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Ammonium induced dysfunction of 5-HT_{2B} receptor in astrocytes

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ABSTRACT

Previously we reported that gene expression of astrocytic 5-HT_{2B} receptors was decreased in brains of depressed animals exposed to chronic mild stress (CMS) (Li et al., 2012) and of Parkinson's disease (Song et al., 2018). Depression is also one of the psychiatric symptoms in hyperammonemia, and astrocyte is a primary target of ammonium in brain *in vivo*. In the present study, we have used preparations of the brains of urease-treated mice and ammonium-treated astrocytes in culture to study gene expression and function of 5-HT_{2B} receptors. The urease-treated mice showed depressive behaviour. Both mRNA and protein of 5-HT_{2B} receptors were increased in the brains of urease-treated mice and in ammonium-treated cultured astrocytes. Further study revealed that mRNA and protein expression of adenosine deaminase acting on RNA 2 (ADAR2), an enzyme catalyze RNA deamination of adenosine to inosine was increased in the brains of urease-treated mice and in ammonium-treated cultured astrocytes. This increase in ADAR2 induced RNA editing of 5-HT_{2B} receptors. Cultured astrocytes treated with ammonium lost 5-HT induced Ca^{2+} signalling and $ERK_{1/2}$ phosphorylation, indicating dysfunction of 5-HT_{2B} receptors. This is in agreement with our previous observation that edited 5-HT_{2B} receptors no longer respond to 5-HT (Hertz et al., 2014). Ammonium effects are inhibited by ADAR2 siRNA in cultured astrocytes, suggesting that increased gene expression and editing and loss of function of 5-HT_{2B} receptors are results of increased activity of ADAR2. In summary, we have demonstrated that functional malfunction of astrocytic 5-HT_{2B} receptors occurs in animal models of major depression, Parkinson depression and hepatic encephalopathy albeit via different mechanisms. Understanding the role of astrocytic 5-HT_{2B} receptors in different pathological contexts may instigate development of novel therapeutic strategies for treating disease-specific depressive behaviour.

1. Introduction

Hyperammonemia develops in several neurological conditions; of which the most frequent is the liver failure, which often triggers cause ammonium neurotoxicity which is manifested as an acute or chronic hepatic encephalopathy (HE) (Brusilow et al., 2010; Butterworth, 2010; Montana et al., 2014). In the central nervous system (CNS), glutamine synthetase localised exclusively in astrocytes neutralises ammonium released into the extracellular space in the course of glutamatergic neurotransmission (Marcaggi et al., 2004). This enzyme plays key role in glutamate homeostasis catalysing conversion of glutamate, accumulated in astroglial cell, into glutamine (Martinez-Hernandez et al., 1977; Rose et al., 2013), which in turn is transported to neurones for subsequent conversion to glutamate and GABA, the well acknowledged glutamine(GABA)-glutamate shuttle (Hertz et al., 1999). Astroglial glutamine synthetase has higher affinity to ammonium than to glutamate and hence pathological increases in NH_4^+ affect glutamate homeostasis. Clinically hyperammonemia is manifested by state of confusion, memory impairments, aberrant sleep, lethargy, somnolence and, in terminal stages, coma (Waniewski, 1992). Previously, we reported pathological effects of ammonium in astrocytes, including up-regulation of gene expression of α2 isoform of Na,K-ATPase (Xue et al., 2010), of the transient receptor potential channel 1 (TRPC1) (Liang et al., 2014) and of the L-type Ca^{2+} channel, $Ca_v1.2$ (Wang et al., 2015). Recently, we have found that chronic treatment with ammonium activates Cav-1/PTEN/PI3K/AKT signalling pathway which decreases

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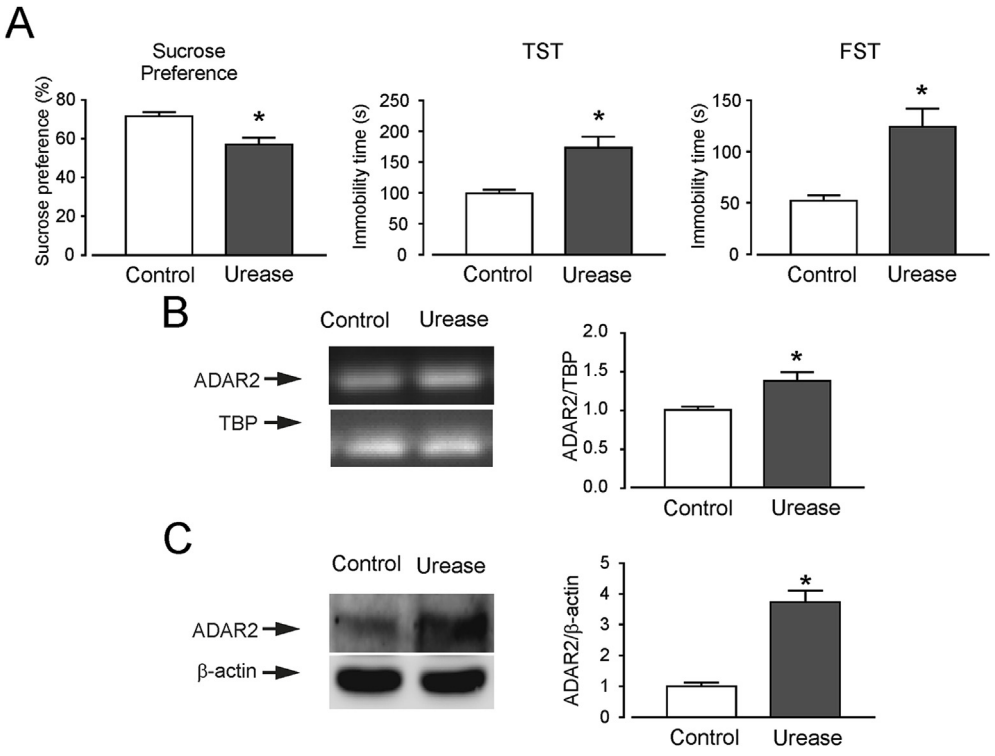

Fig. 1. Depressive behaviour (sucrose preference test, tail suspension test and forced swimming test) and ADAR2 gene expression in brain *in vivo* in animals treated with urease.

A: After 3-days treatment with urease (33 units/kg body weight, i.p.), behaviour tests were performed.

The figure shows results of percentage of sucrose preference in sucrose preference test, the duration of immobility in tail suspension test (TST) and the duration of immobility in forced swimming test (FST). The data are pooled from ten animals in each group.Values were expressed as the mean ± SEM. *Indicates statistically significant ($P < 0.05$) difference from control group at the same treatment period.

B: mRNA expression measured by RT-PCR of ADAR2. A representative experiment showing mRNA for ADAR2 and for TBP, as a housekeeping gene. The size of PCR product of ADAR2 is 203 bp, and that of TBP 236 bp. Similar results were obtained in six independent experiments. Average mRNA expression was quantified as ratio between ADAR2 and the housekeeping TBP gene. Ratios between ADAR2 and TBP in control group were designated a value of one.

C: Protein expression measured by immunoblotting of ADAR2. Band of 90 kDa represents ADAR2 or of 42 kDa β-actin. Similar results were obtained in six aninals in each group. Average protein expression was quantified as ratio between ADAR2 and β-actin. Ratios between ADAR2 and β-actin in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

glycogen synthase kinase 3β (GSK-3β) phosphorylation thus increasing GSK activity with subsequent up-regulation of TRPC1 channel expression in astrocytes (Wang et al., 2017).

Serotonin 5-HT_{2B} receptors are $G_{q/11}$-coupled and are expressed throughout the CNS. Their expression was demonstrated at mRNA level in freshly isolated fractions of neurones and astrocytes labelled by specific expression of distinct fluorescent markers (Lovatt et al., 2004; Li et al., 2012) which allowed fluorescence-activated cell sorting (FACS). The expression of 5-HT_{2B} receptor is higher in astrocytes than in neurones (Li et al., 2012). Activation of 5-HT_{2B} receptor induces phosphorylation of $ERK_{1/2}$ and AKT pathways both mediated via epidermal growth factor (EGF) receptor (EGFR) transactivation (Li et al., 2008); this signalling cascade in turn regulates expression of numerous genes in cultured and freshly isolated astrocytes. These genes include Ca^{2+}-dependent phospholipase A2 ($cPLA_2$), TRPC1, $Ca_v1.2$, and the kainate receptor GluK2, the product of Grik2 gene (Li et al., 2009, 2011a; 2011b, 2012; Du et al., 2014). Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), interacts with 5-HT_{2B} receptor in astrocytes (Hertz et al., 1979; Kong et al., 2002) linking astrocytic 5-HT_{2B} receptors to anti-depressant action of fluoxetine. To probe for anti-depressive action, we used chronic mild stress (CMS), as a model for major depression. The CMS is a short-lasting exposure of experimental animals to moderately stressful experiences, which instigates anhedonia, the latter being a readout of depressive phenotype (Jaggi et al., 2011; Li et al., 2012). Anhedonia coincides with a down-regulation of the 5-HT_{2B} receptor in astrocytes but not in neurones freshly isolated from the *in vivo* brain (Li et al., 2012). Changes in $5HT_{2B}$ receptor expression occurred only in mice that became anhedonic, but were not detected in mice, which escaped depressive phenotype following CMS (Li et al., 2012).

Adenosine deaminases acting on RNA (ADARs) catalyze deamination of adenosine to inosine in double-stranded regions of mRNAs. Because inosine is perceived by the cells as guanosine, this induces changes in amino acids in the translated protein (Bass, 2002). The ADAR family consists of ADAR1, ADAR2 and ADAR3, which all are expressed in the brain (Kawahara et al., 2004). The expression of ADAR1 and ADAR3 is rather low, whereas ADAR2 is expressed in neurones and in glial cells (Köhr et al., 1998). Fluoxetine up-regulates ADAR2 gene expression in astrocytes in the brain *in vivo* (Li et al., 2012). In cultured astrocytes chronic treatment with fluoxetine almost doubled mRNA and protein expression of ADAR2 (Li et al., 2010). This effect is mediated by 5-HT_{2B} receptor since it can be prevented by siRNA 5-HT_{2B} receptor knockout (Li et al., 2010). We selected 8 RNA sites according to RNA editing sites in 5-HT_{2C} receptor (Barbon et al., 2011), and up to 25% become edited at each site after fluoxetine treatment (Li et al., 2012). Among them, editing at 793, 796, 797, 800 and 805 changes amino acid sequence, whereas editing at 798 and 804 was without effect. The edited receptor in COS-7 cells infected with receptor plasmids of edited RNA sites no longer responded to serotonin with an increase in $InsP_3$ (Hertz et al., 2014). Subsequently we found that fluoxetine-induced increase of ADAR2 gene expression only occurred in astrocytes freshly isolated from brains of healthy animals but not from anhedonic animals, suggesting this effect is not related to therapeutic effects of the drug (Dong et al., 2015). The underlying mechanisms of this difference between normal and depressed animals are unknown, since “depressed” cultured astrocytes, a tool convenient for cell signalling studies, are not available.

Depression is one of the psychiatric symptoms in hyperammonemia (Nardelli et al., 2013; Telles-Correia et al., 2015; Mullish et al., 2015). There is a statistically significant relationship between hyperammonemia and anhedonia and loss of energy (Telles-Correia et al., 2015). We hypothesise that both astrocytic 5-HT_{2B}Rs and ADAR2 may be involved in pathogenesis of psychiatric symptoms in hyperammonemia. In the present study, we have examined (i) the effects of chronic treatment with urease on depressive behaviour, ADAR2 gene expression, 5-HT_{2B}R gene expression and editing in the intact brain in mice; (ii) the effects of chronic treatment with ammonium on ADAR2 gene expression, 5-HT_{2B}R gene expression and 5-HT_{2B}R gene editing in

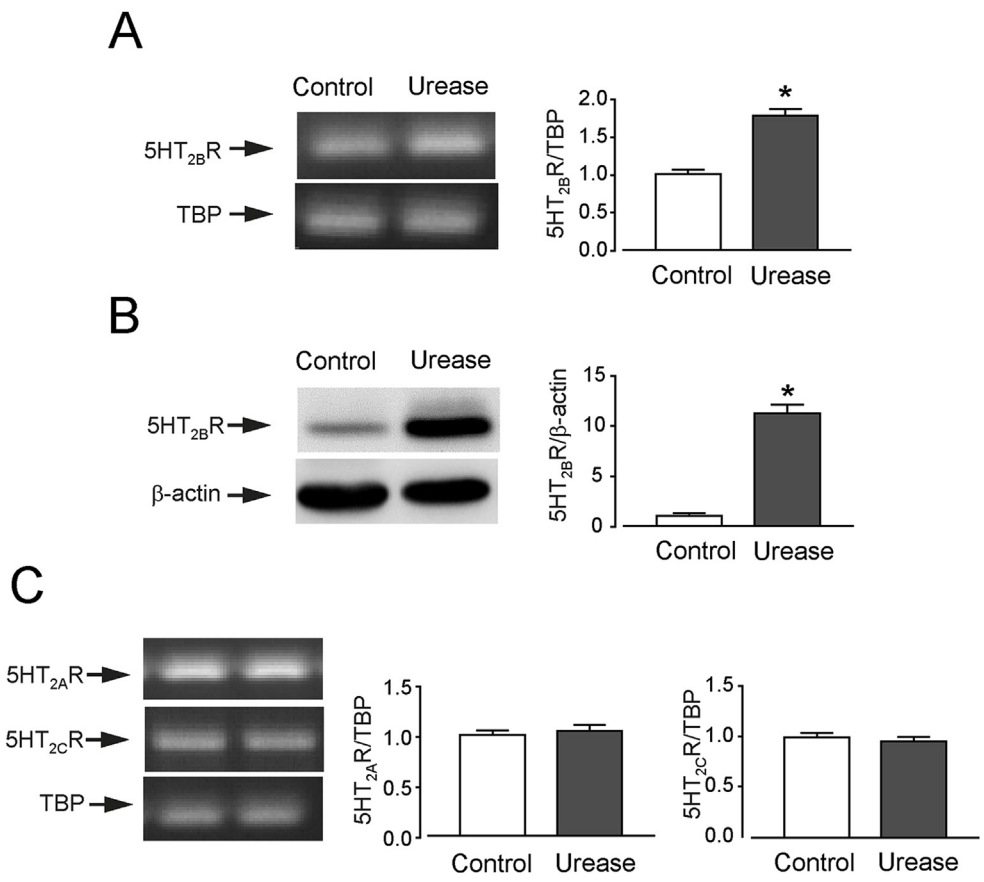

Fig. 2. Gene expression of 5-HT$_2$ receptors in the brain *in vivo* in animals treated with urease.

A: mRNA expression measured by RT-PCR of 5-HT$_{2B}$ receptor. A representative experiment showing mRNA for 5-HT$_{2B}$ receptor and for TBP, as a housekeeping gene. The size of PCR product of 5-HT$_{2B}$ receptor is 370 bp, and that of TBP 236 bp. Similar results were obtained in six animals in each group. Average mRNA expression was quantified as ratio between 5-HT$_{2B}$ receptor and the housekeeping TBP gene. Ratios between ADAR2 and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

B: Protein expression measured by immunoblotting of 5-HT$_{2B}$ receptor. Band of 55 kDa represents 5-HT$_{2B}$ receptor or of 42 kDa β-actin. Similar results were obtained in six animals in each group. Average protein expression was quantified as ratio between 5-HT$_{2B}$ receptor and β-actin. Ratios between 5-HT$_{2B}$ receptor and β-actin in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

C: mRNA expression measured by RT-PCR of 5-HT$_{2A}$ receptor and 5-HT$_{2C}$ receptor. A representative experiment showing mRNA for 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and for TBP, as a housekeeping gene. The size of PCR product of 5-HT$_{2A}$ receptor is 470 bp or 5-HT$_{2C}$ receptor 318 bp, and that of TBP 236 bp. Similar results were obtained in six animals in each group. Average mRNA expression was quantified as ratio between 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and the housekeeping TBP gene. Ratios between 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars.

cultured astrocytes; (iii) the effects of chronic treatment with ammonium on 5-HT-induced extracellular signal-regulated kinase (ERK) phosphorylation and increase of $[Ca^{2+}]_i$ in cultured astrocytes; (iv) the effect of ADAR2 knock-down with ADAR2 siRNA on chronic effects of ammonium on 5-HT$_{2B}$R gene expression and editing, and on 5-HT-induced ERK phosphorylation in cultured astrocytes.

2. Materials and methods

All methods employed in this study have been described in details in our previous publications (Hertz et al., 1998; Kong et al., 2002; Li et al., 2008, 2012). Below we provide brief recapitulation of these methods.

2.1. Animals

Male CD-1 mice 25–30 g of weight were housed in cages on a 12 h light/dark cycle in a temperature-controlled (23–25 °C) colony room with free access to food and water. All experiments were carried out in accordance with the USA National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1978, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University.

2.2. Induction hyperammonemia induction

Sustained hyperammonemia was induced by daily injections of urease (33 units/kg body weight, i.p.) for 3 days (Diemer and Laursen, 1977). The mice were decapitated 24 h after the last dose, and cerebral hemispheres without olfactory bulbs and hippocampi were removed for further analysis.

2.3. Behavioural testing

Mice were subjected to a series of behavioural tests for depression behaviour (tail suspension, forced swim, and sucrose preference); the details of all these tests have been described in our previous papers.

2.4. Cell cultures

Primary cultures of mouse astrocytes were prepared from the neopallia of the cerebral hemispheres of newborn CD-1 mice as previously detailed (Hertz et al., 1998). Cell cultures were grown in Dulbecco's Minimum Essential Medium (DMEM) with 7.5 mM glucose. After 2 weeks *in vitro*, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. These cultures are highly enriched in astrocytes (> 95% purity as judged by expression of glial fibrillary protein- (GFAP-) and glutamine synthetase (Hertz et al., 1985)).

2.5. Knock-down of ADAR2

Transfection was performed as previously described (Li et al., 2008). Transfection solution containing 2 μl Oligofectamine, 40 μl Opti-MEMI, and 2.5 μl siRNA (666 ng) was added to the culture for 8 h. In siRNA (−) control cultures, transfection solution without siRNA was added. Thereafter, 87.5 μl DMEM with 37.5 μl serum was added to the cultures. After 3 days, ADAR2 mRNA and protein expression in cells treated with siRNA was decreased.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

For reverse transcription-polymerase chain reaction (RT-PCR), a cell

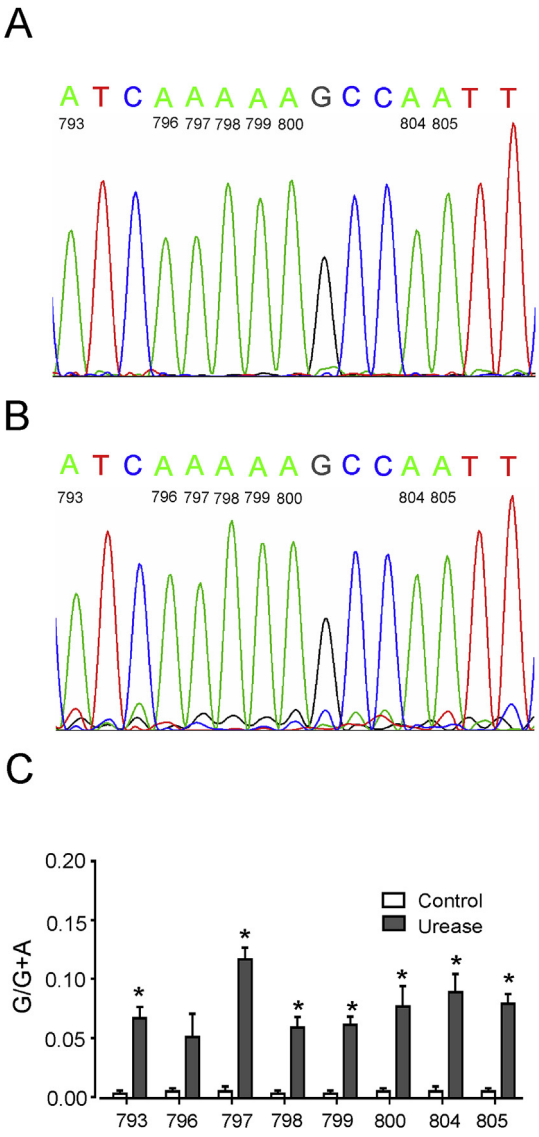

Fig. 3. RNA editing of 5-HT_{2B} receptor in brain *in vivo* in animals treated with urease.

The mice were decapitated 24 h after the last dose of either saline (control; **A**) or urease (**B**), and cerebral hemispheres minus olfactory bulbs and hippocampi were removed for amplification refractory mutation system polymerase chain reaction (ARMS-PCR) analysis of 5-HT_{2B} receptor editing at 793, 796, 797, 798, 799, 800, 804 or 805 sites. Representative Southern blots showing edited (guanosine; G) and unedited (adenosine; A) PCR products at 793, 796, 797, 798, 799, 800, 804 or 805 sites. (**C**) Similar results were obtained from five animals in each group. Standard error of the mean values are indicated by horizontal bars. *Statistically significant ($p < 0.05$) difference from control group.

suspension was prepared by discarding the culture medium, adding Trizol on ice, and scraping the cells off the culture dish. The RNA pellet was precipitated with isopropanol, washed with 70% ethanol, and dissolved in 10 µl sterile, distilled water, and an aliquot was used for determination of the amount of RNA (Kong et al., 2002).

All the details of RT-PCR are described in Kong et al. (2002). mRNA determination was performed in a Robocycler thermocycler with 0.2 µM of sense or antisense and 0.375 U of Taq polymerase for 5-HT_{2A} receptors (forward, AAGCCTCGAACTGGACAATTGATG; reverse, AAG ATTTCAGGAAGGCTTTGGTT′) (Li et al., 2012), for 5-HT_{2B} receptors (forward, CTCGGGGGTGAATCCTCTGA; reverse, CCTGCTCATCACCCT CTCTCA′) (Li et al., 2012), for 5-HT_{2C} receptors (forward, CAGATCA GAAGCCACGTCGA; reverse, GGCTTATAATCGCAGCGCAA′) (Li et al., 2012), for $ADAR_2$ (forward, CGCTTGCTATTTTAGTGCTGCGG; reverse, GCGGTTTTCTTTAACATCAGTGC') (Li et al., 2012), and for TATA box-binding protein (TBP), used as a housekeeping gene (forward, CCACG GACAACTGCGTTGAT; reverse, GGCTCATAGCTACTGAACTG) (el-Marjou et al., 2000). Initially the template was denatured by heating to 94 °C for 2 min, followed by 35 amplification cycles for 5-HT_{2A} receptors, 5-HT_{2B} receptors, 5-HT_{2C} receptors and TBP, and by 40 cycles for ADAR2, each consisting of three periods, the first for 45 s at 94 °C, the second for 45 s at 58 °C for 5-HT_{2A} receptors, 5-HT_{2B} receptors, 5-HT_{2C} receptors, and at 55 °C for ADAR2 and TBP, and the third for 90 s at 72 °C. The final step was extension at 72 °C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, stained with 0.5 µg/ml ethidium bromide, and captured by Fluorchem 5500 (Alpha Innotech Corporation, San Leandro, CA, USA).

2.7. Western blotting

Protein content was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as the standard; for details of experimental procedure see Li et al. (2008).

2.8. Direct PCR sequencing

Polymerase chain reaction amplification was performed with forward and reverse primers for 5-HT_{2B}R, as indicated in Table 1 (Li et al., 2012). Complementary DNA sequencing was carried out by TaKaRa Biotechnology Co., Ltd., and RNA editing efficiencies were calculated by the peak heights.

2.9. Monitoring intracellular Ca^{2+} concentrations

An Olympus 1 × 71 live cell imaging fluorescence microscope (Tokyo, Japan) was used to record the fluorescence intensity of Fura-2-loaded cultured astrocytes. For Fura-2/AM loading, the growth medium was replaced with saline solution (137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 0.5 mM $MgCl_2$ with 10 mM glucose) containing 5 µM Fura-2/AM for 30 min at 37 °C. After washing 2 times with similar saline, the coverslip was perfused with saline with BT100-1J consistent flow pump (Longer Pump, Boading, China) for 3 min. Thereafter, perfusion buffer was switched to solution containing 100 nM 5-HT for 7.7 min. Twenty cells were selected in each coverslip, and three coverslips were averaged in each experimental group.

2.10. Materials

Chemicals for preparation of culturing medium were purchased from Sigma (St. Louis, MO, USA) and horse serum from Invitrogen (Carlsbad, CA, USA). Most chemicals, including dibutyryl cyclic AMP (dBcAMP) and DNase I were purchased from Sigma (St. Louis, MO, USA). Antibodies specific to ADAR2, 5-HT_{2B} receptor, p-$ERK_{1/2}$ or $ERK_{1/2}$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody specific to β-actin was obtained from Sigma-Aldrich (Sigma, USA). The second antibody goat anti-mouse IgG-HRP was purchased from Promega (Madison, USA). The second antibody goat anti-rabbit IgG-HRP and duplex of ADAR2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fura-2AM was obtained from Invitrogen (Carlsbad, CA, USD). Random Hexamer and Taq-polymerase for RT-PCR were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China), and Superscript II from Gibco Life Technology Invitrogen (Grand Island, N.Y., USA).

2.11. Statistics

The differences between individual groups were analyzed by one-

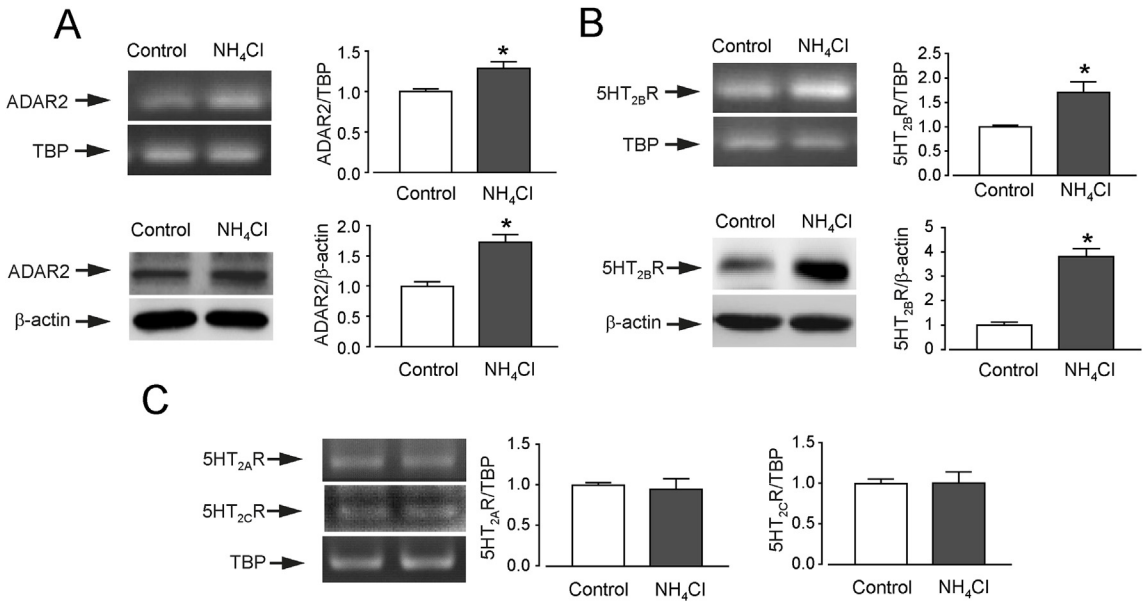

Fig. 4. Gene expression of ADAR2 and 5-HT$_2$ receptors in cultured astrocytes treated with ammonium.

A: *Top:* mRNA expression measured by RT-PCR of ADAR2. A representative experiment showing mRNA for ADAR2 and for TBP, as a housekeeping gene. The size of PCR product of ADAR2 is 203 bp, and that of TBP 236 bp. Similar results were obtained in six independent experiments. Average mRNA expression was quantified as ratio between ADAR2 and the housekeeping TBP gene. Ratios between ADAR2 and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

Bottom: Protein expression measured by immunoblotting of ADAR2. Band of 90 kDa represents ADAR2 or of 42 kDa β-actin. Similar results were obtained in three independent experiments. Average protein expression was quantified as ratio between ADAR2 and β-actin. Ratios between ADAR2 and β-actin in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

B: *Top:* mRNA expression measured by RT-PCR of 5-HT$_{2B}$ receptor. A representative experiment showing mRNA for 5-HT$_{2B}$ receptor and for TBP, as a housekeeping gene. The size of PCR product of 5-HT$_{2B}$ receptor is 370 bp, and that of TBP 236 bp. Similar results were obtained in three independent experiments. Average mRNA expression was quantified as ratio between 5-HT$_{2B}$ receptor and the housekeeping TBP gene. Ratios between 5-HT$_{2B}$ receptor and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

Bottom: Protein expression measured by immunoblotting of 5-HT$_{2B}$ receptor. Band of 55 kDa represents 5-HT$_{2B}$ receptor or of 42 kDa β-actin. Similar results were obtained in three independent experiments. Average protein expression was quantified as ratio between 5-HT$_{2B}$ receptor and β-actin. Ratios between 5-HT$_{2B}$ receptor and β-actin in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group.

C: mRNA expression measured by RT-PCR of 5-HT$_{2A}$ receptor and 5-HT$_{2C}$ receptor. A representative experiment showing mRNA for 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and for TBP, as a housekeeping gene. The size of PCR product of 5-HT$_{2A}$ receptor is 470 bp or 5-HT$_{2C}$ receptor 318 bp, and that of TBP 236 bp. Similar results were obtained in three independent experiments. Average mRNA expression was quantified as ratio between 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and the housekeeping TBP gene. Ratios between 5-HT$_{2A}$ receptor or 5-HT$_{2C}$ receptor and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars.

way ANOVA followed by Fisher's LSD test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Experiments in animals in vivo

3.1.1. Ammonium-induced depression behaviour

In three days after urease treatment, 100% animals in urease group developed depressive behaviour, reflected by reduced consumption of sucrose (Fig. 1A). In three days after urease treatment, sucrose consumption decreased significantly, indicating anhedonia (Fig. 1A; Control: 71.6 ± 2.0%, n = 10; urease: 57.0 ± 3.5%, n = 10; $p < 0.05$). Duration of immobility in tail suspension test similarly significantly increased (Fig. 1A; Control: 99.3 ± 5.9 s, n = 10; urease: 173.1 ± 18.0 s, n = 10; $p < 0.05$). In forced-swimming test (Fig. 1A), injection of urease significantly increased the time of immobility (Control: 52.5 ± 5.4 s, n = 10; urease: 124.7 ± 17.6 s, n = 10; $p < 0.05$).

3.1.2. Up-regulation of ADAR2 gene expression

As shown in Fig. 1B the level of ADAR2 mRNA, normalised to TBP, was increased in cerebral hemispheres from animals after three days of urease treatment (137.8 ± 10.9% of control, n = 6; $p < 0.05$). Protein expression of ADAR2 in urease-treated animals was 373% of that in the control group (Fig. 1C; 373.5 ± 37.0%, n = 6; $p < 0.05$).

3.1.3. Up-regulation of gene expression of 5-HT$_2$ receptors

In cerebral hemispheres from animals after three days of urease treatment, the level of 5-HT$_{2B}$ receptor mRNA, normalised to TBP, increased to 177% of control (177.1 ± 8.6% of control, n = 6; $p < 0.05$) (Fig. 2A), whereas protein expression of 5-HT$_{2B}$ receptor in urease-treated mice was 1121% of control group (1121 ± 85.6% of control, n = 6; $p < 0.05$) (Fig. 2B). In contrast, urease had no effect on mRNA expression of both 5-HT$_{2A}$ receptor (104.0 ± 6.3% of control, n = 6; $p > 0.05$) and 5-HT$_{2C}$ receptor (96.0 ± 4.2% of control, n = 6; $p > 0.05$) (Fig. 2C).

3.1.4. RNA editing of 5-HT$_{2B}$ receptor

Editing of the 5-HT$_{2B}$ receptor RNA were quantified by comparison of the PCR products obtained from cerebral hemispheres of control animals (Fig. 3A) with mice treated with urease for 3 days (Fig. 3B). The ratios between the edited, G-containing and unedited, A-containing isoforms in the PCR products were calculated as shown in Fig. 3C. In control cells, almost no editing was observed in eight selected RNA (Fig. 3C). However, in animals treated with urease for 3 days, the

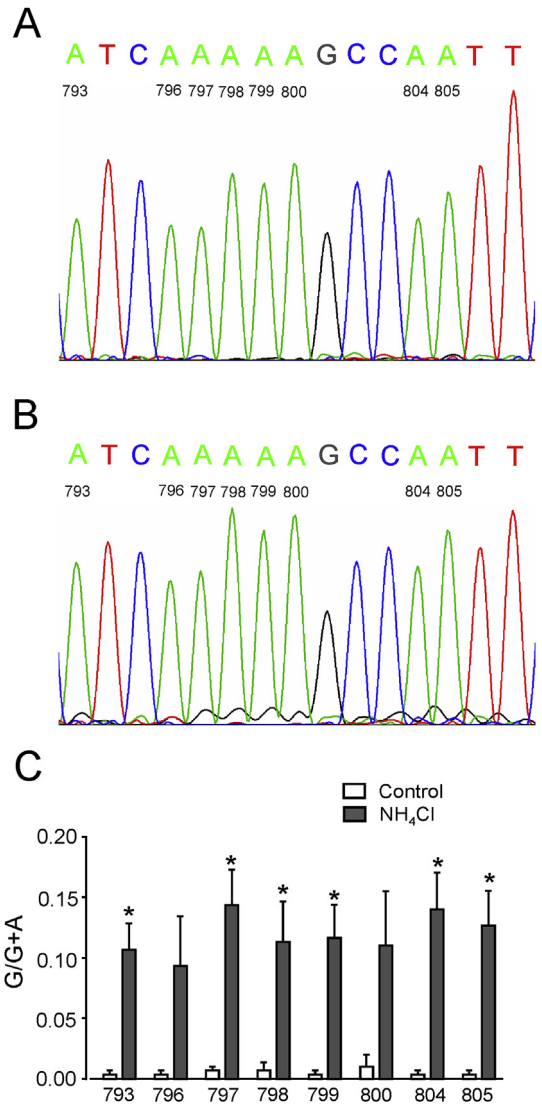

Fig. 5. RNA editing of 5-HT_{2B} receptor in cultured astrocytes treated with ammonium.

After 3 days treatment with either saline (control; **A**) or ammonium (3 mM) (**B**), cells were collected for amplification refractory mutation system polymerase chain reaction (ARMS-PCR) analysis of 5-HT_{2B} receptor editing at 793, 796, 797, 798, 799, 800, 804 or 805 sites. Representative Southern blots showing edited (guanosine; G) and unedited (adenosine; A) PCR products at 793, 796, 797, 798, 799, 800, 804 or 805 sites. (**C**). Similar results were obtained in three independent experiments. Standard error of the mean values are indicated by horizontal bars. *Statistically significant ($p < 0.05$) difference from control group.

frequency of editing at 7 out of 8 RNA sites was significantly increased (Fig. 3C), 7% at site 793 (control: 0.00 ± 0.00; urease: 0.07 ± 0.01; n = 5, $p < 0.05$), 12% at site 797 (control: 0.01 ± 0.00; urease: 0.12 ± 0.01; n = 5, $p < 0.05$), 6% at site 798 (control: 0.00 ± 0.00; urease: 0.06 ± 0.01; n = 5, $p < 0.05$), 6% at site 799 (control: 0.00 ± 0.00; urease: 0.06 ± 0.01; n = 5, $p < 0.05$), 8% at site 800 (control: 0.00 ± 0.00; urease: 0.08 ± 0.02; n = 5, $p < 0.05$), 9% at site 804 (control: 0.01 ± 0.01; urease: 0.09 ± 0.02; n = 5, $p < 0.05$), and 8% at site 805 (control: 0.01 ± 0.00; urease: 0.08 ± 0.01; n = 5, $p < 0.05$), indicating urease-induced 5-HT_{2B} receptor RNA editing.

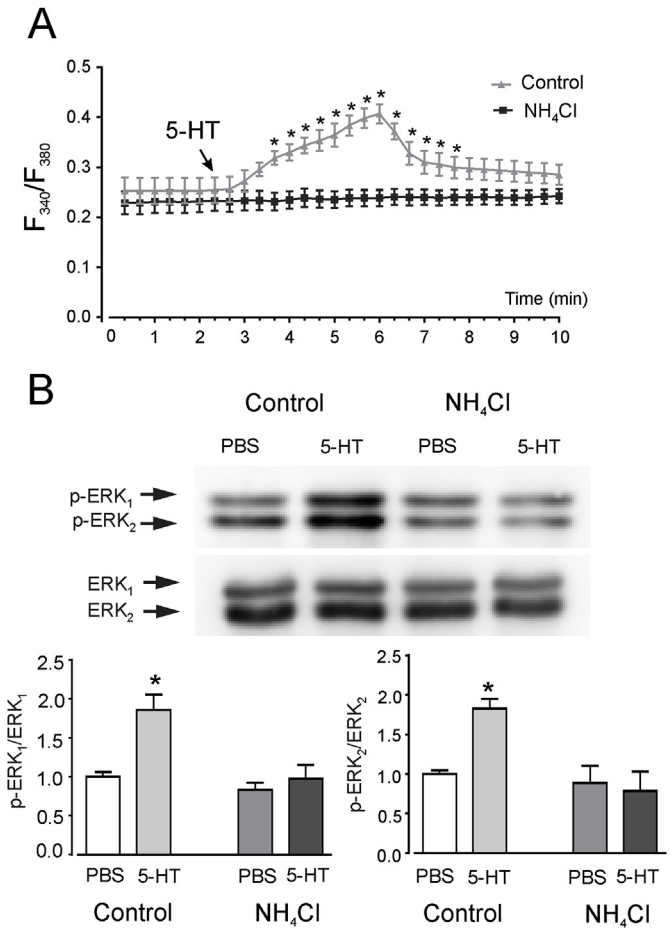

Fig. 6. Effects of 5-HT on $[Ca^{2+}]_i$ and $ERK_{1/2}$ phosphorylation cultured astrocytes treated with ammonium.

A: Representative traces showing the average changes in $[Ca^{2+}]_i$ (340/380) in 20 astrocytes on one coverslip. Results are averages of 340/380 of 7 individual coverslips. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from control group at the same treatment period.

B: Immunoblots from a representative experiment. Bands of 44 kDa represent p-ERK_1 and total ERK_1, or of 42 kDa p-ERK_2 and total ERK_2. Similar results were obtained from four independent experiments. Average ERK phosphorylation was quantified as ratio between p-ERK_1 and ERK_1 or p-ERK_2 and ERK_2. Ratios between p-ERK_1 and ERK_1 or p-ERK_2 and ERK_2 in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from all other groups at the same time period.

3.2. Experiments in vitro

3.2.1. Up-regulation of gene expression of ADAR2

As shown in Fig. 4A, the level of ADAR2 mRNA, normalised to TBP, increased in cultured astrocytes treated with 3 mM ammonium for 3 days (128.6 ± 8.2% of control, n = 6; $p < 0.05$). Protein expression of ADAR2 in ammonium-treated cells was almost doubled compared to the control group (172.8 ± 12.2% of control, n = 3; $p < 0.05$) (Fig. 4A).

3.2.2. Up-regulation of gene expression of 5-HT_2 receptors

In astrocytes treated with 3 mM ammonium for 3 days, the level of 5-HT_{2B} receptor mRNA, normalised to TBP, increased to 171% of control (171.1 ± 21.3% of control, n = 3; $p < 0.05$) (Fig. 4B). Protein expression of 5-HT_{2B} receptor in ammonium-treated cells was 381% of control group (381.5 ± 32.0% of control, n = 3; $p < 0.05$) (Fig. 4B). In contrast, ammonium had no effect on mRNA expression of neither 5-

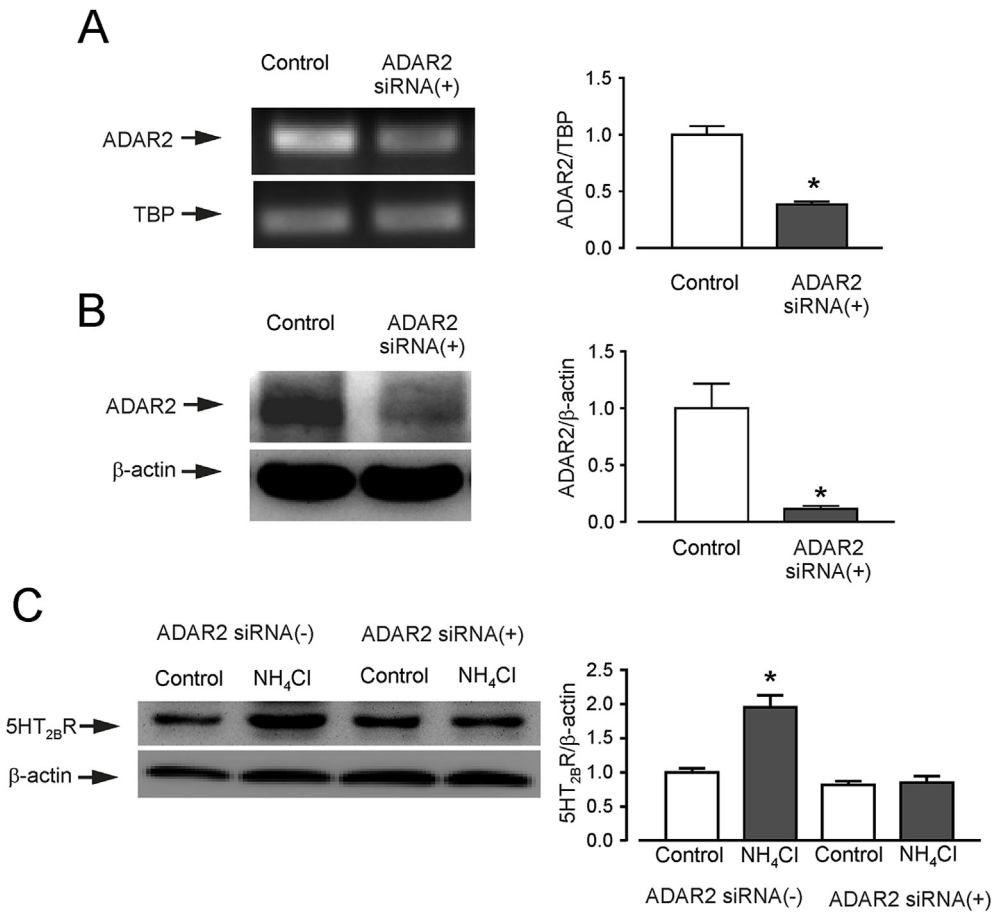

Fig. 7. Down-regulation of 5-HT_{2B} receptor gene by ammonium requires ADAR2 activity in cultured astrocytes.

A: A representative experiment showing mRNA for ADAR2 and for TBP, as a housekeeping gene. The size of PCR product of ADAR2 is 203 bp, and that of TBP 236 bp. Similar results were obtained in five independent experiments. Average mRNA expression was quantified as ratio between ADAR2 and the housekeeping TBP gene. Ratios between ADAR2 and TBP in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from the control group without ADAR2 siRNA.

B: Protein expression measured by immunoblotting of ADAR2. Band of 90 kDa represents ADAR2 or of 42 kDa β-actin. Similar results were obtained in four independent experiments. Average protein expression was quantified as ratio between ADAR2 and β-actin. Ratios between ADAR2 and β-actin in control group were designated a value of one. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from the control group without ADAR2 siRNA.

C: Immunoblots from a representative experiment. Similar results were obtained from 3 independent experiments. Average protein expression of 5-HT_{2B} receptor was quantitated as ratios between 5-HT_{2B} receptor and β-actin. Ratio between 5-HT_{2B} receptor and β-actin in control group were designated a value of one. *Indicates statistically significant ($P < 0.05$) difference from the control group without ADAR2 siRNA.

HT_{2A} receptor (95.0 ± 13.3% of control, n = 3; $p > 0.05$) nor 5-HT_{2C} receptor (100.7 ± 13.8% of control, n = 3; $p > 0.05$) (Fig. 4C).

3.2.3. RNA editing of 5-HT_{2B} receptor

Editing of the 5-HT_{2B} receptor RNA was quantified by comparison of the PCR products obtained from control astrocytes (Fig. 5A) with cells treated with ammonium at 3 mM for 3 days (Fig. 5B). The ratios between the edited, G-containing and unedited, A-containing isoforms in the PCR products were calculated as shown in Fig. 5C. In control cells, no editing was observed in eight selected RNA sites. However, in cells treated with ammonium for 3 days, the frequency of editing at 6 out of 8 RNA sites increased significantly, 11% at site 793 (control: 0.00 ± 0.00; NH_4Cl: 0.11 ± 0.02; n = 3, $p < 0.05$), 14% at site 797 (control: 0.01 ± 0.00; NH_4Cl: 0.14 ± 0.03; n = 3, $p < 0.05$), 11% at site 798 (control: 0.01 ± 0.01; NH_4Cl: 0.11 ± 0.03; n = 3, $p < 0.05$), 12% at site 799 (control: 0.00 ± 0.00; NH_4Cl: 0.12 ± 0.03; n = 3, $p < 0.05$), 14% at site 804 (control: 0.00 ± 0.00; NH_4Cl: 0.14 ± 0.03; n = 3, $p < 0.05$), and 13% at site 805 (control: 0.00 ± 0.00; NH_4Cl: 0.13 ± 0.03; n = 3, $p < 0.05$), indicating ammonium-induced 5-HT_{2B} receptor RNA editing.

3.2.4. Increase of intracellular Ca^{2+} and $ERK_{1/2}$ phosphorylation

As shown in Fig. 6A, application of 100 nM 5-HT caused a significant elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ amounting to 1.71-fold increase above the resting level and lasting for at least 4 min. Three days of treatment with ammonium completely abolished this effect of 5-HT (control: 0.41 ± 0.02, n = 9; NH_4Cl: 0.24 ± 0.02, n = 7; $p < 0.05$).

Exposure to 100 nM of 5-HT also induced an increase of $ERK_{1/2}$ phosphorylation in cultured astrocytes with no difference in total $ERK_{1/2}$ (p-ERK_1: 185.7 ± 20.0% of control; p-ERK_2: 182.5 ± 12.5% of control. n = 4) (Fig. 6B). Chronic treatment with 3 mM ammonium for 3 days also eliminated the 5-HT induced phosphorylation of $ERK_{1/2}$ (p-ERK_1: 97.7 ± 17.9% of control; p-ERK_2: 78.5 ± 24.6% of control. n = 4) (Fig. 6B). As we reported previously, 5-HT at 100 nM stimulates $ERK_{1/2}$ phosphorylation by activation of 5-HT_{2B} receptors but not by of 5-HT_{2A} receptors and of 5-HT_{2C} receptors (Li et al., 2010).

3.2.5. Inhibitory effect of ADAR2 siRNA

To confirm that ammonium-induced dysfunction of 5-HT_{2B} receptor is mediated by ADAR2, astrocyte cultures were treated with ADAR2 siRNA. As shown in Fig. 7, ADAR2 mRNA (Fig. 7A; $ADAR_2$ siRNA: 38.5 ± 2.4% of control group, n = 5, $p < 0.05$) and protein (Fig. 7B; $ADAR_2$ siRNA: 11.4 ± 2.6% of control group, n = 4, $p < 0.05$) were greatly reduced in ADAR2 siRNA treated cells. In ADAR2 knock-down cells, chronic treatment with ammonium failed to up-regulate expression of 5-HT_{2B} receptors (Fig. 7C; in cells treated with $ADAR_2$ siRNA(−), NH_4Cl: 194.9 ± 18.1% of control; in cells with $ADAR_2$ siRNA(+), NH_4Cl: 85.1 ± 9.2% of control; n = 3.).

In addition, in cells treated with ADAR2 siRNA, ammonium had no effect on 5-HT_{2B} receptor RNA editing (Fig. 8A–C), 0% at site 793 (control: 0.01 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 796 (control: 0.00 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 797 (control: 0.00 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 798 (control: 0.00 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 799 (control:

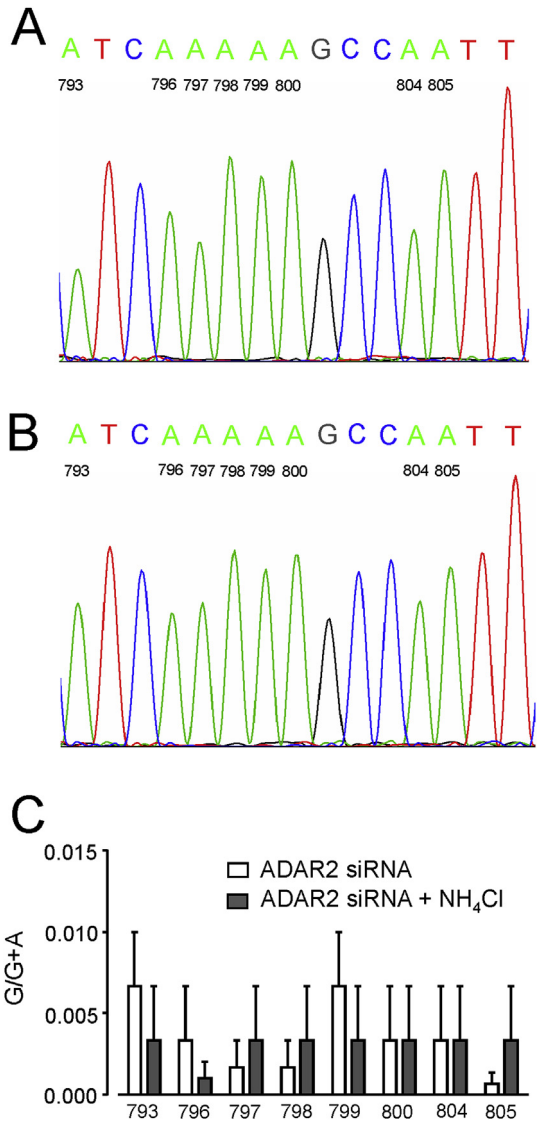

Fig. 8. 5-HT_{2B} receptor editing by ammonium requires ADAR2 activity in cultured astrocytes.

After 3 days treatment with either saline (control; **A**) or ammonium (3 mM) (**B**), cells were collected for amplification refractory mutation system polymerase chain reaction (ARMS-PCR) analysis of 5-HT_{2B} receptor editing at 793, 796, 797, 798, 799, 800, 804 or 805 sites. Representative Southern blots showing edited (guanosine; G) and unedited (adenosine; A) PCR products at 793, 796, 797, 798, 799, 800, 804 or 805 sites. (**C**) Similar results were obtained from three samples in each group. Standard error of the mean values are indicated by horizontal bars. *Statistically significant ($p < 0.05$) difference from control group.

0.01 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 800 (control: 0.00 ± 0.00; ADAR2 siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), 0% at site 804 (control: 0.00 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$), and 0% at site 805 (control: 0.00 ± 0.00; $ADAR_2$ siRNA: 0.00 ± 0.00. n = 3, $p > 0.05$).

As alluded before (cf. Fig. 6A), application of 100 nM of 5-HT triggers an increase in $[Ca^{2+}]_i$. Three days of treatment with ammonium completely abolished this effect of 5-HT (control: 0.39 ± 0.02, n = 7; NH_4Cl: 0.25 ± 0.02, n = 7; $p < 0.05$). However, ammonium had no effect in cells treated with ADAR2 siRNA (siRNA(+) control: 0.39 ± 0.02; siRNA(+) NH_4Cl: 0.38 ± 0.02, n = 7; $p < 0.05$, Fig. 9A).

Furthermore, in the cells with reduced ADAR2 expression, down-regulation of 5-HT-induced $ERK_{1/2}$ phosphorylation by chronic treatment with ammonium was abolished. As shown in Fig. 9B, in cells treated with $ADAR_2$ siRNA(−), 5-HT has no effect on $ERK_{1/2}$ phosphorylation after three days treatment with ammonium (ERK_1: control: 100.0 ± 4.8%; 5-HT: 227.5 ± 15.4%; NH_4Cl: 92.6 ± 4.2%; NH_4Cl +5-HT: 95.3 ± 1.6%. ERK_2: control, 100.0 ± 5.0%; 5-HT: 253.5 ± 10.3%; NH_4Cl: 104.6 ± 9.6%; NH_4Cl +5-HT: 104.9 ± 8.5%. n = 3). However, in cells treated with $ADAR_2$ siRNA(+), the magnitude of the response to 5-HT in control cells and in ammonium-exposed cells were similar (ERK_1: control: 100.0 ± 3.7%; 5-HT: 242.9 ± 1.3%; NH_4Cl: 93.7 ± 4.2%; NH_4Cl +5-HT: 258.6 ± 24.0%. ERK_2: control: 100.0 ± 5.5%; 5-HT: 263.1 ± 14.6%; NH_4Cl: 101.4 ± 6.9%; NH_4Cl +5-HT: 293.6 ± 14.3%,. n = 3).

4. Discussion

Depression is one of the frequent psychiatric symptoms in hyperammonemia and hepatic encephalopathy (Nardelli et al., 2013; Telles-Correia et al., 2015; Mullish et al., 2015). In the present study depressive behaviour was triggered by treating mice with urease, the latter being an acknowledged animal model of hyperammonemia. Similar behavioural phenotype was also obtained in animals treated with thioacetamide-induced acute liver damage (Kawai et al., 2012; Ashkani-Esfahani et al., 2016). The maximal plasma ammonium concentration in urease-injected animals is about 1.5 mM (O'Connor et al., 1984). However, the range of ammonium content in the brain varies from 7.7 to 11 mmol/g wet weight (Butterworth, 2002, 2003). Since the extracellular ammonium concentration is 2–3 times lower than intracellular level (Hindfelt, 1975a; 1975b), the intracellular ammonium concentration can be as high as 3–5 mM, which concentration we used for *in vitro* experiments (Xue et al., 2010; Dai et al., 2013; Wang et al., 2015, 2017).

In the present work, we demonstrated that the increase of ammonium levels up-regulates gene expression of ADAR2 and 5-HT_{2B}Rs, as well as RNA editing of 5-HT_{2B}Rs in the brain *in vivo* as well as in cultured astrocytes *in vitro*. Post-translational mRNA editing mediated by ADAR enzymes which converts adenosines to inosines in coding and noncoding RNA is widespread throughout species and tissues, being particularly prominent in the CNS (Tarik and Jantsch, 2012; Slotkin and Nishikura, 2013). This post-translational editing arguably increases complexity of proteome and is particularly important for development of neural networks (Wahlstedt et al., 2009). Several types of ion channels and neurotransmitter receptors are subjected to ADAR-mediated editing; these in particular include voltage-gated K^+ and Ca^{2+} channels, ionotropic glutamate receptors, $GABA_A$ receptors and serotonin 5-HT_{2C} receptors (Rula et al., 2008; Bhalla et al., 2004; Marion et al., 2004). Failures in ADAR-mediated mRNA editing associated with many deficient brain development and neurological disorders (Tarik and Jantsch, 2012). In particular aberrant or increased editing of 5-HT_{2C} receptors is associated with psychiatric disorders including schizophrenia and major depression (Dracheva et al., 2008). In particular, increased editing of C and C′ site and decreased editing of D site was revealed in victims of suicide linked to major depression (Gurevich et al., 2002). In the present paper we extend the role of ADAR-mediated RNA editing of 5-HT_{2B} receptors in the context of hyperammonemia and related depressive behaviours. The lack of up-regulation of expression and editing of 5-HT_{2B} receptors in ADAR2-knock down cells suggests that these effects of ammonium are mediated through ADAR2 activity. In ADAR2-reduced cells, the suppression of cell responses to 5-HT indicates that the RNA editing of 5-HT_{2B} receptors results in the dysfunction of the receptors, i.e., no response to 5-HT in increase of $[Ca^{2+}]_i$ and phosphorylation of $ERK_{1/2}$. The latter process depends on an increased $[Ca^{2+}]_i$ in cultured astrocytes (Li et al., 2008). Our previous study had shown that 5-HT at concentration of 100 nM stimulates specifically 5-HT_{2B} receptor, without affecting -HT_{2A} or 5-HT_{2C} receptors in astrocytes (Li et al., 2010).

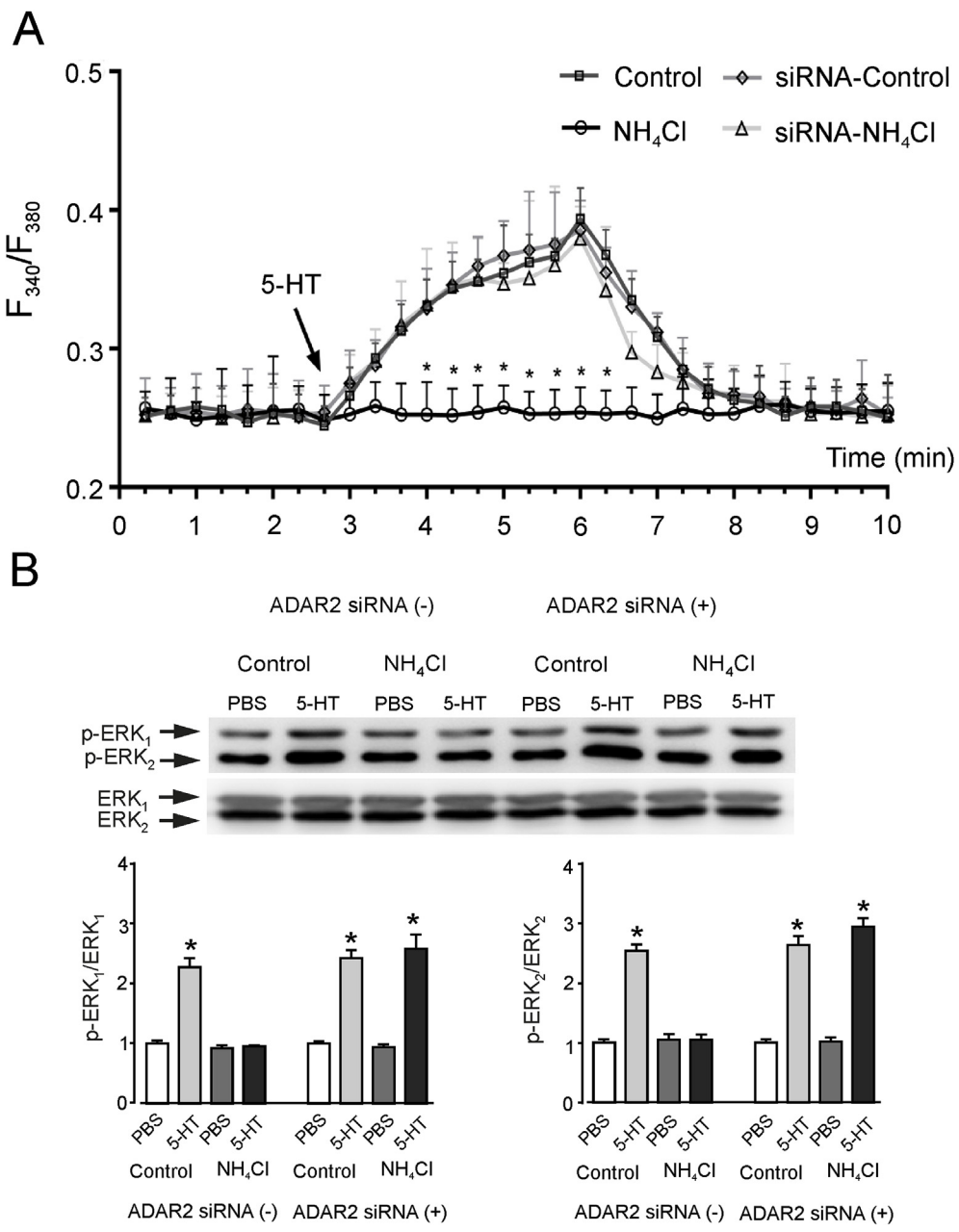

Fig. 9. Effects of ammonium on $[Ca^{2+}]_i$ and $ERK_{1/2}$ phosphorylation in cultured astrocytes require ADAR2 activity in cultured astrocytes.

A: Results are averages of 340/380 of 7 individual samples. S.E.M. values are indicated by vertical bars. *Indicates statistically significant ($P < 0.05$) difference from all other groups at the same treatment period.

B: Cells were incubated for 20 min in serum-free medium in the absence of any drug (control) or in the presence of 100 nM 5-HT. Immunoblot from a representative experiment. Similar results were obtained from 3 independent experiments. Average ERK phosphorylation was quantitated as ratios between p-ERK_1 and ERK_1 and between p-ERK_2 and ERK_2. Ratios between p-ERK_1 and ERK_1 or p-ERK_2 and ERK_2 in control group were designated a value of one. Standard error of the mean values are indicated by vertical bars. *Statistically significant ($p < 0.05$) difference from all other groups for ERK_1 and ERK_2.

Serotonergic transmission is affected in the course of the hyperammonemia. In the both patients and animal models levels of the 5-HT precursor, l-tryptophan, are increased in the plasma and in the brain (Lozeva-Thomas, 2004 and references therein). Although 5-HT concentrations in the brain are not elevated, the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), MAO-A/B activity and gene expression of MAO-A are significantly increased (Lozeva-Thomas, 2004), indicating elevated synthesis and catabolism of 5-HT in HE. Of note, levels of 5-HIAA in the brain parallel arterial ammonium concentrations in the animal model (Lozeva et al., 2004). In contrast to resting 5-HT, high K^+-stimulated release of 5-HT is increased in rats with thioacetamide-induced hepatic encephalopathy (Kaneko et al., 1998). As mentioned above, if anything happened in hyperammonemia 5-HT concentrations are higher but not lower in brain, suggesting that inhibition of 5-HT uptake may not solve the problems in hyperammonemia-induced depression.

As we reported previously, depression cased by CMS as well as depression developed in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) models of Parkinson's disease were associated with a decrease in gene expression of $5\text{-}HT_{2B}R$ in the brain; this decrease was corrected by chronic treatment with fluoxetine (Li et al., 2012; Song et al., 2018). This is in contrast with the present findings that gene expression of $5\text{-}HT_{2B}$ receptor was increased and the same time RNA of $5HT_{2B}Rs$ was edited in astrocytes treated with ammonium as well as in the brain of animals with hyperammonemia. Although there is a decrease of function of astrocytic $5\text{-}HT_{2B}Rs$ in all four depression animal models, the underlying mechanisms of pathogenesis are different, which may also apply to clinical situations and therapeutic strategies. Fluoxetine, as well as other SSRIs act as an agonist of astroglial $5\text{-}HT_{2B}R$; moreover these cells do not express 5-HT transporter (Li et al., 2008; Zhang et al., 2010). Chronic treatment with fluoxetine up-regulates ADAR2 expression and induces $5\text{-}HT_{2B}R$ editing in cultured astrocytes and in astrocytes *in vivo* (Li et al., 2011, 2012; Hertz et al., 2014) but not in depressed animals (Dong et al., 2015), implying that the drug may also have different effects on ammonium treated astrocytes and alleviate clinical hyperammonemia-

associated depression. We had extensively studied signalling cascades linked to ammonium in astrocytes (Dai et al., 2013; Wang et al., 2017). After acute treatment, ammonium and low concentrations of ouabain activate almost identical signal pathway in astrocytes (Haas et al., 2002; Dai et al., 2013). Ammonium acts on α1 subunit of Na,K-ATPase which, in turn stimulates Src and leads to EGFR transactivation. The activation of EGFR and its intracellular signal pathways Raf/MAPK/$ERK_{1/2}$ and PI3K/AKT could regulate multiple gene expression, including ADAR2 in astrocytes. This finding indicates that oubain inhibitor, canrenone, could be a potential therapeutic agent for hyperammonemia depression (Semplicini et al., 1995). We also found that chronic treatment with ammonium increases activity of glycogen synthase kinase 3β (GSK-3β) through Cav-1/PTEN/PI3K/AKT pathway (Wang et al., 2017). Ammonium up-regulates gene expression of Cav-1, increases membrane content of PTEN, decreases activity of PI3K/AKT, and reduces GSK-3β phosphorylation thus enhancing its activity. Since high levels of GSK-3β activity are generally associated with mood disorders (Gould and Manji, 2005), GSK-3β may be implicated in hyperammonemia-induced depression, suggesting that GSK-3β inhibitors, such as lithium salt may be an alternative compound under this circumstance. Although astrocytes are recognised as primary targets for ammonium, we can not make conclusions whether these changes in gene regulation also occur in neurones and other types of brain cells *in vivo*.

In addition to ADAR2 and 5-HT_{2B} receptor, we also reported that chronic treatment with ammonium induced up-regulation of gene expression of α2 isoform of Na,K-ATPase (Xue et al., 2010), of the transient receptor potential channel 1 (TRPC1) (Liang et al., 2014) and of the L-type Ca^{2+} channel, $Ca_v1.2$ (Wang et al., 2015) in astrocytes. Among them, $Ca_v1.2$ is of special interest, because expression of its gene is regulated by fluoxetine and antibipolar drugs in astrocytes and/or neurones (Li et al., 2011; Yan et al., 2013; Du et al., 2014), and $Ca_v1.2$ is claimed to be involved in psychiatric disorders (Casamassima et al., 2010; Bhat et al., 2012).

Up to now, we have found that dysfunction of astrocytic 5-HT_{2B} receptor is involved in (i) experimental major depression, (ii) experimental Parkinson's depression and (iii) depression in hyperammonemia, albeit by different mechanisms. In (i) and (ii), gene expression of 5-HT_{2B}R is decreased whereas in (iii) gene expression of 5-HT_{2B}R is increased while receptor RNA is edited. The understanding of mechanisms of pathogenesis underlying different types of depression may eventually lead to development of new therapeutic modalities as substitute for SSRIs.

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