Original Article

Characterization of intracellular free Ca2+ movements in neural progenitor cells derived from ES cells transfected with MASH1 transcription factor gene

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Intracellular free Ca2+ movements in neural progenitor cells obtained by transfection of embryonic stem(ES) cells with MASH1 transcription factor gene were studied. The MASH1 transfected cells, majority of which were Islet1 positive, have been shown to improve motor functions of hemiplegic mice when transplanted into the injured brain. In this study RT-PCR was conducted to detect Ca2+ channel mRNAs. We found that mRNAs of L-type, N-type and T-type Ca2+ channel mRNAs were expressed in the MASH1 transfected cells. Next, a Ca2+-sensitive fluorescence probe, fluo-3, and a scanning confocal laser microscope were used to detect any changes of intracellular Ca2+ concentration. Enhanced fluo-3 fluorescence was observed when the cells were stimulated with increased extracellular potassium, a depolarization signal. Depletion of extracellular Ca2+ abrogated the increase of fluorescence intensity upon depolarization. A Ca2+ channel blocker, lead, inhibited the increase of fluorescence intensity upon depolarization. A L-type channel inhibitor, nifedipine, but not a N-type channel inhibitor, omega-conotoxin GVIA, reduced the increase of fluorescence intensity upon depolarization. Thapsigargin, which depletes intracellular Ca2+ stores, did not attenuate the depolarization-induced signals. Potassium channel inhibitors, tetraethylammonium and 4-aminopyridine inhibited the fluorescence signals. These results indicate that depolarization-induced intracellular Ca2+ increase is mainly due to inward flow (influx) of Ca2+ via Ca2+ channels and marginally due to the release (mobilization) of Ca2+ from intracellular stores in the MASH1 transfected neural progenitor cells. The calcium signals may regulate various aspects of neural cell function such as authentic cellular activity including neurotransmitter secretion and cell differentiation. Our data add the possibility that calcium signals may play roles even in the ectopically transplanted MASH1-transfected neural progenitor cells reconstituting neural network to improve motor functions of hemiplegic mice.

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Introduction

The limited capacity for structural and functional repair in human brain is partially explained by the inability of mature CNS to regenerate new cellular components[1]. Recently adult brain has been shown to be a regenerative organ that incorporates exogenous neurons to repair the damage[2]. Embryonic stem (ES) cells are considered to be a promising cell source for the neural cell graft[3]. To initiate neural differentiation of ES cells, all-trans retinoic acid (RA) and basic fibroblast growth factor(bFGF) and other factors have often been used[4-10].

We have recently shown that neural progenitor cells were successfully induced by the transfection of ES cells with MASH1 gene and subsequent selection process with G418. Moreover, majority of the neural progenitor cells were Islet1 positive, and the neural cells improved motor functions of hemiplegic mice after transplantation into the damaged brain[11].

Cytoplasmic Ca2+ levels in neurons govern many signaling processes, including neurotransmitter secretion, regulation of membrane excitability, and induction of gene expression[12-15]. Cytoplasmic Ca2+ concentrations are increased by influx of extracellular Ca2+ through plasma membrane Ca2+ channels and by release of Ca2+ from intracellular organelles, especially the endoplasmic reticulum(ER), which serve as Ca2+ stores[16].

Ca2+ channels regulate the cellular functions in nervous system by regulating Ca2+ influx[17]. Several distinct subtypes of neuronal Ca2+ channels(L, N, P/Q, and R) have been identified based on their biophysical and pharmacological properties[18]. These channels consist of four subunits: alpha1, beta, alpha2, and delta; the alpha1 subunit is the pore-forming, voltage-sensing, ligand-binding, and subtype-determining moiety[19]. At least six distinct alpha1 subunits have been cloned for high-voltage-activated Ca2+ channels, encoding alpha1A, alpha1B, alpha1C, alpha1D, alpha1E, and alpha1S phenotypes. Alpha1C, alpha1D, and alpha1S phenotypes encode dihydropyridine-sensitive Ca2+ channels(L-type)[20-21]. Alpha1B encodes omega-conotoxin GVIA-sensitive Ca2+ channels(N-type)[22-23] and alpha1A encodes omega-agatoxin IVA-sensitive Ca2+ channels (P/Q-type)[24-25]. Alpha1E encodes Ca2+ channels resistant to most currently known specific inhibitors (R-type)[26-27]. Four different beta subunits beta1 to beta4 and two different alpha2 subunits serve to regulate assembly and modulate the kinetic parameters of the channels[28-31]. Although Ca2+ signaling[32-33] plays an important role for the regulation of neural cell functions, a little is known with regard to the Ca2+ channel expression and Ca2+ mobilization of neural cells derived *in vitro* from undifferentiated ES cells[34-35].

In this study, we have focused on the Ca2+ channel expression of functional neurons derived from MASH1 transfected ES cells, and thereafter Ca2+ mobilization using Ca2+ sensitive fluorescence probe, fluo-3- acetoxymethyl(AM) and a confocal microscope. Here we found evidence for intracellular Ca2+ increase upon depolarization in the neurons derived from mouse ES cells.

Materials and Methods

1)Cell preparation

The cell source and methods for establishing MASH1 transfected cells has been reported[11]. In brief, the MASH1 cDNA was ligated into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA), which contained a myc-tag, histidine-tag(His-Tag) and G418 selection marker(pMASH1)[11]. Transfection into the undifferentiated ES cells was performed by electroporation(Bio Rad, Hercules, CA), and cells transfected with pMASH1 were selected in medium containing 200 μg/ml G418. After several weeks of growth, stable G418-resistant cells were recovered and maintained in the presence of G418. Transfection with empty vector did not cause morphological changes suggestive of neural cell differentiation, so further characterization was not carried out. This cell population was enriched with Islet1 positive motoneurons[11].

2)RT-PCR

Total RNA extraction, cDNA synthesis and PCR amplification were carried out as previously reported[4,5,11,36]. The primers used were as follows;

β-actin (expected size: 450 bp): sense gatgacgatatcgctgcgctg, antisense gtacgaccagaggcatacagg;

NCAM (203 bp): sense gcaaagcccaaaatcaccta, antisense ttcagggtcaaggaggacac.

Ca2+ channel specific primers;

L-type, alpha1C (473bp) sense tcgtgggtttcgtcattgtca, antisense cctctgcactcatagagggagag;

alpha1D (217bp) sense gagcctcgattagtatagtggaatg, antisense aggatgcagcaacagtccata;

alpha1F (206bp) sense gaagcagcaagatggaagaag, antisense tgtgtggagcgagtagagtg.

P/Q-type, alpha1A (420bp):sense gagcggctggatgacacggaacc, antisense gagctggcgactcaccctggatgtc;

N-type, alpha1B (483bp) sense gaagtagctgaagtcagcc, antisense cttgcgtgtcagcccctgga;

R-type, alpha1E (697bp) sense gagactgtggtgacttttgaggacc,

antisense atagagctatggggcaccatggctt;
T-type, alpha 1G (449bp) sense tacggaggctggagaaaa, antisense gatgatggtggg(a/g)ttgat;
alpha 1H (325bp) sense cgcagactattcacacac, antisense gatgatggtggg(a/g)ttgat;
alpha 1I (364bp), sense ggaaaagaagcgccgtaa, antisense gatgatggtggg(a/g)ttgat[37].

3)Imaging

Intracellular free Ca2+ levels were evaluated using a confocal laser scanning microscope LSM510 (Zeiss, Jena-gottingen, Germany). Briefly, cells were loaded with 1 μM of fluo-3-acetoxymethyl(AM) ester (Dojindo Lab, Kumamoto, Japan) for 30 min at 37°C. Endogenous esterases converted nonfluorescent fluo-3-AM into fluorescent fluo-3. After washing, cells were kept in Ca2+ mobilization buffer containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 5.5 mM D-glucose, 10 mM Hepes (pH 7.3) at room temperature until measurement. Fluo-3 dye was excited with an argon laser at 488 nm and fluorescence emission at $\lambda > 510$ nm was captured every 5 seconds. Nomarski images were recorded simultaneously to avoid focus error. Fluo-3 fluorescence before potassium application was mostly uniform, but often brighter in the center of the cell. In order to depolarize the neurons the following solution was used: 100 mM KCl, 12 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES pH 7.4.

To see the voltage-gated Ca2+ influx, the solution including 100 mM KCl was added onto the cells under investigation and their neighborhood with a large-diameter pipette after recording the base emission intensity. The duration of the application was between 0.8 and 2 seconds. It has been shown under whole-cell current clamp that the application of 100 mM KCl led to a constant membrane potential of -10 mV[38]. We have confirmed the above finding that the MASH1 transfected cells elicited action potential after application of high concentration of KCl, analyzed by patch clamp method (data not shown).

4)Inhibitors

In some experiments Lead Chloride (10 μM)(Sigma, Tokyo, Japan), a Ca2+ channel blocker, was added before KCl stimulation. Ionomycin (4 μM) (Molecular Probes, Eugene, OR) was added at the end of each measurement as a positive control for increase of intracellular Ca2+ level, [Ca2+]i. Addition to the bath of 0.005% saponin in Ca2+ free Ringer's with 10 mM added EGTA rapidly quenched an average of 86% of the whole-cell confocal fluorescence after fluo-3 loading ($n = 4$), indicating a predominantly cytoplasmic localization of the dye[38].

Nifedipine, an L-type Ca2+ channel inhibitor, was obtained from Sigma. Omega conotoxin GVIA, an N-type Ca2+ channel inhibitor, was obtained from Peptide Institute, Osaka, Japan, and was dissolved in Ringer solution.

4-aminopyridine(4-AP), a voltage-sensitive K+ channel inhibitor, and Tetraethylammonium(TEA) Chloride, a non-selective large-conductance Ca-activated and voltage-sensitive K+ channel inhibitor, were from Nacalai Tesque (Kyoto, Japan). Thapsigargin (Sigma), an irreversible inhibitor of sarcoplasmic-endoplasmic reticulum Ca2+ ATPases, was dissolved in 0.02% ethanol. In Ca2+ free solution, CaCl2 was removed.

Results

1)RT-PCR analysis of Ca channel mRNAs expressed on MASH1 transfected cells

MASH1 transfected cells were derived from ES cells transfected with the gene that had been resistant to G418[11,39]. When transplanted into the brain lesion, the cells adapted to the injured brain, restored neural network connection and improved motor functions of the hemiplegic mice.

We thus interested in whether the neural progenitor cells expressed functional Ca2+ channels that were intimately associated with neural cell functions. We examined mRNA expressions of Ca2+ channels by RT-PCR (**Fig.1**). Undifferentiated ES cell expressed mRNAs of N-type, L-type and T-type. MASH1 transfected cells at day 35, which were NCAM positive, expressed mRNAs of N-type, L-type and T-type. Alpha1I (T-type) mRNA has emerged when MASH1 transfected cells were cultured for 35 days.

It was interesting that MASH1 transfected cells 14 days after transfection lacked detectable levels of Ca2+ channel mRNAs, even though they started to express NCAM in some experiments. These results suggested that MASH1 transfected cells expressed mRNAs of neural cell associated Ca2+ channels.

2)Depolarization induced [Ca2+]i in MASH1 cells

We next measured intracellular Ca2+ level, [Ca2+]i, of the MASH1 transfected cells at day 35 by using fluo-3. From the images in **Fig. 2A** it was obvious that fluo-3 was almost equally distributed within the cells. The cells were then imaged again after applying a brief pulse of solution of 100 mM KCl through a micropipette. It has been shown that application of high concentrations of potassium leads to a constant membrane potential of -10 mV when measured under whole-cell current clamp method[38]. We confirmed that the MASH1 transfected cells elicited action potential after application of high concen-

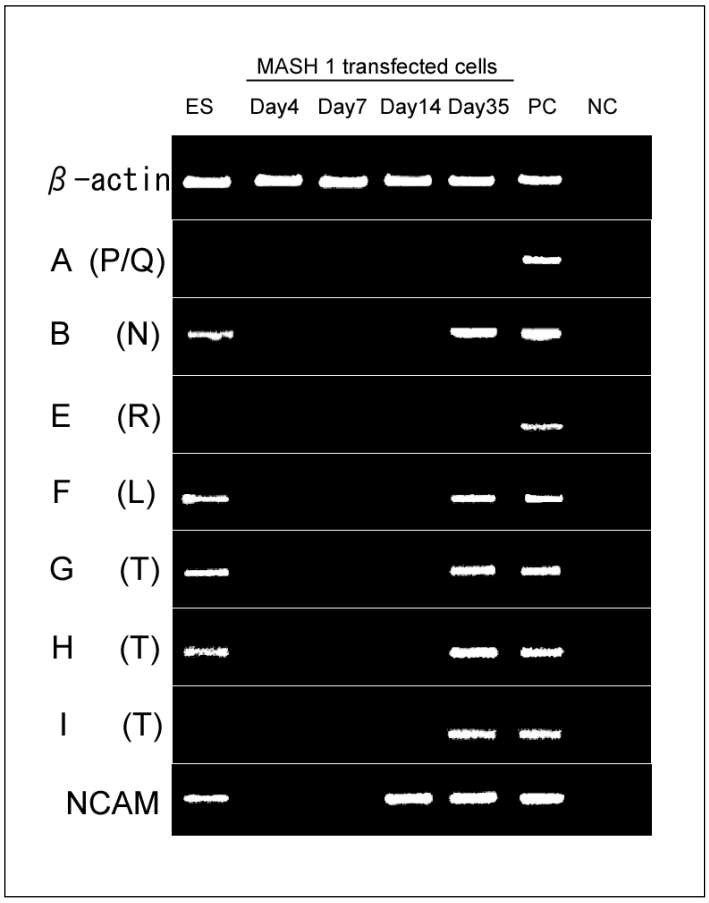

Fig.1 Expression of Ca2+ channel mRNAs in the MASH1 transfected cells by RT-PCR

The neural cells derived from ES cells transfected with MASH1 cDNA at day 4, day 7, day 14 and day 35 were used. Diethylpyrocarbonate (DEPC)-treated water served as negative controls (NC). Adult mouse brain served as positive controls (PC). The MASH1 cells at day 35 expressed mRNAs of N-type, L-type and T-type channels, and NCAM mRNA.

trations of potassium (data not shown). The resulting fluorescence of fluo-3 (**Fig.2B**) was obviously increased. The increase in [Ca2+]i upon depolarization showed a characteristic and well-reproducible pattern. At the end, calcium ionophore ionomycin was introduced for a positive control of Ca2+ influx (**Fig.2C,3A**). When we focused on the cells with angular motor neuron shape (**Fig.2ABC**), the cells responded as well as cells with other shapes to high KCl stimulation. In the absence of extracellular Ca2+, it failed to evoke any change in [Ca2+]i upon depolarization (**Fig.3B**, Ca free). Thus, extracellular Ca2+ is needed to obtain increase in [Ca2+]i upon depolarization in the cells.

To explore whether or not the release of Ca2+ from intracellular stores of Ca2+ is involved in the depolarization induced increase in [Ca2+]i of the MASH1 transfected cells, we examined the effects of thapsigargin, an irreversible inhibitor of sarcoplasmic-endoplasmic reticulum Ca2+-ATPases, on the Ca2+ signals induced by depolarization. Thapsigargin had almost no significant effect on the increase in [Ca2+]i upon depolarization (**Fig.3D**, TG). Treatment of the MASH1 transfected cells with Pb2+ inhibited increase in [Ca2+]i (**Fig.3C**, Pb and **3E**, Pb+TG). Nifedipine moderately suppressed the increase in [Ca2+]i of the cells (**Fig.3F**, Nifedipine). Omega conotoxin + TG seemed to affect marginally the increase in [Ca2+]i of the MASH1 transfected cells (**Fig.3H**, CgTX+TG).

Collectively, with regard to the depolarization induced increase in [Ca2+]i, influx of extracellular Ca2+ through the Ca2+ channels seemed to be more important than that released from intracellular stores in this MASH1 transfected cells.

3)Effects of potassium channel inhibitors, 4-AP and TEA, on depolarization induced [Ca2+]i of the MASH1 transfected cells

We next investigated the contribution of potassium channels to depolarization induced Ca2+ signals of the MASH1 transfected cells. Thus, effects of 4-AP and TEA on the depolarization induced increase in [Ca2+]i of MASH1 transfected cells were studied. Administration of 4-AP and TEA to the cell chamber consistently reduced the peak increase in [Ca2+]i of the MASH1 transfected cells (**Fig.3I**, TEA and **3J**, 4-AP+TEA).

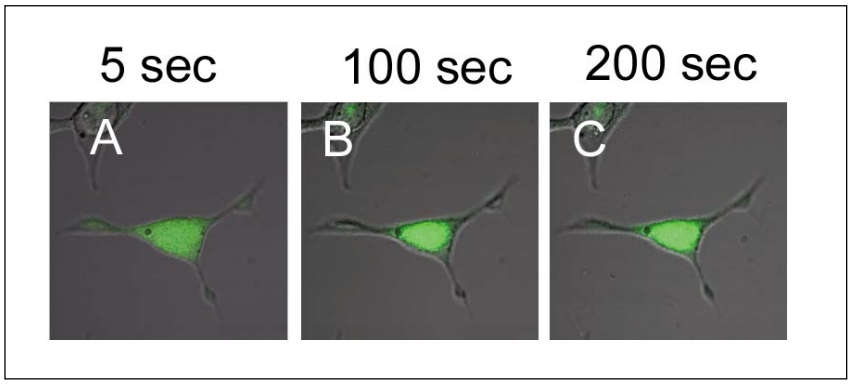

Fig.2 Ca2+ mobilization of MASH1 transfected cells upon KCl induced depolarization

A: Confocal image of MASH1 transfected cells loaded with the Ca2+ indicator dye fluo-3-AM, before depolarization. The basal fluorescence is relatively low and almost homogenous.

B: Stimulation with 100 mM K+ solution induced depolarization of the neurons and elicited increased fluorescence signals.

C: Stimulation with 1 μM ionomycin as a positive control for Ca2+ entry. 5 sec, 100 sec and 200 sec represent time after initiation of confocal microscopic analysis (please refer Fig. 3A, horizontal axis).

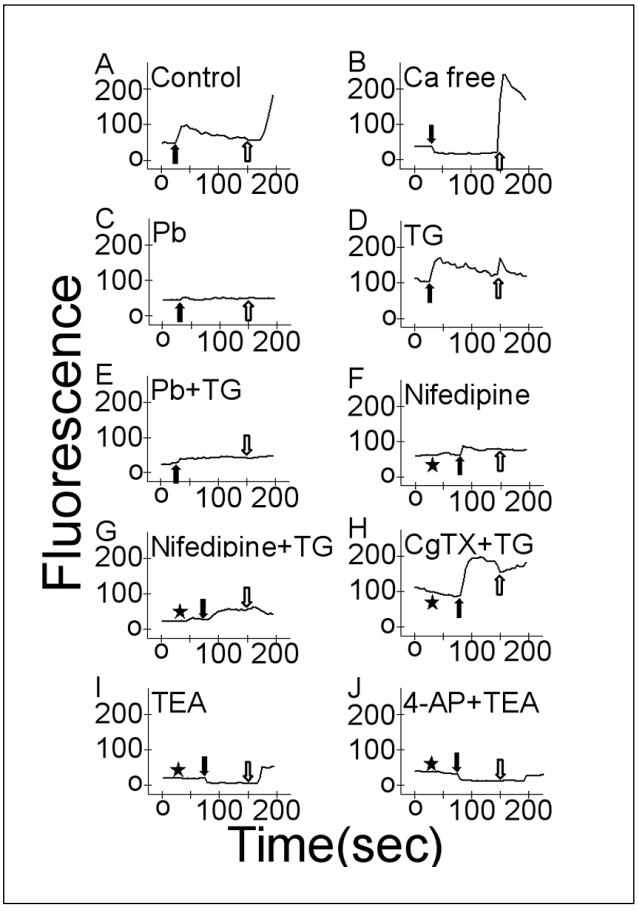

Fig.3 Effects of various inhibitors on Ca2+ mobilization of MASH1 transfected cells upon KCl induced depolarization

A: In the absence of any inhibitor (control), cells were stimulated with introduction of 100mM KCl (indicated by a closed arrow), and then with ionomycin (indicated by an open arrow). Fluorescence intensity of the more than 10 cells was measured, and relative fluorescence intensity of a representative cell was depicted.

B: The cells were stimulated similarly without Ca2+ (Ca free solution).

C: Pb, Lead Chloride 10 μM was added 20 min before KCl stimulation.

D: TG, thapsigargin 1 μM was added 20 min before KCl stimulation.

E: Both Pb and TG were added.

In the presence of various inhibitors (indicated by a star), a high concentration of KCl was added (indicated by a closed arrow). And then, ionomycin was added (indicated by an open arrow).

F: Nifedipine 3 μM.

G: Nifedipine + TG.

H: CgTX, omega-conotoxin GVIA 1 μM + TG.

I: TEA, tetraethylammonium 10 mM.

J: 4-AP, 4-aminopyridine 1 μM.

Discussion

We have previously shown that mouse ES cells transfected with a MASH1 expression vector became apolar, bipolar and multipolar neuron-like appearance and expressed neurofilament middle chain (NFM), β IIItubulin and panNCAM[11]. Half of the MASH1 cells differentiated into Islet1+ neurons. The motor function of hemiplegic mice transplanted with the MASH1 transfected cells gradually improved, and thus, it was suggested that the MASH1 transfected cells were functional *in vivo* as motoneurons. As a next step, we wanted to conduct physiologic characterization of the ES derived neural cells.

In this study, we found expression of T-type, N-type and L-type Ca2+ channels on the MASH1 cells cultured for 35 days after transfection by RT-PCR. The expression was not evident 4,7 and 14 days after transfection, suggesting that T-type, N-type and L-type Ca2+ channel expressions were accompanied by the concomitant differentiation of ES cells into neural lineage. Because the MASH1 transfected cells expressed the Ca2+ channels accompanying simultaneous expression of NCAM, a representative marker of neural cell differentiation[40]. The Ca2+ channels were functionally relevant, because the MASH1 transfected cells showed enhanced fluo-3 fluorescence in response to a high KCl stimulation, and because the increase of [Ca2+]i was reduced when the extracellular Ca2+ was depleted in the solution.

In order to avoid the non-specific effects of Ca2+ channel inhibitors, we first examined roles of extracellular Ca2+ in the Ca2+ signals in response to high KCl. We found that high KCl-induced Ca2+ signals were suppressed by depletion of extracellular Ca2+. Similar dependency of extracellular Ca2+ on an increase in [Ca2+]i was reported in forskolin-induced Ca2+ signals in rat olfactory receptor neurons[41].

We have tested effects of Lead (Pb2+), Nifedipine and Omega conotoxin GVIA as the Ca2+ channel inhibitors on the increase in [Ca2+]i of the MASH1 transfected cells. Lead is a well-known environmental neurotoxicant that causes block of function of voltage-dependent Ca2+ channels[42-43]. Inhibitory effects of acute exposure to Pb2+ on Ca2+ channels have been reported[44-47]. The increase of [Ca2+]i of the MASH1 transfected cells were also sensitive to the addition of Pb2+. Omega conotoxin is a N type Ca2+ channel specific inhibitor. Omega conotoxin + TG seemed to affect marginally the increase in [Ca2+]i of the MASH1 transfected cells, suggesting that N type Ca2+ channels play a minor role for the increase of [Ca2+]i of the MASH1 transfected cells. Nifedipine moderately suppressed the increase in [Ca2+]i of the cells. It is possible that several different types of Ca2+ channels work simultaneously to induce the increase in [Ca2+]i. Nonetheless, a possibility exists that the L-type channels expressed on the MASH1 transfected cells were largely responsible for the increase. Even though Ca2+ influx regulates critical cellular functions in neurons, reports regarding functional roles of Ca2+ influx of the neural cells derived from ES cells were relatively scanty[34-35]. Our present study suggested that rapid increase in [Ca2+]i is associated with the activation of the L-type Ca2+ channels in the MASH1 transfected cells.

In the present study high KCl induced Ca2+ signals were not affected by treatment with thapsigargin. It is suggested that intracellular Ca2+ stores do not play a role in the high KCl induced Ca2+ signals of the MASH1 transfected cells.

Besides other mechanisms, the influx of Ca2+ into embryonic neurons controls growth and differentiation processes. It has been reported that there were remarkable changes of calcium binding proteins and voltage dependent Ca2+ channel subtypes during *in vitro* differentiation of ES cell derived neurons[35]. They reported that ES cell derived neuronal cell maturation proceeded from apolar to bi- and multipolar neurons, expressing all Ca2+ channel subtypes. There was, however, a clear shift in channel contribution to whole cell current from apolar neurons with mainly N-type and L-type channel contribution in favor of P/Q- and R-type participation in bi- and multipolar cells. With regard to our MASH1 transfected cells, majority of which were bipolar cells and had characteristics of motoneurons (Islet1 positive and HB9 positive), they expressed mainly N- type, L-type and T-type channels, almost coinciding with the above report.

Potassium channel blockers inhibited the high potassium induced increase of [Ca2+]i. This finding confirmed that potassium channel was involved in the increase of [Ca2+]i upon high potassium stimulation.

Because forced expression of MASH1 brought about neural differentiation of the undifferentiated ES cells, it is not clear whether Ca2+ channel subtypes expressed on the cells affected subsequent neural differentiation of them.

Based on the present findings obtained in the *in vitro* cell culture, it is reasonable to speculate that the MASH1 transfected cells transplanted into the injured brain may regulate their cellular function as neurons by Ca2+ influx dependent manner mediated by the channels, leading to improved motor functions of hemiplegic mice. The study addressing physiologic characterization of the transplanted ES derived neural cells in the injured brain is awaited.

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