Critical role of type 2 ryanodine receptor in mediating activity-dependent neurogenesis from embryonic stem cells

Hui-Mei Yu a,b, Jing Wen c, Rong Wang a,b, Wan-Hua Shen c, Shumin Duan c, Huang-Tian Yang a,b,*

a Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences & Shanghai Jiao Tong University School of Medicine (SJTUSM), Shanghai 200025, China
b Shanghai Stem Cell Institute of SJTUSM, Shanghai 200025, China
c Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Received 15 May 2007; received in revised form 22 July 2007; accepted 23 July 2007
Available online 4 September 2007

Abstract

Activity-induced neurogenesis via Ca2+ entry may be important for establishing Hebbian neural network. However, it remains unclear whether intracellular Ca2+ mobilization is required and which subtypes of Ca2+ release channels expressed in Ca2+ store organelles are involved in the activity-dependent neurogenesis. Here, we demonstrated that the activity of intracellular Ca2+ signaling, expression of neuronal transcription factor NeuroD, and the rate of neurogenesis were significantly inhibited in neuronal cells derived from embryonic stem (ES) cells deficient in the Ca2+ release channel type 2 ryanodine receptors (RyR2−/−). In wild-type (RyR2+/+) but not in RyR2−/− ES cells, activation of L-type Ca2+ channels, GABAA receptors, or RyRs promoted neuronal differentiation, while inhibition of these channels/receptors had an opposite effect. Moreover, neuronal differentiation promoted by activation of GABA A receptors or L-type Ca2+ channels in RyR2+/+ cells was prevented by RyR inhibitors. No significant difference was detected in the expression level of GABA A receptors and L-type channels between neuronal cells derived from two types of ES cells. Thus, activity-induced Ca2+ influx through L-type Ca2+ channels alone is not sufficient in promoting neurogenesis. Instead, an intimate cooperation of L-type Ca2+ channels with RyR2 is crucial for the activity-dependent neurogenesis induced by paracrine and/or autocrine GABA signaling.

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Keywords: Neurogenesis; Ryanodine receptors; ES cells; L-type Ca2+ channels; GABAA receptors

1. Introduction

Neurogenesis is regulated by a variety of environmental and intracellular signals. Several lines of evidence suggest that intracellular calcium concentration ([Ca2+]i) regulated by Ca2+ entry plays important roles in mediating the neurogenesis and neuronal differentiation [9,15,20,23,26]. Spontaneous Ca2+ transients can be detected in cells as early as a neuronal phenotype can be determined [9] and appear to regulate onset of GABAergic phenotype during neural precursor differentiation [12]. In murine hippocampal progenitor cells, voltage-dependent inward currents present and parallel the progressive expression of functional neuronal phenotypes in vitro [38]. Moreover, neurogenesis is potently enhanced by Ca2+ influx through L-type Ca2+ channels in adult mouse neural stem/progenitor cells [15]. However, it is unclear whether intracellular Ca2+ mobilization is required for the activity-induced neurogenesis and which pathways are involved in the L-type Ca2+ channel-regulated neurogenesis.

The endoplasmic reticulum (ER) represents an important [Ca2+]i store in various tissues. Two types of Ca2+-release
channels are expressed in ER: ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate receptors (IP$_3$Rs), with each type of the channels having three different isoforms [4,5]. In mature neurons, RyRs contribute to the regulation of synaptic transmission and plasticity and learning and memory [4,16]. During murine neurogenesis, all the three isoform of RyRs can be detected in cerebral hemispheres as early as the onset of neurogenesis [17]. However, it is unknown whether intracellular Ca$^{2+}$ signaling regulated by RyRs contributes to early neurogenesis.

Activity-dependent neurogenesis may be important for establishing Hebbian neural network [14,15]. It has been shown that GABAergic activity induces intracellular Ca$^{2+}$ elevation in neural progenitor cells [7]. The effect of GABA on neurogenesis, however, can be either stimulatory [14,19,22,47] or inhibitory [22,30,35], depending on the type of progenitors studied and preparations used. Whether Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) mediated by RyRs [8,42] plays important roles in such activity-dependent neurogenesis and neuronal differentiation and which subtypes of RyRs are involved remains largely unexplored, probably due to the facts that RyRs still lack subtype-specific inhibitors and animals deficient in RyR2 die in early embryonic stage [45].

Embryonic stem (ES) cells have a potential to generate unlimited numbers of cells of all three primary germ layers including neurons, one possible source of transplantable cells. Embryoid bodies (EBs) formed from ES cells resemble early post-implantation embryos and differentiation of ES cells into neuronal cells represent a viable model to study early neuronal developmental paradigms [6,21]. This system is especially useful for identifying gene functions related to early development due to its easy accessibility for genetic manipulation. Using RyR2 deficient (RyR2$^{-/-}$) ES cells combining with multiple approaches, we demonstrated in the present study that Ca$^{2+}$ release through RyR2 plays a critical role in mediating activity-dependent neurogenesis via amplifying intracellular Ca$^{2+}$ signaling triggered by activation of L-type Ca$^{2+}$ channels due to membrane depolarization caused by autocrine and/or paracrine released GABA.

2. Methods

2.1. ES cell culture and neuronal differentiation

RyR2$^{+/+}$ and RyR2$^{-/-}$ R1 ES cells [25,51] were cultivated and differentiated into neuronal cells as previously described [1]. Briefly, undifferentiated ES cells were grown on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF) in ES cell basal medium containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Hycone), 1% non-essential amino acids, 2 mM glutamine, 100 mM β-mercaptoethanol. Differentiation of these two type ES cells into neuronal cells was initiated by a hanging drop method to form EBs in hanging drop medium, containing DMEM with 20% FBS and supplements as described above in ES cell basal medium for 2 days. Then the EBs were incubated in suspension medium, containing the same components as the ES cell basal medium without LIF for 6 days. RA was added during the last 4 days of suspension culture (Fig. 1). After 8 days, EBs were dissociated into single cells and the cells were plated onto gelatin-coated tissue culture dishes or coverslips in neuronal basal medium, containing modified DMEM supplemented with 10% F12, 2% B27, 1% N2, and 100 mg/ml bovine serum albumin (Sigma) for a further 8-day culture. All cultivation medium and other substances for cell cultures were purchased from Gibco BRL except where indicated.

2.2. Immunostaining

EBs were fixed for 45 min at room temperature in 4% paraformaldehyde in 0.1 M PBS, containing (in Mm) 137 NaCl, 2.7 KCl, 5.4 Na$_2$HPO$_4$, 0.56 KH$_2$PO$_4$, pH 7.4, then embedded in OCT (Tissue Tek) and rapidly frozen by CO$_2$. Ebs were taken at 20–25 μm thicknesses. Cells on coverslips were fixed for 15 min in 4% paraformaldehyde and washed three times in PBS [35,43]. Ebs, EB slices and cell cultures were permealized with 0.3% Triton X-100 for 15 min and treated with 10% BSA for 1 h. Each of them were incubated with monoclonal antibodies against Tuj1 (1:1000, Promega,), nestin (1:100, Chemicon), MAP2ab-2 (1:100, Chemicon), RyR2 (1:100, Affinity Bioreagents), α1 subunit of GABAA receptors (1:400, Alomone labs), or GFAP (1:1000, Chemicon) overnight at 4°C. After washing three times in PBS for 10 min, cells were stained for 60 min with the appropriate fluorophore-conjugated secondary antibodies at 1:200 or 1:400 dilution in PBS against specific light chains of mouse or rabbit IgG (Santa Cruz Biotechnology). Cell nuclei were stained with Hoechst No. 33342 (1 mg/Ml) or PI (10 mg/Ml, Sigma) for 10 min. Stained cells were photographed under a Zeiss Axiohot I microscope equipped with epifluorescence optics or a Zeiss LSM510 confocal laser scanning microscope using appropriate fluorescence filters.

2.3. BrdU immunostaining

Cell cultures on coverslips were incubated with BrdU (20 μM, Molecular Probes) for 18 h and then were fixed in 70% EtOH + 30% 50 mM glycine, pH 2.0 solution at −20°C for 20 min [12]. Cells were washed twice with PBS, then incubated with 50 μl of 1:20 diluted mouse anti-BrdU (Alexa Fluor 546 conjugated, Roche) in incubator kits for 30 min.

2.4. Ca$^{2+}$ imaging

Cells were loaded with 2 μM of Fluo4-AM (Molecular Probes) for 20 min at 37°C and then were washed with extra-cellular medium containing (in mM): 140 NaCl, 3 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 10 glucose and incubated for a further 20 min to allow complete de-esterification of the
Fig. 1. Expression of RyR2 during neuronal differentiation of ES cells. (A) The protocol for inducing ES cell differentiation into neural cells. All-trans-retinoic acid (RA) and pharmacological interventions (Pha-I) including various agonists or antagonists were applied from 5 days to 8 days of suspension culture. (B) Representative immunostaining of RyR2 (middle panels, red) and neural precursor marker nestin or mature neuronal marker MAP2ab (top panels, green) in embryoid bodies (EBs) or cells derived from RyR2^{+/+} ES cells. Right panel shows example of evoked action potentials (AP) recorded from 8 + 8 days wild-type neuronal cells. (C) Representative immunostaining of RyR2 (middle panels, red) and nestin or MAP2ab (top panels, green) in EBs or cells derived from RyR2^{-/-} ES cells. Right panel, example of evoked AP recorded from RyR2^{-/-} neuronal cells. (D) RT-PCR analysis of transcripts for RyR1, RyR2, RyR3 and housekeeping gene 28s in RyR2^{+/+} and RyR2^{-/-} cells at various differentiation stages as well as in adult brain. Scale bars in (B) and (C), 20 μm.
2.5. Electrophysiology

Voltage-gated Ca\(^{2+}\) currents and GABA-induced currents were recorded using the whole-cell patch clamp technique with Multi-clamp 700A amplifier (Axon instruments Inc.) [29]. The resistance of the electrode filled with internal solution was 3–9 MΩ. Signals were filtered at 2 kHz and sampled at 10 kHz. Data were analyzed using Clampfit 9.0 (Axon Instruments Inc.). Solutions were rapidly exchanged using a gravity-driven multi-barrel perfusion system. Voltage-gated Ca\(^{2+}\) currents and GABA-induced currents were recorded using previously described solutions. For GABA currents, the intracellular solution contained (in mM): 120 K-Gluconate, 30 KCl, 10 HEPES, 10 Na\(_2\)-phosphocreatine, 4 ATP-Na\(_2\), 0.3 GTP-Na\(_2\), 10 EGTA (pH 7.3). The external solution contained (in mM): 148 NaCl, 3 KCl, 10 HEPES, 3 CaCl\(_2\), 8 Glucose, 2 MgCl\(_2\) (pH 7.3).

2.6. Total RNA isolation and RT-PCR

Undifferentiated ES cells, differentiated cells and adult mouse brains were used to isolate total RNA. Total RNA from each sample was converted to cDNA by using Superscript II reverse transcriptase (Life-Tech) and oligoT (T16, 500 ng) instruction, and 0.4 μl of this was used for each PCR reaction. Semiquantitative RT-PCR reactions were performed with Taq DNA polymerase (Promega). The primer pairs of RyR1: F, 5′-CCACTTTGCGAGTTTGC-3′ and R, 5′-GGTCTGTGGTGAGGCTGA-3′; RyR2: F, 5′-GACGGCAGAGCCA-CCTCACCTGG-3′ and R, 5′-CCTCGACAGAAAATCTGGA-CAACTGG-3′; and RyR3: F, 5′-GGAACCCACCTCAGAAGGC-3′ and R, 5′-TCCCCCACATCACATCA-3′; Hes1: F, CGGCTTGAGCGAGTAGG CAT and R, CGGTGTAAAGCCCTCACA [15]; NeuroD: F, TTTAGGAGGCACG and R, CCAAGAGCAGTAACGACA [15]. Housekeeping gene, 28s, F, AGACAGCCGACTT-GAACTGG and R, TAGGGACAGTGGAATCTCG. DNA was visualized on 1% agarose gel containing ethidium bromide.

2.7. Statistics

Data are presented as mean ± S.E.M. Statistical significance of differences was estimated by one way ANOVA or by Student’s t test or a paired t test, when appropriate (StatSoft, Version 5.1, StatSoft, Tulsa, OK, USA). P<0.05 was considered significant.

All chemicals were from Sigma except BayK 8644 which was from Calbiochem.

3. Results

3.1. Neurogenesis in RyR2\(^{+/+}\) and RyR2\(^{-/-}\) ES cells

Wild-type (RyR2\(^{+/+}\)) and RyR2\(^{-/-}\) R1 ES cells [25,51] were cultured and differentiated into neural lineages by using 1 μM of all-trans-retinoic acid (RA, see Section 2 and Fig. 1A). Both undifferentiated RyR2\(^{+/+}\) and RyR2\(^{-/-}\) ES cells had normal growth characteristics as observed previously [25,51] and formed similar size of EBs during the 8 days of suspension culture before and after addition of RA. RA was added after the first 4 days and left for the last 4 days. The 8 days EBs were dissociated with trypsin and plated for a further 8 days culture (8 + 8 days). Neural precursors, identified by staining with an antibody to the intermediate filament protein nestin [28], were detectable on peripheral cell layers of both RyR2\(^{+/+}\) and RyR2\(^{-/-}\) EBs at 2 days and increased significantly at 5 days (Fig. 1B and C, upper panel). The RyR2\(^{+/+}\) and RyR2\(^{-/-}\) cells with neuronal morphology (Figs. 1A and 2A) were positive for MAP2ab, a specific maker for mature neuron [43], examined at 8 + 0 and 8 + 4 days. Typical action potentials could be detected at 8 + 8 days (Fig. 1B, rightest panel). The time course of the expression of three isoforms of RyR transcripts in ES cell-derived cells was examined. The transcript of RyR2 was detectable from 5 days RyR2\(^{+/+}\) cells and increased significantly with time of differentiation, but it was not detected in RyR2\(^{-/-}\) cells, whereas weak transcript signals of RyR1 and RyR3 were detectable from 8 + 0
in both types of ES cell cultures (Fig. 1D). Consistently, the expression of RyR2 protein was detected by staining with an antibody selective for RyR2 in RyR2<sup>+/+</sup> cells at 5, 8 + 0, and 8 + 4 days, but not in RyR2<sup>−/−</sup> cells (Fig. 1B and C, middle panels). Moreover, the expression of RyR2 colocalized with the expressions of nestin or MAP2ab in RyR2<sup>+/+</sup> group during neuronal differentiation (Fig. 1B and C, bottom panels).
To examine whether RyR2 contributes to the neuronal differentiation, we compared the profiles of neuronal differentiation in cells derived from RyR2+/+ and RyR2−/− ES cells. Cells with oval neuronal morphology were positive for neuronal marker βIII-tubulin (Tuj1+,[13]). The percentage of Tuj1+ cells in RyR2+/+ cultures examined at 8 + 0 days was significantly higher than that in RyR2−/− cultures (Fig. 2A and B). Furthermore, a significant increase in the proportion of Tuj1+ cells in RyR2+/+ cultures was observed when examined at 8 + 4 days or 8 + 8 days compared with that examined at 8 + 0 days (Fig. 2A and B, left panels), while the Tuj1+ cells in RyR2−/− cultures kept at a constant low level at all the three development stages examined (Fig. 2A and B, right panels).

3.2. Activation of RyRs increases neurogenesis in RyR2+/+ but not in RyR2−/− ES cells

To further examine whether the increased neuronal differentiation in RyR2+/+ cells is directly related to RyR function, we analyzed neuronal differentiation by adding ryanodine into suspension medium from 5 days at a low (10 nM) or a high (3 μM) concentration, which is known to stimulate or inhibit the activity of RyRs, respectively [52]. We found that treatment of RyR2+/+ cultures with 10 nM ryanodine resulted in a significant increase in the proportion of Tuj1+ cells at all the three development stages examined, while 3 μM ryanodine decreased the proportion of Tuj1+ cells to a level similar to that found in RyR2−/− cultures. However, similar treatment of RyR2−/− cultures with either the low or the high concentration of ryanodine failed to affect the proportion of Tuj1+ cells at all the three development stages examined (Fig. 2A and B).

Differentiation of neural precursor/stem cells is controlled, to a large degree, by transcription factors with basic helix–loop–helix (bHLH) motif, such as neuronal determination factors (NeuroD-related factors), which mediate terminal differentiation, and inhibitory bHLH proteins (Hes factors), which inhibit neurogenesis [37,40]. To determine whether the difference in neurogenesis between RyR2+/+ and RyR2−/− cells is correlated with the deferential levels of bHLH factors, we examined the expression patterns of NeuroD and Hes1 genes during the early neuronal differentiation from the two types of cells. With increased neuronal differentiation, the expression of NeuroD and Hes1 transcripts in both RyR2+/+ and RyR2−/− cells increased. Furthermore, the expression of NeuroD in RyR2−/− cells was significantly lower than that in RyR2+/+ cells after 8 days suspension culture, while the expression of Hes1 in RyR2−/− cells was significantly higher than that in RyR2+/+ cells after 5 days suspension culture (Fig. 2C).

Since Hes1 plays important roles in gliogenesis, we compared neuron/glia ratio in RyR2+/+ and RyR2−/− ES cells. A significant increase in the proportion of GFAP (astrocyte marker [43]) positive cells, calculated either as percentage of neural cells (neurons plus astrocytes) or percentage of total cells (neural cells plus non-neural cells), was observed in RyR2−/− cells compared with that in RyR2+/+ cells (Fig. 2D). However, the ratio of neural cells as percentage of total cells in RyR2−/− cultures was not significantly different from that in RyR2+/+ cultures examined at 8 + 8 days (right lower panel in Fig. 2D). These results, together with the finding that RyR2 promoted the proliferation of Tuj1+ cells, but not that of Nestin+ cells (Table 1), suggest that RyR2-mediated [Ca2+]i elevation enhance neurogenesis by shifting the differentiation of neural precursors toward the neuronal fate, rather than by simply inhibiting gliogenesis.

3.3. Decreased neurogenesis in RyR2−/− cells at immature neuron stage but not at neural precursor stage

To examine whether the decreased neurogenesis in RyR2−/− cultures results from decreased differentiation of ES cells into neural precursors or from decreased differentiation of neural precursors into neurons, we immunostained RyR2+/+ and RyR2−/− EBs with neural precursor marker nestin and immature neuron marker Tuj1 every 8 h after RA treatment. Within 40 h of RA treatment, only nestin+ cells, but not Tuj1+ cells, were detected and the percentage of nestin+ fraction was similar between RyR2+/+ and RyR2−/− EB cultures (data not shown). At 48 h of RA treatment, a few Tuj1+ cells (Fig. 3A and B, right panel) could be detected and the percentage of nestin+ fraction was similar in both RyR2+/+ and RyR2−/− EB cultures (Fig. 3A and B, left panel). After 56 h treatment with RA, a significant higher proportion of Tuj1+ cells (Fig. 3A and B, right panel), but not of nestin+ cells (Fig. 3A and B, left panel), was detected in RyR2+/+ than in RyR2−/− EB cultures.

To further determine whether the reduced number of Tuj1+ cells in RyR2−/− cultures is due to the decreased neuronal differentiation or decreased cell viability, we examined the percent of nestin positive cells, the proportion of nestin+ or Tuj1+ cells that were incorporated with BrdU (5-bromo2′-deoxy-uridine) added for 18 h, and the percent of cells with pyknotic nuclei identified by Hoechst 33342 staining, an index for cell death [12], in RyR2+/+ and RyR2−/− cultures at 8 + 0 days (Table 1). Neither the proportion of nestin+ cells, nor the BrdU incorporation rate of nestin+ cells had a significant difference between RyR2+/+ and RyR2−/− groups (Table 1), whereas the BrdU incorporation rate of Tuj1+ cells was significantly higher in RyR2+/+ than that in RyR2−/− group (Table 1). The cell viability analyzed by comparing the

### Table 1

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<th>RyR2+/+</th>
<th>RyR2−/−</th>
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<tr>
<td>Percent of precursors (nestin+)</td>
<td>42.4 ± 3</td>
<td>46.1 ± 7</td>
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<tr>
<td>Percent of BrdU+ cells in precursors</td>
<td>87.5 ± 2</td>
<td>87.6 ± 3</td>
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<tr>
<td>Percent of BrdU in neurons (Tuj1+)</td>
<td>22.0 ± 4</td>
<td>11.0 ± 4*</td>
</tr>
<tr>
<td>Percent of neurons displaying pyknotic nuclei</td>
<td>1.4 ± 1</td>
<td>1.9 ± 1</td>
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*P < 0.05 compared with RyR2+/+ group.
ratio of Tuj1+ cells with pyknotic nuclei was similar between two groups (Table 1). Taken together, these results indicate that RyR2 promotes differentiation of neural precursors to neurons and regulates the proliferation of immature neurons, but does not affect the proliferation rate of neural precursors or neuron viability.

RyRs are Ca2+ release channels on ER and contribute to the dynamics of Ca2+ signaling in neurons [4]. It is not clear whether RyR2 contributes to the Ca2+ signaling during early neuronal developmental stage and whether the Ca2+ signaling regulated by RyR2 is required for neurogenesis. We, therefore, measured spontaneous Ca2+ transients [20] in neuronal cells (neural precursors and immature neurons) derived from RyR2+/+ and RyR2−/− ES cells at 8 + 0 days. The neuronal cells, characterized by their oval cell bodies and neuritic outgrowths, are distinct from other cell types in morphology and were confirmed by immunostaining (data not shown). The frequency of spontaneous Ca2+ transients was significantly higher in RyR2+/+ cells than that in RyR2−/− cells (Fig. 4A).

Moreover, the percentage of active cells exhibiting Ca2+ transients within 30-min imaging period was significantly higher in RyR2+/+ cells than that in RyR2−/− cells (Fig. 4B). Thus, RyR2 is functional in regulating spontaneous Ca2+ transients in early-stage of neuronal cells derived from ES cells. To examine whether RyR2 is involved in the stimulation-evoked intracellular Ca2+ activity, we compared the Ca2+ transients induced by ryanodine (100 nM) or caffeine (10 μM), an activator of RyRs, in RyR2+/+ and RyR2−/− neuronal cells. Ryanodine could induce Ca2+ transients in both RyR2+/+ and RyR2−/− cells at 8 + 0 and 8 + 4 days, but the amplitude of Ca2+ transients in RyR2+/+ cells was significantly higher than that in RyR2−/− cells (Fig. 5A and B). Furthermore, the rising velocity of Ca2+ transients induced by ryanodine was slower in RyR2−/− cells at both 8 + 0 and 8 + 4 days than that in RyR2+/+ cells (Fig. 5A and C). The amplitude and rising velocity of ryanodine-induced Ca2+ transients in RyR2+/+ cells examined at 8 + 4 days stage were higher than that examined at 8 + 0 days stage, a result not observed in RyR2−/− cells (Fig. 5B and C). Similar results were obtained in the caffeine-induced Ca2+ transients (Fig. 5D–F).
not detected in RyR2−/− cells (Fig. 6A). The Tuj1+ cells in RyR2−/− cultures in the presence of 20 mM KCl (4.6 ± 0.5%, 5.2 ± 0.4%, and 7.1 ± 1.6% at 8 + 0, 8 + 4, and 8 + 8 days, respectively) was not significantly different from that without high KCl treatment (3.9 ± 0.9%, 3.5 ± 0.5%, and 5.4 ± 0.4% at 8 + 0, 8 + 4, and 8 + 8 days, respectively). Furthermore, the KCl-induced increase in Tuj1+ cells in RyR2−/− cultures was prevented by treatment with high concentration of ryanodine (3 µM) that inhibits RyRs (Fig. 6A and B), indicating that RyR2 is critical for the promotion of neuronal differentiation induced by treatment with 20 mM KCl.

We then directly analyzed the functional relationship of RyR2 and L-type Ca2+ channels by applying BayK 8644 (1 µM), an agonist of L-type Ca2+ channels. We found that in RyR2+/+ cells, BayK 8644 significantly increased Tuj1+ cells at 8 + 0 and 8 + 4 days, an effect prevented by treatment of cultures with high concentration of ryanodine (3 µM, Fig. 6C and D). In contrast, treatment of RyR2+/+ cells with 10 µM of nifedipine, an antagonist of L-type Ca2+ channels, significantly inhibited proportion of Tuj1+ cells, suggesting that tonic activity of L-type Ca2+ channels is important for the neuronal differentiation in ES cells. On the other hand, neither BayK 8644 nor nifedipine had effects on the neuronal differentiation in RyR2−/− cells (Fig. 6C). The Tuj1+ cells in RyR2−/− cultures in the presence of BayK 8644 (4.1 ± 0.6%, 4.5 ± 0.5%, and 6.9 ± 1% at 8 + 0, 8 + 4, and 8 + 8 days, respectively) or nifedipine (3.8 ± 0.4%, 3.7 ± 0.5%, and 5.7 ± 1% at 8 + 0, 8 + 4, and 8 + 8 days, respectively) was not significantly different from control RyR2−/− cultures (3.9 ± 0.9%, 3.5 ± 0.5%, and 5.4 ± 0.4% at 8 + 0, 8 + 4, and 8 + 8 days, respectively). These results demonstrate that activation of L-type Ca2+ channel promotes the neuronal differentiation through its collaboration with RyR2. Lack effect of KCl or BayK 8644 treatment on the proportion of Tuj1+ cells examined at 8 + 8 stage in RyR2+/+ cultures (Fig. 6B and D) suggests that the basal activity of L-type Ca2+ channels is already saturated at this stage in promoting neurogenesis.

To directly estimate the contribution of RyR2 in the elevated [Ca2+]i, induced by activation of L-type Ca2+ channels in differentiating neuronal cells, we compared BayK 8644-induced Ca2+ transients in RyR2+/+ and RyR2−/− cultures. As shown in Fig. 7, the amplitude of Ca2+ transients induced by application of BayK 8644 was significantly higher in RyR2+/+ cells at 8 + 0 and 8 + 4 days than that in RyR2−/− cells (Fig. 7A and B). Consistently, the rising velocity of Ca2+ transients was significantly faster in RyR2+/+ cells than that in RyR2−/− cells either at 8 + 0 or at 8 + 4 days (Fig. 7A and C). The reduced elevation of [Ca2+]i in RyR2−/− cells may not due to the decreased expression or function of L-type Ca2+ channels, because the current density of L-type Ca2+ channels recorded at RyR2−/− cells at 8 + 0 days was not significantly different from that recorded at RyR2+/+ cells (Fig. 7D). Taken together, these data indicate that RyR2 plays important roles in neurogenesis via amplifying [Ca2+]i signaling triggered by activation of L-type Ca2+ channels.

3.4. Activation of L-type Ca2+ channels increases neurogenesis via triggering Ca2+ release from RyR2

Ca2+ entry plays an important role in the activity-dependent neurogenesis in neuronal stem cells [15]. Since RyRs are activated by Ca2+ influx through membrane Ca2+ channels in neurons [11], we examined whether the Ca2+ entry is involved in the neuronal differentiation of ES cells and whether the regulatory effect of Ca2+ influx on neuronal differentiation is functionally coupled to RyR2. To address these questions, we first investigated the impact of activation of voltage-dependent Ca2+ channels on neurogenesis in RyR2+/+ and RyR2−/− cells by applying modest depolarizing level of extracellular KCl (20 mM), together with RA at 5 days. KCl-induced Ca2+ transients in these two kinds of EBs (data not shown). The proportion of Tuj1+ cells in RyR2+/+ cells at 8 + 0 days and 8 + 4 days was significantly increased by treatment with 20 mM KCl (Fig. 6A and B), indicating that Ca2+ influx promotes neuronal differentiation at early developmental stage. The promoting effect of KCl on the early neuronal differentiation observed in RyR2+/+ cells was...
3.5. Activation of GABA<sub>A</sub> receptors promotes neurogenesis through L-type Ca<sup>2+</sup> channel-RyR2 pathway

Activation of GABA<sub>A</sub> receptors is involved in the regulation of proliferation and differentiation of adult neural stem cells [14,18,27,29,47]. We then examined whether such GABAergic regulation is involved in the neurogenesis from ES cells and in particular, if such regulation exists, whether RyR2 is required for this GABAergic activity-dependent regulation of neurogenesis. Immunostaining with anti-α<sub>1</sub> subunit of GABA<sub>A</sub> receptors showed that both neural precursors (nestin<sup>+</sup> cells) and neurons (Tuj1<sup>+</sup> cells) from either RyR2<sup>+/+</sup> or RyR2<sup>−/−</sup> cells at 8 + 0 days expressed GABA<sub>A</sub> receptors (Fig. 8A and B). Electrophysiological recording from either RyR2<sup>+/+</sup> or RyR2<sup>−/−</sup> cells detected apparent GABA-induced currents that could be blocked by GABA<sub>A</sub> receptor antagonist bicuculline (Fig. 8C). Furthermore, statistical analysis did not show significant difference in the density of GABA-induced currents between RyR2<sup>+/+</sup> and RyR2<sup>−/−</sup> cells (Fig. 8C), indicating that RyR2 does not affect the expression and channel activity of GABA<sub>A</sub> receptors. However, treatment of cultures with GABA (100 μM) significantly increased the proportion of Tuj1<sup>+</sup> cells at 8 + 0 days and 8 + 4 days stages in RyR2<sup>+/+</sup> (Fig. 9A left panel and B) but not in RyR2<sup>−/−</sup> cells (Fig. 9A, right panel; summarized data not shown), an effect that could be prevented in the presence of either bicuculline or nifedipine. Moreover, bicuculline or nifedipine inhibited the proportion of Tuj1<sup>+</sup> cells in RyR2<sup>+/+</sup> cultures (Fig. 9A left panel and B) to a level comparable to that seen in RyR2<sup>−/−</sup> cells (Fig. 9A right panel, summarized data not shown), indicating that GABA<sub>A</sub> receptor activation induced by endogenously released GABA significantly promotes the neurogenesis, an effect depending...
Activation of L-type Ca\textsuperscript{2+} channels promotes neuronal differentiation via RyR2. (A) Representative co-immunostaining of Tuj1 (green) and PI (red) in RyR2\textsuperscript{+/+} and RyR2\textsuperscript{−/−} cells pretreated with KCl (20 mM) in the absence (middle panels) or presence (bottom panels) of ryanodine at high concentration (3 \mu M, Ray (H)), respectively, at 8 + 4 days. (B) Summarized data of (A) in RyR2\textsuperscript{+/+} cells at 8 + 0, 8 + 4, and 8 + 8 days. (C) Representative co-immunostaining of Tuj1 (green) and PI (red) in RyR2\textsuperscript{+/+} and RyR2\textsuperscript{−/−} cells pretreated with L-type Ca\textsuperscript{2+} channel agonist BayK 8644 (1 \mu M) or antagonist nifedipine (10 \mu M) in the absence or presence of ryanodine (3 \mu M) at 8 + 4 days. (D) Summarized data of (C) in RyR2\textsuperscript{+/+} cells at 8 + 0, 8 + 4, and 8 + 8 days. All data were from four to six independent experiments in parallel cultures and >500 cells were analyzed per experiment from 6 to 10 areas. *\textit{P}<0.05; **\textit{P}<0.01 compared with control group. Scale bars in (A) and (C), 50 \mu m.

4. Discussion

4.1. Critical role of RyR2-dependent [Ca\textsuperscript{2+}]\textsubscript{i} signaling in neurogenesis

Ca\textsuperscript{2+} elevation in mature neurons can be mediated by multiple pathways, including extracellular Ca\textsuperscript{2+} influx though various ligand- and voltage-gated channels and intracellular Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} stores through IP\textsubscript{3}Rs or RyRs [4,5,49]. IP\textsubscript{3}Rs are activated by the second messenger IP\textsubscript{3}, produced by activation of G-protein coupled receptors. RyRs may be coupled with L-type Ca\textsuperscript{2+} channels in two different ways: type 1 RyR (RyR1) in skeletal muscle is physically linked with L-type Ca\textsuperscript{2+} channels (dihydropyridine receptor, DHPR), which function as a voltage sense and induce
Fig. 7. Involvement of RyR2 in Ca^{2+} transients induced by activation of L-type Ca^{2+} channels. (A–C) Representative traces (A), averaged amplitude (B), and the rising velocity \( V \) for 50% peak (C) of Ca^{2+} transients increased by 1 μM of BayK 8644, an agonist of L-type Ca^{2+} channels, in RyR2^{+/+} \((n = 14, 16)\) and RyR2^{−/−} \((n = 14, 20)\) neuronal cells at 8 + 0 and 8 + 4 days. (D) Current density of L-type Ca^{2+} channels in RyR2^{+/+} and RyR2^{−/−} neural cells at 8 + 0 days \((n = 15\) for each group). \(^* P<0.05\), \(^{**} P<0.01\) compared with RyR2^{+/+} group.

Conformation change and opening of RyR1 in response to membrane depolarization [36,39,45], while RyR2 is activated by extracellular Ca^{2+} influx through the DHPR via CICR mechanism [8,16,42]. The RyRs are involved in the generation of intracellular Ca^{2+} oscillations required for various neuronal functions [2,7,17,34,37,40,46]. Our results indicate that RyR2 plays a key role in mediating spontaneous Ca^{2+} signal and in promoting neuronal differentiation from ES cells. RyR2 is intensively expressed in neural precursors from wild-type ES cells (Fig. 1) and both the frequency of spontaneous Ca^{2+} transients and percentage of cells exhibiting Ca^{2+} transients are significantly lower in RyR2^{−/−} than that in wild-type neural precursors (Fig. 4). Most importantly, the neurogenesis from RyR2^{−/−} cultures was significantly slower than that in wild-type cultures (Fig. 2). Furthermore, in wild-type but not in RyR2^{−/−} cultures, activation of RyRs with caffeine or low concentration of ryanodine promoted neurogenesis (Fig. 2), while inhibition of RyRs with high concentration of ryanodine had an opposite effect (Fig. 2). The result that the percentage of TuJ1-positive cells but not that of nestin-positive cells is significantly decreased in cultures from RyR2^{−/−} ES cells (Figs. 2 and 3) suggests that RyR2-mediated Ca^{2+} signaling promote neurogenesis from neural precursors but not affect generation and/or proliferation of neural precursors from ES cells. Consistent with this notion, we found that the level of transcription factor NeuroD, which promotes neuronal differentiation and maturation [40], was significant decreased, while that of Hes1, which inhibits the neuronal phenotype [37], was significantly enhanced in RyR2^{−/−} cells (Fig. 2). In addition, the decreased population of TuJ1 neuronal cells is associated with an increased population of GFAP^{+} glial cells without change in the total population of neural cells (TuJ1^{+} cells plus GFAP^{+} cells) in RyR2^{−/−} cultures (Fig. 2D). Because neurons and glial cells are generated from a common progenitor [6,21,37], our results suggest that RyR2-mediated [Ca^{2+}]elevation plays
critical role in activity-dependent neurogenesis by shifting the differentiation of neural precursors toward the neuronal fate.

4.2. Critical role of RyR2 in mediating activity-dependent neurogenesis induced by activation of GABA\(_\alpha\) receptors and L-type Ca\(^{2+}\) channels

We found that nifedipine-sensitive Ca\(^{2+}\) current could be recorded at 8 + 0 cultures when neural precursors were dominant. Furthermore, application of nifedipine inhibited proportion of Tuj1\(^+\) cells in wild-type cultures (Fig. 6), suggesting that tonic activation of L-type Ca\(^{2+}\) channels contributes to the neurogenesis from ES cells. L-type Ca\(^{2+}\) channels distribute mainly on cell bodies and proximal dendrites of neurons and may not play important role in neurotransmitter release [10]. However, Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels is more efficient than that though other types of Ca\(^{2+}\) channels in regulating gene transcription and thus plays a critical role in mediating excitation-transcription coupling in neurons [10,13,33]. Consistently, the expression of transcription factor NeuroD and Hes1 important for neural differentiation are up and down regulated, respectively, by activation of L-type Ca\(^{2+}\) channels [15]. Our results that NeuroD and Hes1 were significant decreased and enhanced, respectively, in RyR2\(^{-/-}\) cultures (Fig. 2) suggest that the activity-dependent regulation of these transcription factors depends on RyR2-mediated CICR.

Activation of CICR requires a relatively high level of threshold [Ca\(^{2+}\)], and thus needs a long lasting depolarization...
Fig. 9. Promotion of neuronal differentiation induced by activation of GABAA receptors involves CICR mediated by L-type Ca\(^{2+}\) channels and RyR2. (A) Representative co-immunostaining of Tuj1 (green) and PI (red) in RyR2\(^{+/+}\) and RyR2\(^{-/-}\) cells at 8 + 4 days, pretreated with GABA (100 \(\mu\)M) in the presence or absence of nifedipine (10 \(\mu\)M), an antagonist of L-type Ca\(^{2+}\) channels, or bicuculline (Bic, 100 \(\mu\)M), an antagonist of GABAA receptors. Scale bar, 50 \(\mu\)m. (B) Summarized data showing percentage of neurons (Tuj1\(^{+}\)) in RyR2\(^{+/+}\) cells at 8 + 0, 8 + 4, and 8 + 8 days, under different pretreatments as shown in (A). All data were from four to six independent experiments in parallel cultures and >500 cells were analyzed per experiment from at least 5 areas. (C and D) Representative traces of GABA-induced Ca\(^{2+}\) transients in the absence or presence of bicuculline (C) or nifedipine (D) in RyR2\(^{+/+}\) and RyR2\(^{-/-}\) neural cells at 8 + 0 days. Averaged amplitude (E) and velocity (V) for 50% peak (F) of Ca\(^{2+}\) transients induced by GABA in RyR2\(^{+/+}\) and RyR2\(^{-/-}\) neural cells at 8 + 0 (n = 17, 20) and 8 + 4 days (n = 15, 33). *P < 0.05, **P < 0.01, ***P < 0.001 compared with RyR2\(^{+/+}\) group or with the group indicated.


of the cell [49]. Compared with other types of voltage-gated Ca\(^{2+}\) channels, L-type Ca\(^{2+}\) channels are activated at a more negative potential and are relatively resistant to inactivation [48], making them well-suited to tonic signaling [31,41]. Since neuronal precursors tend to be more easily depolarized than mature neurons [50], L-type Ca\(^{2+}\) channels may be partially opened at rest. The depolarization of neuronal precursors/immature neurons may be induced by cellular milieu, such as elevated extracellular [K\(^{+}\)] during local neuronal activity or voltage changes in active neighboring neurons [24]. In addition, the neuronal precursors may be depolarized by activation of plasma membrane receptors [14,15,19,22,47]. Activation of GABAA receptors opens the ligand-gated chloride channels and causes Cl\(^{-}\) influx in most mature neurons to induce membrane hyperpolarization. However, opening of the same chloride channel by GABA in immature neurons may cause Cl\(^{-}\) efflux and membrane depolarization due to higher intracellular Cl\(^{-}\) concentration in
these neurons [3,30]. Like neural progenitors from embryonic brain [22,30,31] or early postnatal brain [19,35,44], neural precursors from ES cells also express functional GABA_A receptors (Fig. 8) that, when activated, induced intracellular Ca^{2+} elevation and promoted neurogenesis through activation of L-type Ca^{2+} channels (Fig. 9). The result that application of GABA promoted neurogenesis and bicuculline inhibited neurogenesis (Fig. 9) suggests involvement of tonic activation of GABA_A receptors. Thus, the excitation-neurogenesis coupling described in adult neural stem cells involving activation of GABA_A/NMDA receptors and L-type Ca^{2+} channels [14,15,47] is also responsible for the GABA-induced neurogenesis from ES cells. This is consistent with the observation that direct effect of GABA on neural progenitors tends to increase neurogenesis [14,19,22], although some in vivo or organotypic culture studies showed opposite effect by using systemic pharmacological treatments [22,30,35]. The latter phenomenon may be caused by complicated inhibitory effects of GABA on surrounding mature neurons, which may have an indirect inhibition on neurogenesis from neural precursors due to the decreased excitatory input from these mature neurons [14]. Unlike adult neural stem cells or postnatal neural precursors receiving various inputs (either synaptic or non-synaptic) from surrounding mature neurons, activation of GABA_A receptors in neural precursors derived from ES cells may be induced by non-synaptic paracrine and/or autocrine GABA released from precursors and/or immature neurons themselves, similar to that described previously in embryonic [32] and more recently in adult [29] neural precursors/immature neurons.

The most interesting finding in the present study is that neurogenesis induced by activation of GABA_A receptors and L-type Ca^{2+} channels depends critically on the functional RyR2. Of the three RyR isoforms expressed in adult brain, RyR2 is the most abundant [4]. Furthermore, the expression of RyR2 protein, which is detectable as early as the onset of neurogenesis, is earlier and higher than the other two isoforms of RyRs in the brain [17], suggesting important roles of RyK2 in the activity-dependent neurogenesis in the brain. Consistent with this hypothesis, we found that caffeine and ryanodine still evoked low level Ca^{2+} activity in RyR2^{−/−} cultures (Fig. 5), this low level Ca^{2+} activity mediated probably by other subtypes of RyRs may not involved in the activity-induced neurogenesis in ES cells. Thus, antagonist (nifedipine) and agonist (BayK 8644) of L-type Ca^{2+} channels were effective in inhibiting and promoting neurogenesis, respectively, only in wild-type, but not in RyR2^{−/−} cultures (Fig. 6). Similarly, inhibition and promotion of neurogenesis induced by bicuculline and GABA only occurred in wild-type, but not in RyR2^{−/−} cultures (Fig. 9). Furthermore, no significant difference was found between two types of cultures in the expression of GABA_A receptors (Fig. 8A and B), the density of GABA-induced inward current (Fig. 8C), or the density of Ca^{2+} current (Fig. 7D). These results demonstrate that activity-induced Ca^{2+} influx through L-type Ca^{2+} channels, if not powered by CICR mechanism, is not sufficient in promoting neurogenesis. Moreover, although multiple subtypes of IP_3 receptors and RyRs may be expressed in neural precursors, it is RyR2 that plays a key role in mediating the activity-dependent neurogenesis induced by paracrine and/or autocrine GABA signaling.

Conflict of interest

None.

Acknowledgements

This study was supported in part by grants of the National Natural Science Foundation of China (30270656; 30671045; 30321002), The State Major Research Program from the Ministry of Sciences and Technology, China (2006CB0F0900; G200077800) and Programs (06dj14001) from Science & Technology Committee of Shanghai Municipality, China. We thank Dr. Kenneth R. Boheler (NIH/NIA) for providing us R1 mouse ES cells.

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