Permeability properties of a three-cell type *in vitro* model of blood-brain barrier

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Abstract

We previously found that RBE4.B brain capillary endothelial cells (BCECs) form a layer with blood-brain barrier (BBB) properties if co-cultured with neurons for at least one week. As astrocytes are known to modulate BBB functions, we further set a culture system that included RBE4.B BCECs, neurons and astrocytes. In order to test formation of BBB, we measured the amount of ^{3}H-sucrose able to cross the BCEC layer in this three-cell type model of BBB. Herein we report that both neurons and astrocytes induce a decrease in the permeability of the BCEC layer to sucrose. These effects are synergic as if BCECs are cultured with both neurons and astrocytes for 5 days, permeability to sucrose decreases even more. By Western analysis, we also found that, in addition to the canonical 60 kDa occludin, anti-occludin antibodies recognize a smaller protein of 48 kDa which accumulates during rat brain development. Interestingly this latter protein is present at higher amounts in endothelial cells cultured in the presence of both astrocytes and neurons, that is in those conditions in which sucrose permeation studies indicate formation of BBB.

Keywords: blood-brain barrier • cortical neurons • astrocytes • brain capillary endothelial cells • RBE4.B • occludin

Introduction

Blood-brain barrier (BBB) is a selective and dynamic structure, formed by endothelial cells, that allows the selective passage of substances from blood to CNS and vice versa. During brain development, brain capillary endothelial cells (BCECs) gradually acquire the ability to form a selective barrier, and it is known that astrocytes [1] as well as neurons [2] play an inductive role in this process. BCECs are joined together by intercellular tight junctions (TJs) that are responsible for acquisition

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of selective permeability. This latter property is specific of brain TJs and probably depends on the higher density of proteins such as occludin and ZO-1 [3, 4] in brain TJs than in TJs of other tissues. An additional level of regulation seems to rise from variability of occludin. Occludin isoforms, deriving from alternative RNA splicing [5, 6], as well as alternative occludin promotors [6] have been indeed described [for recent review, see: 7]. Alterations of BBB structure and function correlate with neurodegeneration [8] and cancer [9–11]. On the other hand, the existence of a functional BBB challenges treatment of neurodegeneration because drugs have to cross BBB to gain access to brain.

Given the importance of understanding the molecular mechanisms underlying genesis and maintenance of BBB, many authors attempted to set *in vitro* models based on cocultures of brain cells [reviewed in: 12]. Most *in vitro* systems are two-cell type cocultures, including astrocytes and BCECs [1, 13, 14] or, sometimes, BCECs and neurons [2, 15].

We previously described a more complete, three-cell type syngenic *in vitro* model, built up with rat BCECs [RBE4.B cells: 16], astrocytes and neurons [17]. Herein we report that both neurons and astrocytes induce a decrease in the permeability of the BCEC layer to sucrose. These effects are synergic as BCECs cultured with both cell types show lower permeability to sucrose respect to cells cultured with either neurons or astrocytes alone.

By Western analysis, we also identified a protein of about 48 kDa that is recognized by anti-occludin antibodies and accumulates during rat brain development. This protein is present at higher amounts in endothelial cells cocultured with both astrocytes and neurons, where sucrose permeation studies indicate formation of BBB.

Materials and methods

Animals

Wistar rats (Stefano Morini, San Polo d'Enza, Italy) were housed in our institutional care facility under direction of a licensed veterinary. Procedures involving animals were conducted according to the European Community Concil Directory 86/609, OJL 358 1, 12 December 1987.

Cell cultures and establishment of the three-cell type culture system

RBE4.B immortalized rat brain microvessel endothelial cells (BCECs) were a kind gift of Dr. F. Roux [16], under the permission of Neurotech SA (Orsay, France). Cortical neurons, astrocytes and RBE4.B BCECs were cultured as previously described [17]. Briefly, neurons were purified from rat brain cortex at the 16th day of gestation and cultured at the bottom of laminin-coated (2.5 µg/cm^2) wells, in the serum-free Maat Medium (MM) [18]. Astrocytes were purified from brain cortices of two-day old rats, as described by Cole and de Vellis [19], with the modifications reported by Schiera *et al.* [17], and cultured in DME:Ham's F12 (2:1), supplemented with 10% heat-inactivated fetal calf serum. Astrocytes to be used for the three-cell type system were then progressively adapted to MM, and finally plated on the outside of fibronectin-coated (2.5 µg/ml) inserts (translucent, high density type, 23 mm diameter, 0.4 µ pore size, Falcon). After 4 hours, the inserts were placed into the wells where neurons had been already cultured for at least two days. RBE4.B BCECs were plated on collagen I (6 µg/ml) and maintained in DME:Ham's F12 (2:1), supplemented with 10% fetal calf serum. Cells were then progressively adapted to MM and finally plated inside the same inserts, previously coated with collagen IV (3.5 µg/ml), on which astrocytes had been plated two days before. As controls, RBE4.B BCECs were also cultured with either neurons or astrocytes alone and without any other brain cell type. All control- and three-cell type- cultures were fed for 3-5 days with daily changes of MM, in the insert chamber, before running permeability tests or harvesting cells for protein analyses.

Sucrose permeation studies

[^{3}H]Sucrose (Amersham, Cologno Monzese, Italy; specific activity : 6.00 Ci/mmol) was diluted in MM, at a final concentration of 0.5 µCi/ml. An aliquot of this
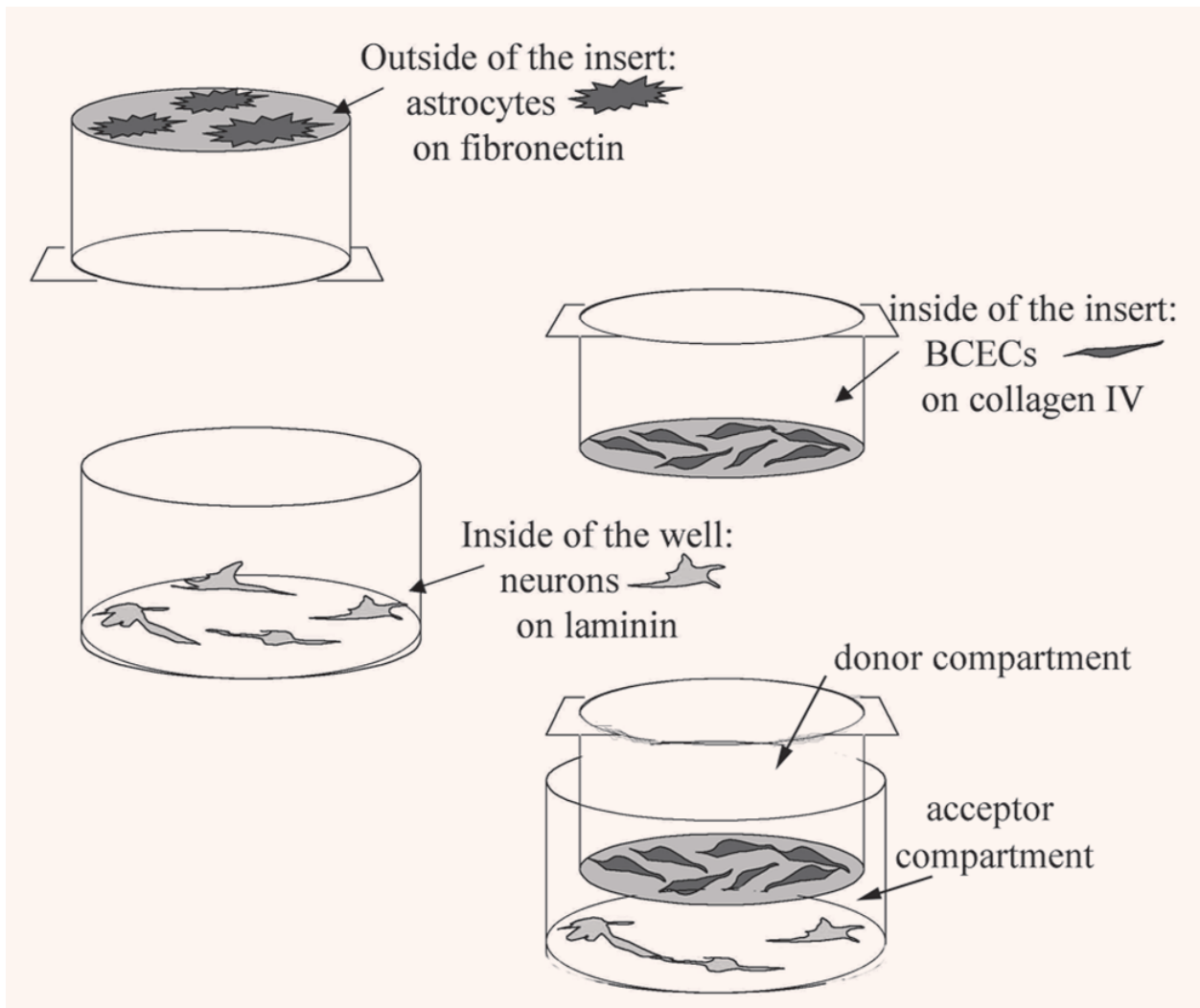
Fig. 1 Schematic drawing of the three-cell type- coculture system. Neurons are cultured at the bottom of the well, also called acceptor compartment. Astrocytes are cultured on the external surface of the inserts and face neurons in the acceptor compartment. BCECs are cultured inside the inserts and face the donor compartment. See the main text for more details.

solution (about 50 μCi of [^{3}H]Sucrose) was then added to each insert (*i.e.* the donor compartment). At different times (30, 60, 120, 240 and 360 minutes) after sucrose addition, 100 μl of medium were collected from both the donor and the acceptor compartment and counted in a liquid scintillation system by using an LS6500 Multipurpose scintillation counter (Beckman Counter TM, USA). [^{3}H] counts/100 μl were used to calculate total counts in the donor- and acceptor- compartment, respectively, as well as the total counts in the whole system (donor plus acceptor compartment). The percent of [^{3}H] counts found in the donor compartment is indicated as "percent of crossing sucrose" throughout the paper.
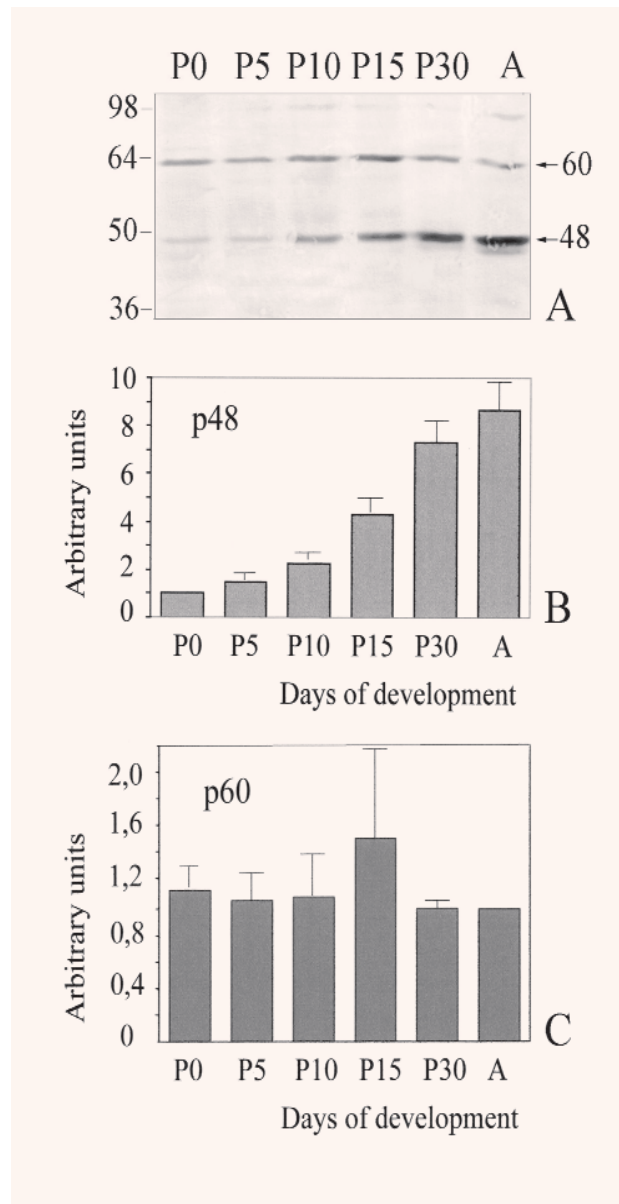
Western analyses

Brain cortices from developing or adult rats were homogenized in homogenization buffer (0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl, 0.5 mM spermine; 0.15 mM spermidine; 2 mM EDTA, and 0.15 mM EGTA), containing the protease inhibitors aprotinin (2 μg/ml), antipain (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), benzamidine (1.0 mM), and phenylmethylsulfonyl fluoride (1.0 mM), all purchased from Sigma. Proteins (20 μg of total cell extracts) were separated by electrophoresis on denaturing 10% polyacrylamide slab gels (SDS-PAGE) and immunoblotted as described
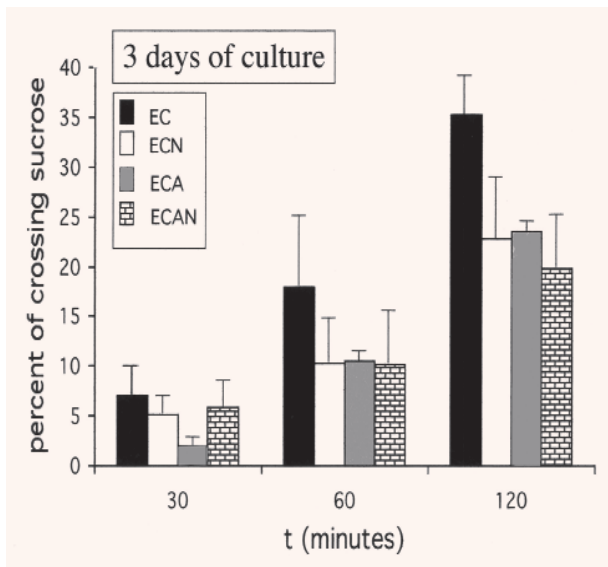
Fig. 2 Percent of [3H]Sucrose that crosses a layer of BCECs cultured for 3 days in serum-free Maat Medium. BCECs were cultured alone (EC) or in the presence of astrocytes (ECA), neurons (ECN) or both cell types (ECAN), for different periods of time (30, 60 and 120 min). Bars indicate the average of at least 3 independent experiments. Standard deviations are indicated.
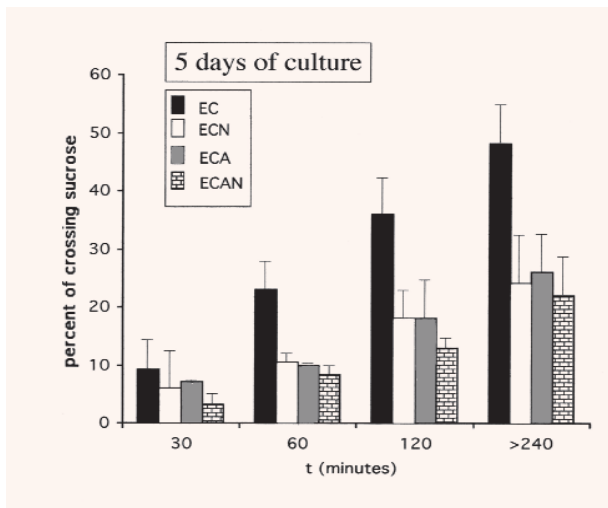
Fig. 3 Percent of [3H]Sucrose that crosses a layer of BCECs cultured for 5 days in serum-free Maat Medium. BCECs were cultured alone (EC) or in the presence of astrocytes (ECA), neurons (ECN) or both cell types (ECAN), for different periods of time (30, 60, 120 and >240 min). Bars indicate the average of at least 3 independent experiments. Standard deviations are indicated.

Fig. 4 Immunostaining with anti-occludin antibodies of total proteins from developing rat brain. Protein extracts (20 μg) were analyzed by Western blot as described under "Materials and Methods". One of these analyses is shown in A. Graphic representation of statistical analysis of data from three different experiments is reported in B (for p48) and C (for p60).
P, postnatal day of development; A, adult brain

elsewhere [2], by using rabbit anti-human occludin antibodies (Santa Cruz, California, USA) directed against an internal region of human occludin (aminoacids 132–411).

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Statistical analyses

As explained above, counts per minute of [^{3}H]Sucrose in both the acceptor and donor compartments were used to calculate percent of sucrose that crossed the RBE4.B BCEC layer. Mean values and standard deviations were calculated from at least three different experiments.

Western blots were scanned and planimetries of scans were used to calculate, by IMAGEJ program, the relative abundance of occludin; values were normalized with respect to the total amount of proteins in each lane. Mean values and standard deviations were calculated from 4 different experiments.

Statistical significance of the differences found in both sucrose permeability assays and western analyses was evaluated by Student t test and calculation of the p value.

Results

Sucrose permeation studies

RBE4.B BCECs, astrocytes and neurons were purified and cultured as described under "Materials and Methods". The final culture system was built up with: i) neurons on laminin, at the bottom of wells, ii) astrocytes, on fibronectin, on the outside of transwell inserts, and iii) RBE4.B BCECs, on collagen IV, on the inside of the inserts. All the cells were fed with serum-free Maat Medium. The complete system was schematically drawn in Fig. 1.

After either 3 or 5 days of culture, [^{3}H]Sucrose was added to the donor compartment and its concentration in both the donor- and acceptor- compartments was measured after different periods of time (30-360 minutes).

We found that RBE.4B BCECs cultured for 3 days with neurons and/or astrocytes (Fig. 2) formed a better barrier than control cells: the percent of crossing sucrose was significantly lower ($p < 0.05$) in all the cocultures with respect to RBE4.B BCECs cultured without other brain cells. On the other hand, no significant difference was noticed that could depend on which brain cell types (neurons, astrocytes or both of them) were used in the coculture system.

When RBE4.B BCECs were cultured with other brain cells for at least 5 days (Fig. 3), the percent of crossing sucrose was always much lower than in the
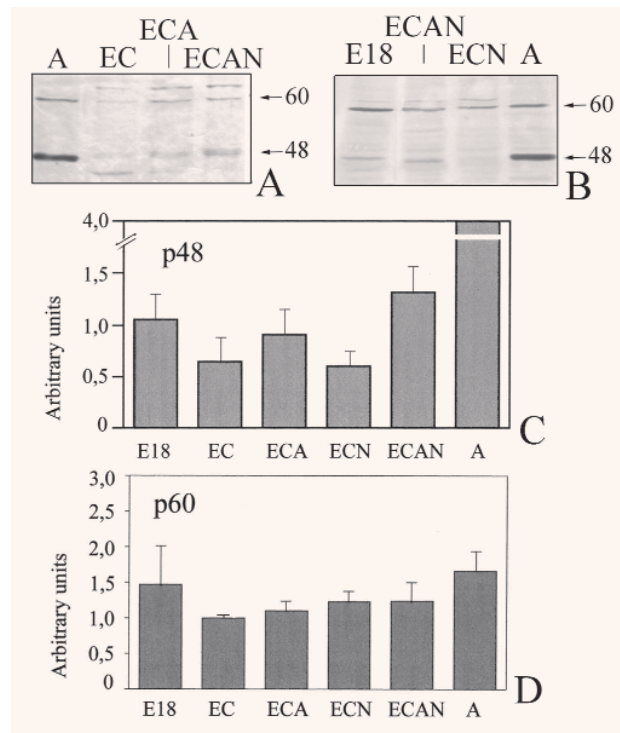
Fig. 5 Immunostaining with anti-occludin antibodies of total proteins from BCECs cultured for 5 days in serum-free Maat Medium. BCECs were cultured alone (EC) or in the presence of astrocytes (ECA), neurons (ECN) or both cell types (ECAN). Two representative western analyses are shown in panels A and B. Graphic representations of the statistical analysis of data from four different experiments are reported in C (for p48) and D (for p60). E18, cell extract from rat brain at the 18th day of fetal development; A, adult rat brain.

case of BCECs cultured alone: *i.e.* about one half, if cocultures included either neurons or astrocytes ($p<0.05$) or about one third, if cocultures included both astrocytes and neurons ($p<0.005$).

Expression of putative occludin in developing rat brain and RBE4.B BCECs cultered alone or with other rat brain cells

As shown in Fig.4, the rabbit anti-human occludin antibodies (Santa Cruz, California, USA), directed against an internal region of human occludin (aminoacids 132-411), recognized as expected a protein of about 60 kDa (p60, Fig. 4A), the concentration of which increases from birth to postnatal day 15 (P15) (Fig. 4C). These antibodies also

immunostain a protein of about 48 kDa (p48, Fig. 4A) whose concentration increases during development even more than that of p60 (Fig. 4B). Moreover, p48, but not p60 (Fig. 5), is enriched in RBE4.B BCECs cultured with both astrocytes and neurons for 5 days, that is in those conditions in which the BCEC layer shows maximal effieciency as a barrier to sucrose passage.

Discussion

Many authors attempted to set *in vitro* models that make use of cocultures of brain cells [reviewed in: 12]. These *in vitro* systems are for most two-cell type cocultures, including astrocytes and BCECs [1, 13, 14] or, sometimes, BCECs and neurons [2, 15]; as a consequence, these models are too simple respect to the *in vivo* situation and are not likely to reproduce many of the cell-cell interactions that control BBB formation and maintenance in the mammalian brain. Moreover, some of these *in vitro* systems rely on endothelial cells that do not belong to the same animal species of the other brain cells used in the system.

We previously described a three-cell type *in vitro* model that includes rat BCECs [RBE4.B BCECs: 16], astrocytes and neurons [17]. Those preliminary experiments had shown that, in the presence of astrocytes, the neuron-induced synthesis and peripheral localization of occludin is precocious (5 days of culture) as compared with cells cultured with neurons only (7 days of culture). That observation suggested an earlier formation of model BBB in the three-cell type system. If this conclusion is correct, BCECs cultured alone or with neurons and/or astrocytes should show different ability to delay the paracellular sucrose passage from the donor to the acceptor compartment in the transwell modified system. In the present study we indeed report that both astrocytes and neurons are independently able to reduce the paracellular passage of sucrose. The effects of the two cell types are additive: when BCECs are cocultured with both astrocytes and neurons, the paracellular flux is reduced to one third. However, the synergic effect is visible only after 5 days of coculture: after three days, indeed, any combination of brain cells added to BCECs induce a similar reduction of the sucrose passage. Interestingly, if we compare histograms of figures 2 and 3, two conclusions could be drawn: i) on average, while the percent of sucrose that crosses the BCEC layer after, for example, 120 minutes is the same at 3 and 5 days of culture, the percent of crossing sucrose is significantly (p<0.01) lower after 5 days of coculture in all the coculture conditions; ii) this effect is more evident for cocultures containing both astrocytes and neurons. These observations suggest that either the quality or/and the intensity of the effects of astrocytes and neurons on BCECs depend on how long the cells are cultured together.

We also found that rabbit anti-human occludin antibodies directed against an internal region of human occludin (aminoacids 132-411), recognized two different proteins, only one of which (p60) had been expected. The second protein (p48) was never described before at our knowledge. This protein increases during development even more than p60 and, more interestingly, is enriched in BCECs cultured with both astrocytes and neurons for 5 days, that is in those conditions in which the BCEC layer shows maximal effieciency as a barrier to sucrose passage. Although it couldn't be excluded that it derives from occludin degradation, its abundance together with the fact that the concentration of p48 changes significantly during development (Fig. 4) and in different culture conditions (Fig. 5), in relation with establishment of a functional barrier, suggests that it has a role to play in this latter event. From this point of view, it has to be underlined that p48 concentration correlates with formation of a barrier much better than that of p60 itself, the concentration of which doesn't show significant differences neither during development nor in BCECs cultured in different conditions.

A further comment concerns the fact that, in our culture system, both neurons and astrocytes exert an effect on BCECs in the absence of cell-cell contacts. This observation suggest that soluble factors are involved. Experiments are now in progress to clarify the molecular mechanisms underlying the reciprocal effects of the three brain cell types.

In conclusion, we describe a novel three-cell type system that could represent a powerful system for studying the molecular mechanisms responsible for BBB formation and maintenance as well as BBB damage in neurodegenerative diseases such as multiple sclerosis. This system could be also quite useful for investigating the ability of a variety of

prodrugs to cross BBB and to gain a functional structure on the trans side of the barrier.

Aknowledgements

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