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Using miniature brain implants in rodents for novel drug discovery

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Abstract

Introduction: There continues to be a need to create an artificial human blood-brain barrier for pharmacological testing and modeling of diseases. Our group has recently vascularized human brain organoids with human iPSC-derived endothelial cells. Other groups have achieved brain organoid perfusion after vascularization with murine endothelial cells.

Areas covered: This review article discusses the remaining hurdles, advantages, and limitations of creating a human organoid blood-brain barrier in rodents for novel drug discovery.

Expert opinion: The creation of a human organoid blood-brain barrier in rodents will be feasible with appropriate molecular and cellular cues. An artificial human blood-brain barrier model may be used for pharmacological testing or for the study of the human blood-brain barrier in development or disease. Potential limitations of the model include an inferior competence of the blood-brain organoid barrier, the immunodeficient environment and low reproducibility due to variations in organoid morphology and vascularization. Despite its limitations, an artificial human blood-brain barrier model in rodents will further our understanding of blood-brain barrier pharmacology, and the field is expected to see significant advances in the next years.

Keywords

Human brain organoid; blood-brain barrier; vascularization; perfusion; human endothelial cells; induced pluripotent stem cells

1. Introduction

Current blood-brain barrier models have limited validity. Our group has recently shown vascularization of human brain organoids with human iPSC-derived endothelial cells *in vitro* and after transplantation into rodents [1]. Successful perfusion has been achieved in brain organoids with murine endothelial cells after transplantation [2]. This article will review the current state of the technology of creating an artificial human organoid blood-brain barrier in rodents and potential pharmacological applications.

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1.1. Why is there a need to create a human blood-brain barrier model if CNS penetration of drugs can be tested in rodents?

Evolutionary conservation of the blood-brain barrier has led to morphological similarities among vertebrates [3]. The blood-brain barrier is composed of tight junctions, pericytes, astroglia foot processes, and basal membranes across vertebrate species, and exhibits a high transepithelial electrical resistance (TEER) and low paracellular flux.

On a molecular level, however, the human blood-brain barrier is uniquely different from the rodent blood-brain barrier. There are significant differences between rodents and humans in the expression of blood-brain barrier proteins such as transporters, tight junctions, and receptors [4–8].

One of these differences involves P-glycoprotein. P-glycoprotein is an ATP-dependent efflux transporter that carries a wide range of drugs across the blood-brain barrier [9] and its substrates such as Verapamil show profound interspecies differences in brain and brain to plasma concentrations in PET studies [9].

There is an innate difference in the blood-brain barrier between species since plasma protein binding and metabolism were not found to explain species-related differences [9]. This also holds true for the difference in blood-brain barrier kinetics between rodents and humans since some drugs can show a manifold difference in brain concentration between the two species [9]. In addition, quantitative-targeted absolute proteomics have verified differences between rodents and humans in the expression of transporters, tight-junctions, and receptors [10,11]. For example, some proteins abundantly expressed in rats such as multidrug resistance-associated protein, organic anion transporter, and organic aniontransporting polypeptide family members, were under the limit of quantification in humans [11].

In summary, there are profound differences between the human and rodent blood-brain barrier which renders extrapolation of rodent blood-brain barrier testing results unreliable with respect to humans.

1.2. Which models of the human blood-brain do currently exist?

An *in vivo* artificial human blood-brain barrier would hold a tremendous advantage over current human blood-brain barrier models. Currently, human blood-brain barrier models rely on *in vitro* modeling of the human blood-brain barrier. Current blood-brain barrier models comprise spheroidals, brain microvessels, transwell-based modeling systems, Matrigel-based or other extracellular matrix-based modeling systems, and micro-fluidics systems [12]. In spheroidal models, brain microvascular endothelial cells (BMECs), astrocytes, and pericytes are able to auto-assemble without scaffolding material [12,13]. However, there is variability in the model, and capillaries are not perfused with blood. Endovascular progenitor cells have self-assembly properties and form tubular structures in Matrigel [14]. Transwell systems are common and widely used. They have the advantage that transport mechanisms can be studied because a semi-permeable membrane separates luminal and abluminal compartments [15]. Several transwell systems have been described either using endothelial cell monoculture [16–19], coculture with pericytes [17], astrocytes [20–22],

differentiated neural progenitor cells [23], or triple cultures [21]. Transwell systems have also been implemented successfully with iPSC-derived vascular endothelial cells [24]. However, one of the disadvantages of the Transwell system and other above-mentioned models is the lack of shear stress. Shear stress has been proven to be important for endothelial function. Endothelial cells are flattened and show an abundance of endocytotic vesicles, micro-filaments, and clathrin-coated pits when exposed to shear stress [25] and they have properties that resemble more closely the *in vivo* behavior [26,27]. Endothelial cells have mechanoreceptors on their apical surfaces that get activated under shear stress [28,29]. Mechanotransduction by endothelial cells is locally generated, direction-dependent, and ligand-specific [30]. Ligands generate locally different shear-induced responses in endothelium depending on how the force is delivered [30]. Mechanotransduction has an effect on the production of vasoactive substances in endothelium [31–33], expression of tight junctions [34–36], cell division [37], and differentiation [38]. Therefore, the absence of shear stress in these models results in important biological changes that render testing of the artificial blood-brain barrier unreliable.

Models that incorporate shear stress are 3D dynamic *in vitro* models of the blood-brain barrier. This technology uses hollow fibers that are coated on the inside with endothelial cells and on the outside with astrocytes [39]. Microfluidic blood-brain barriers on chips enable a real-time study of human endothelial cells in a physiological microenvironment [40–43]. A large number of publications on microfluidic blood-brain barrier systems in recent years illustrates the quest for better blood-brain barrier models [44–48]. However, microfluidic systems are expensive, difficult to implement, require high cell numbers which are difficult to harvest, and there is no visualization of the intraluminal compartment [12].

The problem with *in vitro* systems is that all available cell lines of blood-brain barrier capillary endothelial cells lack sufficiently high transepithelial electrical resistance and lack sufficiently low paracellular permeability. Therefore, there is a need to model a human blood-brain barrier *in vivo*, but it remains unclear whether a high TEER can be achieved in such a model.

1.3. Will human iPSC-derived endothelial cells automatically form a human blood-brain barrier in a human organoid, or will they need additional cues?

The first goal of generating an artificial human blood-brain barrier in a rodent is achieving vascularization of the brain organoid. We have developed iPSCs from the fibroblasts of a patient [49] and subsequently differentiated the patient's iPSCs into endothelial cells and brain organoids [1]. We were able to show vascularization of human brain organoids with human endothelial cells *in vitro* and *in vivo*. The next step is to show the perfusion of human endothelial cells with murine blood. Brain organoid perfusion can be qualitatively analyzed by intra-cardiac injection of DilC12, DilC18 or fluorescein-labeled Dextrans. DilC18 is a hydrophobic carbocyanin that can be used for vessel painting since it labels the endothelial plasma membrane through insertion into the lipid bilayer [50–52]. DilC12 has subsequently been shown to have superior vessel painting qualities [53,54]. The mouse vasculature can be easily visualized with intracardiac DilC12 with liposomes perfusion (Figure 1(a)). Another method of mapping cerebrovascular blood perfusion non-invasively

is optical microangiography [55]. We would expect perfusion of human endothelial cells in an organoid within 2 weeks since Mansour et al. were able to achieve perfusion of murine endothelial cells in such a time frame [2].

While our group was able to show vascularization of the human brain organoid with human endothelial cells, we were not able to show perfusion yet. Figure 1(b) illustrates the vascularization of a small organoid with CD31-positive human endothelial cells (hCD31, green; DAPI, blue). Around the organoid, normal mouse brain perfusion is shown with labeling with DilC12 which was injected into the heart before perfusion (DilC12; orange). Note that there is no double-labeling of hCD31/DilC12 indicating lack of perfusion.

Therefore, the question arises how perfusion of human vasculature can be achieved *in vivo*. VEGF alone has been shown not to be sufficient to achieve perfusion in our experiments. Therefore, different strategies need to be chosen to perfuse the organoid with murine blood. It helps to look at results from other groups working on other organ systems to find a possible answer. The problem may be that VEGF alone does not lead to proliferation or ingrowth of mural vascular cells such as pericytes or α-smooth muscle cells. Alajati et al. showed that VEGF alone is not sufficient to perfuse a subcutaneous model of human endothelial cells [56]. However, when the group supplemented with PDGF-BB, human umbilical artery stem cells or human dermal fibroblasts, they were able to achieve approximately 20–35% perfusion of their xenograft in immunosuppressed mice [56]. Kang et al. [57] showed that human endothelial colony forming cell/mesenchymal progenitor cell-derived mesenchymal vessels could be perfused in a mouse model, even when reimplanted into a new mouse. Therefore, the presence of vascular mural cells besides endothelial cells seems to be mandatory for perfusion of the vasculature, which may also hold true for our human brain organoids.

Another important question concerns the permeability of the artificial blood-brain barrier once perfusion has been established. Tight junction proteins such as Occludin, Claudin, or Tricellulin [58,59] are important to seal the blood-brain barrier. Another protein associated with tricellular tight junctions is Lipolysis-stimulated lipoprotein receptor (LSR) [60]. LSR is a component of paracellular junctions where three epithelial cells meet and is much higher expressed in the CNS than in the peripheral vasculature [61]. There are also other proteins that are exclusively expressed in the cerebral vasculature and not in the peripheral vasculature. Analysis of the human protein atlas, for example, showed that expression was unique to eight proteins in endothelial cells of the CNS compared to peripheral tissues [62]. It is unknown whether ingrowing iPSC-derived endothelial cells will behave like peripheral vasculature or adequately express CNS-specific proteins such as LSR. If iPSC-derived endothelial cells were to express LSR, we would have a better understanding of the mechanism of human blood-brain barrier formation since in this case it is safe to assume that the human brain organoid itself induces expression of these CNS-specific proteins in endothelial cells.

Another question concerning human brain organoids is the maturity and tightness of the human blood-brain barrier. The age of the human brain organoid in our experiments was approximately 7 weeks before animals were euthanized. During post-mortem trypan blue

experiments, however, the fetal blood-brain barrier seemed to be competent only after 12 weeks [63]. Also, tight junction proteins such as occludin or claudin-5 are expressed only by 12 weeks of gestation [64]. It may even take 18 weeks for the fetal tight junction proteins to show the same staining pattern as in adults [65]. Astrocytes are an important component of the human blood-brain barrier [66–69]. However, primary astrocytic encircling does not take place until postpartum week 3 according to rodent studies [70]. Astrocytes may continue to tighten the blood-barrier after birth, even beyond 3 weeks [71]. However, there is also evidence that a blood-brain barrier may be competent without astrocytic encircling since astrocytes form later in fetal development, after the preceding vasculature has already been shown to be competent [70,72]. Besides astrocytes, mural vascular cells such as pericytes and smooth muscle cells also undergo maturation after birth. Pericytes have been described in the postnatal rodent vasculature on day 13 [73]. Pericyte loss leads to a decrease in blood-brain barrier function [74]. Smooth muscle cells regulate the synthesis and turnover of collagen in the vessel wall and determine cerebral blood flow [75]. Both smooth muscle cells and pericytes originate from the cephalic neural crest during development [76]. Smooth muscle cells and pericytes share common expression markers such as PDFR-β, NG2, α-smooth muscle cell actin, desmin, and RGS5 [77], making it at times difficult to tell them apart from one another. Considering the progressive involvement of astrocytes, pericytes and smooth muscle cells in blood-brain barrier maturation during development, it is questionable whether a 7-week old vascularized human brain organoid captures all the physiological processes of a mature adult blood-brain barrier.

It used to be a common understanding that the blood-brain barrier in embryos and newborns is leakier than in adults [78,79]. This may be a misperception that may have arisen from the theory that the fetal human brain does not need a blood-brain barrier because it already is protected by the placenta. Recent and older evidence however points towards adequacy of tight junctions in the fetal human brain [80]. For example, human fetuses from legal abortions that were injected with trypan blue dye within 10 min did not show staining of the brain [63]. Therefore, it is possible that the human organoid blood-brain barrier, while immature, may still be competent like in fetal brain and be useful for pharmacological testing.

1.4. How do you evaluate the human blood-brain barrier in a rodent model?

The next step after successful perfusion will be the comparison of the permeability of the human organoid blood-brain barrier to the human blood-brain barrier to evaluate the model for validity.

Two gold standard experimental methods for measuring the blood-brain barrier are logBB (the concentration of drug in the brain divided by the concentration in the blood) and logPS (permeability surface-area product) [81]. Calculating logBB is feasible in a rodent blood-brain barrier model since the concentration of the drug could be measured in the organoid with a microdialysis probe along with the concentration in the blood through a central line. The permeability surface-area product reaches its limitations in a rodent brain organoid model. The product can be calculated with the Renkin-Crone equation [82,83] and requires knowledge of the cerebral blood flow rate as well as the concentration of the

compound in the brain and in the perfusion fluid. Since currently organoid capillaries are too small to catheterize, calculation of the permeability surface-area product is unpractical. Therefore, calculation of blood/brain drug concentration ratio seems to be the most plausible approach to characterize the organoid artificial blood-brain barrier.

Microdialysis probes have been used in humans to measure the brain concentration of compounds [84–86]. Similarly, a mouse dialysis probe could be used to measure drug concentrations within the organoid to determine the drug brain concentration [87,88]. Microdialysis probes can be divided into four types on the basis of their geometry: concentric, side by side, U-shaped, and horizontal [88]. Since the organoid is usually only 2 mm in size, a horizontal probe would be difficult to insert directly through the center of the organoid. More practical is an insertion into the organoid dorsally under direct visualization. A concentric probe may be most easily used for insertion. Commercially available microdialysis probes can be costly, but numerous protocols exist for the construction of do-it-yourself microdialysis probes [89,90].

Vascularized organoids currently have only been shown to survive in immunosuppressed animals such as NSG mice. Therefore, assessment of the blood-brain barrier is technically more difficult since blood vessels are smaller to catheterize in mice than in rats. In mice, the central line catheter may be placed through the jugular vein into the right atrium of the heart [91,92]. Tail vein sampling could be used to determine the drug concentration in blood at the same time as CNS measurements through the concentric probe are obtained. However, drawing blood from the tail may result in different pharmacokinetic outcomes than drawing blood from central sites [93,94]. Therefore, the central line catheter may best be used for sampling of serum concentrations of medications injected into the tail vein, the peritoneum or administered orally. Figure 2 illustrates a possible experimental design for testing the human blood-brain organoid barrier in a rodent.

The artificial blood-brain barrier could be evaluated with compounds with known behavior across the blood-brain barrier. Drugs with known penetration of the blood-brain barrier, for example, are propranolol, caffeine, antipyrine, carbamazepine, and trazadone. In mice, propranolol could be administered i.p. at a dose of 1.5 mg/kg to 6 mg/kg [95], caffeine at a dose of 6.25 mg/kg to 100 mg/kg i.p. [96], antipyrine at 20 mg/kg i.v. [97], carbamazepine at 20 mg/kg to 40 mg/kg i.p. [98] and trazadone at 5 mg/kg to 10 mg/kg i.p. [99]. Drugs with known poor penetration of the blood-brain barrier are sulpiride [100], epinastine [101], cimetidine [102], quinidine [103], and prazosin [104]. Sulpiride could be administered at a dose of 3 to 10 mg/kg i.p. [105], epinastin at 1 mg/kg s.c. [106], cimetidine at 50 mg/kg i.p. [107], quinidine at 100 mg/kg i.p. [108] and prazosin at 0.5 to 1 mg/kg i.p. [109]. A concentric microdialysis probe placed directly into the center of the transplanted organoid perfused at a rate of less than 3 microliter/minute could obtain serial samples of the concentration of drugs in the CNS interstitial space. At the same time, plasma concentrations could be determined by venous blood sampling from the central line inserted into the jugular vein.

The brain-to-plasma ratio could be used to validate the artificial human blood-brain barrier model and assess its competence. Prazosin, for example, has a brain-to-plasma ratio of 0.17

± 0.02 mL/g [104] which could be calculated from samples obtained from the central line catheter and the brain microdialysis probe. One of the limitations of this approach is that most microdialysis probes take 10 min to generate enough sample for analysis. This renders the determination of a brain-to-plasma ratio more difficult since blood samples are drawn over a shorter period of time and therefore cannot be matched 1:1 with the microdialysis aspirate.

One of the limitations of the human organoid blood-brain barrier model is the lack of a mature thymus and functionally mature T cells. Drugs that augment the immune system to fight a brain tumor organoid, for example, may not be able to be studied if they are thought to function via T cell activation [110]. Also, immune-mediated adverse drug reactions would also likely not become apparent in these models [111]. Another limitation is that blood-brain barrier membrane proteins such as P-glycoprotein and CYP enzymes may be differentially expressed in diseased states [112,113]. Some of these disease states could be modeled with transgenic overexpression in the human brain organoid and endothelial cells, such as overexpression of Aβ in an Alzheimer's disease model. Finally, CNS drugs (especially anti-epileptic drugs) may undergo metabolism in the mouse liver which enables or prevents biotransformation of a useful drug or allows for its detoxification. Since the mouse liver has a different metabolism than human liver, results of an artificial human organoid blood-brain barrier model have to be interpreted with caution. Measurement of the drug serum concentration through a central line should capture the impact of liver metabolism and help with the interpretation of the data. Another problem with the *in vivo* model may be reproducibility. Considering that each brain organoid develops differently and that factors driving human brain organoid development are still poorly understood, it can easily be imagined that there will be a considerable variation among brain microdialysis results. Considering the limitations of the *in vivo* model, it is unclear whether it would be able to replace current *in vitro* blood-brain barrier assays some of which have a proven track record of reproducibility. The proposed human blood-brain organoid barrier model may have a higher validity than *in vitro* assays since it involves testing of a true human blood-brain barrier, but it may have lower reproducibility due to inherent variations in test conditions in this model across experiments.

2. Conclusion

Brain organoid research may soon enable testing of an artificial human blood-brain barrier in a rodent model once the bottleneck of perfusion of iPSC-derived human endothelial cells *in vivo* is overcome. These models will not only allow for pharmacological testing of the blood-brain barrier, they will also enable us to study the blood-brain barrier during development and disease. Like any model, the new model will have limitations. It is unclear whether the human blood-brain organoid barrier will have the tightness of a human blood-brain barrier, and whether results will be reproducible considering the large variation in brain organoid morphology across experiments.

3. Expert opinion

The creation of a human organoid blood-brain barrier should technically be feasible if mural cells are allowed to penetrate the organoid together with endothelial cells considering the experimental results of other investigators in other organs. A combination of endothelial cells with smooth muscle cells has led to perfusion in a subcutaneous rodent model of the human vasculature.

Weaknesses of the human organoid model are a potential discrepancy between the human organoid and human adult brain with respect to the density of astrocyte end-feet encircling, or mural cells coverage of endothelial cells. While I think that perfusion is feasible, it is unknown whether the artificial human blood-brain barrier will have the same permeability as a real human blood-brain barrier. However, even in the setting of a leaky blood-brain barrier, test results could be standardized against multiple reference probes with known permeability across the blood-brain barrier.

The fetal human brain seems to establish a functional blood-brain barrier and express important blood-brain barrier tight junction proteins around 12 weeks of gestation, and it is unclear at the present time which time period a transplanted human organoid with human endothelial cells would require to form a competent blood-brain barrier, if it ever were to form one. It can be imagined that the artificial blood-brain barrier would mostly resemble a fetal brain rather than an adult brain since the organoid resembles more closely a fetal human brain at its early stages. Therefore, interpretation of pharmacological testing results of the organoid blood-brain barrier for adults would need to be performed with great caution.

The creation of a human organoid blood-brain barrier with human endothelial cells continues to hold great promise for pharmacological testing and modeling of blood-brain barrier diseases impacting neurosurgery, neurology, and psychiatry, despite its limitations. The next decade will witness an explosion of applications of the technology and further our understanding of the pharmacology, physiology, and pathology of the human blood-brain barrier.

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Article highlights

- Human brain organoids have been vascularized *in vivo* with human endothelial cells, and perfusion of human brain organoids has been achieved with murine endothelial cells.
- Despite evolutionary conservation among vertebrate species, there are profound molecular differences between the human and rodent blood-brain barrier.
- All available capillary endothelial cell lines lack sufficiently high transepithelial electrical resistance values and sufficiently low paracellular permeability.
- Endothelial cells and VEGF alone are not sufficient to achieve perfusion of human vascularized brain organoids.
- An artificial human blood-brain organoid model will allow for pharmacological testing and study of the human blood-brain barrier during development and disease.
- Potential limitations of the new human blood-brain organoid model may include an inferior competence of the blood-brain barrier, the immunodeficient environment and low reproducibility due to variations in organoid morphology and vascularization.

This box summarizes key points contained in the article.

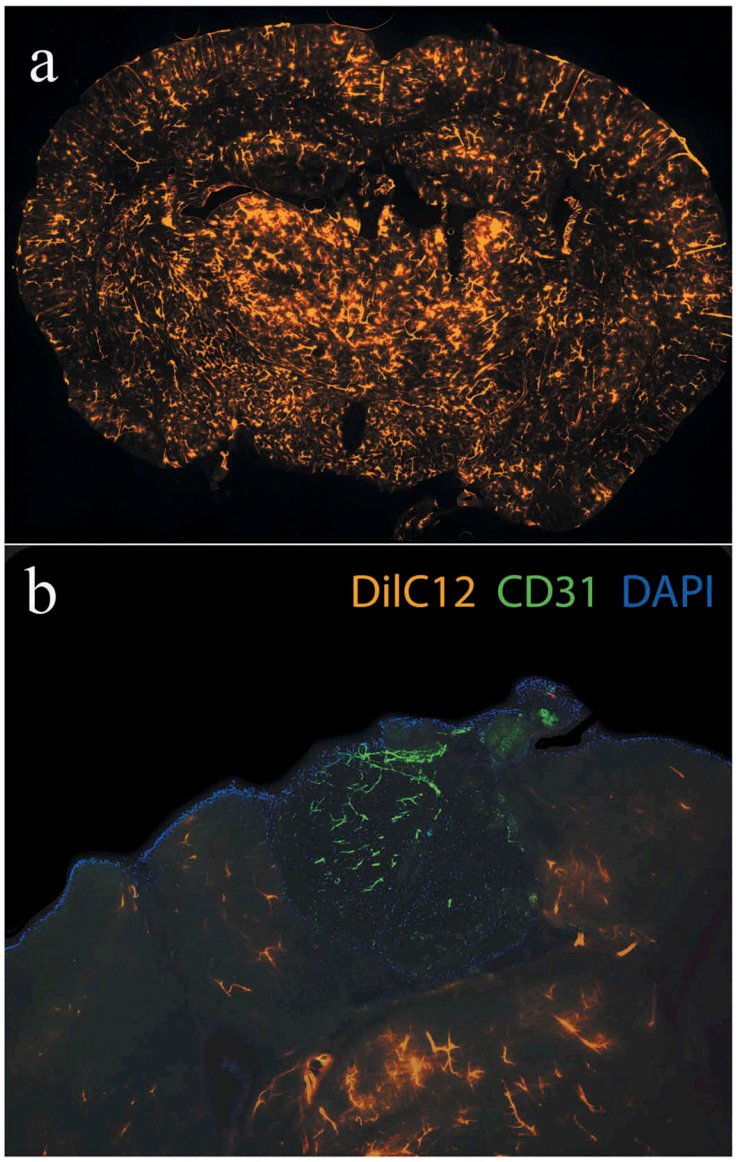

Figure 1.

(a) A coronal section of the mouse brain shows vessel painting of the intracerebral vasculature after intracardiac perfusion with DilC12 (orange). (b) A transplanted vascularized human brain organoid supplemented with VEGF only does not show perfusion after 2 weeks (human CD31: green; DilC12: orange; DAPI: blue).

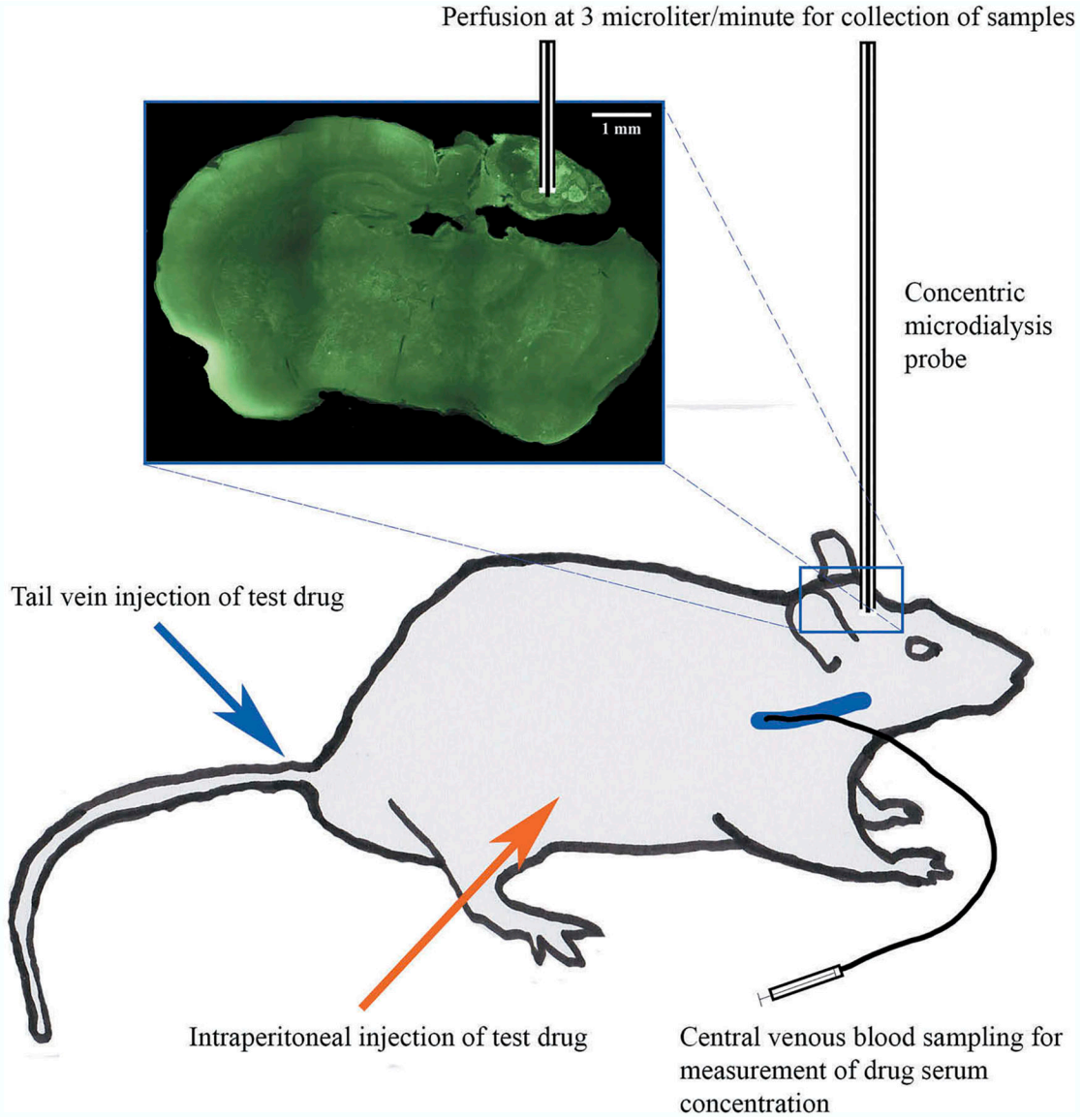

Figure 2.

Model of a human blood-brain organoid barrier in a rodent host. The jugular vein is catheterized for venous blood sampling for measurement of serum drug concentrations. A concentric microdialysis probe is inserted into the previously implanted and vascularized brain organoid (coronal section in blue box, reprinted from [1] with permission from Wolters Kluwer Health). After intravenous tail vein or intraperitoneal injection, the concentration of the test drug in the CNS and serum can be determined by serial sampling from the microdialysis probe and jugular vein catheter for calculation of the blood-brain barrier permeability.