Light-Controlled Astrocytes Promote Human Mesenchymal Stem Cells Toward Neuronal Differentiation and Improve the Neurological Deficit in Stroke Rats

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Astrocytes are key components of the central nervous system (CNS) and release factors to support neural stem cell proliferation, differentiation, and migration. Adenosine 5'-triphosphate (ATP) is one of the key factors released upon activation of astrocytes that regulates the neural stem cell’s function. However, it is not clear whether ATP derived from the depolarized astrocytes plays a vital role in promoting the neuronal differentiation of mesenchymal stem cells (MSCs) in vitro and in vivo. Herein, for the first time, we co-cultured MSCs with light-stimulated-channelrhodopsin-2 (ChR2)-astrocytes, and observed that the neuronal differentiation of MSCs was enhanced by expressing more neuronal markers, Tuj1 and NeuN. The ChR2-astrocyte-conditioned medium also stimulated MSCs differentiating into neuronal lineage cells by expressing more Tuj1 and Pax6, which was blocked by the P2X receptor antagonist, TNP-ATP. Then we found that light-depolarization of astrocytes significantly increased ATP accumulation in their bathing medium without impairing the cell membrane. We further found that ATP up-regulated the Tuj1, Pax6, FZD8 and β-catenin mRNA levels of MSCs, which could be reversed by application of TNP-ATP. Together these in vitro data provided convergent evidence that ATP from light-depolarized-astrocytes activated the wnt/β-catenin signaling of MSCs through binding to the P2X receptors, and promoted the neuronal differentiation of MSCs. Finally but importantly, our study also demonstrated in stroke rats that light-controlled astrocytes stimulated endogenous ATP release into the ischemic area to influence the transplanted MSCs, resulting in promoting the MSCs towards neuronal differentiation and improvements of neurological deficit.

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Key words: astrocytes, ATP, channelrhodopsin-2 (ChR2), mesenchymal stem cells (MSCs), microdialysis, stroke

Introduction

I

schemic stroke is a major cause of permanent disability and mortality due to the neuronal injury and death. It has been suggested that mesenchymal stem cells (MSCs) can have beneficial effects on ischemic brain injury because of their neural differentiation potential (Chen et al., 2013; Mezey and Chandross, 2000; Mezey et al., 2000). In the past 10 years, a wide range of strategies (Deng et al., 2001; Locatelli et al., 2003; Sasaki et al., 2009; Woodbury et al., 2002) have been used to regulate the biological features of MSCs in order to enhance their neuronal differentiation and regenerative effects.

The biological features of stem cells are regulated by their local environmental factors and involve contributions from other cell types (Griswold and Oatley, 2012; Morrison and Spradling, 2008; Scadden, 2006). For example, altered activity of parvalbumin-expressing interneurons was shown to control
the activation and self-renewal mode of quiescent adult neural stem cells (Song et al., 2012); pharmacological stimulation of osteoblasts regulated the proliferation of haematopoietic stem cells (Calvi et al., 2003), confirming that alteration of neighbouring cells’ activity can potentially affect stem cell’s behavior.

Astrocytes are one of the dominant cell types in the CNS, and have been ascribed an important role in controlling the behavior of neural stem cells (Ashton et al., 2012; Horner and Palmer, 2003; Ma et al., 2005; Song et al., 2002). Altering the activity of astrocytes by activation or depolarization resulted in the release of gliotransmitters or neurotrophic factors (Chen et al., 2013; Coco et al., 2003; Huang et al., 2010; Su et al., 2012; Tawfik et al., 2010; Zhang et al., 2007b), which might influence the function of neighbouring stem cells within a local environment.

ATP is one of the key gliotransmitters released from astrocytes (Chen et al., 2013; Coco et al., 2003; Gourine et al., 2010; Zhang et al., 2003, 2007b). Recent studies have shown that astrocytic-ATP played important roles in regulating the proliferation (Lin et al., 2007; Mishra et al., 2006) and migration (Liu et al., 2008; Striedinger et al., 2007; Weissman et al., 2004) of neural stem cells. More studies confirmed that ATP was also involved in regulating the multi-lineage differentiation of stem cells: ATP induces oligodendrocyte progenitors (OPs) to differentiate into oligodendrocytes (Agresti et al., 2005a,b; Cerutti et al., 2011; Kastritis and McCarthy, 1993), promotes adipose-derived MSCs to differentiate towards a glial phenotype (Faroni et al., 2013) and contributes to osteogenic differentiation of MSCs (Carroll and Ravid, 2013; Sun et al., 2013). However, it remains to be seen whether astrocytic depolarization would have beneficial effects on promoting MSCs toward neuronal differentiation in vitro and in vivo, and whether astrocyte-derived ATP upon depolarization is involved in regulating MSCs behavior.

In the present study, we employed the optogenetic approach (Boyden et al., 2005; Zhang et al., 2007a) to demonstrate that selective depolarization of astrocytes via channelrhodopsin-2 (ChR2) have beneficial effects on the neuronal differentiation of neighbouring MSCs in vitro, and found astrocytic-ATP as a molecular signal to regulate the behavior of MSCs. Most importantly, our results also showed that the light-controlled astrocytes promoted the neuronal differentiation of neighbouring MSCs in vivo and improve the repair efficacy in a rat ischemic stroke model.

**Materials and Methods**

**Ethics Statement**

All experiments were approved by the Research Committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, and all experimental procedures involving animals were carried out in strict accordance with the animal use guidelines of the above committee. Experiments were performed under full anesthesia, and every effort was made to minimize animal suffering.

**Primary Culture of Rat Striatal Astrocytes**

The primary culture of rat striatal astrocytes was prepared as described previously (Zhang et al., 2009) with some modification. Small pieces of the striatum of 2-day-old Sprague-Dawley (SD) rats were enzymatically dissected using trypsin 0.25% (Invitrogen, CA), mechanically dissociated, and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, CA) and 1% penicillin/streptomycin (Invitrogen, CA). The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The next day, the cells were washed with phosphate-buffered saline (PBS) twice to exclude floating cells and the culture medium was replaced. After the cells reached confluence, purified astrocytes were obtained by shaking of the 25 cm² flasks for ≥2 h to remove less adhesive cells.

**Human MSCs culture and characterization by flow cytometry**

Human MSCs were kindly donated by the Shenzhen Beike Biotechnology Company. The isolation and culture of MSCs were performed with patient consent as described previously (Wu et al., 2007). Cells cultured between 2 and 5 passages were used in the present study. No significant differences in either renewal or differentiation ability were observed between early and late passages.

To characterize the human MSCs, fluorescence-activated cell sorting (FACS) analysis was performed using antibodies against human CD34 (fluorescein isothiocyanate, FITC-conjugated), CD44 (FITC-conjugated), CD45 (phycoerythrin-conjugated), CD105 (Alexa Fluor® 488), HLA-DR (FITC-conjugated; all from Invitrogen), and CD73 (PE-conjugated; BD Bioscience). Negative control experiments were performed using Alexa Fluor® 488 or 568-conjugated goat anti-mouse antibodies (both from Invitrogen).

**Viral-Mediated Gene Expression in Rat Astrocytes**

Lentiviral vectors (LV) containing humanized glial fibrillary acidic protein (GFAP) promoter and ChR2-eYFP or eYFP gene alone (Fig. 2A) were transfected to the cultured astrocytes with a transfection efficiency of about 92%. LVs carrying the genes were constructed using standard cloning techniques as previously described (Gradinaru et al., 2005).
et al., 2010). High titre LV (>3 x 10^8 TU/mL) was then produced via calcium phosphate co-transfection of 293FT cells with the LVs pCMV-CHR2 and pMD2.G. Twenty-four hours after transfection, the 293FT cells were switched to serum-free medium containing 5 mM sodium butyrate; the supernatant was collected 16 h later and concentrated by ultracentrifugation at 50,000g using a 20% sucrose cushion. The resulting viral pellet was resuspended in PBS at 1/1,000 of the original volume.

**Random access optical stimulation of ChR2-expressing astrocytes and electrophysiological recording**

Electrophysiological recordings in cultured ChR2-expressing astrocytes were performed in standard extracellular solution containing (in mM): NaCl 150, KCl 5, CaCl2 2, MgSO4 2, glucose 10, and HEPES 10 (pH was adjusted to 7.3 ± 0.01 with NaOH, osmolality to 300–310 mOsm/kg with sucrose). The recording pipette was filled with an artificial intracellular saline containing (in mM) KCl 130, MgCl2 2, CaCl2 0.5, Na2ATP 3, EGTA 5, and HEPES 10 (pH was adjusted to 7.3 ± 0.01 with KOH, osmolality to 290 mOsm/kg). Optical stimulation of a single ChR2-positive astrocyte was induced using a customised optical stimulation system (Quick-View-Stim; CBMP, Wuhan, China) as previously described (Liu et al., 2010). Briefly, the laser beam is steered with a two-dimensional acousto-optic deflector, and the intensity and dwell time are finely tuned at each site.

**IMMUNOSTAINING.** Cells were fixed with 4% paraformaldehyde (PFA) and incubated with primary antibodies for 2 h at room temperature. The brain were fixed in 4% PFA at 4°C overnight and cryosectioned at 20 µm per slice. The slices were blocked by goat serum and then incubated with primary antibodies. Alexa Fluor® 488 or 568-conjugated goat anti-rabbit or anti-mouse IgG antibodies (1:500; Invitrogen, CA) were the secondary antibodies. Nuclei were counterstained using Hoechst or DAPI. Immunostaining was performed using the following antibodies: rabbit anti-glial fibrillary acidic protein (GFAP, 1:400; Abcam, MA), mouse anti-Tuj1 (neuron-specific class III β-tubulin, 1:400; Abcam, MA), mouse anti-NeuN (1:100, Millipore, MA), mouse-anti-human-nuclei (human cell-specific antinuclear antibody, ANA, 1: 400; Millipore, MA), and mouse anti-Iba1 (1: 50, Abcam, MA).

**MSCS IN CO-CULTURE WITH GENETICALLY-DEFINED ASTROCYTES AND SELECTIVE OPTICAL STIMULATION OF CHR2-ASTROCYTES.** Co-cultures were established with MSCs grown on top of a monolayer of astrocytes expressing ChR2-eYFP or eYFP alone (Fig. 3B). The cells were grown on poly-lysine-coated cover slips (Sigma) and divided into three groups: control group with MSCs alone; co-culture of MSCs and astrocytes-expressing eYFP alone (As-eYFP + MSC); and co-culture of MSC and astrocytes expressing ChR2-eYFP (As-Chr2 + MSC). The co-cultured astrocytes were optically-stimulated by illuminating the As-eYFP + MSC group and As-Chr2 + MSC group with blue light at 460 to 475 nm using a homemade light-emitting diode (LED) device whose average output power was 1.1 mW/mm² (Fig. 3B).

**IN VITRO NEURONAL INDUCTION OF MSCS.** In vitro neuronal differentiation of MSCs was performed with or without co-cultured astrocytes in the typical neuronal differentiation medium according to the protocol shown in Fig. 3A. The co-cultures were kept in the expansion medium (DMEM with 10% heat-inactivated FBS and 1% penicillin/streptomycin) at 37°C in a humidified 5% CO₂ atmosphere for 3 days. After that, the medium was changed to neurobasal medium (Gibco/Invitrogen) containing 1% B27 (Sigma) supplemented with 50 ng/mL BDNF, 25 ng/mL bFGF, and 500 ng/mL sonic hedgehog (PeproTech Inc.). For optical stimulation groups, the ChR2- or eYFP-astrocytes in the co-cultures received light stimulation for 30 min every three days at ~470 nm. The control group with MSCs alone in the neuronal differentiation medium served as normal neuronal induction group. Immunostaining was performed to examine the neuronal markers expression at day 21. Another co-culture system was set up by collection of the conditioned medium from the As-Chr2 or As-eYFP to incubate MSCs (Fig. 3G). In brief, the astrocyte-conditioned medium was harvested and filtered through a 0.2 µm syringe filter. MSCs were incubated for 21 days in half conditioned medium/half neuronal induction medium, and then the cells were lysed for total RNA extraction to examine the mRNA expression of neuronal markers. The MSCs in complete neuronal induction medium served as the control. MSCs incubated for 24 h in each conditioned medium or DMEM were used to examine cell proliferation by the EdU incorporation assay.

**5-ETHYNYL-2-DEOXYURIDINE (EDU) INCORPORATION FOR THE CELL PROLIFERATION ASSAY.** EdU incorporation for the cell proliferation assay was performed as previously described (Daul et al., 2010; Warren et al., 2009). Briefly, after a 24-h incubation with 10 µM EdU, the assay was conducted using a Click-iTTM Alexa Fluor® 488 EdU Imaging Kit (Invitrogen/Molecular Probes, CA) according to the manufacturer’s protocol. The number of EdU-labelled cells was normalised to the Hoechst-stained cells in all the groups in order to obtain a ratio of EdU-positive cells.

**BIOLUMINESCENCE ASSAY FOR EXTRACELLULAR ATP.** Extracellular ATP content of the samples was determined with the luciferin-luciferase technique using an ATP Bioluminescent Assay Kit (Sigma) in a Synergy™ 4 Multi-Detection Microplate Reader (BioTek Instruments Inc.). The undiluted samples (100 µL) of the bathing medium from the cultured astrocytes were pipetted into a 96-well microplate. Samples (25 µL) of the dialysate from the buffer-perfused brain tissue were mixed with 75 µL ultrapure water. Diluted samples (100 µL) were pipetted into the microplate for ATP analysis. ATP Assay Mix solution (100 µL) was added to each well and the luminescent signal was measured kinetically within 1 min.

**LACTATE DEHYDROGENASE (LDH) ACTIVITY ASSAY.** Cellular injury was assessed by LDH release measurement. The culture medium of astrocytes was collected and centrifuged at 1,000g for 5 min. The activity of LDH that had leaked out from the astrocytes was measured in the supernatants spectrophotometrically using a commercial kit (Cayman Chemical, MI). This colorimetric assay
measures the pyruvate-mediated conversion of 2, 4-dinitrophenylhydrazine into a visible hydrazone precipitate.

**WESTERN BLOT.** Cells were lysed and the total protein was extracted using a commercial lysis buffer (NE-PER® Nuclear and Cytoplasmic Extraction Reagents, Pierce/Thermo Fisher Scientific) containing the protease inhibitor and phosphatase inhibitor cocktail solutions (Roche, Switzerland). Equal amounts of protein samples were loaded onto 10% acrylamide gels, followed by transfer and blotting. Anti-human cyclin D1 (1:500, Cell Signaling Technology), and anti-human β-catenin (1:400, Cell Signaling Technology) were used for immunoblotting. Colour development was achieved using an ECL™ Western Blotting Detection Kit (Pierce/Thermo Fisher Scientific). The band density was assessed and normalised to that of β-actin or GAPDH using Image J software (version 1.43; National Institutes of Health).

**SEMIQUANTITATIVE RT-RCR AND REAL-TIME PCR.** Total RNA was extracted from MSCs using an RNeasy Total RNA Extraction Kit (Qiagen, MD). Complementary DNA (cDNA) was synthesised from 1 μg of total RNA using an oligo (dT)18 primer and M-MuLV Reverse Transcriptase (Fermentas, Canada). cDNA was then amplified using TaqHS DNA polymerase (Takara Bio Inc., Japan). The specific primers and PCR conditions are listed in Table 1. After 38 cycles of PCR amplification, the reaction products were separated on a 2% agarose gel and stained with ethidium bromide. For real-time PCR, analysis was performed in triplicate using LightCycler®480 (Roche, Switzerland) with Thunderbird SYBR qPCR mix (Toyobo) and primers listed in Table 1. Gene expression analysis was performed using relative cycle threshold method, normalized to β-actin expression, and the fold-changes were calculated relative to the control group.

**RAT FOCAL BRAIN ISCHEMIA MODEL.** Transient focal brain ischemia was induced by intraluminal occlusion of the middle cerebral artery (MCA) as previously described (Longa et al., 1989) with minor modifications. Male SD rats (260–300 g) were anaesthetised with sodium pentobarbital (60–70 mg/kg i.p.) and placed in a stereotaxic frame. A microdialysis probe (MER-10 mm guide, 2 mm membrane, Bioanalytical Systems Inc.) was implanted into the cell transplanted area of the right striatum; 1.8 mm away, another hole was drilled in the skull above the ipsilateral striatum for optical stimulation through a fiber-optic cable connected to a laser. Microdialysis samples were collected from the ischemic area before, during and after light stimulation.

The probes were perfused with artificial cerebrospinal fluid (ACSF) (in mmol/L: NaCl 124, KCl 3, CaCl2 2.4, MgSO4 1.3, glucose 10, and HEPES 10, pH = 7.3) for 90 min before beginning the experiment. Samples were collected every 30 min.

**NEUROLOGICAL DEFICITS SCORE.** The neurological status of rats was assessed before, and 1 and 10 days after MCAO. A scale of 0 to 5 was used to assess the motor and behavioral changes after the MCAO (Table 2) as previously described (Rickels, 1993) with minor modifications. In brief, the total score for the assessment was 5, and increasing score indicates greater severity of injury. In preliminary experiments, rats with a 2-4 score showed a stable stroke volume, so these were further used for cell transplantation.

**TRIPHENYL TETRAZOLIUM CHLORIDE (TTC) STAINING AND MEASUREMENT OF INFARCT VOLUME.** At 14 days after MCAO, rats were decapitated under deep sodium pentobarbital anaesthesia after neurological status assessment. The brain was removed and sectioned into 2 mm thick slices from the frontal lobe to the cerebellum. These sections were stained with 2% TTC (Sigma) in normal saline at 37°C for 30 min. The sections were then rinsed in saline for digital scanning. Five adjoining slices of infarction from each animal were scanned and measured with Image J software. The infarct volume was calculated by multiplying the area of infarction with the section thickness and the total number of sections for each animal.

**Statistics**

Data from all of the experiments are expressed as mean ± SEM. In the in vitro experiments, n represents the number of tests and in the in vivo experiments, n represents the number of animals used. One-way ANOVA followed by the Bonferroni post hoc test was used for multiple comparisons. In all cases, significance was established at $P < 0.05$.

**Results**

**Characterization of Rat Astrocytes and Human MSCs in Culture**

In the cultured rat striatal astrocytes, more than 95% of cells were GFAP-positive (Fig. 1A; a), and almost no TuJ1-positive cells were observed (Fig. 1A; d). Cultured human MSCs
<table>
<thead>
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<th>Primers</th>
<th>GenBank accession</th>
<th>Sequence</th>
<th>Predicted length (bp)</th>
<th>Anneal Tm (°C)</th>
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<td>β-actin</td>
<td>NM_001101</td>
<td>240</td>
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<td>5’-CACGATGGAGGGGCCGGACTCATC-3’</td>
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<td>Reverse</td>
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<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CACAGAGACACTGTGAGATGAG-3’</td>
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<td>NM_012226</td>
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<td>Reverse</td>
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<tr>
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</tr>
<tr>
<td>Reverse</td>
<td>5’-CCAGGTCTCTTGCTCTCTC-3’</td>
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<td>5’-GGCACGGTGATCTCTTGGC-3’</td>
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TABLE 2: Neurological Evaluation of Rats After MCAO

<table>
<thead>
<tr>
<th>Score</th>
<th>Evaluation</th>
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<tbody>
<tr>
<td>0</td>
<td>No apparent deficits and the animals extended</td>
</tr>
<tr>
<td></td>
<td>both forelimbs during suspension from the tail</td>
</tr>
<tr>
<td>1</td>
<td>Contralateral forelimb flexion during suspension and no other abnormality</td>
</tr>
<tr>
<td>2</td>
<td>Decreased grip of the contralateral forelimb while tail pulled</td>
</tr>
<tr>
<td>3</td>
<td>Spontaneous movement in all directions when placed on pads; contralateral circling only if pulled by tail</td>
</tr>
<tr>
<td>4</td>
<td>Spontaneous contralateral circling without pulling its tail</td>
</tr>
<tr>
<td>5</td>
<td>Died within 24 h</td>
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</tbody>
</table>

varied in size: flow cytometry analysis showed that most of the cultured MSCs expressed standard MSC surface markers CD44, CD73, and CD105 (Fig. 1B, upper panel), but not the haematopoietic markers CD34, CD45, or HLA-DR (Fig. 1B, lower panel).

**ELECTROPHYSIOLOGICAL PROPERTIES OF CHR2-ASTROCYTES IN CULTURE.** For selective optogenetic stimulation of astrocytes, the cultured astrocytes were infected with a lentiviral Chr2-eYFP construct under GFAP promoter control (Fig. 2A,B). Using whole-cell patch clamp electrophysiology, the astrocytes were held at −80 mV and stepped from −200 mV to +60 mV (10 mV increments; see inset; Fig. 2C). The As-Chr2 displayed typical electrophysiological properties of astrocytes with a mean resting membrane potential of −71 ± 6.7 mV (n = 17; Fig. 2D); mean resting membrane potential of the light stimulated, YFP-infected astrocytes: −75.3 ± 0.9 mV, n = 24, Supp. Info. Fig. 1A), indicating that expression of Chr2-eYFP did not affect their basic physiology. Illumination of Chr2-positive astrocytes with 1 s of blue light triggered rapid depolarising currents that reached a maximal rise rate of 28 ± 19 pA/ms within 5.3 ± 1.8 ms after the light pulse onset (n = 15; Fig. 2E). Mean whole-cell inward currents peaked at 139 ± 32 pA and reached a steady state at 70 ± 18 pA (n = 15, Fig. 2F). After illumination of As-Chr2 with blue light (10 Hz, 50 ms) for 60 mins, 10 Hz light pulse trains could still successfully induce inward currents that displayed inactivation of the peak current and stability of the steady-state current (n = 15, Fig. 2G).

**OPTICAL STIMULATION OF CHR2-ASTROCYTES PROMOTES NEURONAL DIFFERENTIATION OF MSCS.** To examine the efficacy of selectively-activated-astrocytes on the neuronal differentiation of MSCs, we set up a co-culture system where rat astrocytes expressing Chr2 or eYFP alone were used as a feeding layer for MSCs (Fig. 3A,B). After 21 days of neuronal induction, MSCs co-cultured with As-Chr2 exhibited more neuronal marker expression (Fig. 3C–F). The ratio of Tuj1-positive cells was much higher in the MSCs co-cultured with Chr2-astrocytes (Chr2) compared with the MSCs in co-cultured with eYFP-astrocytes (YFP) or in neuronal induction medium alone (CTRL) (n = 7–9, Chr2: 37.5 ± 4.5%; YFP: 11.8 ± 2.4%; Ctrl: 6.3 ± 1.8%, ***P < 0.001); meanwhile, the ratio of NeuN-positive cells was also significantly increased in Chr2 group (n = 8, Chr2: 29.3 ± 4.5%; YFP: 14.8 ± 2.7%; Ctrl: 7.9 ± 1.3%, ***P < 0.001, *P < 0.05), whereas MSCs co-cultured with as-eYFP did not significantly enhance differentiation of neighbouring MSCs into Tuj1 or NeuN-positive cells (P > 0.05). In order to further confirm this neuronal-differentiation-enhancing effect on the MSCs, we next incubated MSCs with half neuronal induction medium and half conditioned medium of As-Chr2 for 21 days (Fig. 3G) and found that the mRNA expression levels of both Pax6 and Tuj1 in MSCs (Chr2 group) were significantly increased compared with the MSCs incubated in the full neuronal induction medium (control group) (**P < 0.01, Fig. 3H,I). On the other hand, incubation in half eYFP-astrocyte-conditioned medium and half neuronal induction medium (eYFP group) for 21 days did not significantly enhance Tuj1 or Pax6 mRNA expression level (P > 0.05 vs. control group; **P < 0.01 vs. Chr2 group, Fig. 3H,I), indicating that As-eYFP conditioned medium and/or neuronal induction medium had no marked effects on promoting the neuronal differentiation of MSCs.

**ATP RELEASED FROM CHR2-ASTROCYTES AFTER LIGHT STIMULATION.** The ATP concentrations in the bathing medium from non-activated-astrocytes (N-ACM), As-YFP or As-Chr2 were then determined. After As-Chr2 were stimulated with 60 min of light, bathing medium ATP was significantly increased to 298.1 ± 61.1% of N-ACM (Fig. 4A, **P < 0.01), which was also significantly higher than As-YFP (Fig. 4A, **P < 0.01). However, bathing medium ATP from As-YFP was unchanged (108.5 ± 27.7% of N-ACM, Fig. 4A, P > 0.05). Release of the cytoplasmic enzyme LDH (a usual indicator of cell lysis) from N-ACM, As-Chr2 or As-eYFP was examined to detect possible cell damage caused by light or virus transfection. No significant difference in LDH activity was detected among these three bathing media (P > 0.05, Fig. 4B). We further examined the time course of extracellular ATP accumulation. The bathing medium ATP of As-Chr2 was significantly increased to 265.4 ± 34.4% of N-ACM within the first 30 mins of light stimulation (Fig. 4C, **P < 0.01), whereas light stimulating As-eYFP for 30 mins failed to elevate extracellular ATP (161 ± 24.9% vs. 100 ±
16.5% of N-ACM, Fig. 4C, \( P > 0.05 \)); the bathing medium ATP of As-ChR2 reached its highest level within 60 mins of light stimulation and remained elevated for 2 h (Fig. 4D, **\( P < 0.001 \)), but 120 min of light stimulation did not further increase it (Fig. 4E, *\( P < 0.05 \)).

**ATP ENHANCED THE NEURONAL DIFFERENTIATION AND ACTIVATED WNT/\( \beta \)-CATENIN SIGNALING OF MSCS.** To directly test the functional effects of ATP on MSC behavior, we first examined the expression profiles of P2X receptor mRNA by the MSCs: MSCs expressed P2X1,

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**FIGURE 1:** Characterisation of cultured rat striatal astrocytes and human mesenchymal stem cells (MSCs). A: Images show staining for glial fibrillary acidic protein (GFAP) and Tuj1 in primary culture: (a) GFAP-positive cells represent astrocytes. (b and e) Nuclei were counterstained with Hoechst; (c) a merged image of a and b shows purity of >95% astrocytes; (f) a merged image of d (Tuj1 staining) and e shows no neuronal cells. The scale bar corresponds to 20 \( \mu \)M. B: Flow cytometry analysis of surface markers on MSCs. The normal MSCs grown in DMEM medium were stained with a fluorescent-conjugated CD34, CD44, CD45, CD73, CD105, and HLA-DR and then analysed. Fluorescence intensity >95% of control was considered positive. The cells had CD44 (+), CD73 (+), and CD105 (+) markers but did not have CD34, CD45, or HLA-DR markers.
and P2X4-7 subtypes (Fig. 5A). MSCs were then incubated for 21 days with full neuronal induction medium (control group) or half neuronal induction medium/half conditioned medium as indicated (Fig. 5B–C): the TuJ1-positive cells ratio was significantly increased when MSCs were incubated with half neuronal induction medium and half ChR2-astrocyte-conditioned medium (ChR2) compared with the MSCs incubated in the full neuronal induction medium (control group) or in half eYFP-astrocyte-conditioned medium/half neuronal induction medium (YFP group); interestingly, this effect of ChR2-astrocyte-conditioned medium on the neuronal differentiation of MSCs was blocked with 30 μM TNP-ATP, the selective ATP P2X receptor antagonist (Burgard et al., 2000) (n = 7–10, CTRL: 11.7 ± 4.8%, YFP: 27.5 ± 5.4%, ChR2: 43.5 ± 9.8%, and ChR2+TNP-ATP: 11.5 ± 1.8%, respectively). Meanwhile, MSCs incubated with 10 μM ATP significantly increased the mRNA expression levels by real-time PCR of both Pax6 and TuJ1, and these effects of ATP on the neuronal lineage commitment of MSCs was also blocked in the presence of TNP-ATP (**P < 0.01, Fig. 5D), indicating that the ATP/P2X system was involved in enhancing the neuronal differentiation of MSCs. Since wnt/β-catenin signaling has been widely suggested to be involved in the regulation of neural stem cell neurogenesis (Hashemi et al., 2011; Lie et al., 2005; Mazumdar et al., 2010; Ota et al., 2012; Qu et al., 2010), we applied 1 to 100 μM ATP to the MSC neuronal induction medium and examined the protein expression level of β-catenin: ATP significantly upregulated the β-catenin protein after 7 days of neuronal induction (Fig. 5E, *P < 0.05), but there was no significant dose-
dependency for β-catenin expression on these ATP concentrations (Fig. 5E). Real-time PCR results demonstrated that 10 μM ATP dramatically increased the wnt target gene expression levels of FZD 8 and β-catenin, compared with control cells (**P < 0.01, Fig. 5F). To further confirm the role of ATP in activating the wnt/β-catenin signaling, we selectively inhibited P2X receptors using 30 μM TNP-ATP: the ATP-induced increase in FZD8 or β-catenin was reversed (1,267 ± 72.5% vs. 84.5 ± 9.1%, **P < 0.01, n = 3, Fig. 5E), suggesting that ATP-activated wnt/β-catenin signaling was through P2X receptors. After exposure of MSCs to the neuronal induction medium plus 10 μM ATP for 3 days, the translocation of β-catenin from the cytosolic part to the nuclei was markedly enhanced (Fig. 5G), indicating an activation of β-catenin by ATP administration to the neuronal induction medium.

We also found that the percentage of EdU-positive cells was significantly increased after 24 h of incubation of MSCs with the As-ChR2-conditioned medium compared with that of MSCs in DMEM alone or in As-eYFP-conditioned medium (ChR2: 43.7 ± 7.6%; eYFP: 30.8 ± 2.5%; control: 31.8 ± 1.7%, **P < 0.01, Supp. Info. Fig. 2A,B). We further confirmed that applying 1 to 100 μM ATP to the MSC expansion medium (DMEM) significantly increased the protein expression of cyclin D1 in a dose-dependent manner, but 100 μM ATP did not further increase the expression of cyclin D1 by the stimulation of 10 μM ATP (*P < 0.05, **P < 0.01, Supp. Info. Fig. 2C).
CO-TRANSPLANTATION OF CHR2-ASTROCYTES AND MSCS IMPROVED NEUROLOGICAL FUNCTIONS AFTER MCAO IN RATS. The cell transplantation protocol and light stimulation protocol are shown in Fig. 6A. Intracerebral transplantation of the cells was performed 1 day after the neurological deficit test (48 h after the MACO) and then 24 h later, the animals received light stimulation at 470 nm with 10 Hz (Fig. 6A). Anti-human-nuclei staining (ANA, red) confirmed that human-derived MSCs resided in the ischemia hemisphere of the rat brain surrounded with the astrocytes expressing ChR2-eYFP (Fig. 6B). The infarct volume at 14 days after MCAO (12 days after intracerebral transplantation and 11 days after light stimulation) was significantly decreased in the MSC + As-ChR2 transplanted group compared with the DMEM control group, MSC transplanted group or MSC + As-YFP transplanted group (Fig. 6C,D, *P < 0.05 or **P < 0.01). Prior to MCAO, there was no neurological deficit in any animals examined (Fig. 6E); 1 day after MCAO, severe neurological deficit (2–4 scores) was observed in the animals, but without any significant difference among the groups (Fig. 6E). The neurological deficits were significantly decreased at 12 days after transplantation of MSC + As-ChR2, compared with the DMEM control-group or MSC-group (Fig. 6E, *P < 0.05). Light stimulation of the ischemic area in the MSC + As-ChR2-treated animals significantly increased the local ATP release from 42.7 ± 8.7 nM.
to 75.7 ± 4.5 nM before and during light on respectively (**P < 0.01), and this increased ATP release could be reversed to the basal level when light was off (Fig. 6F). The ATP concentration in the ischemic area of MSC 1 As-ChR2-treated rats was significantly elevated by optical stimulation compared with MSC 2 or MSC 1 As-eYFP-treated rats (**P < 0.01 or *P < 0.05, Fig. 6F). Further immunohistochemical analysis showed a larger number of TuJ1-positive cells in the injected hemisphere of MSC 1 As-ChR2-transplanted brains compared with MSC-control group or MSC + As-eYFP group (Fig. 6G,H, **P < 0.01 or *P < 0.05); more TuJ1 plus ANA-positive cells were observed in the injected area of MSC + As-ChR2 group compared with MSC or MSC + As-eYFP group (Fig. 6I,J, *P < 0.05).

**THE NUMBER OF IBA1-POSITIVE CELLS IN THE PERI-ISCHEMIC AREA IS NOT ALTERED BY INCREASED EXTRACELLULAR ATP.** In order to test whether increased local ATP in the ischemic area would affect microglia function, we examined the Iba1-immunoreactivity 96 h after

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**FIGURE 5:** The direct effects of ATP on the function of MSCs. A: Sample gel showing RT-PCR determination of P2X isoforms expressed by MSCs. B and C: The effects of conditioned medium on the differentiation of MSCs. The ratio of TuJ1-positive cells was calculated in the MSCs incubated with different conditioned media as defined in the figure. Scale bar = 100 μm, **P < 0.01, n = 7–10. D: qRT-PCR analysis of ATP (10 μM)-treated MSCs show increased gene expression of TuJ1 and Pax6, **P < 0.01, n = 6. E: β-catenin/β-actin analysis in MSCs exposed to the neuronal induction medium plus doses of ATP (0, 1, 10, and 100 μM) by western blot analysis of the total protein extraction. Values are mean ± SEM of three tests. ATP = 0 μM served as control. F: qRT-PCR analysis of ATP (10 μM)-treated MSCs show increased induction of wnt target genes, β-catenin and FZD8, compared with control MSCs. **P < 0.01, compared with control group; ###P < 0.01, compared with ATP-treated group, n = 6. G: Fluorescence image of β-catenin immunostaining in MSCs that had been stimulated for 3 days with neuronal induction medium plus 10 μM ATP.
FIGURE 6: Effects of MSCs co-transplanted with ChR2-astrocytes on the improvement of neurological deficits after middle cerebral artery occlusion (MCAO) in rats. A: Experimental procedures and photostimulation protocol. B: Co-transplantation of MSCs and ChR2-astrocytes into the striatum of stroke rats. Green: astrocytes transfected with ChR2-eYFP; red: immunostaining of anti-human nuclei, indicating the survival of MSCs; blue: cell nuclei. C and D: Brain infarct volume determined by 2,3,5-triphenyltetrazolium (TTC) staining at 14 days after reperfusion from 120 min MCAO. E: Neurological deficit scores in sham (control) animals, MSCs-treated, MSCs co-transplanted eYFP-astrocytes-treated and MSCs co-transplanted ChR2-astrocytes-treated rats. Values are mean ± SEM of five animals of each group, **P < 0.01 or *P < 0.05. F: Measurement of ATP concentrations from the microdialysis samples: optical stimulation of the ischemic area in MSCs-treated, MSCs co-transplanted eYFP-astrocytes-treated and MSCs co-transplanted ChR2-astrocytes-treated rats. Values are mean ± SEM (n = 6 from three rats of each group, **P < 0.01 or *P < 0.05). G and H: Fluorescence images of Tuj1 immunostaining in the ischemia hemisphere of rat brain and the ratio of Tuj1-positive cells (n = 7–9 from four rats of each group); I and J: Fluorescence images of double Tuj1- and ANA-positive immunostaining (n = 6 from three rats of each group). Scale bar 20 μm.
MCAO (24 h after light stimulation). The number of Iba1-positive cells in the peri-ischemic area was counted in 675 × 675 μm fields in three adjacent sections from four rats of each group. There was no significant difference between the four groups as indicated (Supp. Info. Fig. 3A–B, P > 0.05).

Discussion

The main findings of this study were that light-activated astrocytes promoted neuronal differentiation of the neighbouring MSCs in vitro and in vivo (Figs. 3 and 6), and accelerated the functional improvement in stroke rats (Fig. 6). We also found strong evidence that ATP from light-depolarized astrocytes was one of the signals that upregulated the function of MSCs (Figs. 4 and 5): ATP acting via P2X receptors on the MSCs, activates Wnt/β-catenin signaling to enhance the neuronal differentiation (Fig. 5). To our knowledge, this is the first time that the neuronal differentiation capacity of MSCs regulated by optogenetically-stimulated astrocytes has been reported.

Stem cell transplantation is a promising therapeutic strategy for neurodegenerative diseases (Joyce et al., 2010) including stroke. Human umbilical MSCs, which are full of therapeutic potential, are isolated from discarded extra-embryonic tissue after birth (Fan et al., 2010; Wang et al., 2004). MSCs have the prominent advantages of abundant supply, painless collection, and immunosuppression, as well as the potential ability to improve functional deficits after CNS injuries or neurodegenerative diseases (Dezawa et al., 2004; Fu et al., 2004; Koh et al., 2008; Kurozumi et al., 2005; Mitchell et al., 2003). On the other hand, it is generally believed that local environmental elements influence the physiological characteristics of transplanted stem cells (Morrison and Spradling, 2008). It has been proposed that astrocytes, the most abundant cell type in the mammalian brain, control the neurogenic niches of neural stem cells to regulate their function (Jiao and Chen, 2008; Wilhelmsson et al., 2012); human embryonic stem cells (hESCs) enhanced functional neural development via astrocyte coculture (Weick et al., 2010); MSCs, when cocultured with astrocytes, also exhibited neuroectodermal lineage differentiation (Jiang et al., 2003). However, most of these studies were performed only with quiescent astrocytes or astrocyte-conditioned medium, and therefore it remains unknown whether altering the activity of astrocytes would stimulate MSCs toward neuronal differentiation.

In the current study, we applied for the first time an optogenetic approach to understand the role of activated-astrocytes in determining the neuronal commitment of MSCs by selectively stimulating astrocytes in vitro and in a rat ischemic stroke model. The advantage of optogenetic stimulation over pharmacological or electrical stimulation is that the ‘light’ can selectively influence one individual cell population without disturbing the neighbouring cells within a complex neural circuit. The functional expression of light-sensitive ChR2 in cultured astrocytes was confirmed by light-evoked photocurrents (Fig. 2E,F). Membrane depolarization of a ChR2-expressing astrocyte was also successfully induced after 60 mins of light stimulation (Fig. 2G), indicating the stable status of ChR2 during continuous illumination. Our electrophysiological results also showed that cultured astrocytes strongly expressing ChR2 protein did not change their natural properties: the current and voltage were linearly related, with a typical resting membrane potential around −70 mV (Fig. 2D), which is close to the value reported by Duan’s group (Chen et al., 2013).

In the As-ChR2 and MSCs co-culture system, selective photostimulation of ChR2-astrocytes promoted the surrounding MSCs towards a neuronal fate by increasing the expression of Tuj1 and NeuN (Fig. 3C–F). In another co-culture system, bathing MSCs with half light-stimulated-astrocytes-conditioned medium (ChR2) significantly enhanced the mRNA expression of neuronal markers, Tuj1 and Pax6 (Fig. 3H,I). This conditioned medium also accelerated the proliferation of MSCs: the proportion of dividing cells (EdU-positive) was increased after brief incubation of MSCs with the indicated medium for 24 h (Supp. Info. Fig. 2A,B). These results suggested that some factor(s) derived from ChR2-astrocytes under light stimulation may modulate MSCs function.

Many studies have demonstrated that astrocytes release ATP in the CNS (Chen et al., 2013; Coco et al., 2003; Gourine et al., 2010), and extracellular ATP is one of the growth factors that participates in cell proliferation, differentiation, and cell death (Coppi et al., 2007; Di Virgilio et al., 1998; Lin et al., 2007; Sholl-Franco et al., 2010). However, we are not aware of any report describing the direct effects of astrocytic-ATP on the neuronal differentiation of MSCs. In the current study, we found an increase in the extracellular ATP concentrations in the conditioned medium from light-stimulated As-ChR2s (Fig. 4A), which is likely due to the optical activation of them. This conclusion was supported by the findings that no detectable LDH activity (a usual indicator for cell membrane damage) was observed among the cells grown under the indicated conditions (Fig. 4B). The time course of extracellular ATP accumulation further confirmed this conclusion because if the increased extracellular ATP was a non-specific effect due to light or virus transfection-induced damage to the astrocytes, a time-dependent increase in the extracellular ATP concentration would not have been observed during the first 60 mins (Fig. 4C–E).

In order to further assess the specific effects of extracellular ATP on the function of MSCs, we incubated the MSCs with half neuronal induction medium and half ChR2-astrocyte-conditioned medium (ChR2) and found that more MSCs expressed the neuronal lineage marker, Tuj1 (Fig. 5B,C). Application of an antagonist of the ATP P2X...
receptors blocked this neuronal-differentiation-enhancing effect. More importantly, we directly incubated the MSCs with ATP and found the mRNA expression levels of the neuronal lineage markers were upregulated (Fig. 5D). Taking these data together, we suggested that ATP played an important role in stimulating MSCs towards neuronal differentiation. Since wnt/β-catenin signaling was suggested to be involved in neurogenesis and/or neuronal differentiation (Ban et al., 2011; Jiang et al., 2002, 2003; Mezey et al., 2000), we exogenously applied graded doses of ATP to the full neuronal induction medium to grow the MSCs, and the expression of β-catenin, an important downstream molecule in the canonical wnt signaling, was then examined after 7 days of neuronal induction: increased extracellular ATP at 1 to 100 μM stimulated the β-catenin protein production in MSCs (Fig. 5E). Our gene expression experiments further showed that 10 μM ATP treatment enhanced the neuronal lineage differentiation of MSCs by increasing the expression of wnt-targeting genes: FZD8 and β-catenin (Fig. 5F). The nuclear translocation of β-catenin in MSCs in the presence of ATP further confirmed that wnt/β-catenin signaling was activated by extracellular ATP (Fig. 5G). Meanwhile, our results also confirm and extend the previous study mentioned above (Coppi et al., 2007), as we found that after 24 h of incubation, the expression of cell cycle regulatory protein cyclin D1 was increased by exogenous ATP (Supp. Info. Fig. 2C).

Extracellular ATP exerts its effects through two classes of purinergic P2 receptors, the ionotropic P2X receptors and G-protein-coupled P2Y receptors (Liu et al., 2008; Mishra et al., 2006). Previous studies have proposed the involvement of P2Y receptors in the nuclear accumulation of β-catenin (Ortega et al., 2008) and regulation of β-catenin expression (Mastrangelo et al., 2012). However we are not aware of any reports which have examined the role of P2X receptors in the regulation of wnt/β-catenin signaling. Seven P2X receptor subtypes (P2X1-7) have been cloned from mammalian tissues (Khakh and North, 2006; Ralevic and Burnstock, 1998). Therefore, in this study, we examined the mRNA expression profile of P2X receptors by human umbilical MSCs (Fig. 5A) and the involvement of P2X receptors in regulating the wnt target genes. Blockade of P2X receptors by the potent selective inhibitor, TNP-ATP (Burgard et al., 2000), reversed the ATP-induced upregulation of wnt target genes: FZD8 and β-catenin (Fig. 5F), strongly suggesting the involvement of P2X receptors in the regulation of the wnt/β-catenin signaling. However, further investigation would be needed to study the molecular pathways underlying ATP activated wnt/β-catenin signaling via P2X receptors.

Our data do not exclude the possibility that other component(s) in the MSC niche containing the ChR2-astrocytes could have synergistically enhanced the effects of ATP. For example, astrocyte-derived wnts might also be involved in the regulation of stem cell functions as previously suggested by Gage’s group (Li et al., 2005). Therefore, further study would be needed to identify other factors that interact with ATP in the regulation of MSC function. β-catenin was also suggested to play an essential role in regulating mitotic events (Huang et al., 2007), suggesting links between cell cycle regulators and the canonical wnt signaling (Davidson and Niehrs, 2010). However, further investigation is needed to fully elucidate whether and how wnt/β-catenin signaling is linked to ATP-induced up-regulation of cyclin D1.

Lastly, our results showed that co-transplantation of ChR2-astrocytes and MSCs into the ischemic core of the stroke rats significantly improved the neurological deficit after light stimulation of the ChR2-astrocytes (Fig. 6C,D), which might be due to an increase in the total number of TuJ1 positive cells in the local area (Fig. 6G,H). Our results also suggested that light-stimulated astrocytes enhanced the transplanted MSCs differentiating into neuronal cells in the ischemic area of the stroke rats by increased co-expression of TuJ1 and ANA (Fig. 6L,J). Furthermore, we also demonstrated that light stimulation of ChR2-astrocytes released more ATP into the ischemic area (Fig. 6F), which might mediate the differentiation of neighbouring MSCs into neuronal cells. However, further experiments would be needed to identify whether ChR2-astrocytes under light stimulation also prevent the survived neurons from dying or promote endogenous neurogenesis after stroke.

In conclusion, our study indicated that light-activated-astrocytes modulated the MSCs toward neuronal differentiation in vitro and in vivo. We also demonstrated that astrocytic-depolarization helps to improve the efficacy of MSC-based regeneration in the rat stroke model. It is noteworthy that light stimulation of astrocytes via ChR2 could release ATP and/or other factors to trigger the intracellular wnt/β-catenin signaling, and consequently influence the behavior of neighbouring MSCs. Therefore, our findings may provide a new therapeutic strategy for stroke.

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References


