression of ADAR1 in the hippocampus and frontal cortex of social isolation stress mice in this study support our hypothesis. These results suggest that ADAR1 involves in social isolation stress-induced pathological. Although clarifying the detail mechanism need further study, our findings benefit better understanding the mechanisms of social isolation stress-induced cognitive deficits epigenetically.

**ADARs distribution characters and functions**

Three ADARs have been described in mammalian cells (ADARs 1-3), ADARs highly express in the nervous system and lay in the nuclear of the neuron chiefly (Chen et al., 2000). The expression level of ADAR1 mRNA is constant in the development, whereas ADAR2 expression level increases markedly (Chen et al., 2000). ADAR3 is expressed in the brain only and works as a regulatory role that inhibits the activities of ADAR1 and ADAR2.

There are two predominant isoforms including p110 and p150 for ADAR1 (Figure 5). p150 is composed of multiple RNA-binding motifs (21, 24), Z α and Z β (two copies of a Z-DNA-binding motif) in the N-terminal region of the protein, the constitutive p110 lacks the
N-terminal 295 amino acids including the Z-binding motif (George et al., 2005). p110 and p150 are generated through transcription by alternative promoters (George and Samuel, 1999). So far, the influences of social isolation stress on ADARs protein levels and expression patterns in the hippocampus and frontal cortex are not known. ADARs may be involved in changes induced by social isolation stress. To test this hypothesis, we observed the expression pattern changes of ADAR1 isoforms in social isolation stress mice and found an obvious increased protein expression of ADAR1 (p110) in the social isolation stress treatment mice (Figure 3). In our study, the p110 isoform was easily identified; however, p150 was hard to be detectable in control and social isolation mice in relatively low expression (data not shown). That is consistent with the reported finding that p150 expression is extremely low in the brain. p150 is an interferon (IFN)-inducible protein isoform, whose expression is controlled by the IFN-inducible promoter. ADAR1 (p150) expression augments through elevated IFN level (George & Samuel, 1999) and is induced by IFN or pathogen infection (Toth et al., 2006). These findings suggest that different roles of ADAR1 (p150) between IFN/pathogen infection and social isolation stress. On the contrary, the protein expression of p110 was increased in the hippocampus and the cortex significantly in response to social isolation stress (Figure 4). We found that ADAR1 immunoreactivity was predominantly detected in the pyramidal cell layer of the frontal cortex and hippocampus in both control and social isolation mice, in the meanwhile, the number of detectable ADAR1 immunoreactivity positive cells were increased in the social isolation stress groups significantly (Figure 3). There were wide distribution of ADAR1 positive cells existed in CA1, dentate gyrus and hilus, especially in the pyramidal cells. In frontal cortex, the ADAR1 immunoreactivity positive cells displayed almost all layers from molecular layer to multiform layer. Based on the close role of pyramidal cells in the cognition ability (Elston et al., 2001), we focused on counting the ADAR1 positive immunoreactivity cells in layer 5. We found the high expression of ADAR1 positive immunoreactivity cells, which may be one of reasons to be involved in regulation of the social isolation stress-induced cognition deficits. In hippocampus, we also found the wide distribution of ADAR1 positive cells existed in CA1, dentate gyrus and hilus, especially in the pyramidal cells. Our findings suggested the ADAR1 played an active role in the neuron
pathway based on the high expression in the pyramidal cells, a main projecting neuron with rich axons and dendrites.

The function of morphological distribution character of ADAR1 in the hippocampus and frontal cortex in the social isolation stress is still unknown. The deletion and mutation of ADAR gene influence the biological phenotype exceedingly, composing of ataxia, seizure, and neuron degeneration. ADAR activities deficient mice exhibit defects in nervous system, decreased tolerance to stress and failure of hematopoiesis (Tseng et al., 2013). All of above published reports combined with our findings support the hypothesis that ADAR1 involves in modifying social isolation stress-induced cognitive deficits.

**Recovering cognitive deficits by re-socialization**

In order to observe the recovery effect of social isolation stress-induced cognitive deficits from the re-socialization, we also analyzed the re-socialization group in adolescent period. Interestingly, we found that both cognitive dysfunction and abnormal expression of ADAR1 were recovered by re-socialization in adolescence. Those results may be related to the strong plasticity in the adolescence. Environment stress produces much more strong and lasting effects on the behavior and neurophysiology character in young animals than those in adult animals (Forbes & Dahl, 2005). If the living environment in the key period of the development of the rodents is changed, the animal shows the abnormal behavior in the adulthood (Kilts, 2001). Our findings suggest that re-socialization adolescentsly can be considered to be an efficient cognitive enhancer probably capable to compensate for assumed abnormal expression of ADAR1 during isolated rearing. Moreover, isolated mice with re-socialization can recover the abnormal expression of ADAR1 after the short term isolation stress, which suggested that ADAR1 may involve in the mechanism of modifying the social isolation induced cognitive dysfunction in an early period of stress.

**Conclusions**

Taken together, this study demonstrate that was ADAR1 altered in hippocampus and frontal cortex of the cognitive decline mice induced by social isolation stress, which give the clue to clarify social isolation stress-induced cognitive deficits in the epigenetic level. However, how the ADARs family members maintain their homeostasis for regulating the RNA editing
plasticity and inflammation in the cognitive dysfunction caused by social isolation stress had not been elucidated in parallel at both behavioral and molecular levels. Our future study will focus on the possible related mechanisms, which are the basis of the study on social environment and body-mind healthy in human beings.

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References


Figure Legends

Figure 1: **A: Experimental design for groups divided** - The isolated healthy male Kunming mice were rearing individually in a standard condition sized cage for 2, 4 and 8 weeks regarded as SI 2W (isolation of 2 weeks), SI 4W (isolation of 4 weeks) and SI 8W (isolation of 8 weeks) since post-weaning (3 weeks old). Re-socialization group mice (SI 2WR) were returned to their 4 littermates’ gregarious group until adult after 2 weeks isolation. Control groups were the same age mice without isolation stress (C 2W, C 4W and C 8W).

**B: Objection Recognition Test** - The diagram shows the apparatus and cubic objects A, B and C used in this test. The cubic objects (A, B and C, 5×5×5 cm) for recognition are woody blocks. Objects A and B with black color and object C with black and white pattern were used for recognition in the tests. The process of ORT was designated with the sample and the test trial. during the sample trial, object A and object B were placed oppositely. In the test trial, object C was instead of object B.

**C: Objection Location Test** - The diagram shows the apparatus and cubic objects B and C’ used in this test. the cubic objects B and C’ were the same as objects A and B in the ORT.
the sample trial, objects B and C’ were put in the similar location in the ORT. In the test trial, object C’ was moved to locate at the opposite direction toward the object B.

**D: Discrimination index in the objection recognition test and the objection location test** - SI 2W, SI 4W and SI 8W represent 2, 4 and 8 weeks social isolation rearing since postnatal 21 days; C 2W, C 4W and C 8W represent the same age mice in the control groups without isolation stress; SI 2WR represents re-socialization group (Figure 1A). *P<0.05 refers to isolation groups vs the same age mice without isolation in the control groups. (n=10/group).

Figure 2: **Spontaneous alternation times in T maze in Kunming mice** - SI 2W, SI 4W and SI 8W represent 2, 4 and 8 weeks social isolation rearing since postnatal 21 days; C 2W, C 4W and C 8W represent the same age mice in the control groups without isolation; SI 2WR represents re-socialization group (Figure 1A). *P<0.05 refers to isolation groups vs the same age mice without isolation in the control groups. (n=10/group).

Figure 3: **Immunohistochemical analysis of ADAR1 positive cells in the brain of Kunming mice** - (A) The frontal cortex and hippocampus sections were analyzed. The figures were adapted from mice brain atlas of Paxinos and Watson (1997). Black boxes indicated the analyzed area and magnification pictures were presented as follows. (B.C) Double staining with 1% thionine (sigma) and anti-ADAR1-Ab (P110). The representative images were shown. Arrow 1 pointed at the ADAR1 immunoreactive positive neurons. (thioning staining is a kind of nissl staining, which was benefit for marking the neurons). Interestingly, not all the neurons expressed ADAR1 (arrow 2) and glia cells also expressed ADAR1 (arrow 3). The number of ADAR1-immunoreactive positive neurons was counted in the sequential cutting sections (Analyzed sections in frontal cortex is from Bregma 1.18mm for 4 sections, analyzed sections in hippocampus is from Bregma -2.18mm for 4 sections, 16 μm per section.) and got the averages, the analyzed areas were focused on frontal cortex in the internal pyramidal cell layer 5, the analyzed areas were focused on hippocampus are hilus and CA1. The analyzed square was 10000 μm². Averages were measured. Scale bar = 50 μm. (D) The number of ADAR1 immunoreactive positive cells in the frontal cortex and hippocampus of
Kunming mice were analyzed. Data were expressed as the mean ± SEM and were analyzed by two-way ANOVA followed by Tukey’s post hoc testing.

SI 2W, SI 4W and SI 8W represent 2, 4 and 8 weeks social isolation rearing mice since postnatal 21 days; C 2W, C 4W and C 8W represent the same age mice without isolation in the control groups; SI 2WR represents re-socialization group (Figure 1A). *P<0.05 refers to isolation groups vs the same age mice without isolation in the control groups. (n=5/group)

Figure 4: The results of Western blot in the hippocampus and frontal cortex of Kunming mice - The optical density ratio of ADAR1 divided by GADPH was measured. The data was expressed as the mean ± SEM and analyzed by two-way ANOVA followed by Tukey’s post hoc testing. (A): The protein expression of ADAR1 by western blot; (B): The analyzed data.

SI 2W, SI 4W and SI 8W represent 2, 4 and 8 weeks social isolation rearing since postnatal 21 days; C 2W, C 4W and C 8W represent the same age mice in the control groups without isolation; SI 2WR represents re-socialization group (Figure.1 A). *P<0.05 refers to isolation groups vs the same age mice without isolation in the control groups. #P<0.05 refers to re-socialization group vs 4 weeks isolation group. (n=5/group).