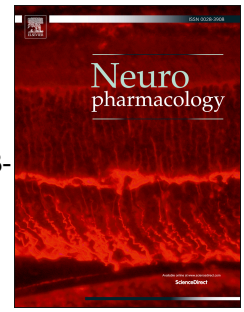

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Credit author statement

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Kaiyang Ma: Data curation, Writing- Original draft preparation.
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Kaiyang Ma: Writing- Reviewing and Editing

Lactate enhances Arc/arg3.1 expression through Hydroxycarboxylic acid receptor 1-β-arrestin2 pathway in astrocytes

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ABSTRACT

In recent years, with the discovery and research of lactate-specific receptor HCAR1(hydroxycarboxylic acid receptor 1), lactate is not only as a product of Glycolysis in astrocytes, but also as a signaling molecule which has gradually received attention. Studies have found that lactate can be used as an intercellular signaling molecule involved in synaptic plasticity, and so that peripheral administration of lactate can produce antidepressant effects. Here, we focus on HCAR1 on the most widely distributed astrocytes in the brain, found and verified that lactate could cause Arc/arg3.1 protein overexpression in astrocytes through HCAR1. However, the expression of Arc/arg3.1 does not depend on the Gi protein pathway of HCAR1, and we found that lactate enhanced the expression of Arc/arg3.1 protein through the HCAR1- β -arrestin2 pathway. In summary, lactate acts on HCAR1 of astrocytes. It enhances the expression of MAPK-dependent Arc through β-arrestin2, thereby reducing the influx of calcium ions when astrocytes are exposed to glutamate damage, achieving the role of protecting astrocytes and indirectly enhancing the absorption of glutamate by astrocytes. These results also demonstrate that HCAR1 in the brain is a potential therapeutic target in an experimental in vitro model of glutamate damage, which is strongly associated with many neurodegenerative diseases.

INTRODUCTION

In the central nervous system, astrocytes are the most widely distributed type of cells in the brain and the largest in glial cells. Under normal circumstances, astrocytes mainly play a role in providing information processing, nutrition, and protection for neurons. They also participate in the process of signal transmission across synapses; and are also sites of neurotransmitter metabolism such as glutamic acid. The main site of glucose hydrolysis in the brain[1] .As a product of glycolysis in astrocytes in the brain, lactate has been discovered in recent years in addition to itself as a product of glycolysis. Lactic acid has also been discovered for its potential signal molecule function and role as a fuel for cell functions. Evidence supports the role of lactate as an intercellular signaling molecule involved in synaptic plasticity. In recent studies, it has been found that peripheral administration of L-lactate can produce antidepressant effects [2], and
can even promote resilience to stress and rescued social avoidance and anxiety by restoring hippocampal class1 histone deacetylase levels and activity, specifically HDAC2/3[3].

L-lactate is produced in the brain by glycolysis of neurons and astrocytes or from the periphery through the blood-brain barrier to the brain. It is hypothesized that neurons use L-lactate as a supplemental fuel. Regardless of whether or not it is used as a substrate fuel, L-lactate may also have a signaling effect in the brain [4]. As a specific receptor for lactate, HCAR1 undoubtedly plays a vital role in lactate signaling. It was first discovered in fat cells that lactic acid can activate HCAR1 and thereby inhibit lipolysis in adipocytes, and down-regulate cAMP through the action of Gi protein[5]. It was subsequently discovered that lactate could inhibit the activation of NLRP3 by β-arrestin2, thereby inhibiting the inflammation in fat cells[6]. Studies have shown that HCAR1 is selectively activated in vivo by lactate in the concentration range (approximately 0.1-3.0mM)[7]. Under physiological conditions, the concentration of lactate in the brain is less than ~ 1.5 mmol / L. In addition, exercise and the intake of exogenous HCAR1 agonists can activate receptors on cerebral blood vessels and brain cells[8], Therefore, lactic acid in the brain concentration range is sufficient to activate HCAR1 and may have a series of biological effects.

There is evidence that HCAR1 mRNA and protein were localized to the hippocampus, neocortex, and cerebellum[8], widely distributed. Some studies support the astrocyte-neuron lactate shuttle (ANLS) is necessary for long-term memory[9], so more research will focus on lactate and neurons, especially the emergence of the ANLS hypothesis[10]. Astrocytes are the most widely distributed type of cells in the brain. However, the effect of lactate on astrocytes has rarely been studied. Therefore, in this experiment, Therefore, in contrast, in this experiment, the effects of lactate and HCAR1 in the brain are gathered on astrocytes. We consider whether astrocytes can act on lactic acid produced by glycolysis like adipocytes, or whether lactic acid in the brain has a positive effect on the most widely distributed astrocytes.

As we know, glutamate is an excitatory neurotransmitter involved in neural development, synaptic plasticity, and plays an important role in learning and

memory[11]

However, it is well known that glutamate-induced excitotoxicity is one of the important causes of brain damage, especially a common feature of many central nervous system diseases, such as brain injury, stroke, neurodegenerative diseases including Alzheimer's disease, Huntington's disease, and Parkinson's disease[12]. Excessive activation of glutamate receptors can cause neuron damage or death through glutamate excitotoxicity, which may cause neuron dysfunction[13]. Therefore, the normal maintenance of neuron survival and function requires rapid removal of glutamate, and astrocytes are one of the main cell types responsible for glutamate uptake[14]. AMPA receptors are also abundantly expressed on astrocytes. The presence of these AMPA receptors also makes astrocytes sensitive to the increase of extracellular concentrations of glutamate[15]. Excessive activation of these AMPA receptors can also cause toxicity in astrocytes due to excessively high glutamate concentrations. Therefore, reducing the sensitivity of AMPA receptors can reduce glutamate damage to astrocytes.

Our study found that lactate can promote the expression of Arc/arg3.1 in astrocytes, the protein also has a variety of roles in synaptic plasticity. The protein of Arc plays a key role in synaptic plasticity by promoting endocytosis of AMPA receptors[16], and the expression of Arc/arg3.1 can reduce calcium influx caused when astrocytes are in the face of glutamate damage[17].

Therefore, the significance of our study is to explore the lactate signal through HCAR1-β-arrestin2 pathway, which can enhance the expression of Arc/arg3.1 in astrocytes. Our findings show that the lactate signal pathway is reduced glutamate damage to astrocytes in vitro experiments, whose reduction of glutamate damage may show HCAR1 is a potential therapeutic target for brain damage caused by glutamate-damaged.

Materials and methods

Animals

C57BL/6J mice (male, 3-month old) were obtained from the Animal Resource Centre of Nanjing Medical University. β-arrestin2−/−mice (male, 3-month old) were obtained from Gang Pei’s laboratory. Mice were bred and maintained in the Animal Resource Centre of Nanjing Medical University. Mice had free access to food and water in a room with an ambient temperature of 22 °C ± 2 °C and a 12:12–hour light/dark cycle. Neonatal mice within 3 d were used. All animal procedures were performed in strict accordance with the guideline of the Institutional Animal Care and Use Committee of Nanjing Medical University.

Reagents

L-lactate was purchased from Aladdin.PD98059 was purchased from TOCRIS. SB203580 were purchased from Selleck(#s1076). PTX was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cck-8 were purchased from Selleck. Anti-Arc Ab was purchased from Santa Cruz (#sc-17839, 1:500). Anti-GPR81 Ab was purchased from Absin (#abs133145, 1:500). Anti-GPR81 Ab was purchased from Bioss (#bs-16265R, 1:500). Anti-β-arrestin1 Ab was purchased from CST (#3857,

1:1000). Anti-β-arrestin2 Ab was purchased from Proteintech(#10171-1-AP, 1:1000). Anti-GAPDH Ab was purchased from Santa Cruz (#SC-32233, 1:1000). GFAP was purchased from Millipore (#MAB360),3AM-Flou was purchased from Invitrogen(#F1242)

Primary astrocyte cultures

The whole brain of the neonatal mice that aged 1–3 d was removed and separated from the meninges and basal ganglia. Tissues were digested with 0.25% trypsin (Amresco, Solon, OH, USA) at 37 ° C and terminated with medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific) and 1% penicillin / streptomycin. Cells were then seeded on 120 cm petri dishes (Sigma). After 24h, the medium was changed every 3 days. When the bottom of the culture dish was 90% confluent, the cells were seeded in a six-well plate at a concentration of 1.5×10^5 / ml for cultivation. In general, primary cultured astrocytes cultured for 7-10 days can be used in our experiments.

Cell transfection

SiRNA targeting HCAR1 or negative control (NC) siRNA (Jima, Shanghai, China) was transfected in mouse primary cultured astrocytes by using Lipofectamine RNAiMAX Reagent(Invitrogen, Life Technologies) in OPTI-MEM reduced serum medium (Gibco) according to the manufacturer's instructions. siRNA duplexes used were as follows:

GPR81-mus-1215(sense): UCACCUACCUGAACAGUAUTT
GPR81-mus-1215 (antisense): AUACUGUUCAGGUAGGUGATT
GPR81-mus-402 (sense): UCCUCUCUCCAACUGCUAUTT
GPR81-mus-402 (antisense): AUAGCAGUUGGAGAGAGGATT
GPR81-mus-942 (sense): GGUGGCACGAUGUCAUGUUTT
GPR81-mus-942 (antisense): AACAUGACAUCGUGCCACCTT
NC (sense): UUCUCCGAACGUGUCACGUTT
NC (antisense): ACGUGACACGUUCGGAGAATT

Six hours later, the transfection mixture was removed and cells were further incubated with normal medium for additional 48 h.

Plasmids of pcDNA3.1-β-arrestin2 were transfected to astrocytes by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions provided. Cells were collected for western blotting analysis after 48 h transfection.

Cellular treatments: Transfected cells were used by treating with 0.5mM of L-lactate for 1h. In addition, transfected cells were stimulated by 100uM of L-glutamate for 24h to cause glutamate damage and treating with 0.5mM of L-lactate for 1h at last.

Western blotting analysis

Cells were lysed in buffer (Bio-Rad) according to the manufacturer's instructions. The protein concentration was measured using a Micro BCA kit (Beyotime, Shanghai, China). Protein separation using polyacrylamide TGX gel (Bio-Rad, Hercules, California, USA) by sodium lauryl sulfate polyacrylamide gel electrophoresis, and then transferring the protein to a polyvinylidene fluoride (PVDF) membrane on. After blocking with 10% milk, the PVDF membrane was incubated with the various

specific primary antibodies described above in a Tris Buffered Saline with Tween 20 at 4 ° C overnight. The membrane was washed and incubated in the corresponding secondary antibody (1: 1000, KPL) for 1 hour at room temperature. Proteins were visualized and detected by an enhanced chemiluminescence western blot detection reagent (Pierce, Thermo Fisher Scientific) and analyzed using the Image Quant ™ LAS 4000 imaging system (G.E. Healthcare, Pittsburgh, PA, USA).

Immunohistochemical staining and Immunofluorescence staining

Brain slices were rinsed carefully in PBS followed by 3% H_2O_2 for 30 min to quench the endogenous peroxidase activity then incubated with 0.3 % Triton X-100 in PBS supplemented 5% BSA for 1 h. After that, the sections were incubated with specific primary antibodies (rabbit anti-GPR81 Ab, 1:500) in PBS containing 5% BSA at 4 °C overnight. After extensive washing, brain slices were incubated with secondary antibodies for 1 h at room temperature. Finally the slides were incubated with Diaminobenzidin for 5 min. For Nissl staining, the slides were soaked in CV solution (0.1 g cresyl violet, 99 ml H2O and 1 % acetic acid 1 ml) for 30 min at room temperature then dehydrated with alcohol and xylene. The brain slices were observed under stereomicroscope (Olympus)

Brain slices were rinsed with 0.1 M phosphate-buffered salin (PBS) and fixed with 4% paraformaldehyde, followed by block with PBS containing 5% bovine serum albumin (BSA), then incubated with the primary antibody (anti-GPR81 Ab, 1:500, anti-GFAP Ab, 1:1000) at 4 °C overnight. After washing, were exposed to secondary antibody (Goat anti-Rabbit，Alexa Fluor 488, 1:1000, # A11008, Invitrogen; Alexa Fluor 555 goat anti-mouse IgG, 1:1000, #A21422, Invitrogen) for 1 h at room temperature. After washing and treatment with Hoechst, Brain slices were observed under stereomicroscope (Olympus, Tokyo, Japan).

RNA Reverse-Transcription and Quantitative R.T. -PCR Analysis

Total cellular RNA was extracted by using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA (1 μg) of each sample was reverse transcribed into cDNA, amplified using PrimeScriptTMRT Master Mix (Takara, RR036A, Takara Biotechnology, China) according to the manufacturer's instructions. RT-PCR was performed using QuantiTect Green PCR Kit (Qiagen, Germany) and ABI 7300 StepOneTM Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences used in this study are listed. After adding primers and template DNA to the master template, the thermal cycling parameters of PCR are as follows: 95 ° C for 3 minutes, 40 cycles: 55 ° C for 30 s and 95 ° C for 15 s, and the melting curve from 60 to 95 ° C to ensure the amplification of a single product. In each sample, the GAPDH gene was used as an endogenous control to normalize differences in total RNA amounts.

Flow Cytometry Analysis

Primary astrocytes were split onto six-well culture plates at 1.5×10^5 per ml, and dilute an 3AM-Flou aliquot of DMSO stock solution (4 mM) to a final concentration of 4μM in the buffered physiological medium of choice. After primary astrocytes treated with glutamate and L-lactate, cells usually are incubated with the AM ester for 40 minutes at 37°C. Specific experimental operations are performed according to the

instructions of 3AM-Flou. Before fluorescence measurements are commenced, cells should be washed in an indicator-free medium such as PBS. Flow cytometric analyses with Guava easy Cyte System 8 (Millipore25801, Hayward, CA, United States).

Primary astrocytes were treated with L-lactate for 1h after damaging by glutamate. Apoptosis of cells was assessed by staining cells with Annexin V/PI (Invitrogen, CatV13242) at 37◦C for 30min according to the manufacturer's instructions. The cells were then for flow cytometric analyses with Guava easy Cyte System 8 (Millipore25801, Hayward, CA, United States).

Statistical Analysis

All data are presented as the means ± SEM and were collected and analyzed in a blinded manner. Statistical analysis was performed using Student's t-test. Two-way ANOV A was used when the genotype and treatment were considered as two independent variables. The tests used are indicated in the figure legends. In all studies, n indicates the number of samples per group, and cases in which P-values < 0.05 were considered statistically significant.

RESULTS

Figure 1. HCAR1 widely exists in the brain and forms a huge HCAR1 system network

Studies have shown that HCAR1 mRNA was localized to the hippocampus, neocortex, and cerebellum. Therefore, the expression of HCAR1 in different brain regions was first verified by immunohistochemistry. HCAR1 is widely expressed in the cortex, corpus callosum, hippocampus, cerebellum, and choroid (Figure 1, A), which proves that there is a huge network of HCAR1 in the brain. This system network must have a certain signal transmission effect on the role of lactate in the brain. Astrocytes are a type of glial cells with the largest number of cells in the brain, so the research of this signaling effect is focused on astrocytes. As shown in figure1, HCAR1 was detected by immunofluorescence detection of the hippocampal brain region. Co-standardization of HCAR1 with astrocyte marker GFAP proves the presence of HCAR1 in astrocytes (Figure 1, B), which provides the experimental basis for studying the effect of lactic acid on HCAR1 in astrocytes.

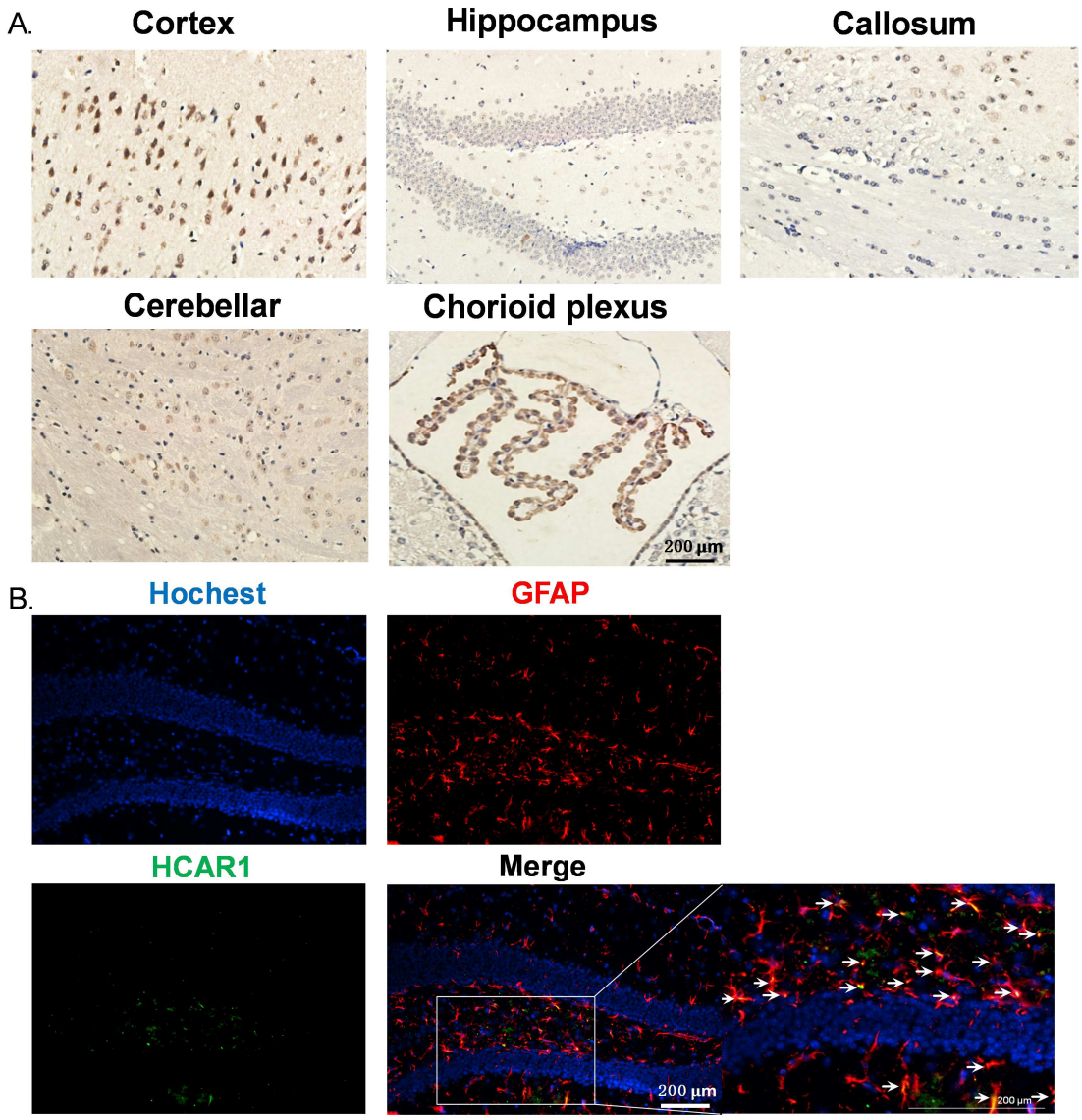

Figure 1. The expression of HCAR1 was detected in different brain regions, HCAR1 are widely present in the brain and form a huge network of HCAR1 systems. (A) Immunohistochemistry of mouse brain tissue showed widespread expression of HCAR1 in the cortex, corpus callosum, hippocampus, cerebellum and choroid. Scale bar:200um(B)The hippocampal D.G. region was selected for fluorescence co-standardization of GFAP and HCAR1, which showed that HCAR1 was expressed in astrocytes. Scale bar:200um

Figure2 Lactate causes significant upregulation of Arc/arg3.1 in astrocytes among numerous neurotrophic factors

In order to verify the effect of lactate on astrocytes, we first determine the effective concentration of L-lactate. Under physiological conditions, the concentration of lactate in the brain should not be higher than 1.5mM, and it should not be higher than 20mM after strenuous exercise. Therefore, the concentration range from 0 to 20mM was selected for the Cell viability test, and the optimal concentration of 0.5mM was

finally selected (Figure2, A). There is evidence to prove that L-lactate has a protective effect on neurons after treating it for 1h. Therefore, the effect of L-lactate on astrocytes was also observed in this test by the same condition. Studies have shown that stimulation of lactate in astrocytes can cause some mRNA levels of neurotrophic factors and synaptic plasticity related factors increase. Our studies also verified this by quantitative RT-PCR analysis and found that the mRNA level of synaptic plasticity-related Arc/arg3.1 has a significant upward trend (FIgure2, B). It was also Arc/arg3.1 protein level that L-lactate caused to increase When treated with 0.5mM for 1h (Figure 2, C). Interestingly, this increasing trend does not change with the time of treating with 0.5mM L-lactate.

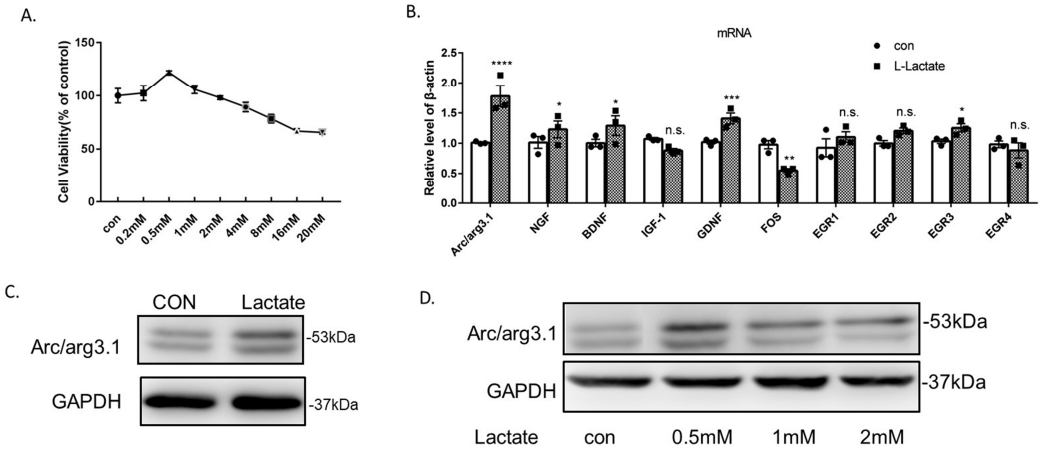

Figure 2. Lactate causes significant up-regulation of the Arc / arg3.1 level among many trophic factors in astrocytes. (A) The CCK-8 test found that treating with 0.5 mM concentration of L-lactate for 1h had the strongest ability to increase the value of primary cultured astrocytes without damaging effects. It was determined that the 0.5 mM concentration was the optimal concentration. (B) After treating with 0.5 mM lactate to primary cultured astrocytes for 1 h, quantitative RT-PCR analysis of RNA-seq indicated increase genes such as ARC and the other. values are means ±SEM *$P<0.05$,**$P<0.01$, *** $P<0.001$, $^{n.s.}P> 0.05$, VS control, n=3. (C) Western blot analysis was performed to access Arc / arg3.1 expression in astrocytes. (D) Western blot analysis verified that the expression level of Arc / arg3.1 in the 0.5-2 mM concentration range did not change with treating with L-lactate for 1h.

Figure 3 Lactate causes upregulation of Arc/arg3.1 expression through HCAR1

To determine the expression of Arc/arg3.1 treating with L-lactate in astrocytes is related to HCAR1.We first obtained the U87 cell line with GPR81 / HCAR1 knockout by Crispr-case9 technology（Figure3，A）. It is worth mentioning that when HCAR1 is knocked out, there is a significant difference in morphology and size compared with U87 cell lines under normal conditions（Figure3，A）. Subsequently, we showed that HCAR1 knocked out significantly reduced Arc/arg3.1 protein expression in U87 cell lines (Figure 3, B) These results indicate that the up-regulation of Arc protein expression caused by L-lactate is related to HCAR1. To further confirm this result, we used siRNA to inhibit HCAR1 expression in primary cultured astrocytes specifically.

We explored the optimal siRNA sequence of HCAR1 with 120 nM concertation (Figure 3, C) We found that the increasing tendency for Arc/arg3.1 protein expression to be significantly canceled when HCAR1 was silenced, even after treating mouse primary cultured astrocytes with 0.5mM L-lactate for 1h (Figure3, D). These results indicate that L-lactate stimulates astrocytes to up-regulate Arc protein expression through HCAR1.

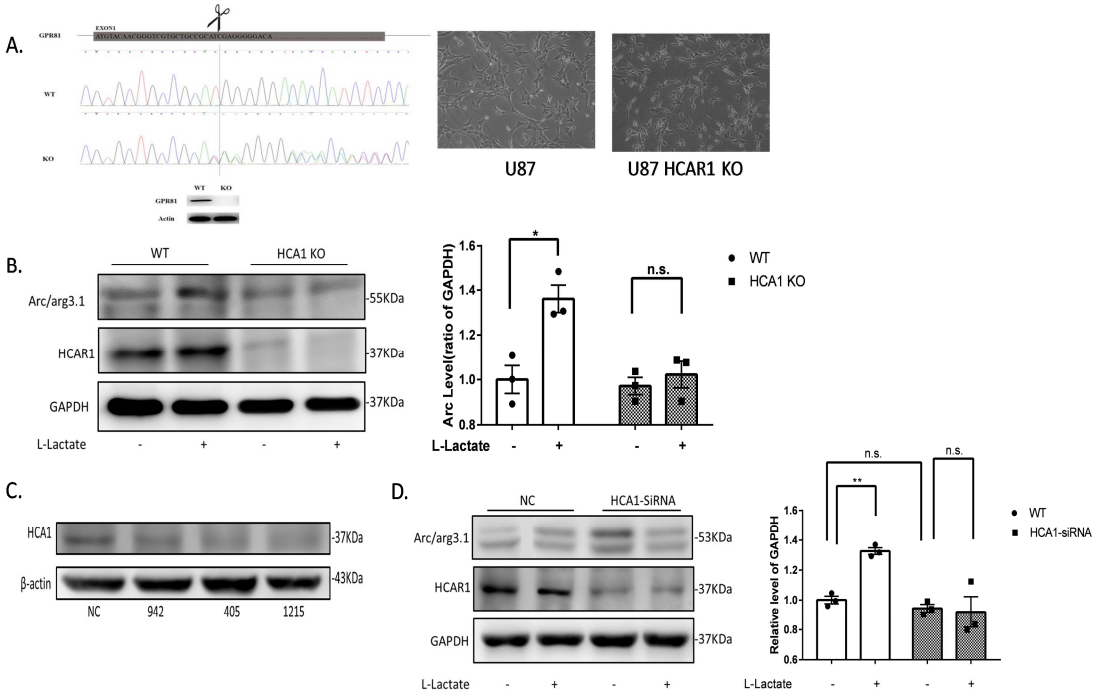

Figure 3. Lactate causes ARC / arg3.1 up-regulation via HCAR1. (A) U87 with GPR81 (HCAR1) knockout stable cell line obtained by Crispr-case9 technology. Cell observation at 20x microscope. (B) HCAR1 knockout stable cell line was treated with 0.5mM lactate for 1 h, Western blot analysis was performed to access Arc / arg3.1 expression, values are means±SEM **P* <0.05, $^{n.s.}$*P*> 0.05 vs.con,n=3 (C) Three groups of HCAR1-sRNA sequences were detected by W.B. experiment, The 1215 group was selected as the best HCAR1 interference sequence (D).primary cultured astrocytes were treated with 0.5 mM lactate for 1 h, and Western blot analysis was performed to access Arc / arg3.1 expression , values are means±SEM ***P* <0.01, $^{n.s.}$*P*> 0.05 vs.con, n = 3.

Figure 4. Lactate enhances Arc / arg3.1 expression mediated by β-arrestin2

HCAR1 is a G Protein-Coupled Receptors (GPCR) of the Gi protein pathway, which causes CAMP down-regulation. Our results in Figure 2 verify that L-lactate can stimulate astrocytes to cause Arc/arg3.1 expression through HCAR1. In fact, some studies have shown that the expression of Arc depends on the upregulation of cAMP[18]. This indicates that the expression of Arc/arg3.1 in astrocytes may not be via the Gi protein pathway, but may be caused by a Noncanonical pathways. Therefore, we treated primary cultured astrocytes with 1uM PTX which is Gi protein inhibitor for 1h, and we found that there was no significant effect on Arc protein expression, when L-lactate was given for 1h after the Gi protein pathway was inhibited, We

attribute another possibility to the signaling function of β-arrestin, which is the noncanonical pathways of β-arrestin by GPCR. We verified this hypothesis by β-arrestin1 knockout and β-arrestin2 knockout mice primary cultured astrocytes. The results showed that compared with wild type primary cultured astrocytes, the up-regulation of Arc/arg3.1 expression in β-arrestin2 knockout mice primary cultured astrocytes was canceled after treated with L-lactate under the same conditions as before（Figure4，A）. However, astrocytes from β-arrestin1 knockout mice and astrocytes from W.T.(wild type) mice had no significant changes after being treated with L-lactate under the same conditions as before (Figure 4, B).After the transfection of the β-arrestin2 plasmid into wild type mice, it was found that the expression of Arc/arg3.1 was significantly up-regulated when β-arrestin2 was overexpressed into primary cultured astrocytes (Figure 4, C). These results indicated that L-lactate mediates Arc/arg3.1 protein expression through β-arrestin2.

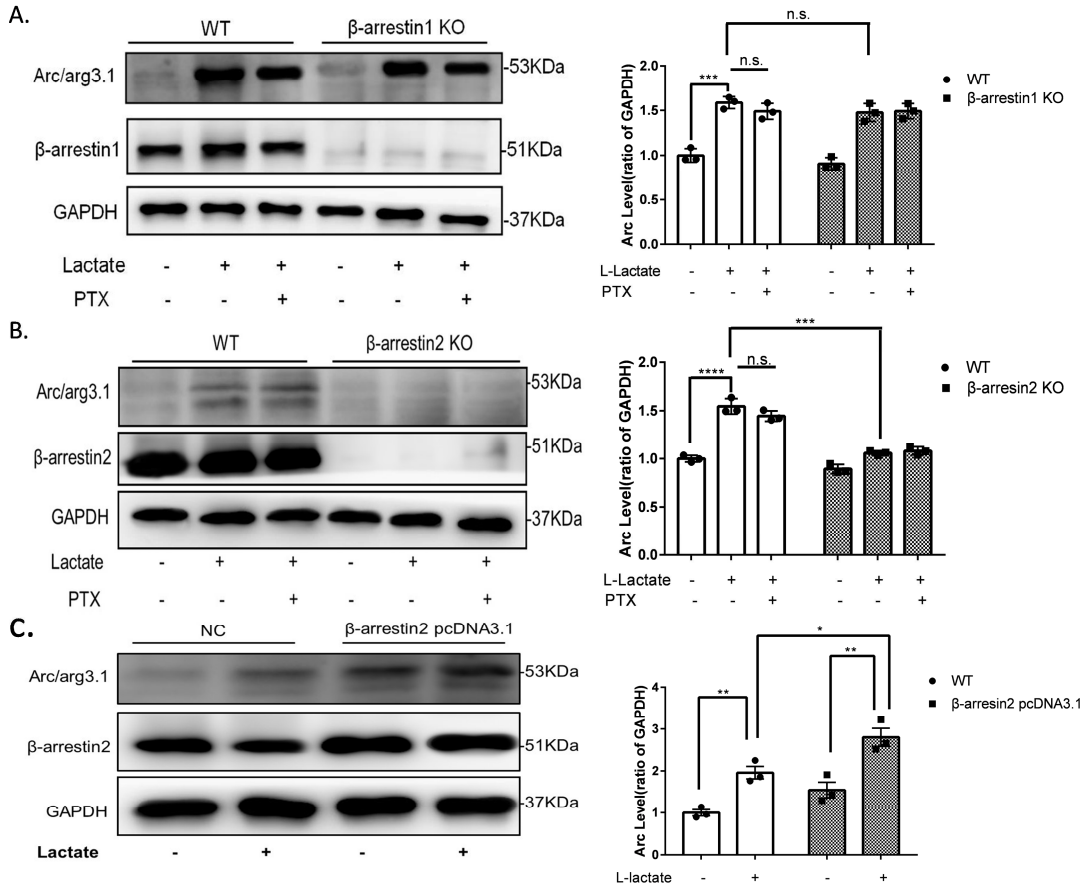

Figure 4. Lactate mediates upregulation of Arc / arg3.1 through β-arrestin2,but not mediated through the Gi protein pathway of HCAR1. (A) (B)primary cultured astrocytes of wild type and β-arrestin1 / β-arrestin2 knockout mice were pretreated with 0.1uM PTX for three hours and then treated with 0.5mM L-lactate for 1h , Western blot analysis was performed to access Arc / arg3.1 expression, values are means± SEM *** P <0.01, $^{n.s.}P$> 0.05, VS. control， n=3. (B) β-arrestin2 overexpression mice primary cultured astrocytes which transfected β-arrestin2 plasmid treated with 0.5 mM L-lactate for 1 h, Western blot analysis was performed to access Arc / arg3.1 expression ,values are means± SEM *P＜0.05,**P＜0.01, VS.

control，n=3 as determined by two-way ANOVA

Figure 5 Lactate enhances MAPK-dependent Arc / arg3.1 expression through the β-arrestin2-MAPK pathway

Numerous studies show that Arc/arg3.1 expression depends on MAPKs activation [18] To verify this result, we chose two MAPK inhibitors PD980590 and SB203580 to inhibit MAPK activation. Our results show that the trend of increase of Arc/arg3.1 expression disappeared when pretreating astrocytes with MAPK inhibition (Figure5, AB)

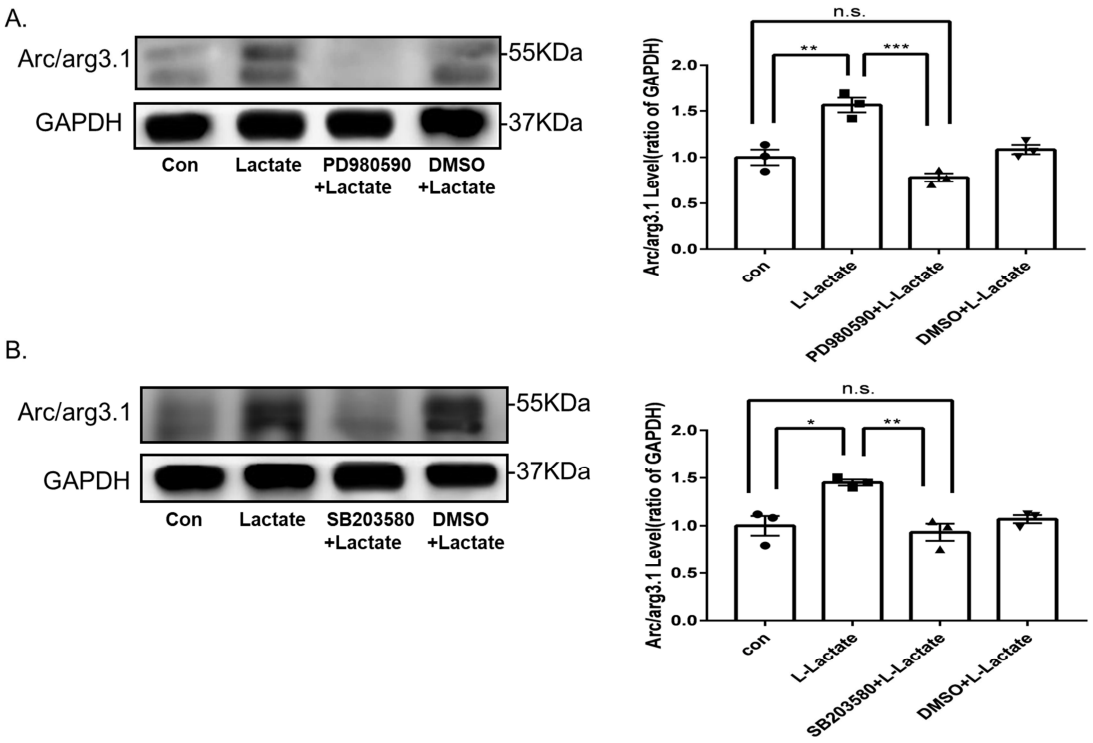

Figure 5 Lactate enhances MAPK-dependent Arc / arg3.1 expression through the β-arrestin2-MAPK pathway（A）Primary astrocytes of wild type were pretreated with 1uM PD 980590 for three hours and then treated with 0.5mM L-lactate for 1h ， Western blot analysis was performed to access Arc / arg3.1 expression.(*P＜0.05,**P＜0.01, ****P＜0.0001 VS. control，n=3)（B）Primary astrocytes of wild type were pretreated with 1uM SB203580 for three hours and then treated with 0.5mM L-lactate for 1h ， Western blot analysis was performed to access Arc / arg3.1 expression.(**P＜0.01, ***P＜0.001，$^{n.s.}P$>0.05 VS. control，n=3)

Figure6. Lactate enhances Arc/arg3.1 expression through HCAR1-β-arrestin2 pathway and reduces calcium influx of astrocytes caused by glutamate damage

The transcription of the immediate early gene Arc is necessary for various forms of learning and memory[19]. The transcription of Arc can lead to the endocytosis of AMPA receptors, which in turn regulates the homeostasis of synaptic plasticity by regulating Long-term potentiation (LTP) and long-term depression (LTD) in neuronal activity[20]. Taking into account this characteristic of Arc/arg3.1, when the transcription and expression of Arc/arg3.1 in astrocytes cause endocytosis of AMPA

receptors, as one of the iontropic glutamate receptors (iGluRs), endocytosis of AMPA receptors inevitably reduces the influx of calcium ions in the face of glutamate damage, there is no doubt that this phenomenon will protect astrocytes from glutamate damage caused by some neurodegenerative diseases. Thereby indirectly enhances the glutamate uptake in primary cultured astrocytes and protects neurons. To verify the effect of lactate on calcium influx in astrocytes with faced of glutamate damage, we used siRNA to interfere with the expression of HCAR1 on primary cultured astrocytes in mice, and then used the Glutamate damage astrocyte model which is pretreated with 100uM L-glutamate for 24h. After treating with 0.5mM L-lactate for 1h, we detected the calcium ion by highly Calcium Probe flou-3AM Fluorescence. L-lactate reduces the influx of calcium ions in the case of glutamate damage, and this phenomenon is canceled when HCAR1 expression is disturbed (Figure 6, A) In the same way, Calcium Probe were performed on primary cultured astrocytes of W.T. mice and β-arrestin2 knockout mice to detect the calcium ion. The results showed that the phenomenon of reduced calcium influx also on primary cultured astrocytes from β-arrestin knockout mice was canceled in the case of glutamate damage (Figure 6, B). These results also indicated that lactate enhances the expression of Arc/arg3.1 through the HCAR1-β-arrestin2 pathway in astrocyte. Our data have shown that lactate can reduce calcium ion influx caused by glutamate damage, so we speculate that lactate can reduce the damage when astrocytes are in the face of glutamate damage. Therefore, mice's wild-type primary astrocytes, HCAR1 disrupted astrocytes and β-arrestin2 knockout primary astrocytes were treated with 0.5mM L-lactic acid for 1h after damaging with glutamate. Flow cytometry results using Annexin V/PI indicated that lactate reduces apoptosis induced by glutamate damage in astrocytes, besides, interference with HCAR1 expression and β-arrestin2 knockout abolished this protection (Figure6 E).

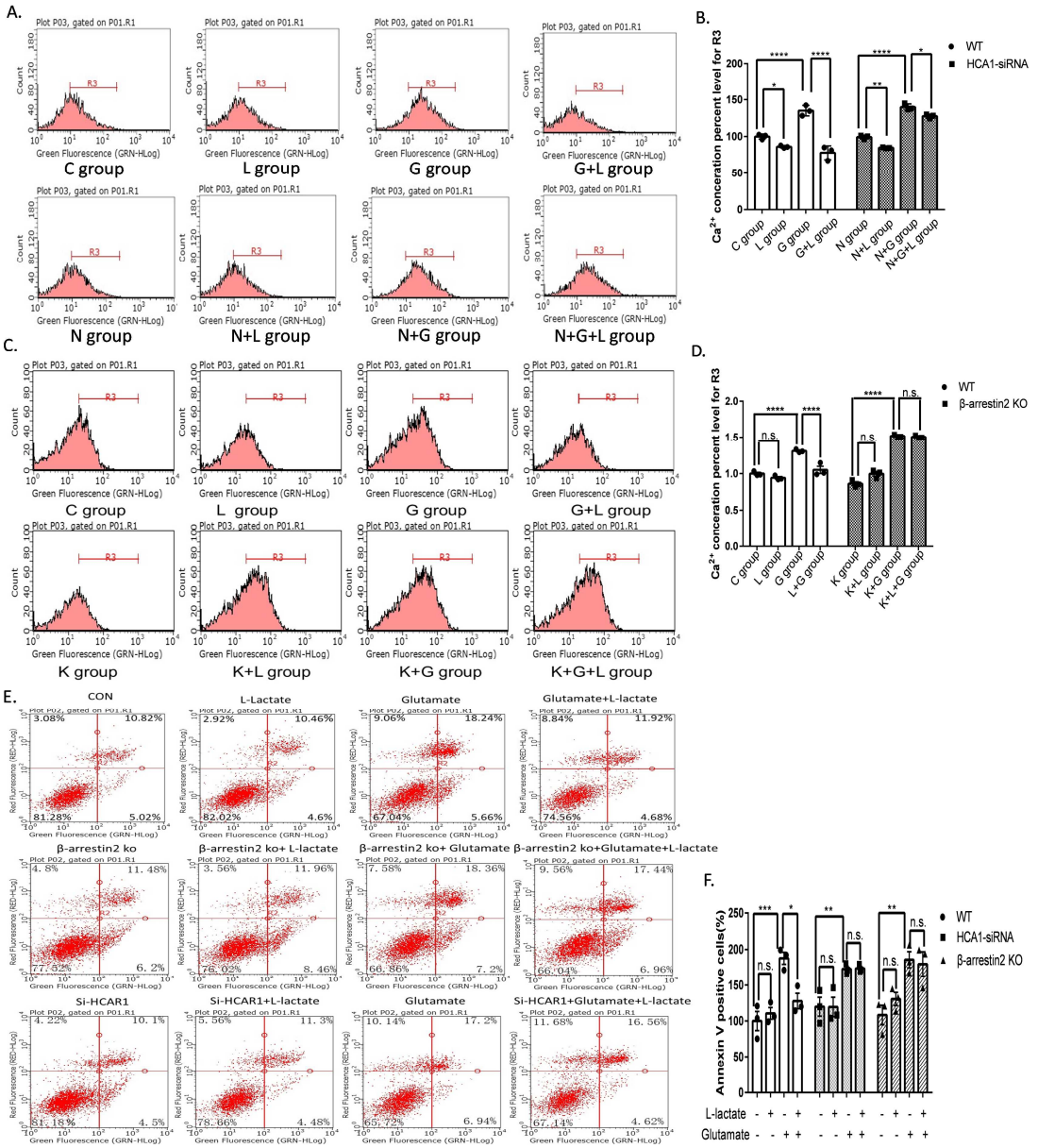

Figure6. A.C group (Control group)，L group (L-Lactate group)， G group (L-Glutamic acid group)， G+L group (Lactate + L-Glutamic acid group)， N group (SiRNA- HCAR1 group)，N+L group (SiRNA- HCAR1 +L-Lactate group)， N+G group (SiRNA HCAR1 +L-Glutamic acid group)，N+G+L group (SiRNA HCAR1 + Lactate+ L-Glutamic acid group); Fluorescence detection of calcium ion probe flou-3Am by cell flow cytometer and analyzed data was showed in B .C. C group (Control group)，L group (L-Lactate group)，G group (L-Glutamic acid group)，G+L group (Lactate + L-Glutamic acid group)， K group (β-arrestin2 ko group)，K+L group (β-arrestin2 ko +L-Lactate group)， K+G group (β-arrestin2 ko +L-Glutamic acid group)，K+G+L group (β-arrestin2 ko + Lactate + L-Glutamic acid group); Fluorescence detection of calcium ion probe flou-3Am by cell flow cytometer and analyzed data was showed in D. E. Mice's W.T. primary astrocytes, HCAR1 disrupted astrocytes which followed by siRNA transfection 48h and β-arrestin2 knockout

primary astrocytes were treated with 0.5mM L-lactate for 1h.Apoptosis was assessed by Annexin V/PI staining and flow cytometry, The data for analysis in F are presented as a percentage of the cell population. Data in (B, D, F) were shown as mean ±SEM. *P<0.5, **P<0.01, ***P<0.001, ****P<0.0001, $^{n.s.}P$>0.05,VS.control， n=3 as determined by two-way ANOVA.

DISCUSSION

Astrocytes are the main site of glycolysis in the brain, and lactate as a product of glycolysis is no longer known only as a waste of glycolysis. With the discovery of the NALS hypothesis and the discovery of HCAR1, the role of lactate as a substrate fuel and signal molecule has also been gradually verified and studied[21] .In the hippocampus, lactate converted by glycogen plays a vital role in providing neurons with substrate fuel and memory formation[22]. In addition to the lactate produced by glycolysis in the brain, lactate taken from the body during exercise or from foods can enter the brain through the blood-brain barrier. There is evidence that regular exercise can improve hippocampal-dependent learning and memory dysfunction induced by type 2 diabetes, and this improvement may be mediated by ANLS[23]. These results undoubtedly indicate that lactic acid has an important role in improving and treating some neurodegenerative diseases. However, a lot of research has focused on the connection between ANLS or lactate and neurons. At the same time, astrocytes, as a type of cells that support and protect neurons, are widely present in the brain. But the protective effect of lactic acid on astrocytes is rarely mentioned. We demonstrate for the first time that lactate also has a protective effect on astrocytes. Starting from astrocytes, we studied the mechanism by which lactate may improve neurodegenerative diseases.

HCAR1 was first reported as a specific receptor for lactic acid in the 1990s. The earliest functional discovery was to prove that lactic acid receptor G protein-coupled receptor 81 (GPR81, also known as HCA1 or HCAR1) can promote adipocytes. Storage of lipids, inhibition of fat breakdown and activation of NLRP3 in inflammatory bodies[24]. It was later found in the brain of mammals, including the cerebral cortex and hippocampus, to be activated by physiological concentrations of lactate and HCAR1 agonist 3,5-dihydroxybenzoate[25] ,which is to reduce cAMP levels and thus help optimize cAMP concentrations[26]. HCAR1 in the brain is concentrated on the post-synaptic membrane of excitatory synapses and is enriched at the blood-brain barrier. By activating HCA1, lactate can act as a volume transmitter, linking neuronal activity, cerebral blood flow, energy metabolism and the availability of energy substrates, including responses that save glucose and glycogen[27]. Our results are shown as Figure1 and previous reports also indicated that lactate might have a signaling effect through receptor activation in a wide range of central nervous systems[28] Therefore, considering the extensive expression and various protective effects of HCA1 in the brain, we associate HCA1 with astrocytes which are also widespread. We indicate that HCAR1 is present in astrocytes, and lactate can enhance the transcription and expression of an immediate early gene Arc / arg3.1 by activating HCA1 in astrocytes, which can reduce the large of calcium ion influx during

glutamate damage. These results indicated HCAR1 might be an important target for improving neurotoxicity caused by glutamate damage. We found and verified for the first time that lactate could increase Arc / arg3.1 expression through HCA1 in astrocytes. As a GPCR, once activated, it will be coupled to the Gi protein and HCA1 will bind to lactate. As GPCRs, HCAR1 couples with heterotrimeric Gi proteins; generate second messengers once activated by agonists. It also reduces cAMP concentration. The transcription and expression of Arc/arg3.1 depend on the activation of cAMP and MAPK. It is considered that β-arrestins (including β-arrestin1 and β-arrestin2) will be recruited after HCA1 binds to its ligand. β-arrestins can also activate downstream signaling or induce desensitization and internalization of receptors[29]. For example, regulating the activation of MAPKs is also called the non-classical pathway of GPCR.

Arc protein can cause the endocytosis of AMPA receptors in cells to regulate the homeostasis of LTP and LTD[30], in contrast, AMPA receptor is one of the three major ionic glutamate receptors. Reduced glutamate sensitivity and weakened calcium internalization, AMPA receptor endocytosis leads to a reduction in the sensitivity of cells to glutamate and a decrease in calcium influx[15], and the increased expression of Arc/arg3.1 in astrocytes undoubtedly also reduces the sensitivity of astrocytes to glutamic acid, protected astrocytes from glutamic acid damage caused by increase of glutamic acid to a certain extent. Of course, We did prove it through our results. We also believe that this can indirectly enhance glutamate uptake by astrocytes, and thus may also protect neurons from glutamate damage.

Therefore, we think our findings have important implications for metabolic brain disease. We know that the causes of metabolic brain disease are various. The most common causes include ischemia, systemic diseases, and toxic substances, which eventually cause neurological diseases or neurodegenerative diseases[31]. So metabolic brain disease can also be defined as a systemic disease with diffuse brain damage[32], which affects the homeostasis of the brain. We consider from the perspective of glutamate in the brain, glutamate in the brain causes excitatory toxicity of neurons to aggravate brain damage when hypoxic-ischemic brain damage occurs. So that increasing intracellular Ca^{2+} concentration leads to neuronal damage, which causes a range of brain diseases[33]. At the same time, glutamate became a toxic substance causing metabolic brain disease. Our findings suggest that the administration of an appropriate amount of lactate can reduce Ca^{2+} influx of astrocytes and reduce glutamate damage of astrocytes. It will indirectly enhance glutamate uptake by astrocytes, reduce glutamate concentration in the brain, indirectly reduce glutamate excitotoxicity of neurons, save brain damage and improve brain metabolic diseases. Therefore, our findings show that lactate can be as a potential therapeutic drug, which has important neuropharmacological effects on metabolic brain diseases.

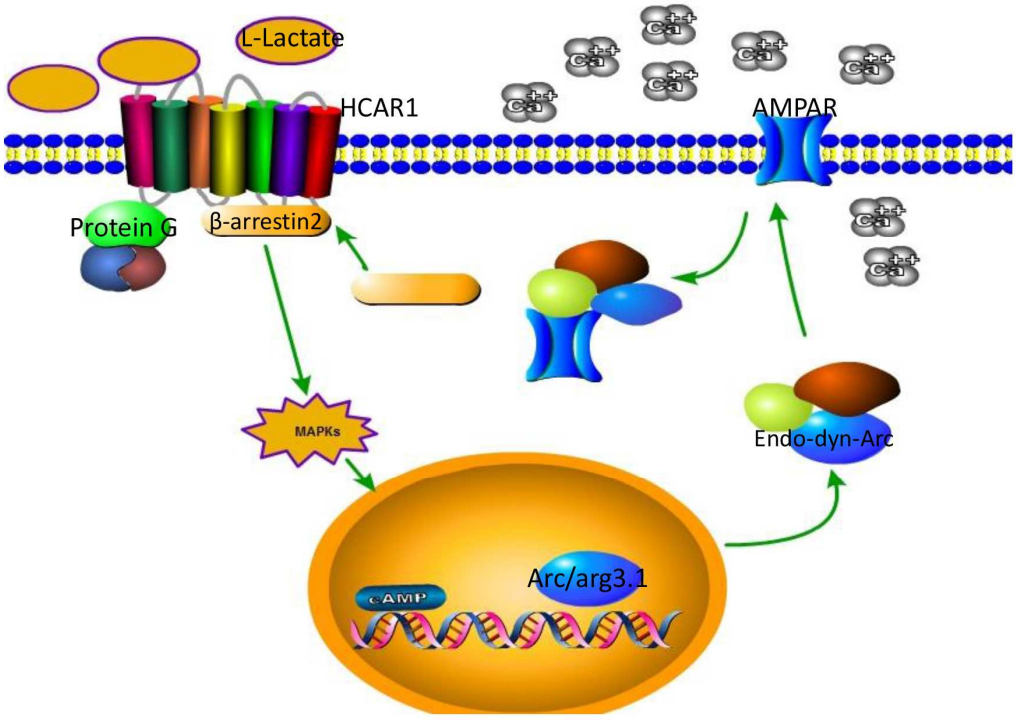

Figure 7 Schematic diagram of the HCA1 enhances Arc / arg3.1 expression and reduces calcium influx through β-arrstin2. A simulation of lactate in astrocytes enhances MAPK-dependent Arc/arg3.1 expression through the HCA1-β-arrestin2 pathway and reduces extracellular calcium ion influx in glutamate damaged Astrocyte Model.

In summary, our study shows that lactate enhances MAPK-dependent Arc / arg3.1 expression through the HCA1-β-arrestin2 pathway in astrocyte, as shown in Figure 7, which probably provide an experimental basis for lactate as a therapeutic or protective drug in neurodegenerative diseases or brain injury caused by glutamate damage. In addition, we also indicate that HCA1 is an essential target for future studies to reduce neurotoxicity toxicity. Therefore, it can be used as an important target for the treatment of neurodegenerative diseases. But this study has some limitations, and there are fewer in vivo experiments of mice. Of course, we still found and proved a new mechanism for the protective effect of lactate in the brain, especially on astrocytes. It also suggested that proper exercise and supplementation of lactate such as fruits can increase the content of lactate in the brain and activate lactate specific receptor HCAR1.HCAR1 helps to improve and prevent a series of neurodegenerative diseases such as neurotoxicity caused by glutamate damage, Alzheimer's disease, depression and so on.

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Conflict of interest

All authors claim that there are no conflicts of interest.

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Article reference: NP_NEUROPHARM-D-20-00087
Article title: Lactate enhances Arc/arg3.1 expression through Hydroxycarboxylic acid receptor 1-β-arrestin2 pathway in astrocytes

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Highlights

•Lactate as a signaling molecule enhances the expression of Arc/arg3.1 in astrocytes

•Lactate can reduce glutamate damage in astrocytes through HCAR1-β-arrestin2 pathway

•Hydrocarboxylic acid receptor 1 is a significant receptor for improvement and treatment of brain damage