# An ADAR1 inducer attenuated the effects of social isolation on depressive-like behavior and ADAR1 (p110) in BALB/c mice

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## Introduction

Social isolation induces depressive-like behavior in animals and humans by impacting RNA editing, but the detailed mechanisms are still unknown. The purpose of this study was to explore how an ADAR1 (RNA-editing enzyme) inducer and inhibitor may impact the isolation-induced depressive-like behavior of mice and to identify new therapeutic targets for the development of an effective solution for the recovery from depressive-like behavior in socially isolated animals and humans.

## Methods

Twenty-one-day-old BALB/c mice with and without isolation treatment were evaluated for depressive-like behavior by open-field tests, tail suspension tests, and forced swimming tests. Immunohistochemistry and Western blots were used to measure the immunoreactivity and protein expression of ADAR1 (p110). In addition, the isolated mice were treated with an ADAR1 inducer (IFN-γ) or inhibitor (EHNA). The performance of both treatments on the behavior of and ADAR1 (p110) expression in isolated mice was examined.

## Results

Both the immunoreactivity and protein expression of ADAR1 (p110) in the prefrontal cortex decreased in isolated BALB/c mice with depressive-like behavior compared to those of the age-matched, gregarious BALB/c mice. Additionally, the treatments with ADAR1 inducer or inhibitor improved or aggravated depressive-like behavior in isolated mice, respectively. Furthermore, the ADAR1 inducer returned the immunoreactivity and protein expression of ADAR1 (p110) back to the normal level.

## Conclusion

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## Abstract

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**Key words:** ADAR1; Depressive-like behavior; BALB/c mice; Social isolation

## Introduction

Social isolation refers to the fact that individuals are isolated from or separated from others or society, causing a lack of all or partial interpersonal relationships, which is a kind of psychosocial stressor (Barratt RL et al., 2011;O'Keefe LM et al., 2014; Khodaie B et al., 2015). Studies have shown that animals or human beings that are in a state of social isolation for a long time will exhibit depressive-like behavior (Evans IEM et al., 2017; Kosofsky BE et al., 1987; Ge L et al., 2017; Chow PI et al., 2017; Li X et al., 2013). It is known that depressive-like behavior is related to abnormal brain development, abnormal serotonin levels, and neuroinflammation (Miragai AS et al., 2018; Xin F et al., 2018; Han L et al., 2018). However, the mechanism of depressive-like behavior induced by social isolation stress has not yet been fully elucidated. Experience of early social isolation will affect rodent brain development and its structure and function, as well as adult behavior (Heim C et al., 2004; Mirescu C et al., 2004; Rapoport JL et al., 2005). It has been reported that social isolation stress leads to decreased dendric density of pyramidal neurons in the cortex (Silva-Gómez AB et al., 2003; Varty GB et al., 1999; Ibi D et al., 2008), reduced formation of neurons and synapses, and changes in the prefrontal cortex dopamine and serotonin systems (Hall FS et al., 1998; Muchimapura S et al.,

2003; Heidbreder CA et al., 2000). Notably, depression patients had abnormal RNA editing of serotonin receptors (Lyddon R et al., 2013). Our recent study found that social isolation not only led to abnormal behavior (An D et al., 2017) but also impacted ADAR1 (p110) immune reactivity and protein expression in the brains of Kunming mice and BALB/c mice (Chen W et al., 2016). Additionally, we found that 5-HT2cR antagonist and 5-HT2cR inverse agonist can rescue isolation-induced abnormal behavior partially mediated by ADAR1 (p110) expression and Htr2c RNA editing (Yu WZ et al., 2018). Therefore, we hypothesized that ADAR1, as an upstream part of serotonin regulation, could be used as an upstream target to intervene in abnormal behavior caused by social isolation stress. To demonstrate that, an ADAR1 inducer and inhibitor were used to treat BALB/c mice with social isolation to explore the action of ADAR1 on depressive-like behavior in isolated BALB/c mice in this study. This study will provide new ideas for the prevention and control of related human depressive-like behavior caused by social isolation stress.

## Materials and Methods

### Animal groups and drug administrations

Healthy male BALB/c mice (n=60) at postnatal 21 days (15 ± 5) g were obtained from the Dalian Medical University Laboratory Center (Dalian, Liaoning, China). The mice lived in the animal house at 21 ± 1°C and with humidity at 55 ± 5%. The mice were randomly divided into 6 groups (n=10 mice/group, Fig. 1). The gregarious mice (5 mice/cage) were reared in plastic cages (290×178×160 mm, Beijing Heli Technology Development Co. Ltd. China) treated with physiological saline (20 ml/kg, i.p.) and were labeled as the GH group (gregarious-house group, n=10). The other group of mice were reared singly and treated with physiological saline (20 ml/kg i.p.) for 4 weeks and were labeled as the SI group (social isolation group, n=10). Additionally, based on our pilot study, ADAR1 inducer (IFN-γ, $5.0\times10^4$ U/20 ml/kg, i.p.) and inhibitor (EHNA, 10 mg/20 ml/kg, i.p.) treatments were examined, and the treated groups were labeled as the SI+IFN-γ group (n=10/group) and the SI+EHNA group (n=10/group), respectively. The age-matched gregarious mice treated with ADAR1 inducer (IFN-γ, $5.0\times10^4$ U/20 ml/kg, i.p.) or inhibitor (EHNA, 10 mg/20 ml/kg, i.p.) were labeled as GH+IFN-γ and GH+EHNA groups (drug treatment alone groups, n=10/group), respectively. All experimental procedures were approved by the Tab of Animal Experimental Ethical Inspection.

### Open-Field Test (OFT)

The open-field test was used to evaluate the nervous and autonomous behavior of experimental

animals in the new environment. The box of the open field was 50 cm×50 cm×40 cm. The inner wall was black, and the bottom surface was divided into 25 lattices (10 cm×10 cm). The analysis system number started from left to right and from top to bottom. Numbers 7, 8, 9, 12, 13, 14, 17, 18 and 19 were in the central region, and the remaining numbers were in the peripheral area; number 13 was in the middle region. The light was approximately 40 lx. The background noise of laboratories was below 65 dB. During the experiment, the tail of the mouse was pinched and placed in the number 13 lattice. The behavioral analysis system began to take videos and automatically recorded the activity of the experimental mice for 5 min. The time the mice stayed in the central region and the total distance of the movement were analyzed. After each experiment, 75% alcohol was used to wipe the box to avoid disturbance by odor or excreta.

**Tail Suspension Test (TST)**

The tail suspension test was used to evaluate the despair state of the mice. The mouse in tail suspension attempts to escape but is unable; then, the mouse abandons the struggle and exhibits an immobile state. Animal immobility time was recorded to reflect the depression state. The tail of the mice was suspended with the head 5 cm from the table. The mice struggled upside down, and after a period of time, stopped struggling because of "despair" and appeared discontinuous inactivity. Video footage was recorded for 6 min. The mice displayed passive suspension with limb immobility. The immobility time in 4 min was analyzed by the double blind method.

**Forced Swimming Test (FST)**

The forced swimming test is also used to evaluate the desperate state of mice. The mice were forced to swim in a transparent glass container with warm water kept at 22 ± 1°C. Each mouse was tested separately, and the swimming tests were arranged in each group in a random order to ensure that the test time of each group was unbiased. The video data were recorded for 6 min, and the total time of drift time and absolute immobility time in 4 min of video were analyzed with a double blind method.

**Immunohistochemistry**

After anesthesia, the mice were perfused with 1% and 4% paraformaldehyde. Then, the brains were incubated in 4% paraformaldehyde and phosphate-buffered saline (PBS) with 20% sucrose at 4°C overnight. Sixteen-micrometer thick slices were cut by a microtome-cryostat. After that, the prefrontal cortex slices were rinsed with PBS and then incubated in 1% bovine serum albumin. Afterwards, ADAR1-Ab (p110) (1:100, Proteintech, USA) was put on the slices at 4°C overnight. Then, the slices were washed with PBS, and the avidin-biotin complex was

put on the slices at room temperature for 2 h. Diaminobenzidine (DAB) was used to detect the positive signals of ADAR1 (p110). Negative control slices were incubated with PBS only. Image analysis was used for the quantification of the results (n=5/group).

**Western blot analysis**

An extraction kit (Keygen Biotech, China) was used to extract the protein of the prefrontal cortex. A BCA protein assay kit (Keygen Biotech, China) was used to measure the protein concentration. The denatured proteins (30 mg per sample) were loaded onto 7.5% sodium dodecyl sulfate-polyacrylamide (SDS) gels. Afterwards, the proteins were transferred to polyvinyl difluoride (PVDF) membranes and blocked for 1 h with 5% bovine serum albumin. After that, the membrane with the proteins was immunoblotted with the primary antibody ADAR1-Ab (1:1000, Proteintech, USA). After washing the membranes with Tris-buffered saline containing Tween 20 (TBST), horseradish peroxidase-labeled secondary antibody (anti-goat 1:5,000; ZSJQ-BIO Company, China) was incubated for 2 h at room temperature in a dark room. BIO-RAD (Hercules, USA) gel analysis software was used to measure the infrared band signals. Then, stripping buffer was used to strip the membranes. Subsequently, the membranes were washed in TBST and probed with GADPH-Ab (1:1,000, Beyotime Company, China). After washing with TBST, horseradish peroxidase-labeled secondary antibody (anti-mouse, 1:5,000; ZSJQ-BIO Company, China) was incubated with the membranes. ADAR1 (p110) protein was normalized to the internal control GADPH (n=5/group).

**Statistical Analyses**

GraphPad Prism 5.0 (San Diego, CA, USA) and IBM SPSS Statistics 21.0 (Aramonk, NY, USA) were used for statistical analyses in this study. All data expressed as the means±SD were analyzed by using Tukey's post hoc test. A t-test was used to analyze the variance for the groups with and without isolation, as well as the groups with and without drug treatment. Two-way ANOVA was used to determine whether there was an interaction between social isolation and drug treatment (two independent variables) on depressive-like behavior and ADAR1 expression (dependent variable) among mice. The results of behavior analyses, immunohistochemistry, and Western blot were analyzed by using Tukey's post hoc test. The data for ADAR1 inducer (IFN-γ) and inhibitor (EHNA) treatments were obtained from two separate analyses. The results were considered statistically significant at $p$-value $<0.05$ (n=10/group in behavior tests, n=5/group in immunohistochemistry staining and Western blot analysis).

## Results

**ADAR1 inducer (IFN-γ) recovered decreased autonomous behavior and exploratory behavior of socially isolated BALB/c mice**

In the open-field test (Fig. 2, A and B), total distance (GH: 29203.21 ± 10500.03, SI: 13030.24 ± 5333.06; *p*-value 0.0043) and the time spent in the central region (GH: 3.30 ± 1.71, SI: 0.98 ± 1.41; *p*-value 0.0024) were significantly reduced for isolated mice compared with the control group. Compared with that of the isolated group, total distance (SI: 13030.24 ± 5333.06, SI+IFN-γ: 31323.69 ± 14396.41; *p*-value 0.0019) and the time spent in the central region (SI: 0.98 ± 1.41, SI+IFN-γ: 4.01 ± 1.38; *p*-value 0.0003) were significantly increased for isolated mice treated with ADAR1 inducer, while there was no significant difference in total distance and the time spent in the central region between isolated mice and isolated mice treated with ADAR1 inhibitor. The results suggested that for social isolation in BALB/c mice, autonomous and exploratory behavior decreased in the new environment, and the ADAR1 inducer recovered this behavior.

**ADAR1 inducer (IFN-γ) recovered increased despair behavior of isolated BALB/c mice**

In the tail suspension test and the forced swim test (Fig. 3, A and B), compared with that for the control group, the immobility time for isolated mice (TST, GH: 21.70 ± 10.76, SI: 49.60 ± 21.16; *p*-value 0.0023; FST, GH: 17.70 ± 11.93, SI: 51 ± 26.82; *p*-value 0.0028) increased significantly. Compared with that of the isolated group, the immobility time for isolated mice treated with the ADAR1 inducer (TST, SI: 49.60 ± 21.16, SI+IFN-γ: 16.40 ± 13.75; *p*-value 0.0020; FST, SI: 51.00 ± 26.82, SI+IFN-γ: 26.40 ± 18.26; *p*-value 0.0453) was significantly reduced, but there was no significant difference in the immobility time of isolated mice treated with the ADAR1 inhibitor. The results showed that BALB/c mice increased their desperation in the unavoidable limited environment after social isolation stress, and ADAR1 inducers alleviated those symptoms.

**ADAR1 inducer (IFN-γ) recovered the decreased ADAR1 (p110) immunoreactivity in the prefrontal cortex of isolated BALB/c mice**

Figure 4 shows that compared with the that of control group, the optical density of ADAR1 (p110)-positive signals in the prefrontal cortex (GH: 0.023 ± 0.003, SI: 0.008 ± 0.001; *p*-value < 0.0001) in the social isolation mice significantly decreased. Compared with that of the isolated mice, optical density values of ADAR1 positive signals in prefrontal cortex (SI: 0.008 ± 0.001,

SI+IFN-γ: 0.015 ± 0.003; *p*-value < 0.0001) increased significantly for isolated mice treated with the ADAR1 (p110) inducer, but there was no significant difference in ADAR1 (p110) optical density between isolated mice and isolated mice treated with the ADAR1 (p110) inhibitor.

**ADAR1 inducer (IFN-γ) recovered the decreased protein expression of ADAR1 (p110) in the prefrontal cortex of isolated BALB/c mice**

Figure 5 shows that compared with that of the control group, ADAR1 (p110) protein expression of the prefrontal cortex (GH: 1.00 ± 0.00; SI: 0.44 ± 0.08; *p*-value < 0.0001) in the socially isolated mice was significantly decreased. Compared with that of the isolation group, ADAR1 (p110) protein expression of the prefrontal cortex (SI: 0.44 ± 0.08; SI+IFN-γ: 0.84 ± 0.05, *p*-value 0.0014) for isolated mice treated with the ADAR1 inducer increased significantly, but there was no obvious difference in ADAR1 (p110) protein expression between isolated mice and isolated mice treated with the ADAR1 inhibitor.

## Discussion

**Social isolation induced depressive-like behavior**

It has been reported that social isolation induces stress and depressive-like behavior in humans and animals (Fone KC & Porkess MV, 2008). Four weeks of social isolation induced depressive-like behavior in C57BL/6J mice (Koike H et al., 2009; Dang YH et al., 2015;) and ICR mice (Benavides-Varela S et al., 2015). These reports are consistent with our findings that BALB/c mice showed depressive-like behavior after 4 weeks of social isolation. The prefrontal cortex is one of the most sensitive brain regions closely related to the pathogenesis of depression, and it is also the main target area of antidepressant drugs (McEwen BS, 2008; MacQueen G & Frodl T, 2011). It is worth noting that the frontal cortex is vulnerable not only to social isolation stress but also to depression-related brain activity (Papp M et al., 2018). Therefore, the present study focused on the related changes in the frontal cortex region of BALB/c mice.

**Abnormal ADAR1 expression in the prefrontal cortex induced by social isolation and its recovery**

ADAR1 belongs to the ADAR family; ADAR family members interact with one another to participate in the RNA editing of precursor mRNA to adenosine (A) and inosine (I) in gene posttranscriptional processes (Fritzell K et al., 2017). ADAR1 has two subtypes (Fig. 6),

ADAR1 (p110) and ADAR1 (p150); ADAR1 p110p150 contain a conservative catalytic deaminase domain (deaminase motif, DM) at the C end. There are 3 double-stranded RNA binding domains (dsRBDs) at the N end. ADAR1 (p150) has a nuclear export signal (NES) Z alpha domain and Z beta domain at the N end, while ADAR1 (p110) has only a Z beta domain at the N end. ADAR1 (p150 and p110) has a nuclear localization signal (NLS). ADAR1 is widely distributed in the central nervous system (Liscovitch N et al., 2014; Rybak-Wolf A et al., 2015). ADAR1 mRNA expression is constant in the forebrain neocortex during postnatal development (Schmauss C et al., 2010). Our previous study found that ADAR1 (p110)-positive signals were distributed widely in almost all layers (from the molecular layer to the multiform layer) of the frontal cortex. Additionally, the number and immune reactivity of ADAR1 (P110)-positive signals were significantly abnormally increased in the frontal cortex of isolated mice compared to age-matched gregarious control mice and were recovered by resocialization (Chen W et al., 2016). Interestingly, in this study, we found that the ADAR1 inducer was effective in decreasing ADAR1 (p110) protein expression and immunoreactivity in the prefrontal cortex to a normal level in isolated mice. This finding suggested that the ADAR1 inducer is a potential therapeutic target for social isolation stress-related disorders.

**The ADAR1 inducer recovered depressive-like behavior induced by social isolation and its possible mechanism**

It is known that there are several ways to alleviate social isolation-induced abnormal behavior, including resocialization (Maisonnette S et al., 1993), drug treatment (Jones AC et al., 2011, Yu WZ et al., 2018), and electroacupuncture (Manni L et al., 2009). In the present study, we found that the ADAR1 inducer was effective in recovering from depressive-like behavior (Patent Application Number: 201810220277.3); on the contrary, the ADAR1 inhibitor (EHNA) aggravated depressive-like behavior compared with that of the isolated model group. Research on the function of ADAR indicates that ADAR-deficient mice exhibit defects in the nervous system and a decreased tolerance to stress (Hsiao MN et al., 2013). Additionally, we found that, compared with isolated mice treated with physical saline, isolated mice treated with the ADAR1 inducer (IFN-γ) showed an increased expression of ADAR1 in the frontal cortex. These results suggest that ADAR1 is involved in regulating the formation of depressive-like behavior in mental disorders. It is noteworthy that ADAR1 expression did not significantly change in the ADAR1 inducer- or inhibitor-treated group. The reason for this may be that EHNA is a competitive inhibitor of ADAR1, which can competitively inhibit the intrinsic activity of ADAR1, but it cannot directly regulate its expression. IFN-γ has been proven to induce the high expression of ADAR1at the cellular level (Patterson JB & Samuel CE, 1995). Another reason is that the homeostatic state of editing-dependent and editing-independent mechanisms maintain

the normal state of ADAR1 expression. Based on our findings and the literature, there is evidence that ADAR1 expression in the prefrontal cortex is involved in the pathogenesis and recovery mechanism of isolation-induced depressive-like behavior.

It is possible that social isolation can aggravate the nonhomeostatic function of ADAR1 (p110), which can cause abnormal expression of the target gene under both dependent and independent editing mechanisms, leading to depressive-like behavior in the animals. Moreover, the ADAR1 inducer, 5-HT2cR antagonist and inverse agonist (Yu WZ et al., 2018), and resocialization (Chen W et al., 2016) are effective in relieving the negative impact of social isolation on ADAR1 expression, bringing animals' behavior back to normal (Fig. 7). In the future, we will further focus on the mechanisms and metabolic pathways of the ADAR1 regulator and conduct in-depth research downstream of the target gene of ADAR1 to reveal the mechanism related to social isolation stress, which leads to abnormal behavior of humans and experimental animals, to provide a theoretical basis for the prevention and treatment of stress caused by abnormal behavior or mental illness.

## Conclusion

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## Acknowledgements

This study was supported by the National Natural Science Foundation of China (31201724 and 81471373), China scholarship council (201408210227).

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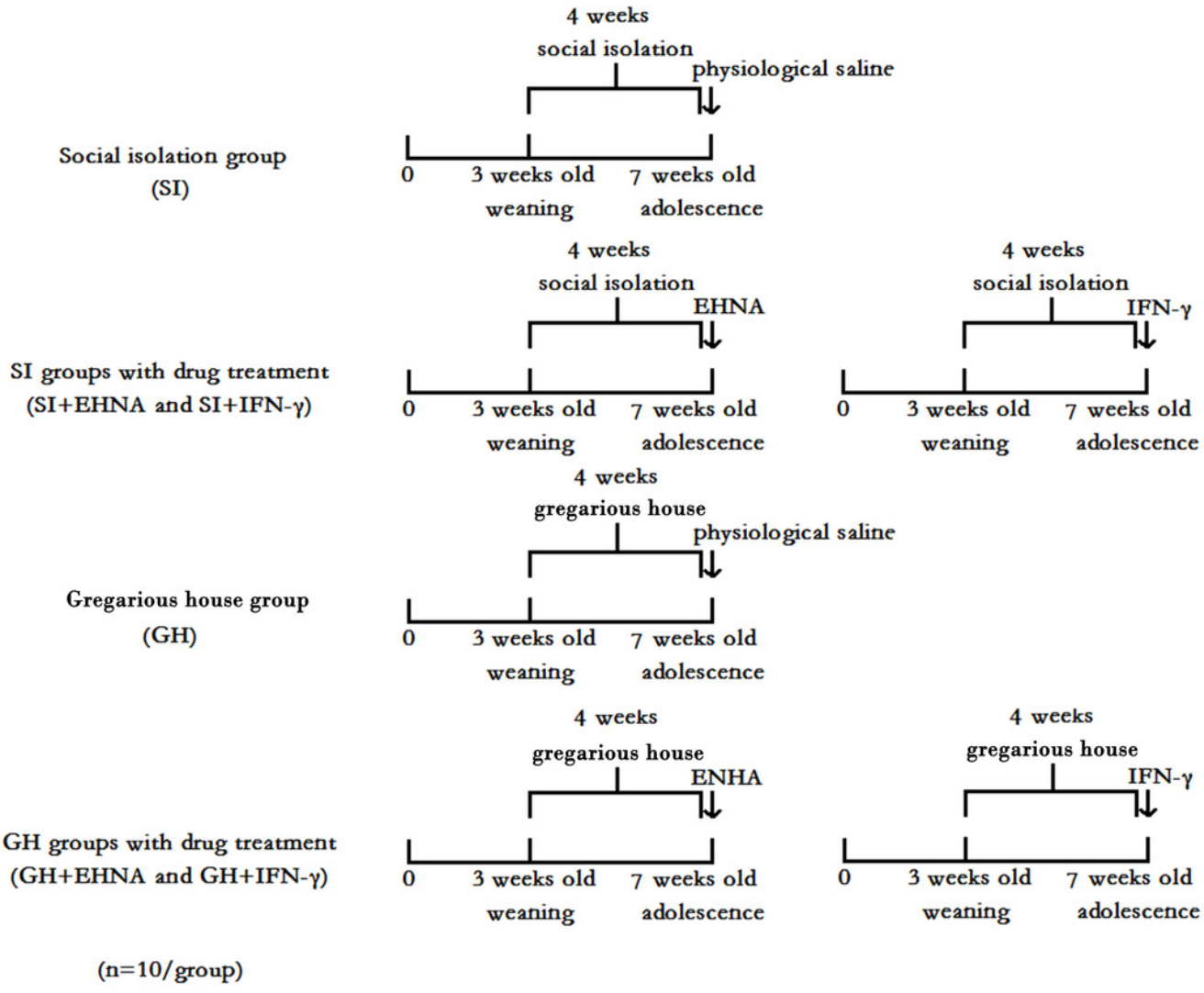
425

# Figure 1

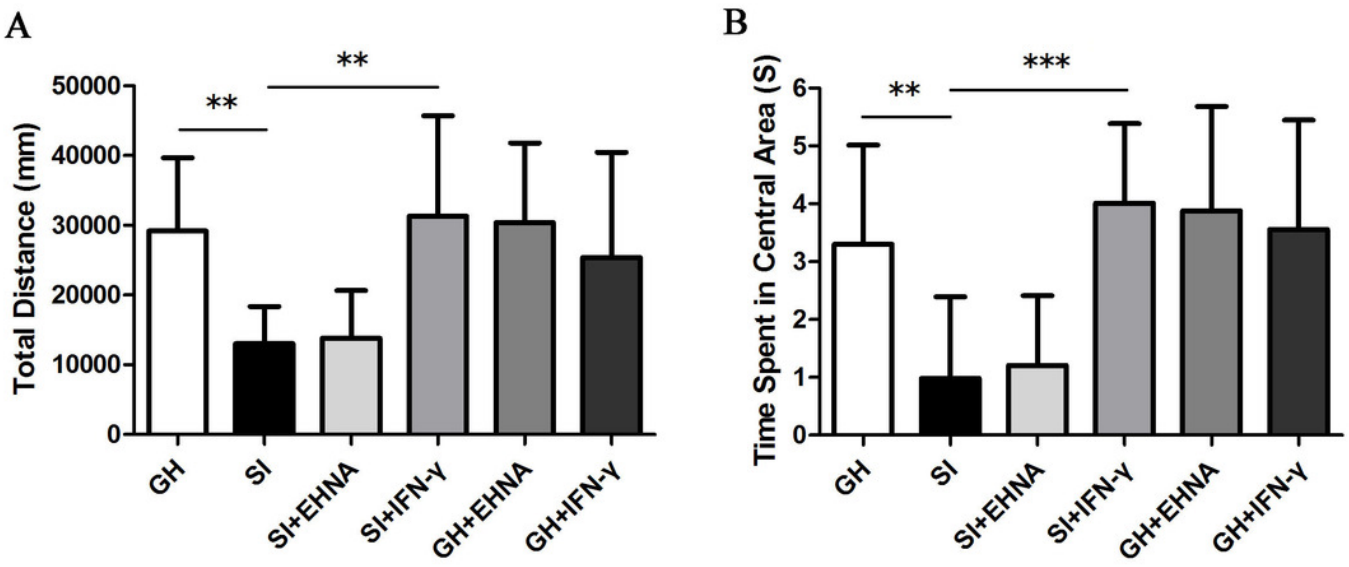
Groups of mice with and without 4 weeks social isolation followed by treatments with ADAR1 inducer (IFN-γ) / inhibitor (EHNA)



# Figure 2

ADAR1 inducer (IFN-γ) recovered decreased autonomous behavior and exploratory behavior of social isolation BALB/c mice

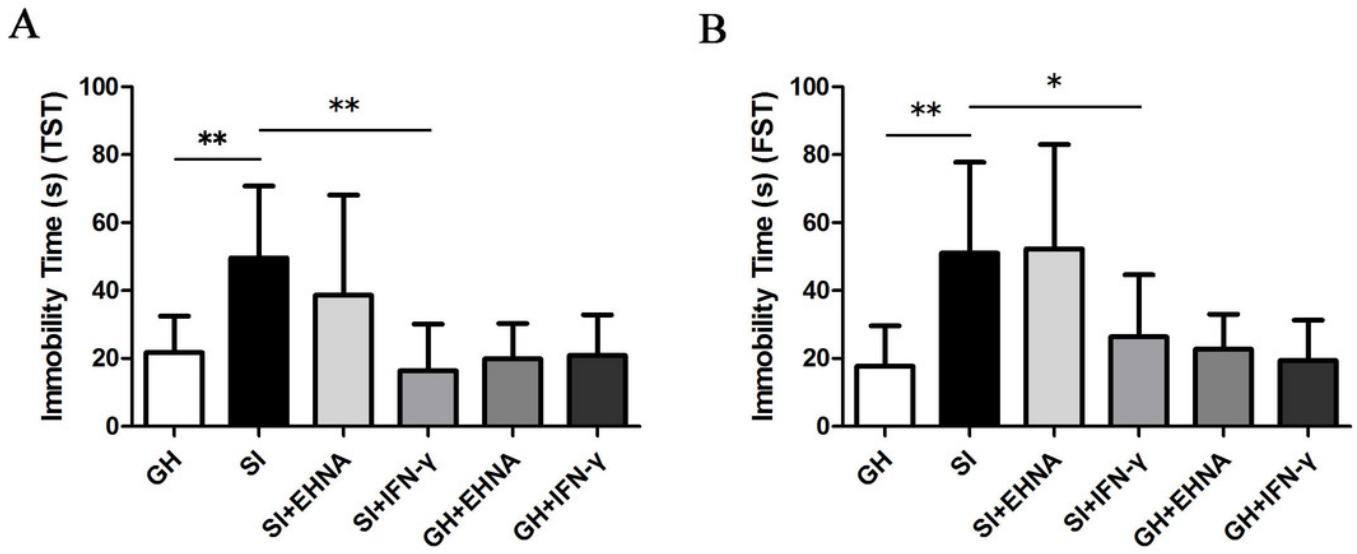
(A) Total distance of the mice's movement; (B) Time the mice spent in the central region. IFN-γ/ EHNA represent ADAR1 inducer/inhibitor; data represents mean±standard deviation; $^{**}p < 0.01$,$^{***}p < 0.001$; (n=10/ group).



# Figure 3

ADAR1 inducer (IFN-γ) recovered increased despair behavior of social isolated BALB/c mice

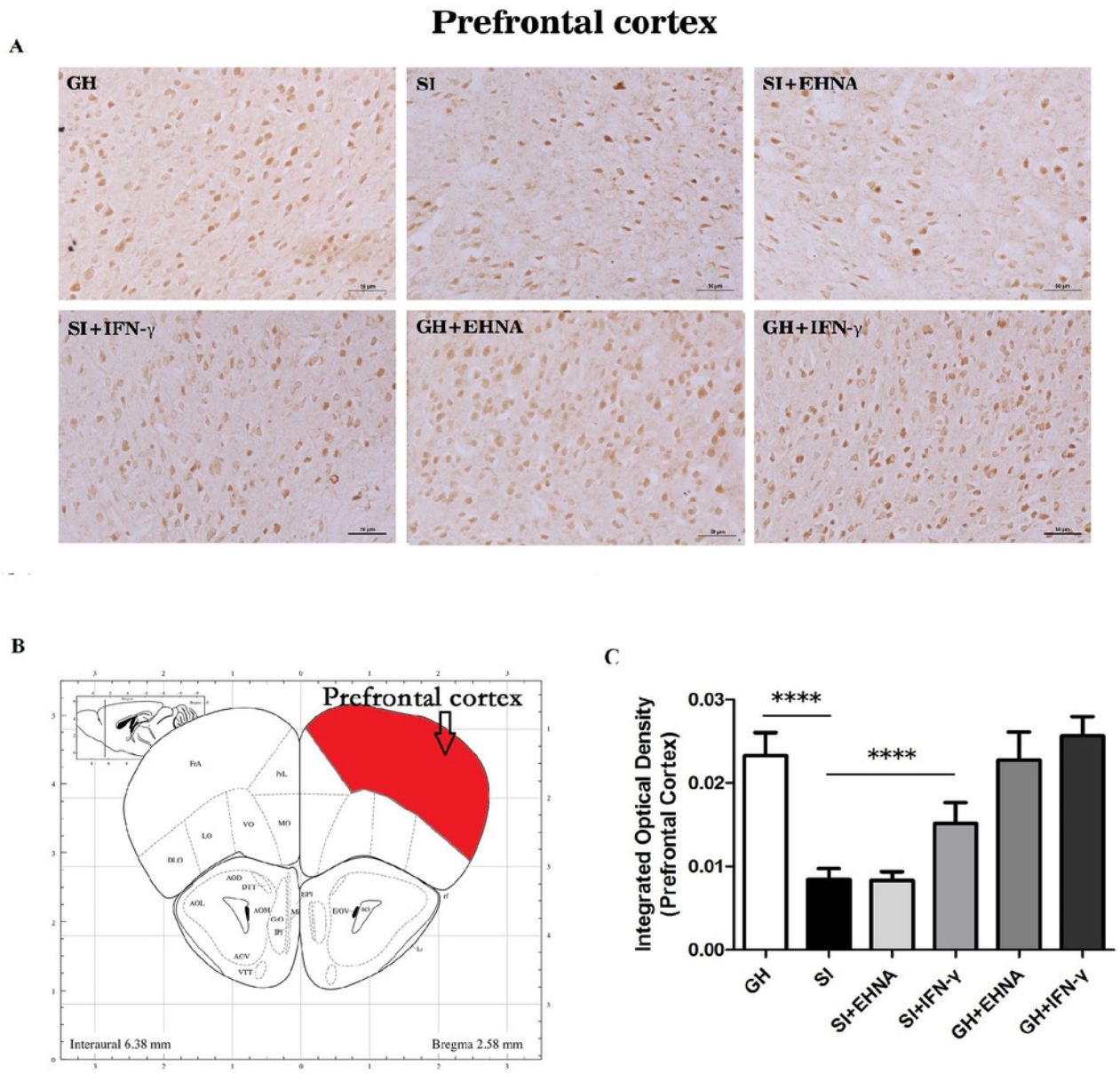
(A) Immobility time in tail suspension test (TST); (B) Immobility time in forced swimming test (FST). IFN-γ/ EHNA represent ADAR1 inducer/inhibitor; data represents mean±standard deviation; *$p$< 0.05, **$p$< 0.01; (n=10 / group).



# Figure 4

ADAR1 inducer (IFN-γ) recovered the decreased ADAR1 (p110) immunoreactivity in prefrontal cortex of isolated BALB/c mice

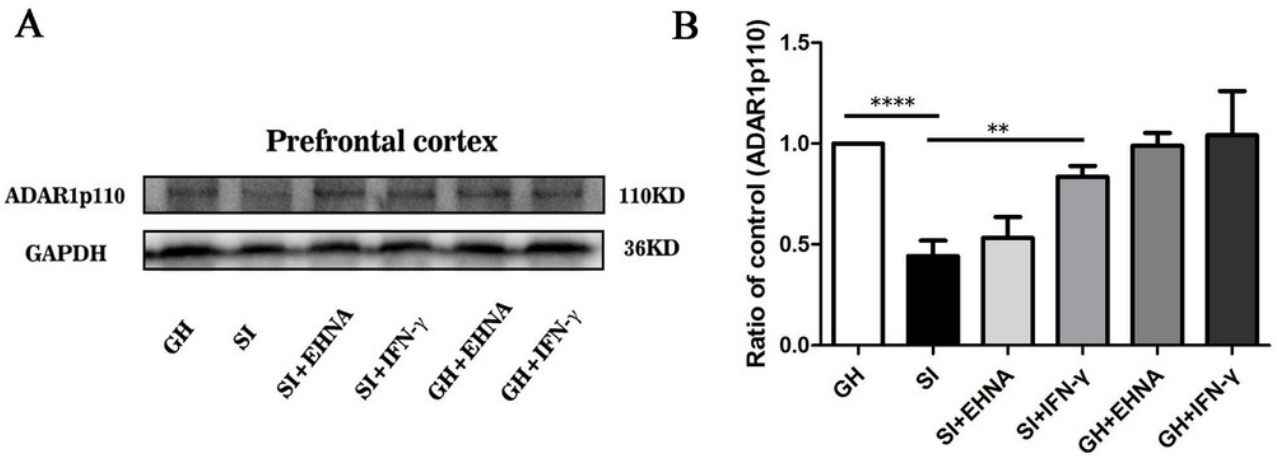
(A) Immunohistochemical staining pictures of ADAR1 (p110) positive signals in prefrontal cortex; (B) Schematic map of prefrontal cortex; (C) The optical density values of ADAR1 (p110) positive signals in prefrontal cortex. IFN-γ/ EHNA represent ADAR1 inducer/inhibitor; data represents mean±standard deviation; ****$p$< 0.0001; (n=5 / group).



# Figure 5

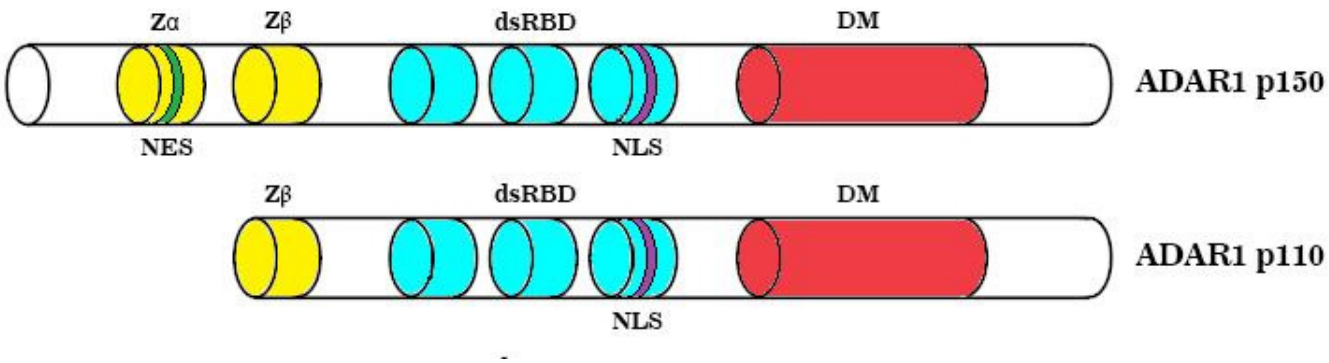
ADAR1 inducer (IFN-γ) recovered the decreased protein expression of ADAR1 in prefrontal cortex of social isolation BALB/c mice

(A) ADAR1 (p110) protein expression of prefrontal cortex; (B) The statistical results for normalized ADAR1 (p110) protein expression by internal control GADPH; IFN-γ/ EHNA represent ADAR1 inducer/inhibitor; data represents mean±standard deviation; $^{**}p < 0.01$, $^{****}p < 0.0001$; (n=5 / group).



# Figure 6

Structure pattern diagram of ADAR1 and its subtypes



# Figure 7

The possible mechanism on ADAR1 inducer recovering the depressive-like behavior induced by social isolation