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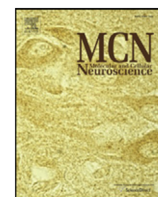

Mesenchymal stem cells enhance GABAergic transmission in co-cultured hippocampal neurons

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A B S T R A C T

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent stem cells endowed with neurotrophic potential combined with immunological properties, making them a promising therapeutic tool for neurodegenerative disorders. However, the mechanisms through which MSCs promote the neurological recovery following injury or inflammation are still largely unknown, although cell replacement and paracrine mechanisms have been hypothesized. In order to find out what are the mechanisms of the trophic action of MSCs, as compared to glial cells, on CNS neurons, we set up a co-culture system where rat MSCs (or cortical astrocytes) were used as a feeding layer for hippocampal neurons without any direct contact between the two cell types. The analysis of hippocampal synaptogenesis, synaptic vesicle recycling and electrical activity show that MSCs were capable to support morphological and functional neuronal differentiation. The proliferation of hippocampal glial cells induced by the release of bioactive substance(s) from MSCs was necessary for neuronal survival. Furthermore, MSCs selectively increased hippocampal GABAergic pre-synapses. This effect was paralleled with a higher expression of the potassium/chloride KCC2 co-transporter and increased frequency and amplitude of mIPSCs and sIPSCs. The enhancement of GABA synapses was impaired by the treatment with K252a, a Trk/neurotrophin receptor blocker, and by TrkB receptor bodies hence suggesting the involvement of BDNF as a mediator of such effects.

The results obtained here indicate that MSC-secreted factors induce glial-dependent neuronal survival and trigger an augmented GABAergic transmission in hippocampal cultures, highlighting a new effect by which MSCs could promote CNS repair. Our results suggest that MSCs may be useful in those neurological disorders characterized by an impairment of excitation versus inhibition balance.

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Introduction

Mesenchymal stem cells (MSCs) are considered a leading candidate for neurological regenerative therapy due to their immunological properties (Marigo and Dazzi, 2011) and neuroprotective features (Kassis et al., 2011), including the capability of releasing neurotrophic factors (Miller et al., 2010), combined with the relative feasibility of isolation from several tissues (Caplan, 2007). Bone marrow MSCs are adult non-hematopoietic cells capable to differentiate to mesodermal lineages (Gregory et al., 2005; Jiang et al., 2003; Reyes and Verfaillie, 2001). In addition, it has been shown that MSCs can trans-differentiate to endothelial, neuronal and glial cells [(Phinney and Prockop, 2007; Ross and Verfaillie, 2008); and refs. therein], although this remains a controversial issue (Miller et al., 2010; Montzka et al., 2009). Is has been hypothesized that the therapeutic efficacy of MSCs is due to their ability to selectively target damaged areas and release a wide array of trophic factors that drive the endogenous cell repair. Moreover, there is growing evidence that MSCs are linked to pericytes, considered as potential mesenchymal stem or progenitor cells (Feng et al., 2010).

The cellular and molecular basis of MSC-induced functional recovery is not well understood. Thus, a better understanding of the mechanisms

Abbreviations: AraC, arabinoside C; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; DIV, day in vitro; ELISA, enzyme-linked immunosorbent assay; GDB, gelatin dilution buffer; GFAP, glial fibrillary acidic protein; MEM, minimum essential medium; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; Syt_{ecto} mAb, monoclonal antibody directed against the intravesicular domain of the synaptic vesicle protein synaptotagmin I; Syp, synaptophysin; vGAT, vesicular GABA transporter; vGlut-1, vesicular glutamate transporter-1.

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underlying MSC-induced trophic effects, including the identification of the mediator(s) driving the neural repair, is a necessary prerequisite to consider the usefulness of MSCs in the cell therapy for neurodegenerative diseases (Karussis et al., 2008; Ross and Verfaillie, 2008; Uccelli et al., 2008). Here we set up a simplified experimental *in vitro* co-culture system allowing to study the mutual interactions occurring between MSCs and hippocampal neurons in comparison with co-cultures of neurons with astrocytes, mimicking the physiological cell-to-cell interaction occurring in the CNS. The experimental system does not enable any direct cell-to-cell contact between neurons and co-cultured MSCs or astrocytes, hence intercellular communication only occurs through substances released by either cell type into the shared culture medium. Our results show that the proliferation of glial cells induced by MSCs is necessary for the morphological and functional differentiation of co-cultured hippocampal neurons. In addition, we show that MSCs enhance the inhibitory neurotransmission *in vitro*, through the release of mediators, including the BDNF neurotrophin, acting in a paracrine manner on GABAergic pre-synapses.

Results

MSCs support the morphological and functional differentiation of co-cultured hippocampal neurons

To evaluate whether MSCs support the *in vitro* differentiation of CNS neurons, 'sandwich' co-cultures were established where neurons from embryonic day 18 (E18) rat hippocampi were grown on top of a feeding monolayer of MSCs isolated from femoral and tibial bone marrows of adult rats (Lennon and Caplan, 2006). MSC stem-like features were characterized as shown in Supplementary Fig. 1 both for MSCs alone and MSCs grown with neurons. As a control, co-cultures between hippocampal neurons and cortical astrocytes, mimicking the 'physiological' brain environment, were established as described (Banker and Goslin, 1998; Kaech and Banker, 2006). In both co-cultures, the two cell types were kept physically separated by paraffin spacers so to avoid any direct cell-to-cell contact (Bartlett and Banker, 1984b). Fig. 1A shows that β-III tubulin-positive hippocampal neurons grown on MSCs acquired a morphological phenotype following a time scale similar to those grown on astrocytes. In fact, 3 DIV neurons exhibited a polarized morphology characterized by the presence of few dendrites and one longer axon (Banker and Goslin, 1998), and 10 DIV neurons formed a dense network making difficult to distinguish the neuritic processes belonging to each individual cell (Bartlett and Banker, 1984b).

To assess whether the morphological differentiation concurred with the acquisition of functional properties, hippocampal neurons grown on MSCs or astrocytes were compared for their ability to undergo exo-endocytotic recycling of synaptic vesicles using an immunocytochemical assay. The analysis was performed by evaluating the internalization of a monoclonal antibody directed against the intra-vesicular domain of the synaptic vesicle protein synaptotagmin I (Matteoli et al., 1992) into the lumen of the synaptic vesicles fusing with the neuronal plasmalemma, following 5 min depolarization with 50 mM KCl (Fig. 1B). The immunofluorescence staining of internalized Syt_{ecto} mAb was measured together with that of synaptophysin, that labels total synaptic vesicles, on cultures at 3, 7 and 10 DIV. The quantitative analysis (Fig. 1C) shows that the internalization of Syt_{ecto} mAb occurred in hippocampal neurons grown on either MSCs or astrocytes at all examined times, although the percentage of recycling synaptic vesicles was significantly less in 3 DIV neurons grown on MSCs ($60.2 \pm 6.6\%$; $p<0.05$; $n=3$), thus suggesting that neurons developed the release machinery slower on MSCs than on astrocytes.

To further compare neuronal activity in the two co-cultures during development, the Ca^{2+} conductance through presynaptic voltage-gated Ca^{2+} channels, namely N- and P/Q-types, was measured over time in culture by using whole-cell patch-clamp in the presence of 5 μmol/L nifedipine to block L-type Ca^{2+} channels. Inward Ca^{2+} currents recorded at 0 mV from voltage-clamped hippocampal neurons displayed a rapid activation and a slow and incomplete inactivation with Ba^{2+} (Fig. 1D), both typical features of functional high voltage-activated (HVA) N, and/or P/Q-type Ca^{2+} channels in hippocampal neurons (Toselli and Taglietti, 1992). Ion currents with qualitatively similar features were elicited at all time points in neurons grown on MSCs or astrocytes, and their amplitudes significantly increased between 3 and 11 DIV. Noteworthy, Ca^{2+} current amplitude was higher in 11 DIV neurons grown on MSCs (Fig. 1D and E; neurons on astrocytes ($n=18$): -1.94 ± 0.34 pA/pF; neurons on MSCs ($n=16$): -4.51 ± 0.68 pA/pF; $p<0.01$), showing that MSCs support the expression of fully functional N- and/or P/Q-like Ca^{2+} channels in co-cultured hippocampal neurons as efficiently as astrocytes, or even better. Furthermore, in a fraction (about 30%) of neurons grown either on MSCs or astrocytes, in addition to a HVA current component, inward currents displayed a low voltage-activating (LVA) and fast inactivating current component (Fig. 1F) with similar features than T-type Ca^{2+} currents already described in hippocampal neurons (Toselli and Taglietti, 1992). As expected, T-type currents became negligible upon step depolarization from a holding potential of −50 instead of −70 mV (data not shown). Fig. 1G shows the averaged Ba^{2+} current density/voltage (I/V) relationships obtained from 11 DIV neurons on astrocytes ($n=13$) and MSCs ($n=21$). Barium currents derived from neurons displaying either HVA currents only, or mixed HVA and LVA currents were pooled together. The shoulder in the voltage range between −60 and −30 mV of I/V relationships accounts for LVA current activation. The average T-type current density measured at −40 mV, a voltage value at which HVA current contamination is still negligible, was -0.44 ± 0.13 pA/pF in neurons on astrocytes, and -0.64 ± 0.15 pA/pF in neurons on MSCs. These results show that neurons grown on MSCs or astrocytes not only express N- and/or P/Q- but also T-type Ca^{2+} channels, hence making co-cultured hippocampal neurons potentially capable to generate Ca^{2+} spikes upon stimulation.

Altogether our data suggest that MSCs sustain both morphological and functional *in vitro* differentiation of hippocampal neurons.

MSCs stimulate astrocytic proliferation to guarantee neuronal survival

Double immunofluorescence staining of hippocampal cultures using antibodies directed against the neuron-specific β-III tubulin and the glial fibrillary acidic protein (GFAP) to specifically label astrocytes, showed the presence of a low percentage of 'contaminating' glial cells in both types of co-cultures already at 3DIV, with a slightly higher percentage of the ratio between GFAP-positive and DAPI-positive cells in MSC vs. astrocyte co-culture (15 vs. 7%, respectively). Interestingly, 7 and 10 DIV hippocampal cultures on MSCs maintained a higher percentage of GFAP-positive cells than those on astrocytes (respectively 7 DIV = 30 vs. 15%; 10 DIV = 34 vs. 27; Fig. 2A). The glial increment was also achieved when neurons were maintained in the presence of MSC-conditioned medium vs. astrocyte-conditioned medium, (data not shown). The enhanced astrocytic proliferation was confirmed by western immunoblotting analysis of GFAP expression in hippocampal extracts from both co-cultures (Fig. 2B). In order to directly estimate the MSC-induced glial proliferation, co-cultures of hippocampal astrocytes and MSCs were set up to perform two different spectrophotometric assays of cell growth, based on the cellular conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, and on crystal violet incorporation, both showing about a double increment of hippocampal glial density induced by MSCs, as reported in Supplementary Fig. 2.

The increased density of astrocytes proved to be essential for the MSC-promoted survival of hippocampal neurons since four-day *in*

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