Fluoxetine improves behavioural deficits induced by chronic alcohol treatment by alleviating RNA editing of 5-HT2C receptors

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Fluoxetine improves behavioural deficits induced by chronic alcohol treatment by alleviating RNA editing of 5-HT_{2C} receptors

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Abstract

The alcoholism and major depressive disorder are common comorbidity, with alcohol-induced depressive symptoms being eased by selective serotonin re-uptake inhibitors (SSRIs), although the mechanisms underlying pathology and therapy are poorly understood. Chronic alcohol consumption affects the activity of serotonin 2C receptors (5-HT_{2C}R) by regulating adenosine deaminases acting on RNA (ADARs) in neurons. Astrogliopathic changes contribute to alcohol addiction, while decreased release of ATP from astrocytes can trigger depressive-like behaviours in mice. In this study, we discovered that chronic alcohol treatment increased editing of RNA of 5-HT_{2C}R via up-regulating the expression of ADAR2, consequently reducing the release of ATP from astrocytes induced by 5-HT_{2C}R agonist, MK212. Moreover, SSRI antidepressant fluoxetine decreased the expression of ADAR2 through the transactivation of EGFR/PI3K/AKT/cFos signaling pathway. The increased release of astroglial ATP by MK212 which was suppressed by chronic alcohol consumption, and reduction in ADAR2 activity eliminated the RNA editing of 5-HT_{2C}R increased by alcohol *in vitro* and recovered the release of ATP from astrocytes induced by MK212. Meanwhile, fluoxetine improved the behavioural and motor symptoms induced by alcohol addiction and decreased the alcohol intake. Our study suggests that the astrocytic 5-HT_{2C}R contribute to alcohol addiction; fluoxetine thus can be used to alleviate depression, treat alcohol addiction and improve motor coordination.

Key words: 5-HT_{2C} receptor; chronic alcohol addiction; ADAR2; ATP; astrocytes

1. Introduction

Alcohol abuse, characterized by excessive drinking and inability to control alcohol consumption, reflecting serious psychological dependence, is a widespread addictive disorder (Grant et al., 2015). Concomitant occurrence of alcoholism and major depressive disorder (MDD) is common, while symptoms of depression induced by alcohol abuse can gradually disappear with the alcohol abstinence (Pettinati et al., 2013). The mechanism(s) underlying the role of alcohol addiction in formation of depressive symptoms is poorly understood. Clinical management of alcohol-induced depressive symptoms mostly relies on selective serotonin re-uptake inhibitors (SSRIs) (O'Donnell and Shelton, 2011), with the therapeutic effects developing in 3-4 weeks after therapy commencement; the relevant pharmacological mechanisms remain unclear.

Serotonin 2C receptors (5-HT_{2C}R), are Gq-protein coupled receptors widely expressed in neurones and astrocytes in the central nervous system (CNS). After chronic exposure to alcohol, the activity of 5-HT_{2C}R can be modified by adenosine deaminases acting on RNA (ADARs), which convert adenosine (A) to inosine (I) at five sites of 5-HT_{2C}R mRNA; this process is associated with the expression of 24 potential edited isoforms (Wang et al., 2000a, 2000b). Aberrant activity of 5-HT_{2C}R was suggested to link to the alcohol addiction and the neuropsychiatric disorders (Werry et al., 2008; Watanabe et al., 2014). The mRNA editing of neuronal 5-HT_{2C}R in mice can be also triggered by stress as well as by exposure to SSRIs (Englander et al., 2005). Alcohol abuse, however, can affect not only neuronal networks but also act

on astrocytes, which are primarily responsible for homeostasis and catabolism of central neurotransmitters (Kaczor et al., 2015; Crawford et al., 2012; Lindberg et al., 2018). According to our previous studies, dysfunction of astrocytes contributes to pathophysiology of MDD, while SSRI antidepressant fluoxetine selectively acts as an agonist of 5-HT_{2B}R in astrocytes; this action being linked to fluoxetine anti-depressive activity (Li et al., 2008, 2009, 2011, 2017; Li et al., 2019).

Recent studies demonstrated that decreased secretion of ATP from astrocytes can induce the depressive-like behaviours in mice, while fluoxetine exposure was shown to increase the level of extracellular ATP in rodents (Cao et al., 2013; Kinoshita, 2018). Astrocytes can release ATP in response to physiological stimulation (Hertz et al., 2015), and hence we analyzed the relationship between astroglial ATP release and editing of serotonin receptors in the context of alcohol addiction and fluoxetine therapy.

2. Materials and methods

2.1. Animals

The CD-1 mice were purchased from Charles River, Beijing, China. For *in vitro* experiments, newborn mice were used for making primary cultures. For animal experiments, 10 - 12 weeks old male mice weighting about 25 g were used; animals were raised in standard housing conditions (22 ± 1℃; light/dark cycle of 12/12h), with water and food available *ad libitum*. All experiments were performed in accordance with the US National Institutes of Health Guide for the Care and Use of

Laboratory Animals (NIH Publication No. 8023) and its 1978 revision, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, No. [2019]059.

2.2. Materials

Primary antibodies against ADAR2, 5-HT_{2C}R, 5-HT_{2B}R, β-actin and cFos, were purchased from Santa-Cruz Biotechnology (Santa, CA, USA). Primary antibodies raised against NeuN and GFAP was purchased from Thermo Fisher Scientific (CA, USA). Secondary antibodies for western blot, antimouse IgG HRP conjugated, was purchased from Promega (Madison, WI, USA). Fluor-conjugated secondary antibodies were purchased from Abcam (Cambridge, MA). Most chemicals, including MK212 (2-Chloro-6-(1-piperazinyl)-pyrazine hydrochloride), SB204741 (N-(1-Methyl-1H-5-indolyl)-N'-(3-methyl-5-isothiazolyl) urea), LY294002(2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine), SB242084 (6-chloro-5-methyl-1-[2-(2-methylpyridyl-3-oxy)-pyrid-5-yl carbamoyl] indoline) and AG1478 (N-[(2R)-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl]-Ltryptophan methylamide), were purchased from Sigma (St Louis, MO, USA). ATP assay kits were purchased from Beyotime Biotechnology (Shanghai, China). TUNEL cells apoptosis assay kits were purchased from Roche (Mannheim, Germany).

2.3. Alcohol addiction model and drug treatment

As described previously (Nuutinen et al., 2011), 24 mice were randomly divided into two groups to be fed either with alcohol or water. Mice in alcohol addiction model group were exposed to gradually increasing alcohol concentrations: 3% for 5 days, 7% for 5 days, 11% for 5 days, 15% for 7 days and 20% for 7 days. Alcohol solution was only water source for alcohol group. After this protocol, alcohol preference test was performed. Subsequently, mice feed with water or alcohol were further randomly divided into two groups with intraperitoneal injection (i.p.) of fluoxetine or normal saline (NS) for one week after which behavioral tests were performed.

2.4. Primary culture of astrocytes

As described previously (Li et al., 2011; Xia et al., 2018), newborn mice were used to isolate astrocytes. The neopallia of cerebral hemispheres were isolated, dissociated and filtered. Isolated astrocytes were grown in Dulbecco's Minimum Essential Medium (DMEM) with 7.5 mM glucose supplemented with 20% horse serum. Astrocytes were incubated at 37 ℃ in a humidified atmosphere of CO_2/air (5:95%). The astrocytes were stained for the astrocytic marker GFAP and neural marker NeuN followed by incubation with DAPI solution at 1:1000 for detecting the purity of the primary cultures of astrocytes. Images were captured with a confocal scanning microscope (DMi8, Leica, Germany).

2.5. ADAR2 RNA interfering

As described previously (Li B. et al., 2018 and Li X. et al., 2019), the cultured astrocytes were incubated in DMEM without serum for half day before transfection. A transfection solution containing 2 μl oligofectamine (Promega, Madison, WI, USA), 40 μl MEMI, and 2.5 μl ADAR2 siRNA (666 ng) was added to the culture in every well for 8 h. In the siRNA-negative control cultures, transfection solution without siRNA was added. Thereafter, DMEM with three times serum was added to the cultures. ADAR2 siRNA duplex chains were purchased from Santa Cruz Biotechnology (CA, USA).

2.6. Western blotting

As described previoulsy (Li et al., 2008), protein concentrations of samples were detected by Lorry method, with bovine serum albumin as the standard. In brief, each sample contained 50 μg protein, which were applied on slab gels of 10% polyacrylamide. After transfer to nitrocellulose membranes, TBS-T (Tris Buffered Saline with Tween 20) containing 5% skimmed milk powder was used to block samples for 2 h. Then, the primary antibodies were utilized to incubate the nitrocellulose membranes for 2 h, which were specific to $5HT_{2B}R$, $5HT_{2C}R$, cFos and ADAR2. Samples were incubated for another 2h with the corresponding secondary-antibodies. Staining was visualized by ECL detection reagent, and images were acquired with an electrophoresis gel imaging analysis system.

2.7. PCR sequencing

As described previoulsy (Li et al, 2011; Yue et al., 2019), polymerase chain reaction amplification was performed with 5-HT_{2C}R primers, forward (5' agatatttgtgccccgtctgg 3') and reverse (5' aagaatgccacgaaggaccc 3'). The amplified samples were then used for cDNA sequence test. Complementary DNA sequencing was carried out by TaKaRa biotechnology Co. Ltd, and RNA editing efficiencies were calculated by the peak heights.

2.8. ATP content assay

The culture medium was collected after incubating cells with or without 4 mg/ml alcohol and 1 μM fluoxetine for 24 h; subsequnetly cells were treated with 30 μM MK212 (a selective agonist of 5-HT_{2C}R) or NS (normal saline) for 15 min,. As described previoulsy (Wang et al, 2007), ATP assay kit (Beyotime Biotechnology, Shanghai, China) was used to detect ATP concentration. ATP standard samples were prepared with same culture medium. 20 μl samples or standard ATP samples were added into opaque 96-well plates. 100 μl ATP assay reagent were added into 96-well plates to mix with samples for 5 min. Chemoluminescence intensity was detected by fluorescence reader (Infinite M200 Pro, Tecan, Switzerland).

2.9. Alcohol preference test

As described previoulsly (Nuutinen et al, 2011), alcohol drinking percent indicates alcohol preference. After 12 h of food and water deprivation, mice were provided with two pre-weighed bottles, including one bottle that contained 20 % alcohol and a

second bottle filled with water, for 2 h. The percentage preference was calculated according to the following formula: % preference = (alcohol intake/(alcohol + water intake)) × 100 %.

2.10. Open field test

The open field test is an anxiety-based test, as previously described (Xia et al, 2017). The mice were placed in the centre square of an open field box (60 × 60 × 40 cm) divided into nine squares, and behaviours were recorded for 6 min. The parameters used for analysis included the total travel distance and time spent in the central area.

2.11. Tail suspension test

As described previously (Xia et al, 2017), comparing the immobility time in tail suspension test reflects despair-like behaviour. Mice were suspended by their tails at 20 cm from ground for 6 min. The time of immobility was recorded to calculate percentage.

2.12. Rotating rod test

As per our previous description (Xia et al, 2017), time of staying on rotating rod indicates motion balance ability. Each mouse was placed on a rotating bar, which was set to a rotation speed of up to 20 rpm during the test. The time spent on the rotating bar was recorded as the latent period. The latency before falling was recorded using a

stopwatch, with a maximum of 90 s.

2.13. Hematoxylin-eosin (HE) staining

After fixation with 4% paraformaldehyde, cells were washed twice by double distilled water for 5 min. Subsequently cells were stained with hematoxylin solution for 5 min and 1% eosin solution for 3 min. The number of cells was imaged by a microscope (Axio Scan Z1, Zeiss, Germany).

2.14. TUNEL staining

As described previoulsy (Li et al, 2016), cultured astrocytes were fixed with 4% paraformaldehyde. The cells were permeabilized with 4% bovine serum albumin (BSA) for 1 hr. Apoptosis in the astrocytes was detected via TUNEL assays (cell death detection; Roche, Mannheim, Germany), according to the manufacturer's protocol. Then, the astrocytes were stained for the astrocytic marker GFAP followed by incubation with DAPI solution at 1:1000. Images were captured with a confocal scanning microscope (DMi8, Leica, Germany). The apoptotic cells were expressed as a percentage of the TUNEL-positive cells among the DAPI-positive cells. The positive control by pretreatment with BSA was shown in supplementary figure 1.

2.15. Statistics

The differences among multiple groups were analyzed by one-way or two-way analysis of variance (ANOVA) followed by a Tukey post hoc multiple comparison

test for unequal replications using SPSS 20.0 software. All statistical data in the text are presented as the mean ± SEM, the value of significance was set at $p < 0.05$.

3. Results

3.1. Alcohol increases expression of cFos and ADAR2 in cultured astrocytes

As compared with control group, the protein expression of cFos was increased to 212.50 ± 24.71% at 4 mg/ml ($n = 6$, $p = 0.020$) and to 271.07 ± 44.53% at 8 mg/ml ($n = 6$, $p = 0.021$) (Fig. 1A and 1B), the protein level of ADAR2 was also up-regulated to 212.36 ± 18.66% ($n = 6$, $p = 0.016$) and to 356.89 ± 30.83% ($n = 6$, $p = 0.003$) at these two doses of alcohol (Fig. 1C). Based on these results, 4 mg/ml alcohol was used for the subsequent experiments. As shown in Fig. 1D and E, alcohol exposure did not significantly change the number of astrocytes stained with HE and observed in the bright field. The number of astrocytes in every mm^2 is about 12.04 ± 1.37. In the TUNEL assay (Fig. 1F), the ratio of TUNEL+/DAPI+ in alcohol group was 86.02 ± 22.76% of control group ($n = 6$, $p = 0.715$; Fig. 1G), this difference however did not reach statistical significance. This concentration of alcohol did not evoke apoptosis. The purity of the primary cultures of astrocytes was more than 95% (Fig. 1H), as our previous studies (Li et al., 2008; Yue et al., 2019).

3.2. Fluoxetine suppresses expression of cFos and ADAR2 in cultured astrocytes

The treatment with fluoxetine with or without alcohol did not change the protein expression of 5-HT_{2C}R in astrocytes (Fig. 2A and 2B). However, exposure of 1 μM

fluoxetine to primary cultured astrocytes decreased expression of both cFos and ADAR2 to 47.36 ± 4.38% (n = 6, p = 0.022) and 58.38 ± 3.51% (n = 6, p = 0.002) respectively (compared to the control; Figs. 2C and 2D). After treatment of cultures with 4 mg/ml alcohol for 1 day, the protein expression of cFos in alcohol plus fluoxetine group was decreased to 83.40 ± 2.11% of alcohol plus NS group (n = 6, p = 0. 030) (Fig. 2C), and the protein expression of ADAR2 in alcohol plus fluoxetine group was decreased to 83.12 ± 1.84% of alcohol plus NS group (n = 6, p = 0.009) (Fig. 2D). Conversely, the expression of 5-HT_{2B}R were not effected by the treatment with alcohol or fluoxetine (Fig. 2E). The level of 5-HT_{2B}R treated with fluoxetine was 93.36 ± 7.14% of NS group (n = 6, p = 0.537), the expression of 5-HT_{2B}R in alcohol plus fluoxetine group was 112.94 ± 4.94% of alcohol plus NS group (n = 6, p = 0.309) (Fig. 2F).

Effects of fluoxetine on cFos and ADAR2 expression was antagonized by several inhibitors of cellular signaling cascades (Fig. 3A). As shown in Fig. 3B and 3C, SB204741 (SB), an inhibitor of 5-HT_{2B}R, increased the expression of cFos and ADAR2 to 117.08 ± 10.18% (n = 6, p = 0.242) and 91.73 ± 7.97% (n = 6, p = 0.370) of control group. The PP1, a selective antagonist of Src, increased the level of cFos and ADAR2 to 114.78 ± 10.19% (n = 6, p = 0.271) and 88.08 ± 8.54% (n = 6, p = 0.320) of control group. The AG1478 (AG), a specific inhibitor of EGFR, enhanced the expression of cFos and ADAR2 to 130.04 ± 19.74% (n = 6, p = 0.189) and to 71.70 ± 3.28% (n = 6, p=0.130) of control group, but there was no significant difference between fluoxetine in combination with AG group and control group

regarding the expression of cFos or ADAR2. The LY294002 (LY), an inhibitor of AKT phosphorylation, changed the level of cFos and ADAR2 to 87.57 ± 15.93% (n = 6, p = 0.333) and 101.84 ± 5.78% (n = 6, p = 0.469) of control group.

As shown in Fig. 3A, the administration of alcohol still significantly increased the expression of cFos and ADAR2 to 207.02 ± 7.74% (n = 6, p = 0.007) and to 256.68 ± 17.89% (n = 6, p=0.007) of control group. Fluoxetine could also decrease these levels of cFos and ADAR2 elevated by alcohol to 161.56 ± 8.66% (n = 6, p = 0.043) and to 175.09 ± 10.39% (n = 6, p=0.038) of control group. However, pre-treatment with SB, PP1, AG and LY, the co-administration of alcohol and fluoxetine increased the level of cFos to 209.15 ± 12.54% (n = 6, p = 0.010), to 217.64 ± 10.95% (n = 6, p = 0.007), to 219.55 ± 11.73% (n = 6, p = 0.007) and to 206.73 ± 13.64% (n = 6, p=0.013) of control group, separately (Fig. 3B). As the same treatments, compared to control group, the level of ADAR2 were also increased to 270.05 ± 15.98% (n = 6, p = 0.004) in SB pretreated alcohol plus fluoxetine group, to 277.61 ± 14.26% (n = 6, p = 0.003) in PP1 pretreated group, to 248.38 ± 12.90% (n = 6, p = 0.004) in AG pretreated group and to 254.48 ± 16.42% (n = 6, p=0.006) in LY pretreated group, separately (Fig. 3C).

3.3. Alcohol and fluoxetine have opposite effects on RNA editing of 5-HT_{2C}R

As shown in Fig. 4A and 4B, three subunits of ADAR catalyze editing of adenosine (A) to inosine (I) which is recognized as guanosine (G) at specific five sites of pre-mRNA (A-E). The representative mRNA sequencing images at D site, the peak of

adenosine (A) was green, the peak of guanosine (G) was black (Fig. 4C). The ratios between the edited G-containing and unedited A-containing isoforms at site D of 5-HT_{2C}R were determined. In the control group, RNA editing ratio between edited (G) and the sum of edited and unedited 5-HT_{2C}R (G+A) was 77.08 ± 1.09% (n = 6). Fluoxetine decreased the ratio of G/G+A to 88.58 ± 1.48% of control group (n = 6, p = 0.001) at 1 μM, the treatment with alcohol increased the percentage of G/G+A to 104.54 ± 0.58% of control group (n = 6, p = 0.024) at 4 mg/ml. However, the administration of fluoxetine suppressed this editing ratio to 95.38 ± 0.79% of alcohol group (n = 6, p = 0.003), there was no significant difference between control group and alcohol plus fluoxetine group (Fig. 4D). The ratios of G/(G+A) for the other four RNA editing sites were shown in Fig. 4E, there were no significant difference among different groups.

Meanwhile, we also measured the RNA editing conditions in the primary cultured astrocytes (Fig. 4F). The ratio of G/(G+A) in fluoxetine group was decreased to 51.02 ± 4.71% of control group (n = 6, p = 0.006), alcohol increased this ratio to 238.35 ± 7.20% of control group (n = 6, p < 0.001), fluoxetine could decrease this increased ratio by alcohol to 142.80 ± 6.33% of control group (n = 6, p = 0.015). However, after the pre-treatment with ADAR2 siRNA duplex, the ratios of G/(G+A) in these four groups were totally suppressed, and there was no significant difference (Fig. 4G). RNA interfering efficiency was shown in supplementary figure 2, after using ADAR2 siRNA duplex, the protein level was decreased to 4.99 ± 3.33% of control group (n = 6, p < 0.001).

3.4. Alcohol and fluoxetine have opposite effect on 5-HT_{2C}R-stimulated release of ATP

In the primary culture of astrocytes, MK212 (a selective agonist of 5-HT_{2C}R) increased the level of extracellular ATP to 14.80 ± 0.59 nmol/g ($n = 6$, $p < 0.001$), which was 175.88 ± 6.97% of control group bathed normal saline (NS) (Fig. 5A). The selective antagonist of 5-HT_{2C}R (SB242084) completely abolished the level of ATP increased by MK212 to 99.67 ± 5.42% of control group (Fig. 5A). After pre-incubation with 1 μM fluoxetine for 1 day, MK212 enhanced the level of ATP to 197.42 ± 14.74% of control group ($n = 6$, $p < 0.001$), the exposure of astrocytes to fluoxetine alone changed extracellular ATP concentration, and there was no significant difference between two MK212 group of NS and fluoxetine groups (Fig. 5B). In the presence of alcohol for 1 day, the extracellular level of ATP was 94.85 ± 6.78% ($n = 6$, $p = 0.577$), in the cells exposed to alcohol and fluoxetine ATP level was 94.1 ± 6.69% ($n = 6$, $p = 0.523$), compared with NS group without alcohol (Fig. 5B). Comparing with NS group treated with alcohol, the level of ATP induced by MK212 was increased to 120.37 ± 7.43% ($n = 6$, $p = 0.101$) (Fig. 5B), there was no significant difference. After treatment with alcohol plus fluoxetine, MK212 however stimulated ATP release to 146.09 ± 5.46% of NS group ($n = 6$, $p < 0.001$) (Fig. 5B).

Moreover, after pretreatment with ADAR2 siRNA duplex, as comparison to control group, MK212 could significant increase the release of ATP from astrocytes to 218.45 ± 15.54% ($n = 6$, $p < 0.001$) in control plus NS group, to 201.78 ± 13.99% ($n = 6$, $p <$

0.001) in control plus fluoxetine group, to 228.47 ± 16.75% (n = 6, p < 0.001) in alcohol plus NS group, to 224.79 ± 17.15% (n = 6, p < 0.001) in alcohol plus fluoxetine group. However, there was still no significant difference between any MK212-induced group treated with ADAR2 siRNA duplex and any MK212-induced group in control group treated without siRNA duplex (Fig. 5B). And the relevant average mean in ever group were also shown in supplementary Table 1.

3.5. Fluoxetine improves alcohol-impaired behaviour

The gradually increased dose of alcohol was used for building an addiction model, whereas mice in control group were fed with water for 29 days (Fig. 6A). Subsequently "alcoholic" mice were divided into two groups, which were injected with NS or fluoxetine (1 mg/kg dose, i.p.) for one week; meanwhile treatment with alcohol was continued (Fig. 6A). At the end of all tests, the weight of mice was also measured, there was no significant difference between groups (Fig. 6B). Before the injection of fluoxetine, the ratio of alcohol preference was increased to 128.85 ± 5.98% of control group (n = 12, p = 0.011) on 29th day (Fig. 6C). After the combined administration of alcohol with fluoxetine for one week, the preference ratio in alcohol plus fluoxetine group was decreased to 86.12 ± 2.17% of alcohol plus NS group (n = 6, p = 0.004), the alcohol plus NS group was increased to 156.74 ± 6.38% of control group (n = 6, p < 0.001) (Fig. 6D). For checking movement capacity by rotating rod test, alcohol reduced the time of mice standing on the rod to 30.32 ± 3.05% of control group (n = 6, p<0.001), the treatment with fluoxetine increased the dwell time

reduced by alcohol to 60.65 ± 10.55% of control group (n = 6, p = 0.017) (Fig. 6E). In the open field test, compared with control group, the distance was increased to 130.42 ± 8.57% in alcohol plus NS group (n = 6, p = 0.019). However, this distance in alcohol plus fluoxetine was decreased to 82.82 ± 5.76% of alcohol plus NS group (n = 6, p = 0.045) (Fig. 6F). As to another measured indicator in open field test, time spent in the centre was suppressed to 43.81 ± 7.14% of control group (n = 6, p = 0.007), fluoxetine increased this time to 98.07 ± 12.81% of control group (n = 6, p = 0.469) (Fig. 6G). In the open field test, treatment with fluoxetine did not significantly change the travelled distance and staying time in centre area (Fig. 6F and 6G). Measuring mice depressive-like behaviour by tail suspension test, the recorded immobility time was increased by alcohol to 206.21 ± 16.11% of control group (n = 6, p = 0.002), fluoxetine decreased the immobility time to 68.45 ± 9.13% of alcohol group (n = 6, p = 0.012) (Fig. 6H).

4. Discussion

In this study, we discovered that the treatment with alcohol increased the expression of ADAR2, which promoted RNA editing of 5-HT_{2C}R in astrocytes; an increased pool of edited 5-HT_{2C}R inhibited the release of ATP induced by MK212. Fluoxetine at 1 μM selectively stimulated 5-HT_{2B}R and transactivated EGFR, which by means of Src, initiated PI3K/AKT pathway and down-regulated the expression of cFos and ADAR2, which ultimately suppressed RNA editing ratio of 5-HT_{2C}R induced by alcohol and rescued the release of ATP stimulated by MK212. In

experiments *in vivo*, in mouse alcohol addiction model, administration of fluoxetine decreased the alcohol preference, while simultaneously improving motor coordination, anxiety and despair-like behaviour (Fig. 7).

Reduced release of ATP from astrocytes is related to depressive-like behaviors (Cao et al., 2013). We found that alcohol decreased the release of ATP by up-regulating expression of ADAR2 which promoted RNA editing of 5-HT_{2C}R thus rendered this receptor dysfunctional. Fluoxetine down-regulated the expression of ADAR2 and hence rescued ATP secretion by recovering the function of 5-HT_{2C}R. The reduced ATP release from astrocytes may be due to the dysfunction of mitochondria. Alcohol withdrawal decreases the conversion of ADP to ATP in TCA cycle and inhibits ATP synthase in order to impede mitochondrial function via stimulating excitatory neurotransmitter glutamate in Purkinje cells (York and Biederman, 1991; Dohrman et al., 1997). Moreover, the neurone-derived glutamate induced by stress triggers the release of ATP from astrocytes (Iwata et al., 2016; Ferrini and De Koninck, 2013). However, the effect of chronic alcohol addiction on the regulation of ATP in neural cells is still unclear; although the decreased ATP level may contribute to the development of alcohol addiction.

RNA editing of 5-HT_{2C}R via ADARs has been linked to alcohol preference (Watanabe et al., 2014). Mice with specific ADAR2 deletion in nucleus accumbens were resistant to depressive or anxiety-like behaviours associating with low alcohol intake (Shirahase et al., 2019). Administration of fluoxetine could decrease mRNA editing ratio of 5-HT_{2C}R enhanced by alcohol by suppressing the expression of

ADAR2 in astrocytes, thereby improving the behavioural phenotypes associated with chronic alcohol addiction in mice. In the present study we found that stimulation of 5-HT_{2C}R using selective agonist increases the release of ATP from astrocytes. Arguably, stimulation of 5-HT_{2C}R triggers the release of intracellular Ca^{2+} via PLC/IP3 signalling pathway thus stimulating secretion of ATP (Burnstock, 2007). Alternatively activation of 5-HT_{2C}Rs may recover mitochondrial function thus increasing the expression of ATP synthase and elevating the level of ATP, as was shown in NRK-52E cells (Rasbach et al., 2010).

After abolishing the expression of ADAR2 in astrocytes, the release of ATP from astrocytes reduced by alcohol was re-increased by 5-HT_{2C}R agonist, MK212. This demonstrated alcohol suppressed the astrocytic ATP release via increasing the expression of ADAR2 and the RNA editing of 5-HT_{2C}R. And the chronic alcohol treatment, fluoxetine totally suppressed the RNA editing ratio of 5-HT_{2C}R at D site, and the mice also similar behavioral performance in the associated anxiety or depressive relevant tests. According to the previous reports that fluoxetine only plays antidepressant role via selectively stimulating astrocytic 5-HT_{2B}R (Peng et al., 2012 and 2014) and our present results that fluoxetine completely abolished the expression of ADAR2 and the RNA editing of 5-HT_{2C}R increased by chronic alcohol treatment, we speculate that fluoxetine decreased the expression of ADAR2 via specially stimulating astrocytic 5-HT_{2B}R and the followed Src/EGFR/AKT/cFos signaling pathway, and suppressed astrocytic RNA editing ratio of 5-HT_{2C}R which was increased by chronic alcohol treatment *in vivo*.

Treatment with various concentration of fluoxetine regulates the expression of cFos through the opposite effects of PI3K/AKT and MAPK/ERK signalling pathways in astrocytes. The cFos is a transcription factor which regulates expression of several genes, such as caveolin-1 and BDNF (Li et al., 2017 and 2019; Ren et al., 2018). Here we found that fluoxetine at 1 μM suppressed the expression of ADAR2 increased by alcohol by regulating cFos through Src-mediated transactivation of EGFR and the downstream PI3K/AKT signalling pathway in astrocytes. Moreover, Src phosphorylation of EGFR at Y845 site is regulated by PI3K/AKT pathway (Nair and Sealfon, 2003).

In conclusion, our results suggest that fluoxetine at low concentration has protective effects on the locomotor impairment and mental malfunction induced by alcoholism. Fluoxetine stimulates 5-HT_{2C}R and the release of ATP via decreasing the RNA editing controlled by ADAR2 on astrocytes. In addition, fluoxetine is beneficial for suppressing the alcohol intake and preventing alcohol addiction.

Conflict of interest

The authors have no conflicts of interest to disclose.

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References

Burnstock, G., 2007. Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. 87, 659–797. https://doi.org/10.1152/physrev.00043.2006.

Cao, X., Li, L.P., Wang, Q., Wu, Q., Hu, H.H., Zhang, M., Fang, Y.Y., Zhang, J., Li, S.J., Xiong, W.C., Yan, H.C., Gao, Y.B., Liu, J.H., Li, X.W., Sun, L.R., Zeng, Y.N., Zhu, X.H., Gao, T.M., 2013. Astrocyte-derived ATP modulates depressive-like behaviors. Nat. Med. 19, 773–777. https://doi.org/10.1038/nm.3162.

Crawford, D.C., Jiang, X., Taylor, A., Mennerick, S., 2012. Astrocyte-derived thrombospondins mediate the development of hippocampal presynaptic plasticity in vitro. J. Neurosci. 32, 13100-13110. https://doi.org/10.1523/JNEUROSCI.2604-12.2012.

Dohrman, D.P., West, J.R., Pantazis, N.J., 1997. Ethanol reduces expression of the nerve growth factor receptor, but not nerve growth factor protein levels in the neonatal rat cerebellum. Alcohol Clin. Exp. Res. 21, 882–893. https://doi.org/10.1111/j.1530-0277.1997.tb03853.x.

Englander, M.T., Dulawa, S.C., Bhansali, P., Schmauss, C., 2005. How stress and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. J. Neurosci. 25, 648-651. https://doi.org/10.1523/jneurosci.3895-04.2005.

Ferrini, F., De, K.Y., 2013. Microglia control neuronal network excitability via BDNF signalling. Neural. Plast. 2013, 429815. https://doi.org/10.1155/2013/429815.

Grant, B.F., Goldstein, R.B., Saha, T.D., Chou, S.P., Jung, J., Zhang, H., Pickering, R.P., Ruan, W.J., Smith, S.M., Huang, B., Hasin, D.S, 2015. Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry 72, 757-766. https://doi.org/ 10.1001/jamapsychiatry.2015.0584.

Hertz, L., Xu, J., Song, D., Du, T., Li, B., Yan, E., Peng, L., 2015. Astrocytic glycogenolysis: mechanisms and functions. Metab. Brain. Dis. 30, 317-33. https://doi.org/10.1007/s11011-014-9536-1.

Iwata, M., Ota, K.T., Li, X.Y., Sakaue, F., Li, N., Dutheil, S., Banasr, M., Duric, V., Yamanashi, T., Kaneko, K., Rasmussen, K., Glasebrook, A., Koester, A., Song, D., Jones, K.A., Zorn, S., Smagin, G., Duman, R.S., 2016. Psychological Stress Activates the Inflammasome via Release of Adenosine Triphosphate and Stimulation of the Purinergic Type 2X7 Receptor. Biol. Psychiatry. 80, 12–22. https://doi.org/10.1016/j.biopsych.2015.11.026.

Kaczor, P., Rakus, D. Mozrzymas, J.W., 2015. Neuron-astrocyte interaction enhance GABAergic synaptic transmission in a manner dependent on key metabolic enzymes. Front. Cell. Neurosci. 9, 120. https://doi.org/10.3389/fncel.2015.00120.

Kinoshita, M., 2018. Anti-Depressant Fluoxetine Reveals its Therapeutic Effect Via Astrocytes. EBioMedicine 32, 72-83. https://doi.org/10.1016/j.ebiom.2018.05.036.

Li, B., Zhang, S., Zhang, H., Nu, W., Cai, L., Hertz, L., Peng, L., 2008. Fluoxetine-mediated 5-HT2B receptor stimulation in astrocytes causes EGF receptor transactivation and ERK phosphorylation. Psychopharmacology (Berl) 201, 443-458. https://doi.org/10.1007/s00213-008-1306-5.

Li, B., Zhang, S., Li, M., Hertz, L., Peng, L., 2009. Chronic treatment of astrocytes with therapeutically relevant fluoxetine concentrations enhances cPLA2 expression secondary to 5-HT2B-induced, transactivation-mediated ERK1/2 phosphorylation. Psychopharmacology (Berl) 207, 1-12.

https://doi.org/10.1007/s00213-009-1631-3.

Li, B., Zhang, S., Li, M., Hertz, L., Peng, L., 2011. Fluoxetine affects GluK2 editing, glutamate-evoked Ca(2+) influx and extracellular signal-regulated kinase phosphorylation in mouse astrocytes. J. Psychiatry Neurosci. 36, 322-338. https://doi.org/10.1503/jpn.100094.

Li, B., Qi, S., Sun, G., Yang, L., Han, J., Zhu, Y., Xia, M., 2016. Leptin suppresses adenosine triphosphate-induced impairment of spinal cord astrocytes. J. Neurosci. Res. 94, 924-35. https://doi.org/10.1002/jnr.23795.

Li, B., Jia, S., Yue, T., Yang, L., Huang, C., Verkhratsky, A., Peng, L., 2017. Biphasic Regulation of Caveolin-1 Gene Expression by Fluoxetine in Astrocytes: Opposite Effects of PI3K/AKT and MAPK/ERK Signaling Pathways on c-fos. Front. Cell. Neurosci. 11, 335. https://doi.org/10.3389/fncel.2017.00335.

Li, B., Ren, J., Yang, L., Li, X., Sun, G., Xia, M., 2018. Lithium Inhibits GSK3β Activity via Two Different Signaling Pathways in Neurons After Spinal Cord Injury. Neurochem. Res. 43, 1-9. https://doi.org/10.1007/s11064-018-2488-9.

Li, X., Liang, S., Li, Z., Li, S., Xia, M., Verkhratsky, A., Li, B., 2019. Leptin Increases Expression of 5-HT2B Receptors in Astrocytes Thus Enhancing Action of Fluoxetine on the Depressive Behavior Induced by Sleep Deprivation. Front. Psychiatry 9, 734. https://doi.org/ 10.3389/fpsyt.2018.00734.

Lindberg, D., Andres-Beck, L., Jia, Y.F., Kang, S., Choi, D.S., 2018. Purinergic Signaling in Neuron-Astrocyte Interactions, Circadian Rhythms, and Alcohol Use Disorder. Front. Physiol. 9, 9. https://doi.org/10.3389/fphys.2018.00009.

Nair, V.D., Sealfon, S.C., 2003. Agonist-specific transactivation of phosphoinositide 3-kinase signaling pathway mediated by the dopamine D2 receptor. J. Biol. Chem.; 278, 47053–47061. https://doi.org/10.1074/jbc.M303364200.

Nuutinen, S., Lintunen, M., Vanhanen, J., Ojala, T., Rozov, S., Panula, P., 2011. Evidence for the Role of Histamine H3 Receptor in Alcohol Consumption and Alcohol Reward in Mice. Neuropsychopharmacology 36, 2030-2040. https://doi.org/10.1038/npp.2011.90.

O'Donnell, J.M., Shelton, R.C., 2011. Drug Therapy of Depression and Anxiety

Disorders, in: Brunton, L.L., Chabner, B.A., Knollmann, B.C. (Eds), Goodman & Gilman's The Pharmacological Basis of Therapeutics. McGraw-Hill Medical, Ohio, pp. 397-415.

Pettinati, H.M., O'Brien, C.P., Dundon, W.D., 2013. Current status of co-occurring mood and substance use disorders: a new therapeutic target. Am. J. Psychiatry 170, 23-30. https://doi.org/10.1176/appi.ajp.2012.12010112.

Peng, L., Huang, J., 2012. Astrocytic 5-HT(2B) receptor as in vitro and in vivo target of SSRIs. Recent Pat. CNS Drug Discov. 7, 243-53. https://doi.org/10.2174/157488912803252078.

Peng, L., Gu, L., Li, B., Hertz, L., 2014. Fluoxetine and all other SSRIs are 5-HT2B Agonists - Importance for their Therapeutic Effects. Curr. Neuropharmacol. 12, 365-379. https://doi.org/10.2174/1570159X12666140828221720.

Rasbach, K.A., Funk, J.A., Jayavelu, T., Green, P.T., Schnellmann, R.G., 2010. 5-hydroxytryptamine receptor stimulation of mitochondrial biogenesis. J. Pharmacol. Exp. Ther. 332, 632-639. https://doi.org/10.1124/jpet.109.159947.

Ren, J., Li, X., Sun, G., Li, S., Liang, S., Li, Z., Li, B., Xia, M., 2018. Protective effect of leptin-mediated caveolin-1 expression on neurons after spinal cord injury. Cell Calcium 76, 122-128. https://doi.org/10.1016/j.ceca.2018.11.003.

Shirahase, T., Watanabe, Y., Tsujimura, A., Kwak, S., Yamamoto, T., Kanamura, N., Tanaka, M., 2019. Ethanol Preference and Drinking Behavior Are Controlled by RNA Editing in the Nucleus Accumbens. Front. Behav. Neurosci. 12, 331. https://doi.org/10.3389/fnbeh.2018.00331.

Wang, Q., O'Brien, P.J., Chen, C.X., Cho, D.S., Murray, J.M., Nishikura, K., 2000a. Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. J. Neurochem. 74, 1290-1300. https://doi.org/10.1046/j.1471-4159.2000.741290.x.

Wang, Q., Khillan, J., Gadue, P., Nishikura, K., 2000b. Requirement of the RNA editing deaminase ADAR1 gene for embryonic erythropoiesis. Science 290, 1765-1768. https://doi.org/10.1126/science.290.5497.1765.

Wang, Y., Pu, P., Le, W., 2007. ATP depletion is the major cause of MPP+ induced

dopamine neuronal death and worm lethality in α-synuclein transgenic C. elegans. Neurosci. Bull. 23, 329-335. https://doi.org/10.1007/s12264-007-0049-3.

Watanabe, Y., Yoshimoto, K., Tatebe, H., Kita, M., Nishikura, K., Kimura, M., Tanaka, M., 2014. Enhancement of alcohol drinking in mice depends on alterations in RNA editing of serotonin 2C receptors. Int. J. Neuropsychopharmacol. 17, 739-751. https://doi.org/10.1017/s1461145713001545.

Werry, T.D., Loiacono, R., Sexton, P.M., Christopoulos, A., 2008. RNA editing of the serotonin $5HT_{2C}$ receptor and its effects on cell signalling, pharmacology and brain function. Pharmacol. Ther. 119, 7-23. https://doi.org/ 10.1016/j.pharmthera.2008.03.012.

Xia, M., Yang, L., Sun, G., Qi, S., Li, B., 2017. Mechanism of depression as a risk factor in the development of Alzheimer's disease: the function of AQP4 and the glymphatic system. Psychopharmacology (Berl) 234, 365-379. https://doi.org/10.1007/s00213-016-4473-9.

Xia, M.., Li, X., Yang, L., Ren, J., Sun, G., Qi, S., Verkhratsky, A., Li, B., 2018. The ameliorative effect of fluoxetine on neuroinflammation induced by sleep deprivation. J. Neurochem. 146, 63-75. https://doi.org/ 10.1111/jnc.14272.

York, J.L., Biederman, I., 1991. Hand movement speed and accuracy in detoxified alcoholics. Alcohol Clin. Exp. Res. 15, 982–990. https://doi.org/ 10.1111/j.1530-0277.1991.tb05199.x.

Yue, T., Li, B., Gu, L., Huang, J., Verkhratsky, A., Peng, L., 2019. Ammonium induced dysfunction of 5-HT_{2B} receptor in astrocytes. Neurochem. Int. 129, 104479. https://doi.org/10.1016/j.neuint.2019.104479.

Figure Legends

Figure 1. The dose response of alcohol on the expression of cFos and ADAR2 in astrocytes.

(A-C) Primary cultured astrocytes were incubated in absence of alcohol (0 mg/ml as control group, which was treated with normal saline) or in the presence of 2, 4 or 8 mg/ml alcohol for 24 hours. **(A)** Representative western blots showing protein levels of cFos, ADAR2 and β-actin (a housekeeping protein). Similar results were obtained from six independent experiments. Average expression of cFos or ADAR2 was quantitated as ratio between cFos and β-actin (**B**), or ratio between ADAR2 and β-actin (**C**). Data are shown as mean ± SEM, n = 6. *Indicates statistically significant ($p<0.05$) difference from control group; **indicates statistically significant ($p<0.05$) difference from any other group. **(D and E)** Primary cultured astrocytes were incubated in presence of normal saline (control group) or 4 mg/mL alcohol for 24 h. **(D)** Representative images showing HE staining. **(E)** The average number of cells in per mm^2 was presented as mean ± SEM, n = 6. **(F)** Apoptosis as detected by the TUNEL assay. Astrocytes were immunostained with anti-GFAP antibody (red), and cell nuclei were stained with DAPI (blue). Bar 20 μm. **(G)** Percentage of cell death was determined by the ratio of TUNEL+ and DAPI+ cells. Data are represented as mean ± SEM, n = 6. (**H**) The purity of the primary cultured astrocytes, the cells were stained with DAPI (blue), anti-GFAP (red), anti-NeuN (green) antibodies.

Figure 2. Fluoxetine suppressed the expression of cFos and ADAR2 increased by alcohol.

Pre-treated with normal saline (NS; control) or with 4 mg/ml alcohol for 30 minutes, the primary cultures of astrocytes were exposed to NS or 1 μM fluoxetine for 24 hours. **(A)** Representative western blots showing protein levels of 5-HT_{2C}R, cFos, ADAR2 and β-actin. Similar results were obtained from six independent experiments. Average expressive of 5-HT_{2C}R, cFos and ADAR2 was quantified as ratio between 5-

$HT_{2C}R$ and β-actin (**B**), ratio between cFos and β-actin (**C**), or ratio between ADAR2 and β-actin (**D**). Data represent mean ± SEM, n = 6. *Indicates statistically significant ($p < 0.05$) difference from any other group; **indicates statistically significant ($p < 0.05$) difference from control (Ctrl) and alcohol (Alc) groups. (**E**) Representative western blots showing protein levels of 5-$HT_{2B}R$ and β-actin. Similar results were obtained from six independent experiments. Average expressive of 5-$HT_{2B}R$ was quantified as ratio between 5-$HT_{2B}R$ and β-actin (**F**).

Figure 3. The regulation of alcohol and fluoxetine in the expression of cFos and ADAR2. Pre-treated with normal saline, 400 nM SB204741 (SB; an inhibitor of 5-$HT_{2B}R$), 10 μM PP1 (an inhibitor of Src), 10 μM AG1478 (AD; an inhibitor of EGFR), 10 μM LY294002 (LY; an inhibitor of p-AKT) for 30 minutes, primary cultured astrocytes were treated with NS, 1 μM fluoxetine, 4 mg/mL alcohol or alcohol with fluoxetine for 24 hours. **(A)** Representative western blots protein levels of cFos, ADAR2 and β-actin. Similar results were obtained from six independent experiments. Average expression of cFos and ADAR2 was quantified as ratio between cFos and β-actin (**B**), or ratio between ADAR2 and β-actin (**C**). Data are shown as mean ± SEM, n=6. *Indicates statistically significant ($p<0.05$) difference from control group; **indicates statistically significant ($p<0.05$) difference from control group, fluoxetine group or alcohol group.

Figure 4. Fluoxetine decreased RNA editing percentage of 5-$HT_{2C}R$ enhanced by alcohol *in vivo* and *in vitro*.

The mice were feed with water or different concentration of alcohol for 36 days, then the mice were intraperitoneal injected (i.p.) with normal saline (NS) or fluoxetine (1 mg/kg/day) in the last week. (**A**) The pre-mRNA of 5-$HT_{2C}R$ can be edited by ADARs at five sites named A-E. (**B**) One representative mRNA sequencing data in control group. (**C**) The representative mRNA sequencing images at D site *in vivo*, the peak of adenosine (A) was green, the peak of guanosine (G) was black. The ratio of G and G+A was shown at D site (**D**) and other four sites (**E**). (**F**) The representative

mRNA sequencing images at D site *in vitro*, the ratio of G and G+A was shown at D site (**G**). Data are shown as mean ± SEM, n = 6. *Indicates statistically ($p<0.05$) significant difference from any other group; **indicates statistically ($p<0.05$) significant difference from control group and alcohol group; #indicates statistically ($p<0.05$) significant difference from control group.

Figure 5. Fluoxetine enhanced the release of ATP decreased by alcohol via stimulating 5-HT_{2C}R in astrocytes.

(**A**) Pre-treated with normal saline (NS; control) or 1 μM SB242084 (a selective antagonist of 5-HT_{2C}R) for 30 minutes, cultured astrocytes were treated with NS or MK212 (a selective agonist of 5-HT_{2C}R) for 15 minutes, the extracellular level of ATP was measured and shown as nmol/g. (**B**) Pre-treated with NS or 4 mg/ml alcohol for 30 minutes, cultured astrocytes were treated with NS or 1 μM fluoxetine for 24 h; extracellular ATP levels were measured and normalized by the protein level of samples after the administration of NS or MK212 for 15 minutes. Data are shown as mean ± SEM, n = 6. *Indicates statistically significant ($p<0.05$) difference from any other group except for each other; **indicates statistically significant ($p<0.05$) difference from any other group.

Figure 6. Fluoxetine improved the behaviour patterns induced by chronic alcohol addiction.

(A) Mice were feed with water or different concentration of alcohol for 36 days, then the mice were intraperitoneal injected (i.p.) with normal saline (NS) or fluoxetine (1 mg/kg/day) in the last week. On the 30^{th} and 37^{th} day, alcohol preference test and behavioural measurements were performed. **(B)** The weight of mice was measured at the end of all treatments. Alcohol preference test was checked before the injection of fluoxetine (**C**) and after injection for one week (**D**). In rotarod test, the time dwelling on the rotarod was recorded **(E).** In the open field test, the travelled distance (**F**) and the time spent in the central area (**G**) were calculated. In tail suspension test, the time of immobility was determined **(H)**. Data are shown as mean ± SEM, n = 6. *Indicates

statistically significant ($p < 0.05$) difference from any other group; **indicates statistically significant ($p < 0.05$) difference from control plus NS group and alcohol plus NS group.

Figure 7. Fluoxetine alleviates alcohol effects by decreasing the expression of ADAR2 in astrocytes.

The chronic alcohol addiction increased the expression of ADAR2, which stimulated editing of mRNA of 5-HT_{2C}R and made this receptor dysfunctional. Stimulating 5-HT_{2C}R with a selective agonist (MK212) increased the level of ATP released from astrocytes. Chronic alcohol treatment reduced the MK212-induced ATP release via upregulating the RNA editing effect of ADAR2. The decreased release of ATP from astrocytes could induce depressive like behaviours. However, fluoxetine at 1 μM transactivated Src-dependent EGFR and the downstream PI3K/AKT signalling pathway via stimulating 5-HT_{2B}R, thereby inhibiting the expression of cFos and ADAR2. Fluoxetine decreased the RNA editing percentage of 5-HT_{2C}R mRNA by suppressing the expression of ADAR2, hence recovering the function of 5-HT_{2C}R impaired by alcohol. As a result, fluoxetine increased the MK212-induced release of ATP, which is decreased in chronic alcohol addiction. The recovery of ATP release from astrocytes may explain positive effect of fluoxetine on mice behavioural deficits triggered by chronic alcohol abuse.

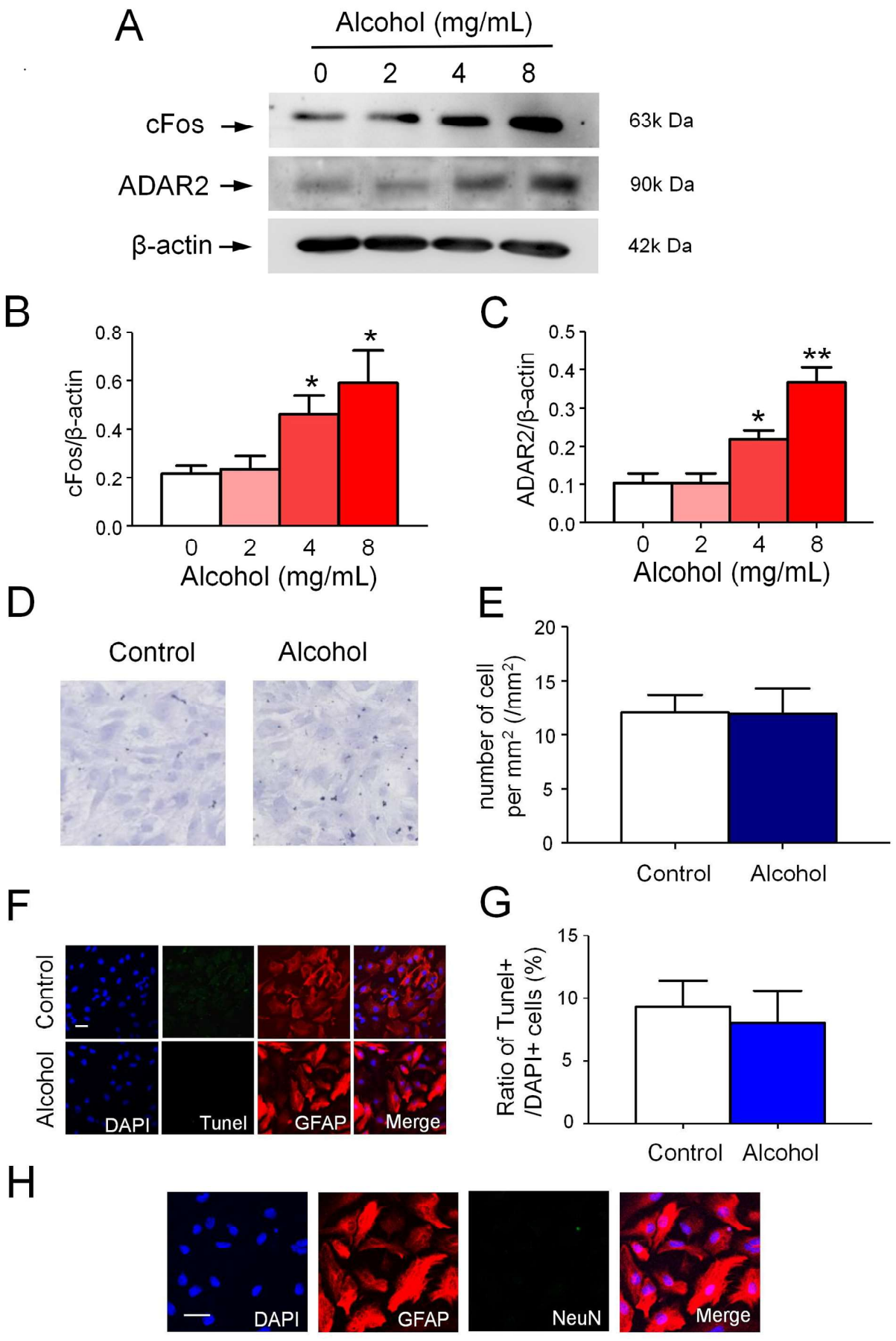
Supplementary Figure 1. Positive control of apoptosis as detected by the TUNEL assay.

After the pretreatment with BSA, the positive reagent DNAase was used to trigger cell apoptosis and measured by TUNEL assay (green color), then the sample was stained with DAPI (blue color).

Supplementary Figure 2. The expression of ADAR2 after RNA interfering.

After treating with normal saline (control), ADAR2 siRNA negative control (ADAR2 siRNA (-)), ADAR2 siRNA duplex (ADAR2 siRNA (+)) for 3 days, the protein

expression of ADAR2 in primary cultured astrocytes was measured. (**A**) Representative western blots protein levels of ADAR2 and β-actin. Similar results were obtained from six independent experiments. Average expression of ADAR2 was quantified as ratio between ADAR2 and β-actin (**B**). Data are shown as mean ± SEM, n=6. *Indicates statistically significant ($p<0.05$) difference from any other group.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ADAR2
β-actin
63k Da
90k Da
42k Da
B
cFos/β-actin
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0 2 4 8
Alcohol (mg/mL)
C
ADAR2/β-actin
0.0 0.1 0.2 0.3 0.4 0.5
0 2 4 8
Alcohol (mg/mL)
D
Control
Alcohol
E
number of cell per mm² (/mm²)
0 5 10 15 20
Control Alcohol
F
Control
Alcohol
DAPI
Tunel
GFAP
Merge
G
Ratio of Tunel+ /DAPI+ cells (%)
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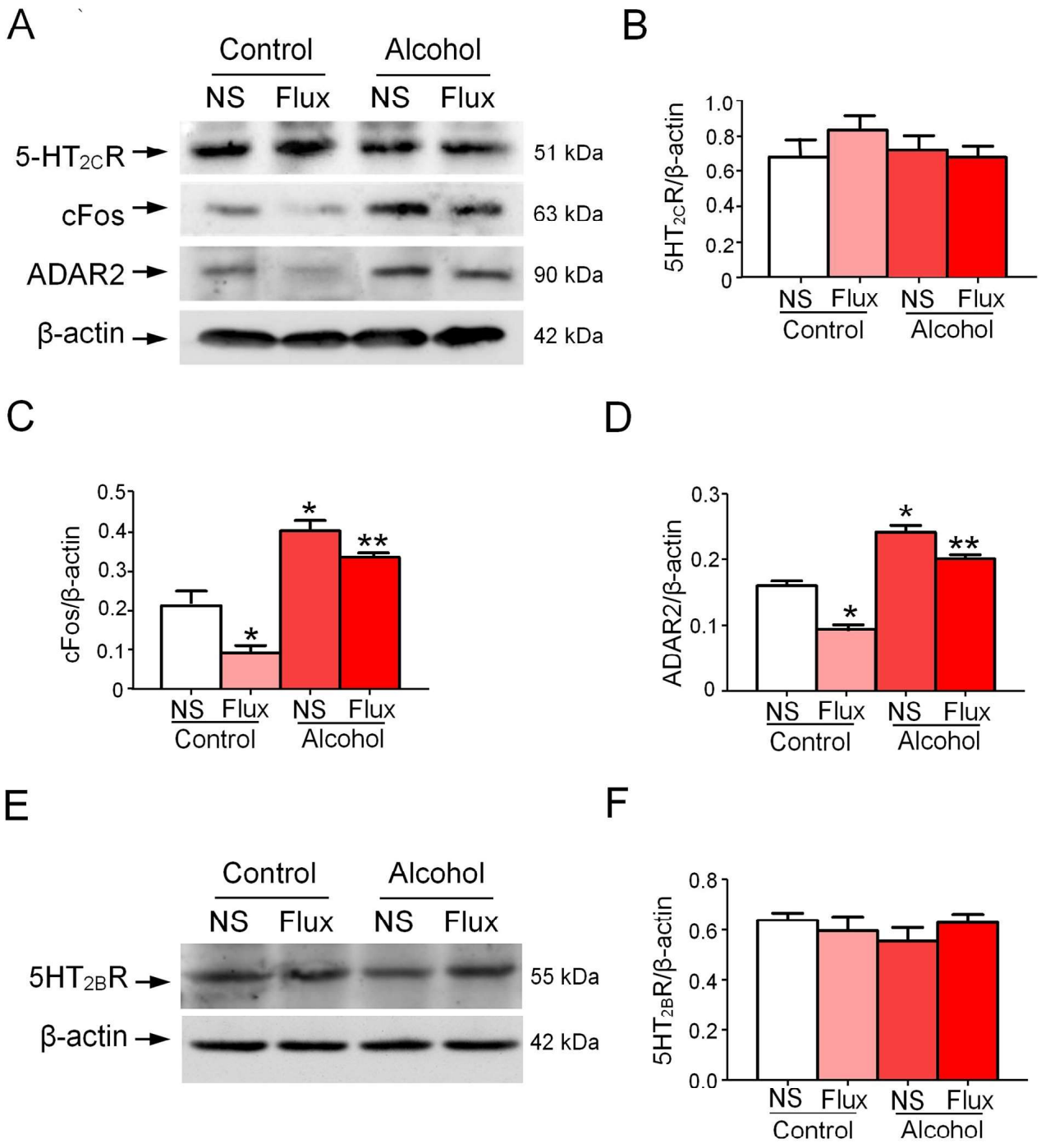
Control Alcohol
H
DAPI
GFAP
NeuN
Merge

Fig. 1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Control
Alcohol
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Flux
NS
Flux
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cFos
ADAR2
β-actin
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63 kDa
90 kDa
42 kDa
B
5HT2CR/β-actin
1.0
0.8
0.6
0.4
0.2
0
NS
Flux
NS
Flux
Control
Alcohol
C
cFos/β-actin
0.5
0.4
0.3
0.2
0.1
0
*
*
**
NS
Flux
NS
Flux
Control
Alcohol
D
ADAR2/β-actin
0.3
0.2
0.1
0
*
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NS
Flux
NS
Flux
Control
Alcohol
E
Control
Alcohol
NS
Flux
NS
Flux
5HT2BR
β-actin
55 kDa
42 kDa
F
5HT2BR/β-actin
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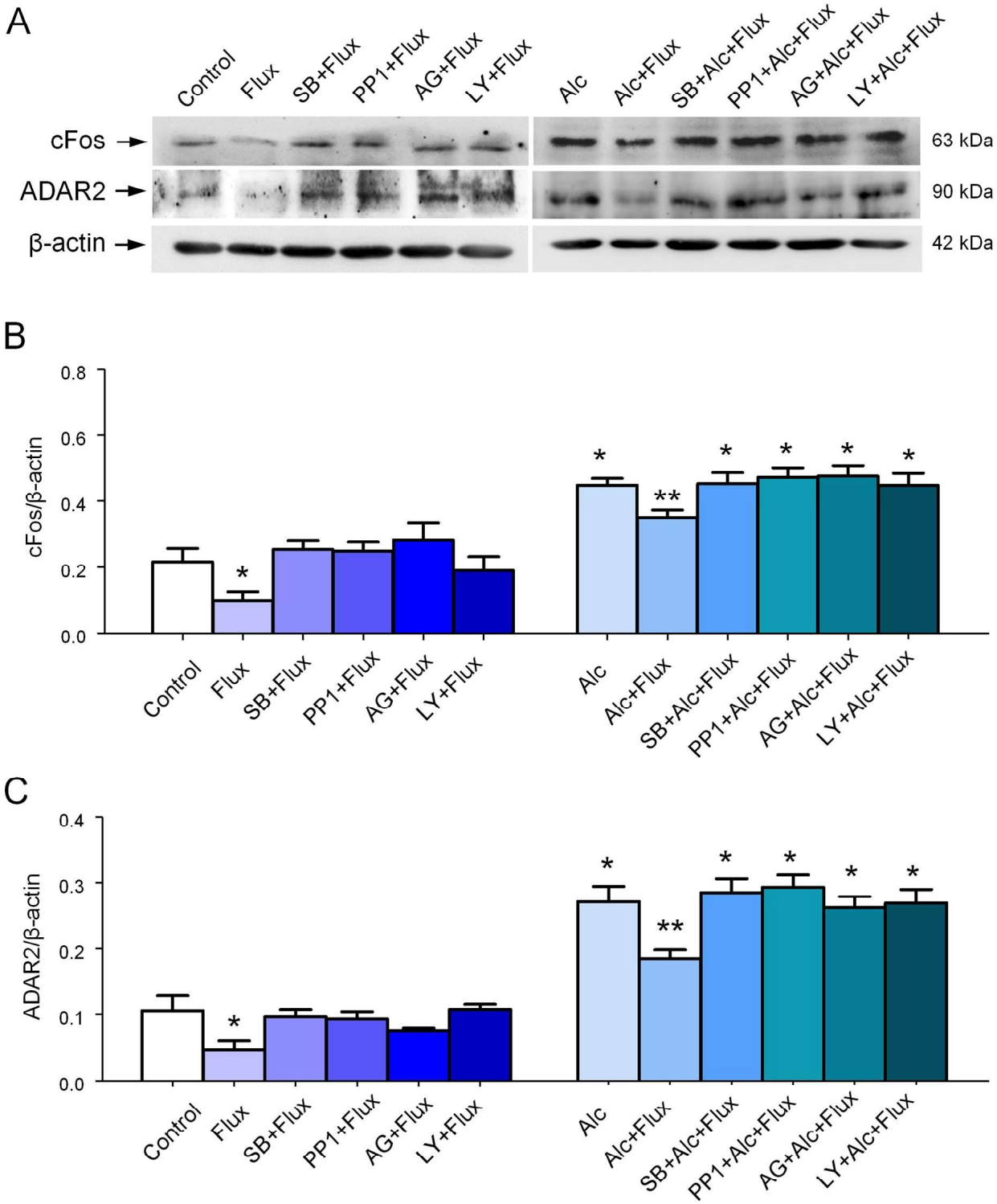
0.4
0.2
0.0
NS
Flux
NS
Flux
Control
Alcohol

Fig. 2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Flux
SB+Flux
PP1+Flux
AG+Flux
LY+Flux
Alc
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SB+Alc+Flux
PP1+Alc+Flux
AG+Alc+Flux
LY+Alc+Flux
cFos
ADAR2
β-actin
63 kDa
90 kDa
42 kDa
B
cFos/β-actin
0.0
0.2
0.4
0.6
0.8
C
ADAR2/β-actin
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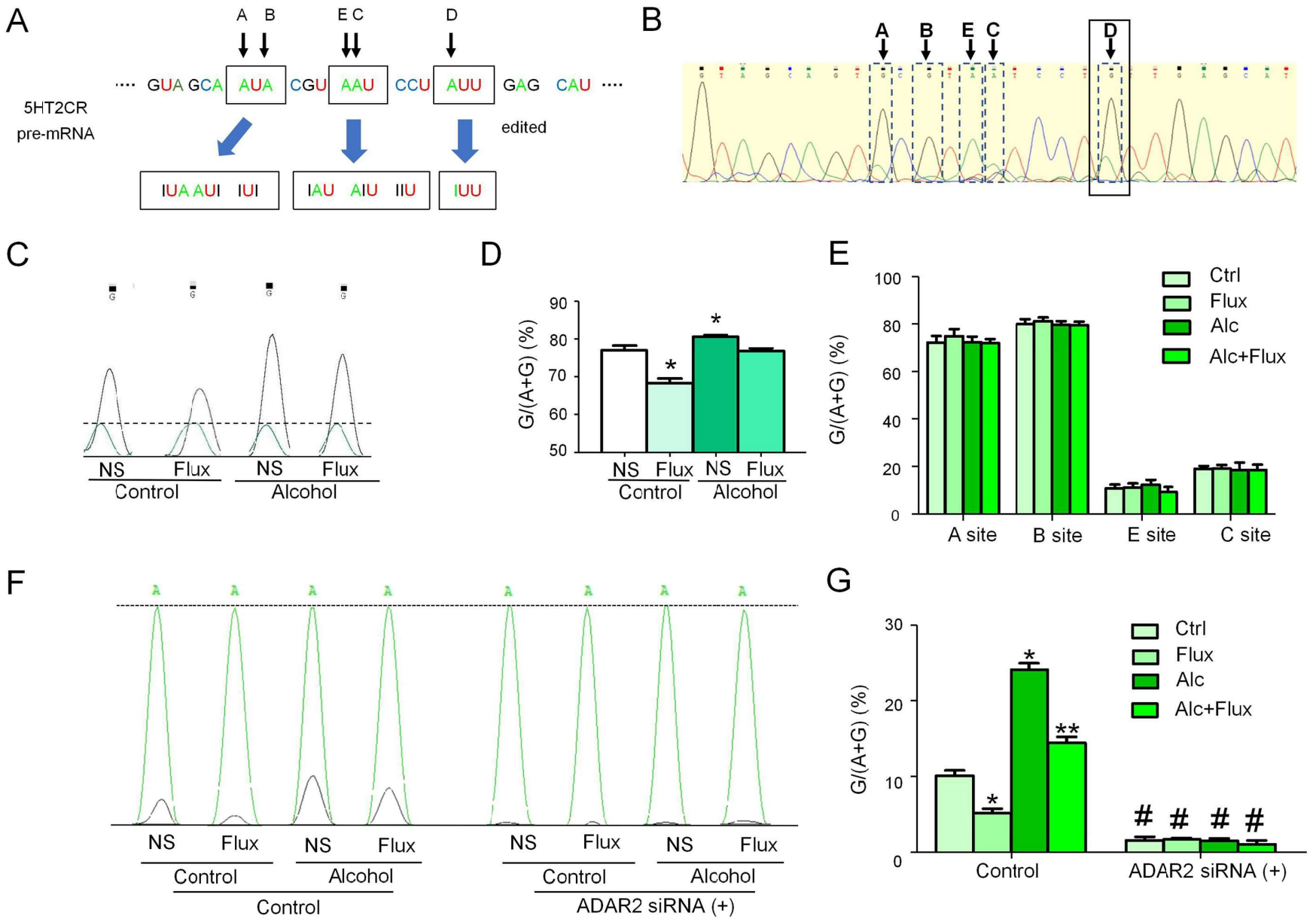
0.1
0.2
0.3
0.4

Fig. 3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E C
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pre-mRNA
edited
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IAU AIU IIU
IUU
B
A B E C D
C
NS Flux NS Flux
Control Alcohol
D
G/(A+G) (%)
90
80
70
60
50
NS Flux NS Flux
Control Alcohol
E
G/(A+G) (%)
100
80
60
40
20
0
Ctrl
Flux
Alc
Alc+Flux
A site
B site
E site
C site
F
NS Flux NS Flux NS Flux NS Flux
Control Alcohol Control Alcohol
Control ADAR2 siRNA (+)
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10
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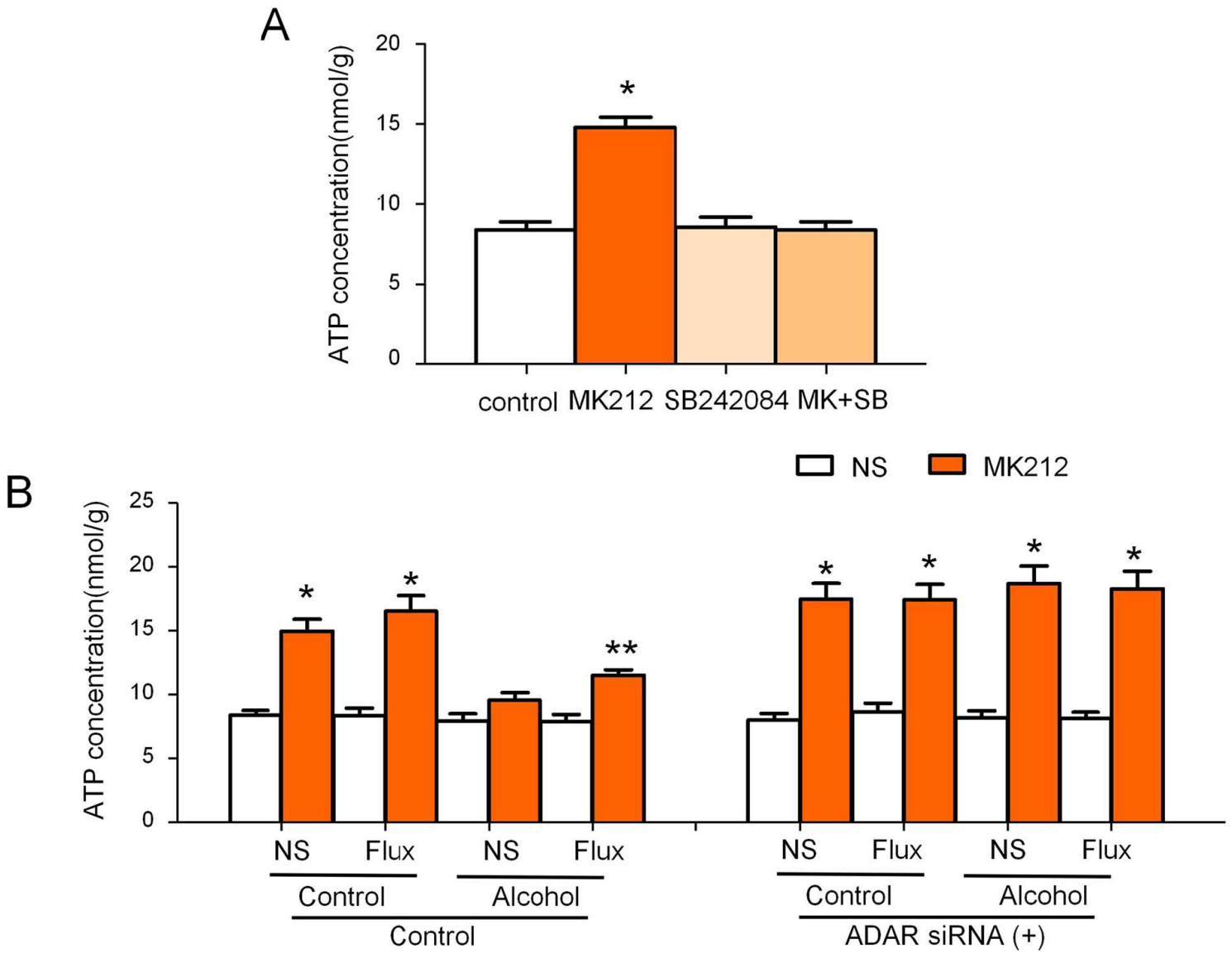
Flux
Alc
Alc+Flux
Control
ADAR2 siRNA (+)

Fig. 4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15
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5
0
*
control
MK212
SB242084
MK+SB
B
NS
MK212
ATP concentration(nmol/g)
25
20
15
10
5
0
*
*
**
*
*
*
*
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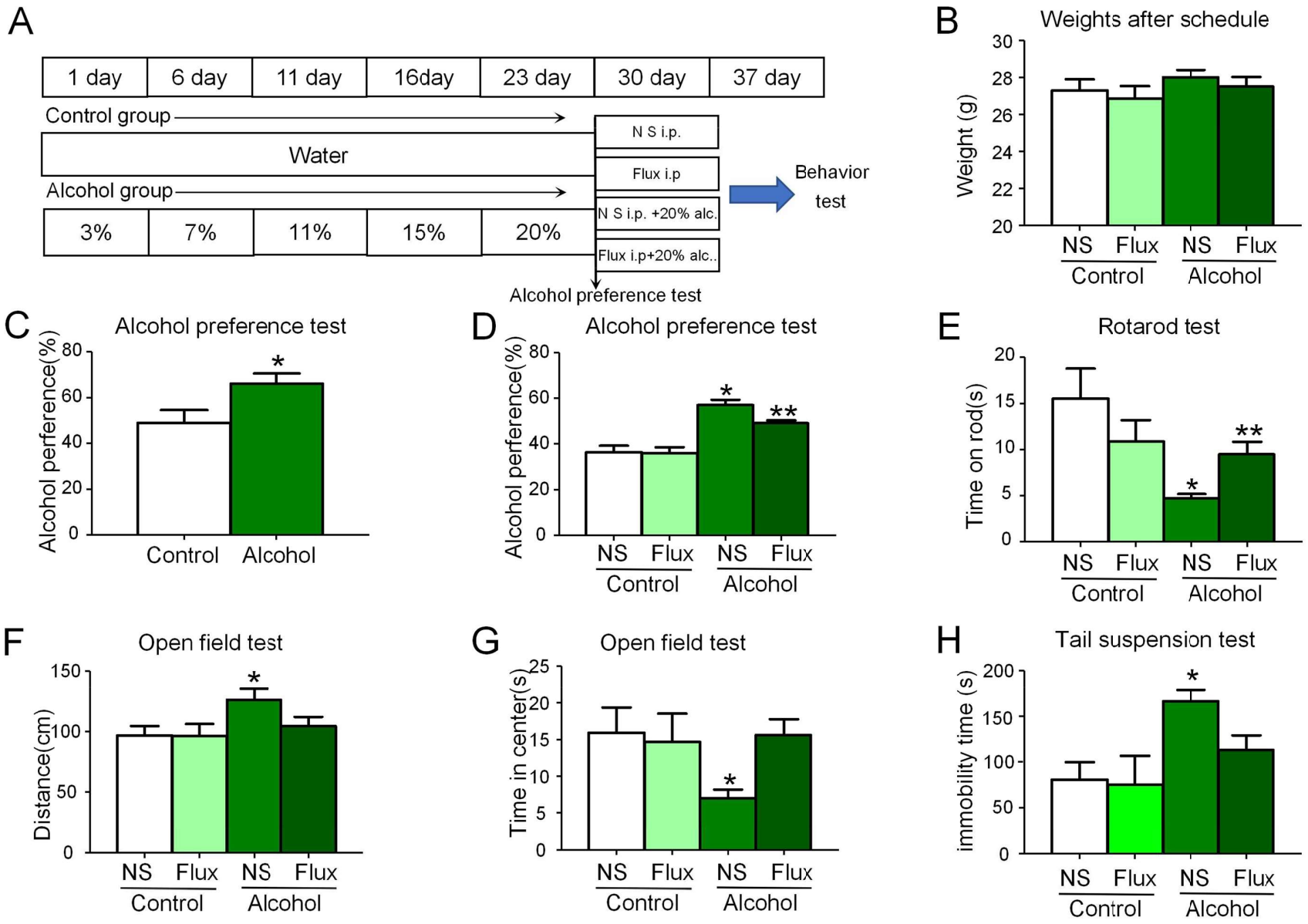
NS
Flux
NS
Flux
NS
Flux
Control
Alcohol
Control
Alcohol
Control
ADAR siRNA (+)

Fig. 5


A
1 day
6 day
11 day
16day
23 day
30 day
37 day
Control group
Water
Alcohol group
3%
7%
11%
15%
20%
N S i.p.
Flux i.p
N S i.p. +20% alc.
Flux i.p+20% alc..
Behavior test
Alcohol preference test
B
Weights after schedule
Weight (g)
NS
Flux
Control
Alcohol
C
Alcohol preference test
Alcohol perference(%)
Control
Alcohol
D
Alcohol preference test
E
Rotarod test
Time on rod(s)
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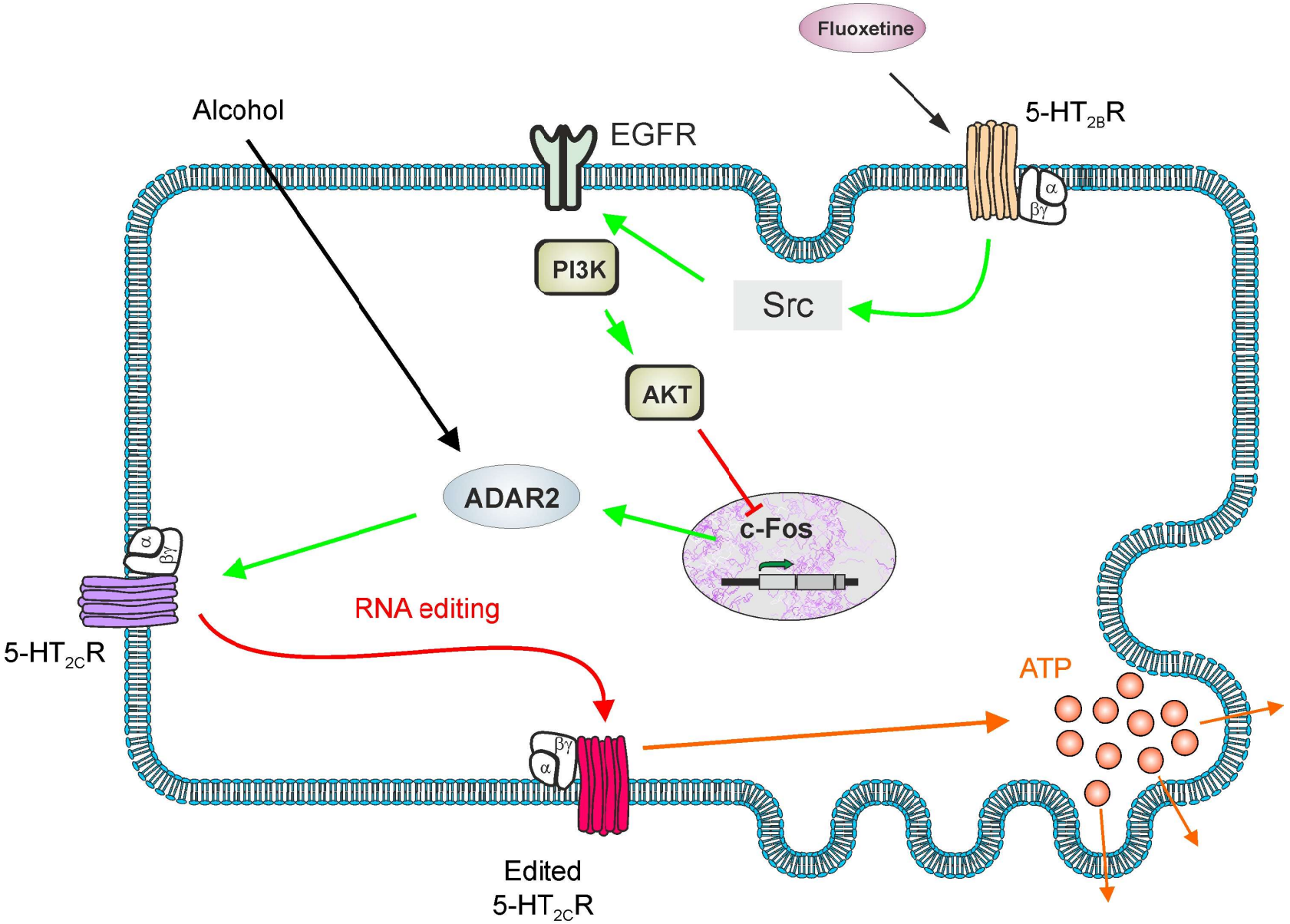
Open field test
Distance(cm)
G
Open field test
Time in center(s)
H
Tail suspension test
immobility time (s)

Fig. 6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EGFR
5-HT2BR
α
βγ
PI3K
Src
AKT
ADAR2
c-Fos
α
βγ
RNA editing
5-HT2CR
ATP
βγ
α
Edited
5-HT2CR

Fig. 7

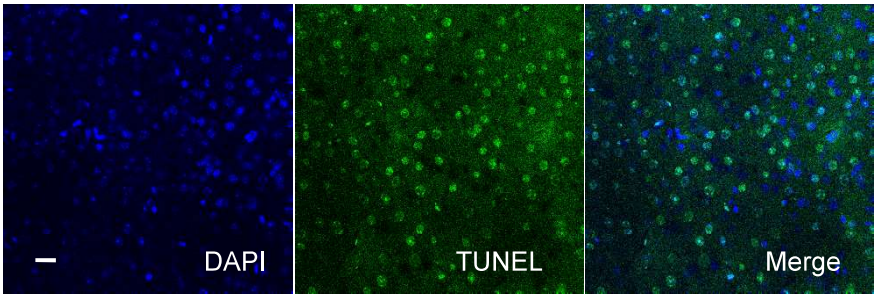
Author statement

All experiments were performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and its 1978 revision, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, No. [2019]059.

Supplementary Table 1. The astrocytic ATP level induced by fluoxetine and/or alcohol pretreated with or without ADAR2 siRNA duplex.

			Mean±SEM (nmol/g)
Control	NS	NS	8.36±0.38
		MK212	14.92±0.96*
	Flux	NS	8.35±0.57
		MK212	16.50±1.23*
	Alc	NS	7.93±0.57
		MK212	9.54±0.0.59
	Alc+Flux	NS	7.87±0.56
		MK212	11.50±0.43**
ADAR siRNA (+)	NS	NS	7.99±0.52
		MK212	17.45±1.24*
	Flux	NS	8.63±0.70
		MK212	17.41±1.21*
	Alc	NS	8.18±0.53
		MK212	18.68±1.37*
	Alc+Flux	NS	8.12±0.50
		MK212	18.25±1.39*

Supplementary Figure 1.



Supplementary Figure 2.