

DEVELOPING *in vitro* blood–brain barrier (BBB) models that closely mimic the natural state is important for theoretical and practical applications, including drug development. We previously developed an *in vitro* BBB model based on co-culturing endothelial cells with glia in the presence of flow on hollow fiber tube culture substrates. We now report that this dynamic *in vitro* BBB (DIV-BBB) can be successfully used to co-culture differentiated serotonergic neurons in the presence of a BBB. These neurons demonstrated fluoxetine-sensitive serotonin (5HT) uptake and depolarization-induced release of [<sup>3</sup>H]5HT. Our results demonstrate that the DIV-BBB is a suitable model for culturing of neurons in a quasi-physiological microenvironment and in the presence of a high-resistance, stereoselective BBB. *NeuroReport* 10:3725–3731 © 1999 Lippincott Williams & Wilkins.

## A new model of the blood–brain barrier: co-culture of neuronal, endothelial and glial cells under dynamic conditions

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### Introduction

The mammalian blood–brain barrier (BBB) comprises microvascular endothelial cells (EC) that acquire specialized properties upon exposure to yet unknown factors present in the CNS, and presumably secreted by neighboring glia [1]. Among the crucial properties of BBB endothelia is the capacity to act as an impenetrable barrier isolating the brain from systemic influences, while simultaneously constituting pathways for transport of nourishment to neurons and clearance of potentially toxic substances from the brain [2]. Thus, the BBB does not constitute a passive cellular layer with exclusion properties, but rather consists of a specialized multicellular tissue with complex physiological properties. Modern understanding of many neurological disorders has pinpointed the failure of brain endothelial mechanisms in the etiology of a variety of CNS disorders [3], warranting additional insight into BBB function in health and disease.

We have learned more about brain microvascular endothelial cell physiology from *in vitro* experiments than by direct observations *in situ*, which is almost impossible with today's techniques [3,4]. Some of the salient properties of EC in culture (e.g.

tight junctions) are greatly enhanced by exposure to glial factors or glia themselves [5]. Other phenotypic aspects of the BBB have been more difficult to reproduce *in vitro*, since low trans-endothelial electrical resistance (TEER) values and abnormally high permeability characterized both monoculture and co-culture BBB models [3,6–8]. Surprisingly, it was not until recently that emphasis was placed on the possibility that flow may constitute an important factor regulating gene expression and physiology of the BBB in addition to glial influences [9].

EC *in vivo* are exposed to shear stress generated by the flow of blood across their apical surfaces. Both short- and long-term changes occur in cerebral arterioles in response to intraluminal flow [9–11]. Stanness *et al.* have recently developed a new dynamic *in vitro* model of the BBB (DIV-BBB) characterized by a tridimensional, pronectin-coated hollow fiber structure that enables co-culturing of EC with glia [12–15]. In the hollow fiber apparatus, EC are seeded intraluminally and exposed to flow. Under these conditions, EC develop a morphology that closely resembles the endothelial phenotype *in situ* [9]. In the presence of glia, EC develop a BBB-specific phenotype including low permeability to potassium, negligible extravasation of proteins, and

the expression of a glucose transporter and induce the expression of BBB-specific ion channels and stereoselective transporters [12,14,15].

One of the limitations of all existing *in vitro* models of the BBB is the lack of neuronal targets in the abluminal compartment of the *in vitro* model itself (see [16]). In spite of the wealth of information suggesting that normal brain astrocytes participate in the induction and maintenance of the BBB, it is still not known whether neuronal elements favor, or disrupt, BBB formation. Direct contacts between neurons and brain capillaries have been described recently in human brain [17], but the functional significance of these contacts is presently unknown. At least two possibilities exist: BBB-neuronal contacts are part of mechanisms of neurovascular regulation of cerebral blood flow, or these cell-cell interactions modulate BBB function. The latter issue is difficult, if not impossible, to study *in situ* due to the challenge of dissecting neuronal *vs* glial effects. We thus have decided to investigate these complex interactions in a well controlled BBB-like environment, and took advantage of the existing DIV-BBB model to directly assess the feasibility of neuronal-glial-endothelial co-culture.

We have used a rodent DIV-BBB model (rat brain EC co-cultured with primary rat neocortical astrocytic cultures [18]) with B14 cells (an immortalized rat neuronal cell line that can be differentiated into a serotonergic phenotype) added to the abluminal glial surface to investigate whether the presence of neurons affects the maintenance of the BBB. This neuronal-glial-endothelial culture system allowed us to assess neuronal function by classic neurochemical methods and test the integrity of the BBB from both intraluminal and abluminal surfaces.

## Materials and Methods

*Isolation and culture of rat brain microvascular cells and rat neocortical astrocytes:* Rat brain microvascular EC (RBMEC) and rat brain astrocytes (RBA) were isolated from rat brain and the primary cells were cultured as described previously [15,18,19]. RN46A cells were developed by immortalizing rat embryonic raphe neurons with a temperature sensitive SV40 retrovirus. The B14 cell line was derived from RN46A cells by stable transfection with the gene for BDNF, and was obtained from Scott Whittemore (University of Miami) [20,21]. These cells secrete BDNF enhancing their survival and serotonergic differentiation. Cells were grown as described previously [20,21]. The temperature was changed to 39°C to induce B14 biochemical and morphological differentiation into a serotonergic phenotype (Fig. 1B). Cells were differentiated for

7–9 days. By day 4, cells began to sprout neuritic processes. To induce cells to fully differentiate biochemically they were depolarized with KCl (40 mM) between days 5 and 7. By day 7 cells were fully differentiated morphologically (Fig. 1B).

*Hollow fiber apparatus:* Cells were co-cultured using 150 µm diameter hollow fiber tubes (capillary vessels) inside a sealed chamber (extracapillary space, ecs) accessible by ports (CELLMAX QUAD; see [4,12–15]). RBMEC were seeded intraluminally and allowed to establish themselves for 0–11 days before astrocytes were introduced into the ecs surrounding the capillaries. Undifferentiated B14 cells were loaded into the ecs surrounding the capillaries 20–30 days after RBMEC were seeded ( $\sim 2 \times 10^6$  cells). Cells were allowed to grow for 2 days at 33°C (B14 permissive growth temperature). The medium was changed to differentiation medium and the temperature raised to 39°C.

*Morphology and immunocytochemistry:* For microscopic examination, cells were fixed by intracapillary perfusion and treated as described [15]. Sections were incubated with antibodies to GFAP (1:200), neurofilament (1:1000), or serotonin transporter (SERT, 1:1000, a gift from Dr Mark Brownfield, University of Wisconsin), incubated with a Cy3-conjugated secondary antibody for 1 h at 37°C, washed  $3 \times 10$  min in PBS–0.03% Triton-X100, rinsed in deionized water and coverslipped.

*[<sup>3</sup>H]5HT uptake:* Uptake was measured in the DIV-BBB by replacing medium in the ecs with medium containing 5 µCi [<sup>3</sup>H]5HT and 10 µM pargyline. Cells were allowed to take up label for  $\sim 1$  h at 37°C in the absence of flow and the labeled medium was replaced with unlabeled medium three times; the sum of the counts contained in these samples represented the [<sup>3</sup>H]5HT not undergoing cellular uptake (5HT lost in Fig. 3). After each wash, a baseline sample from the ecs was taken: [<sup>3</sup>H]5HT present in the last of these samples constituted the basal pre-depolarization value. Specific uptake was defined as  $\mu\text{Ci}_{\text{added}} - \mu\text{Ci}_{\text{lost}}$ . The flow rate was adjusted to 4 ml/min; the ecs was challenged with 18 mM KCl and samples taken after appropriate intervals (see Fig. 4). Fluoxetine (5 µM) was added after the labeling and 10 min before KCl challenge. Statistical comparisons were made using two tailed Student's *t*-test.

*Permeability measurements:* A known concentration of the tracer under investigation ([<sup>14</sup>C]sucrose, [<sup>3</sup>H]D-Asp or [<sup>3</sup>H]L-Asp) was dissolved directly into the media bottle and perfused intraluminally at

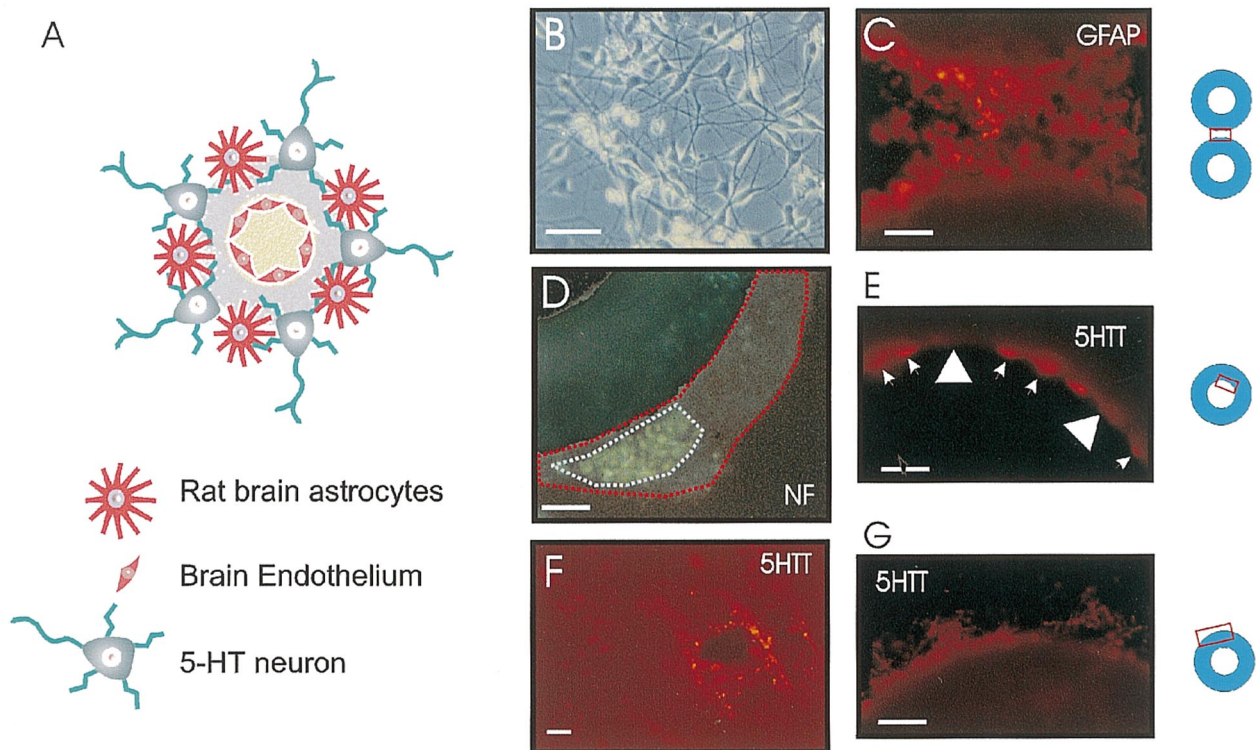


FIG. 1. Morphological appearance and immunocytochemical properties of neuronal, glial and EC in the DIV-BBB. (A) Schematic representation of the cellular partitioning used. RBMEC were seeded intraluminally and, owing to the narrow porosity of the hollow fibers ( $\sim 0.5\ \mu\text{m}$ ), cellular migration from and to the ecs was obstructed. Glia and neuroblasts were seeded abluminally. (B) Morphological appearance of B14 cells after full differentiation in monolayer culture as described previously [21]. Cells were grown for 4 days at  $33^\circ\text{C}$ , and then changed to differentiation growth conditions as described in Materials and Methods. Note the neuronal appearance of the cells, and the extensive neurite outgrowth. (C) Astrocytes grown in the abluminal compartment of the DIV-BBB apparatus stained intensely for the astrocyte-specific marker GFAP. In this micrograph, two contiguous capillaries are shown, as indicated in the schematic drawing (bar =  $40\ \mu\text{m}$ ). (D) Immunoreactivity for 68 kDa neurofilament protein (NF) revealed that clusters of neurons (white dotted line) were growing within glial layers (negative stain, red outline in the figure). (E–G) SERT immunoreactivity was present in intraluminally grown EC (E) as well as in clusters of neurons (G), and was not diffusely localized as was the case for GFAP immunostaining to astrocytes. The micrograph in F shows the pattern of immunoreactivity for the SERT determined in a coronal section of the rat neocortex. Note the dotted appearance of immunosignal in proximity to the inner, endothelial layer of a large penetrating pial vessel. Similarly, immunoreactivity was observed in most EC in the DIV-BBB (open arrows in E), but a small percentage of cells were immunonegative (larger arrowheads). Neuronal cells grown in the abluminal compartment stained robustly for SERT immunoreactivity, confirming their serotonergic nature; immunostaining was only apparent in the abluminal compartment when B14 cells were included.

a rate of  $4\ \text{ml}/\text{min}$ . Samples were taken from the ecs or the lumen as described [15]. Permeability of the tracer under study was determined by measuring the radioactive content of a sample removed from the ecs or lumen media and counting by liquid scintillation at 30% efficiency. The permeability/surface product was calculated as previously described [15]. Statistical comparisons were made using two tailed Student's *t*-test.

## Results

*Immunocytochemical characterizations of endothelial, glial and neuronal cells in the DIV-BBB:* A schematic diagram of the cellular configuration used to load the DIV-BBB is shown in Fig. 1A. Figure 1B shows the morphological appearance (phase contrast) of differentiated B14 cells following exposure to differentiation media and depolarization in monolayer culture. Figure 1B–G shows cell growth in the

DIV-BBB following prolonged culturing of neurons, glia and RBMEC. Robust growth of GFAP positive (GFAP<sup>+</sup>) astrocytes was observed (Fig. 1C), and neuronal neurofilament positive (NF<sup>+</sup>) cell clusters developed within glial elements (Fig. 1D); the area comprised by the white dotted lines represent NF immunoreactivity, while glia growth is outlined in red.

B14 cells express plasma membrane SERT. In the DIV-BBB, expression of SERT immunoreactivity (Fig. 1E–G) was not confined to differentiated 5HT neurons but was also found in RBMEC grown intraluminally. Note that not all RBMEC were positive for SERT-like immunoreactivity (the larger arrowheads indicate a weakly stained EC and a cell devoid of SERT immunoreactivity). To assess whether expression of SERT molecules in RBMEC constituted a cell culture artifact, rat brain sections were immunostained with the same antibody (Fig. 1F); diffuse immunoreactivity delineated a

blood vessel in the sensory cortex suggesting that SERT-like immunoreactivity may also be associated with EC *in situ* and not only in DIV-BBB culture conditions.

**Induction of BBB properties:** Permeability to various radioactive tracers was determined in the tricultural cultures as described previously [15]. The membrane-impermeant compounds [ $^{14}\text{C}$ ]sucrose and [ $^3\text{H}$ ]D-Asp permeated across the neuron-containing DIV-BBB reluctantly (Fig. 2A), as expected if intact BBB properties could be maintained. [ $^3\text{H}$ ]L-Asp traversed the endothelial barrier at a significantly higher rate than the D-stereoisomer (Fig. 2B–D). This significant difference in permeability between aspartate stereoisomers confirmed the quasi-physiological permeability levels for [ $^{14}\text{C}$ ]sucrose (Fig. 2D;  $n = 4$ ).

**Uptake and release of serotonin in the DIV-BBB:** To promote uptake of radiolabeled 5HT by B14 cells,  $5\ \mu\text{Ci}$  [ $^3\text{H}$ ]5HT was added to the ecs. The effectiveness of this procedure was determined by quantifying the amount of radioactivity that was recovered upon wash-out of the extracellular media. As shown in Fig. 3, robust uptake of [ $^3\text{H}$ ]5HT was achieved. On average,  $31\ 527 \pm 1330$  c.p.m. of [ $^3\text{H}$ ]5HT were added to the ecs and  $1828 \pm 151$  were recovered during washout, giving a total uptake efficiency of  $94.2 \pm 0.3\%$  ( $n = 6$ ;  $p < 0.01$ ).

Neuronal cells respond to depolarizing stimuli with release of neurotransmitter. KCl (18 mM) was applied to the ecs, and media were removed and assayed for released [ $^3\text{H}$ ]5HT (Fig. 4A). After a delay of 5–10 s basal levels of [ $^3\text{H}$ ]5HT increased to a plateau level that lasted for several hundred seconds. When a similar procedure was performed in a cartridge containing EC and glia but no

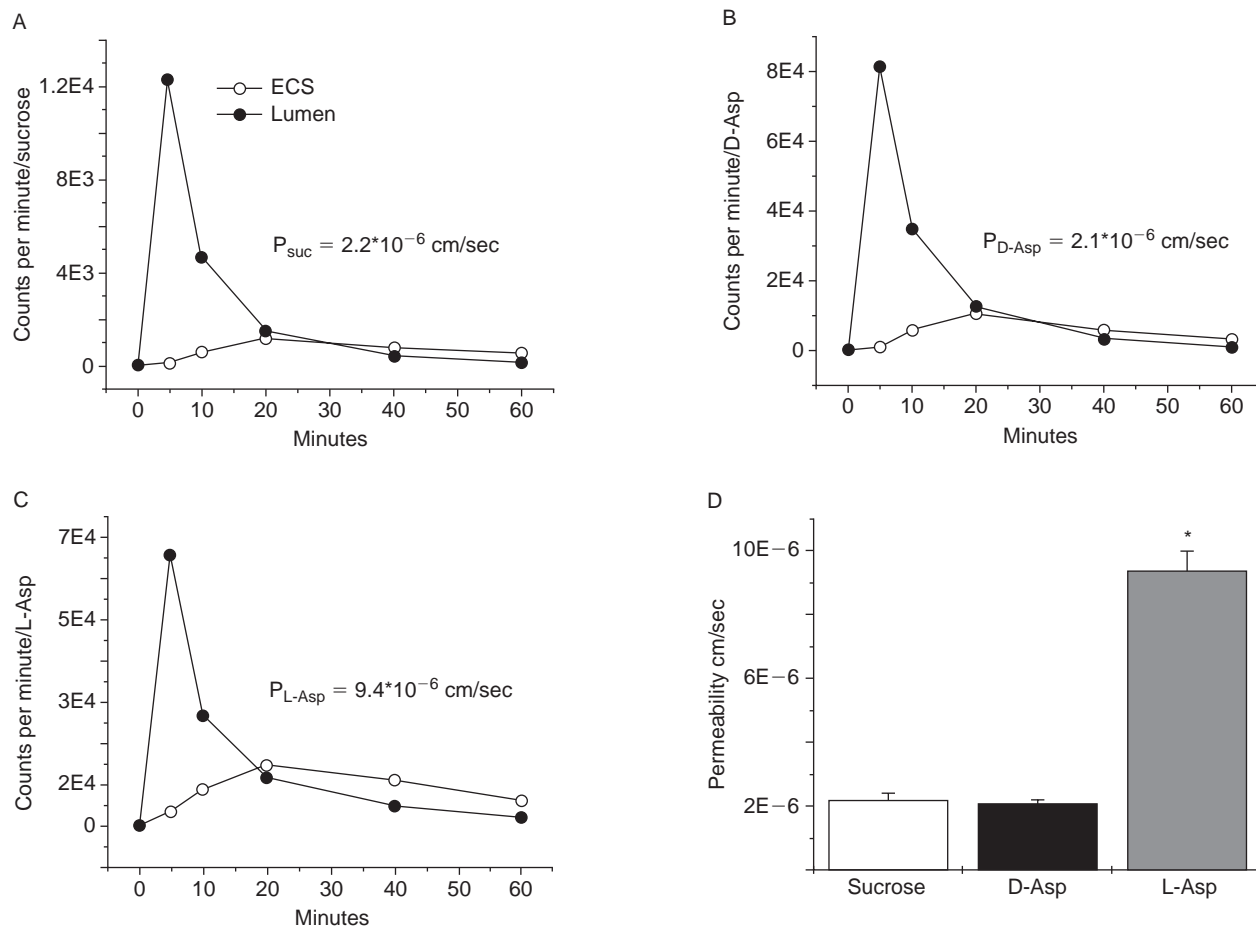


FIG. 2. Transendothelial permeability properties of neuronal-endothelial-glia co-cultures. (A) Low sucrose permeability is hallmark of integrity of *in vivo* and *in vitro* blood-brain barriers. The permeability to [ $^{14}\text{C}$ ]sucrose ( $^{14}\text{C}$ Suc) was assessed 2–3 weeks following co-culture of B14 cells and glia on the abluminal side of a DIV-BBB containing intraluminal rat brain microvascular endothelial cells. Permeability to intraluminal sucrose was exceedingly low, as expected for a fully developed BBB. (B,C) Stereoselective properties of the neuronal DIV-BBB. L-Aspartate (L-Asp) is normally transported across the BBB, while the D-isomer (D-Asp) is excluded. In the absence of barrier, their permeability was equivalent depending exclusively on mobility, charge and mol. wt [19]. Note that in a fully differentiated BBB  $P_{\text{L-Asp}}$  was significantly higher than  $P_{\text{D-Asp}}$ , as previously demonstrated for a bicellular configuration (EC + glia). (D) Cumulative response of four different cartridges containing tricultural DIV-BBB cultures. L-Asp had greater permeability than D-Asp. \*  $p < 0.05$ .

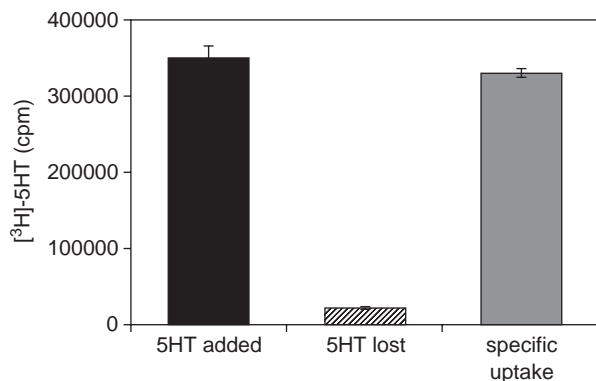


FIG. 3. Neuronal uptake of serotonin (as [<sup>3</sup>H]5HT) in the neuronal DIV. Note that only a fraction of the radiolabeled monoamine was lost following incubation for 15 min ( $n=6$ , significantly less than [<sup>3</sup>H]5HT added; \* $p<0.01$ ). Loading of the cells with trace amounts of labeled 5HT was performed on a background concentration of  $\sim 1 \mu\text{M}$  unlabeled 5HT present in the cell culture medium.

neuronal cells, there was no significant basal or KCl-evoked [<sup>3</sup>H]5HT release ( $n=2$ ).

We further assessed the presence of physiological mechanisms of 5HT uptake by exposing these cells to fluoxetine, which inhibits 5HT reuptake at the SERT. The early portion of depolarization-induced release was not significantly affected by this treatment; however, released [<sup>3</sup>H]5HT continued to accumulate in the presence of fluoxetine during the later phase of depolarization ( $n=4$ , Fig. 4B).

## Discussion

The goals of the present study were two-fold: to demonstrate the feasibility of the DIV-BBB as a cellular and structural substrate for neuronal growth and differentiation, and to study the possible influ-

ence of neurons on the development of an *in vitro* model of the BBB. The main finding resulting from this work is that it is possible to maintain neuronal cells *in vitro* in a model of their natural environment and complex interrelationship with the ubiquitous cellular structure that normally shields neuronal cells from blood-borne influences, the BBB. We have shown that differentiated neuronal cells from an immortalized cell line derived from embryonic rat midbrain progenitor cells maintain their serotonergic phenotype when grown in the DIV-BBB: this was assessed by immunocytochemical and physiologic testing. Uptake-release experiments with [<sup>3</sup>H]5HT also indirectly demonstrated that these cells develop functional presynaptic specialization. Fluoxetine, a selective SERT inhibitor, impaired the reuptake of 5HT released by early phase depolarization by KCl, increasing measured release (Fig. 4B). Taken together, these results demonstrate that precursor cells capable of expressing a serotonergic neuron phenotype can grow and differentiate in the abluminal compartment of the DIV-BBB. Glial cells did not contain SERT immunoreactivity, and did not appear to participate in high affinity [<sup>3</sup>H]5HT uptake and release. The BBB characteristics of the EC cells in the DIV-BBB were not impaired by the presence of the B14 serotonergic cells, as indicated by the permeability experiments (Fig. 2). It is possible that neurites from the B14 cells can grow through the pores in the walls of the hollow fiber capillaries and interact with the EC, but it is important to note that any such interactions did not interfere with BBB function. Indeed, this would model the *in situ* case, where neurons do contact brain capillaries [17]. Finally, we have also demonstrated that endothelial cell differentiation may occur in the presence of

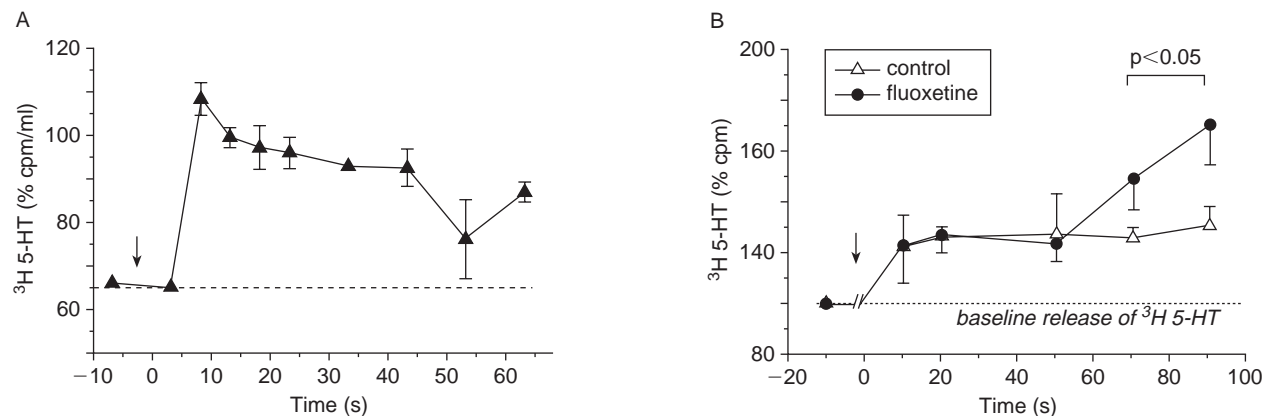


FIG. 4. Neuronal uptake of 5HT is blocked by serotonin-specific uptake inhibition by fluoxetine. (A) Depolarization-induced release of serotonin in the DIV-BBB. Neurons were loaded with [<sup>3</sup>H]5HT and 18 mM KCl was applied to induce release (arrows). A rapid increase of measurable monoamine was determined immediately following application of elevated potassium. Serotonin levels remained high throughout the experiments ( $n=6$ ). (B) Fluoxetine prevents uptake of 5HT in B14 neurons grown in the DIV-BBB ( $n=3$ ). Note that following an initial plateau, serotonin levels surged again in the presence of this uptake inhibitor; the time scale for this plot differs from (A) and the early increase was not detected by the sampling intervals chosen for these long-term experiments.

abluminal glia and neurons, since, as observed *in vivo* [22], RBMEC developed SERT immunoreactivity in the DIV-BBB.

**Relevance and comparison with other tissue culture methods:** Techniques for growing animal and human cells on plastic surfaces, or in suspension, have contributed significantly to the development of biomedical science. The technology has improved through the decades, partly as a result of interest in the methods, rather than through its direct application. Only recently with the development of stem cells and fetal tissue culturing, the exciting prospect of chronic implants of neural tissue for therapeutic purpose may become reality [24]. A major limitation of most currently available tissue culture systems derives from the original focus on growth-permissive media, and comparably little attention to the topographic aspects of tissue growth *in situ*. It has become apparent, however, that full differentiation into a phenotype comparable to the *in vivo* situation can be achieved only when a number of conditions are met. These include exposure to differentiating factors secreted by neighboring cells often from different origin and the respect and preservation of physiological orientation and polarity, among others [4].

The principles behind the development of the DIV-BBB are described elsewhere [3,4,13–15], but can be summarized as follows: (1) full differentiation of a BBB phenotype requires EC to be in proximity to perivascular glia (abluminally) and exposed to intraluminal flow; (2) flow modulates the pattern of endothelial cell growth and gene expression [9]; (3) glia influence endothelial cell development by secretion of yet unidentified factors, and these effects are enhanced by topographic proximity between glial and EC [13]. The original studies by Stanness, Ott and others, however, did not include neuronal cells in the design of the DIV-BBB. Since neurons are the principal functional component of the brain, we decided to expand the use of the DIV-BBB by adding neuronal progenitor cells to the abluminal compartment of the DIV-BBB.

A similar *in vitro* modeling attempt was performed by Stoppini and colleagues [16]. In their elegant model, brain slices were co-cultured onto a support of EC grown in Transwell filters, preserving the morphology and neuronal circuitry of the explanted tissue. However, such EC monolayers fail to reproduce many of the crucial properties of the BBB *in situ*, such as low permeability to small molecules and absent expression of key proteins that have been previously used to define brain microvascular EC immunologically [3,4,15]. Expression of these proteins may even be necessary for the functional BBB

properties to manifest. An obvious further development would be the growth of organotypic brain slices in the DIV-BBB in order to have the benefits of each model approach in studying neuron–BBB interactions.

**Serotonergic markers expression in cultured neuronal and EC:** The primary mechanism for termination of monoaminergic neurotransmission is through reuptake of released neurotransmitter by Na<sup>+</sup>,Cl<sup>-</sup>-dependent plasma membrane transporters. While SERT is predominantly or exclusively expressed by serotonergic neurons, SERT expression by non-neuronal cells is still poorly understood [2,5]. Our experiment revealed expression of SERT in both DIV-BBB-cultured and *in situ* RBMEC.

Although one recent report has detected SERT in brain astrocytes and in primary astrocyte culture [25] using RT-PCR and [<sup>3</sup>H]5HT uptake, another study found no SERT immunoreactivity in glial cells [23]. Even if present, the level of SERT expression in astrocytes is very low in comparison to SERT in serotonergic neurons, and its physiological relevance is uncertain. It is also difficult to rule out contamination of differentially purified astrocyte membrane vesicle preparations by serotonergic synaptosomes in that study. In our experiments, while DIV-BBB RBMEC displaying BBB properties were readily stained by SERT immunocytochemistry, the abluminal layer of astrocytes was devoid of SERT immunoreactivity. Furthermore, the uptake of [<sup>3</sup>H]5HT was low in the absence of B14 neuronal cells, and there was no depolarization-induced [<sup>3</sup>H]5HT release. We are currently performing experiments to study this issue further and to determine whether the detection of SERT in primary astrocyte cultures is an artifact of the monolayer growth conditions or is also detectable in the DIV-BBB model.

In conclusion, we have developed a co-culture system that models the BBB by maintaining intraluminal flow and allowing growth of parenchymal glia and neurons on the abluminal side of hollow fibers. This allows for in depth studies of drug delivery paradigms *in vitro*, and provides a tool for studies of neuronal–glial–endothelial interactions at the blood–brain interface.

## References

1. Janzer RC and Raff MC. *Nature* **325**, 253–257 (1987).
2. Cordon-Cardo C, O'Brien P, Casals D *et al.* *Proc Natl Acad Sci USA* **86**, 695–698 (1989).
3. Grant GA, Abbott NJ and Janigro D. *NIPS* **13**, 287–293 (1999).
4. Janigro D, Leaman S and Stanness KA. *Pharmacol Sci Technol Today* **2**, 7–12 (1999).
5. Lathera J and Goldstein GW. Brain microvessels and microvascular cells *in vitro*. In: Pardridge WM, ed. *The Blood–Brain Barrier Cellular and Molecular Biology* New York: Raven Press, 1993: 1–25.

6. Pardridge WM, Triguero D, Yang J and Cancilla PA. *J Pharmacol Exp Ther* **253**, 884–891 (1990).
7. Pirro JP, Di Rocco RJ, Narra RK and Nunn AD. *J Nucl Med* **35**, 1514–1519 (1994).
8. Abbott NJ and Romero IA. *Mol Med Today* **2**, 106–113 (1996).
9. Ott MJ, Olson JL and Ballermann BJ. *Endothelium* **3**, 21–30 (1995).
10. Bevan S, Lindsay RM, Perkins MN and Raff MC. *J Physiol* **82**, 327–335 (1987).
11. Yuan Y, Granger HJ, Zawieja DC and Chilian WM. *Am J Physiol* **32**, H641–H646(1992).
12. Janigro D, Stanness KA, Nguyen TS *et al.* Possible role of glia in the induction of CNS-like properties in aortic endothelial cells: ATP-activated channels. In: Bellardinelli L and Pelleg A, eds. *Adenosine and Adenine Nucleotides* Boston: Nijhoff, 1996: 85–96.
13. Pekny M, Stanness KA, Eliasson C *et al.* *Glia* **22**, 1–11 (1997).
14. Stanness KA, Guatteo E and Janigro D. *Neurotoxicology* **17**, 481–496 (1996).
15. Stanness KA, Westrum LE, Mascagni P *et al.* *Brain Res* **771**, 329–342 (1997).
16. Dupont S, Robert F, Muller D *et al.* *Proc Natl Acad Sci USA* **95**, 1840–1845 (1998).
17. Estrada C and DeFelipe J. *Cerebr Cortex* **8**, 193–203 (1998).
18. McKhann GM, D'Ambrosio R and Janigro D. *J Neurosci* **17**, 6850–6863 (1997).
19. Janigro D, Nguyen TS, Gordon EL and Winn HR. *Am J Physiol* **270**, H1423–H1434 (1996).
20. Eaton MJ, Staley JK, Globus MYT *et al.* *Dev Biol* **170**, 169–182 (1995).
21. Eaton MJ and Whittemore SR. *Exp Neurol* **140**, 105–114 (1996).
22. Brust P, Bergmann R and Johannsen B. *Neurosci Lett* **194**, 21–24 (1995).
23. Sur C, Betz H and Schloss P. *Neuroscience* **73**, 217–231 (1996).
24. Brundin P, Bjorklund A and Lindvall O. *Prog Brain Res* **82P**, 707–714 (1990).
25. Hirst WD, Price GW, Rattray M *et al.* *Neurochem Int* **33**, 11–22 (1998).

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