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Continuous Monitoring Reveals Protective Effects of *N*-Acetylcysteine Amide on an Isogenic Microphysiological Model of the Neurovascular Unit

Isabelle Matthiesen, Dimitrios Voulgaris, Polyxeni Nikolakopoulou, Thomas E. Winkler,* and Anna Herland*

Microphysiological systems mimic the in vivo cellular ensemble and micro-environment with the goal of providing more human-like models for biopharmaceutical research. In this study, the first such model of the blood-brain barrier (BBB-on-chip) featuring both isogenic human induced pluripotent stem cell (hiPSC)-derived cells and continuous barrier integrity monitoring with <2 min temporal resolution is reported. Its capabilities are showcased in the first microphysiological study of nitrosative stress and antioxidant prophylaxis. Relying on off-stoichiometry thiol-ene-epoxy (OSTE+) for fabrication greatly facilitates assembly and sensor integration compared to the prevalent polydimethylsiloxane devices. The integrated cell-substrate endothelial resistance monitoring allows for capturing the formation and breakdown of the BBB model, which consists of cocultured hiPSC-derived endothelial-like and astrocyte-like cells. Clear cellular disruption is observed when exposing the BBB-on-chip to the nitrosative stressor linsidomine, and the barrier permeability and barrier-protective effects of the antioxidant *N*-acetylcysteine amide are reported. Using metabolomic network analysis reveals further drug-induced changes consistent with prior literature regarding, e.g., cysteine and glutathione involvement. A model like this opens new possibilities for drug screening studies and personalized medicine, relying solely on isogenic human-derived cells and providing high-resolution temporal readouts that can help in pharmacodynamic studies.

1. Introduction

The blood-brain-barrier (BBB) maintains the homeostasis of the brain by tightly regulating the influx and outflux of everything from metabolites to pathogens and therapeutics. The healthy BBB consists of a layer of brain microvascular endothelial cells (BMEC) closely interlinked by membrane protein complexes.^[1,2] This tight barrier is supported structurally and metabolically by pericytes and astrocytes embedded in an underlying layer of extracellular matrix (ECM), and further interacts with nearby neurons and microglia. With its central role in homeostasis, understanding the BBB is critical to shedding light on disorders and developing new therapeutics.

In vivo studies of the BBB are often complex and unreliable (or even impossible, at least in humans) due to the difficulty in accessing the BBB without impairing its function. Static in vitro studies recreating the BBB are more accessible and have been performed with several different cell sources, typically on

microporous membranes suspended inside standard tissue culture wells (e.g., transwells).^[1,2] More recently, engineering approaches to create dynamic in vitro models using microfluidics have emerged (i.e., BBB-on-chip).^[3] To date, ≈20–30 distinct BBB-on-chip models have been reported, attempting to better mimic biological cues such as ECM or fluid flow.^[4,5] The most common underlying design is to employ microfluidics cast in poly(dimethylsiloxane) (PDMS), with a porous cell growth support separating two rectangular channels. These approaches have shown great promise, with for instance the application of shear stress inducing more in vivo-like morphology in certain BBB cell models, or the cellular compartmentalization shedding light on metabolic coupling between BMEC, astrocytes, pericytes, and neurons.^[6,4] An additional advantage of these microengineered systems is the potential for direct integration of sensors to monitor, e.g., neural activity or barrier integrity.^[7]

In this work, we present a BBB-on-chip—illustrated in Figure 1a,b—that builds upon such prior work and combines three crucial advantages: cell ensembles with in vivo-like

I. Matthiesen, D. Voulgaris, Dr. T. E. Winkler,^[†] Prof. A. Herland
Division of Micro- and Nanosystems
KTH Royal Institute of Technology
Malvinas Väg 10 pl 5, Stockholm 100 44, Sweden
E-mail: thomas.winkler@tu-braunschweig.de; aherland@kth.se

D. Voulgaris, Dr. P. Nikolakopoulou, Prof. A. Herland
AIMES, Center for Integrated Medical and Engineering Sciences
Department of Neuroscience
Karolinska Institute
Solnavägen 9/B8, Solna 171 65, Sweden

The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/sml.202101785>.

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^[†]Present address: Institute for Microtechnology & Center of Pharmaceutical Engineering, Technische Universität Braunschweig, Franz-Listzt-Straße 35a, 38106 Braunschweig, Germany

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Figure 1. Overview of our microphysiological system and study design. a) Photograph of our hiBBB-on-chip system, with the vascular (magenta) and perivascular (green) compartments perfused with colored solutions above and below the permeable membrane with its interdigitated electrodes (gold). The dotted red box indicates the b) schematic cross-section of our system, showing the cell coculture, microfluidic materials, and how the electrodes are used to measure barrier integrity (blue). The various materials utilized are indicated in the legend below and further discussed in the Introduction. P1,^[8] P2,^[9] and P3^[10] refer to hiPSC differentiation protocols. c) Experimental timeline and experimental conditions for our study. Abbreviations: off-stoichiometry thiol-ene-epoxy (OSTE+), polycarbonate (PC), gold (Au), human induced pluripotent stem cells (hiPSC), long-term self-renewing neuroepithelial-like stem cells (lt-NES), hiPSC-derived brain microvascular endothelial-like cells (hiBMEC), hiPSC-derived astrocyte-like cells (hiAstro), Linsidomine (SIN-1), *N*-acetylcysteine amide (NACA).

functionality, truly continuous barrier integrity monitoring, and a hydrophilic structural material that facilitates simple fabrication and integration of sensors. To date, the combination of just two such advances in barrier-on-chip systems remains elusive.

One of the most important functional characteristics in any BBB model is proper integrity of the endothelial layer, where standard assays include permeability of hydrophilic tracer molecules (e.g., fluorescent dyes) and transendothelial electrical resistance (TEER). TEER relies on electrodes placed on either side of the biological barrier to measure the electrical resistance across.

In frog and rat BBB, *in vivo* TEER has been measured in the range of 1.5–8 k Ω cm².^[11] Conversely, *in vitro* culture of primary or immortalized BMEC (human and animal) rarely exceeds 0.5 k Ω cm². Only over the past decade, human induced pluripotent stem cells (hiPSC) have emerged as an alternative to avoid interspecies variation and achieve *in vivo* biological function *in vitro*.^[1] Several protocols have been published for hiPSC-derived BMEC-like cells (hiBMEC) that achieve up to 4 k Ω cm² as monocultures. As mentioned earlier, *in vivo* BBB function is reliant also on BMEC interactions with other cell types, with, e.g., tight junction formation being induced by neural progenitor cells.^[2] Indeed, coculture systems with astrocytes and/or pericytes have proven to more fully recapitulate BBB characteristics from P-glycoprotein activity to disease phenotypes.^[1,2] On-chip models have contributed to this research starting with a report of hiBMEC coculture with primary rat astrocytes.^[12] For our system, we rely on hiBMEC cocultured with astrocyte-like cells derived from the same hiPSC line (hiAstro; Figure 1b).^[8,10] This approach of isogenic coculture can uniquely enable patient-specific disease and treatment models. Only three other BBB-on-chip studies to date employ multi-cellular hiPSC-derived models (hiBBB);^[13–15] most continue to rely on primary or immortalized cells, or just one hiPSC-derived cell type.

TEER is an obvious candidate for sensor integration into BBBs-on-chips, and has been demonstrated in multiple systems, including two of the hiPSC models mentioned above.^[13,14] These examples, however, continue to provide measurements only once per day, losing out on a wealth of temporal information. In the aforementioned works (and also non-hiPSC ones like them), this is often due to reliance on manually inserted wire-based (rather than microfabricated) electrodes and associated difficulties in creating long-term incubator-stable interfaces. One prior BBB-on-chip system has demonstrated TEER with minute-scale resolution; however, the study relied on primary cells and recorded only ≈ 12 h of treatment-phase data.^[16] Another study of note, modeling the placenta barrier with a cancer cell line monoculture, has recently employed interdigitated electrodes for electric cell–substrate impedance sensing (ECIS) with subminute resolution.^[17] Both these systems, however, suffer from complex fabrication and assembly of 5 to 9 layers (discussed further in a later paragraph).

It is worth briefly laying out the differences between TEER and ECIS here.^[18] TEER is based on electrodes placed away from the cells on opposing sides of the barrier, making it almost exclusively sensitive to barrier integrity. In on-chip systems, results are also impacted by electrode placement (with optimal placement obstructing the optical path), but generally, capture a global average.^[19] With ECIS, the (often interdigitated) electrodes are placed directly underneath the cell layer in direct contact. This similarly picks up on barrier integrity, but—due to the much closer electrode/cell interaction—signals also include information on cell attachment, spreading, and similar (depending on the chosen frequency, and complex-to-real number conversion).^[18] Conversely, however, ECIS information is generally only sensitive to cellular response at or near the electrodes. Although providing more complex information, it is a proven tool for cell & barrier stimulus-response analysis. An important advantage of ECIS is its coplanar electrode geometry, which allows for simpler incubator-suita

ble chip-to-world interfaces. For our hiBBB-on-chip, we implemented ECIS with microfabricated interdigitated electrodes in direct contact with the hiBMEC (Figure 1b) to gain an electrical measure of hiBBB tightness. We can thus, for the first time, gain barrier integrity information with <2 min temporal resolution in a hiPSC-based microphysiological system.

Last but not least, extant BBB-on-chip systems are based almost exclusively on PDMS or commercialized plastic devices.^[5] The latter are inherently fixed designs not suited to customization with, e.g., sensors. Assembly of PDMS-based microphysiological systems—especially with sensor electrodes involved—on the other hand requires chemical adhesives or physical pressure sealing between the 5+ layers which generally results in long fabrication times and poor reproducibility.^[16,20] Less common approaches such as glass (as with the ECIS-integrated system mentioned above)^[17] or 3D printing suffer from similar sensor integration problems. Many of these methods also necessitate the use of thick layers (PDMS, physical adhesives, gaskets) that put the cells outside the working distance of high-resolution objectives. PDMS in particular is moreover suboptimal for on-chip cell culture due to its hydrophobicity and strong absorption of small molecules (such as pharmaceuticals).^[21] To overcome these challenges in our hiBBB-on-chip, we employ off-stoichiometry thiol-ene-epoxy (OSTE+), a commercially-available polymer system combining the ease-of-fabrication of PDMS, the hydrophilicity and tightly crosslinked (i.e., nonabsorbing) nature of thermoplastics, as well as intrinsic bonding capabilities to a range of plastics and metals.^[22] It allows us to simplify fabrication to essentially a 3-layer process and to achieve excellent imaging capabilities. OSTE+ has previously been demonstrated for microfluidic cell culture,^[22] but never before with multi-day culture of sensitive hiPSC-derived cells.

In this work, we employed our hiBBB-on-chip to study nitrosative stress (i.e., reactive oxygen/nitrogen species, RONS) and its prevention. An excess of RONS, produced, e.g., by microglia in response to cytokines, is a common neuroinflammatory pathway implicated in Alzheimer's, multiple sclerosis, schizophrenia, and other pathologies.^[23–25] Previous studies in traditional cell culture models showed that such RONS stress damages BBB integrity by attacking tight junction proteins ZO-1 and claudins.^[26,27] To counteract the effects of nitrosative stress, drugs and antioxidants like N-acetylcysteine (NAC) have been studied clinically as treatment, amelioration, or prevention for RONS-related disorders.^[28] To date, the biological effects of RONS have not been investigated in barrier-on-chip systems, with the exception of the simplest ROS (H₂O₂) as a stressor in primary- or cell line-based endothelial models.^[29–31] There are no reports using NAC or any of its derivatives in an on-chip system. Our study is the first to venture into RONS, specifically applying them in the perivascular space where they would be naturally produced by activated M1 (pro-inflammatory) microglia.^[32] We rely on the chemical RONS generator linsidomine (SIN-1); it and its prodrug find application both in vivo and in vitro.^[33–35] SIN-1 generates superoxide and nitric oxide (which further combine to peroxynitrite; thus mimicking microglia activation),^[32,36] over the course of a few hours.^[37] On the vascular side, we applied N-acetylcysteine amide (NACA),^[38] a more lipophilic (and thus more bioavailable and

BBB-permeable)—albeit much less widely used—version of the parent molecule NAC, both proven to reduce neurotoxicity.^[39–41]

2. Results and Discussion

2.1. Device Design

Our system, displayed in Figure 1a,b, utilized a vertically stacked channel geometry common in barrier-on-chip systems. A track-etched polycarbonate (PC) membrane—patterned with interdigitated gold electrodes for ECIS measurements—served as the cell culture support between the two mirror-symmetric channels. To circumvent separate bonding and alignment of the membrane and the fluidics, we implemented a single-step molding process that first encapsulates the membrane inside (semicrosslinked) open-channel OSTE+ fluidics (Figure S9, Supporting Information). A similar approach has been previously published for PDMS,^[42] and we adapted it here for the first time to OSTE+. Unlike the PDMS process, subsequent channel enclosure (with a commercial polycarbonate chip-to-world interface, and a polycarbonate film) was simply facilitated by the epoxy-based bonding properties inherent to semicrosslinked OSTE+. Besides process simplification, our approach offers two additional advantages. First, it allowed us to produce a unique channel profile with gradual sidewall curvature towards the membrane, which eliminates the typical sharp corners that present likely cell barrier failure points. Second, the top and bottom closures from high-quality thermoplastics mean this is the only material in the optical path. This eliminates scattering and/or autofluorescence issues associated with microfluidic molding processes in general, and previous OSTE-based systems in particular.^[22] Additionally, since the bottom polycarbonate film has a thickness akin to a #0 coverglass, imaging of cells on the membrane is possible with even <300 μm working distance objectives (e.g., ≈NA 1.2 oil immersion). We note that we attempted further improving the optical qualities by replacing PC with TOPAS or Zeonor; however, these materials proved unable to bond with OSTE+ without further modification (coverglasses, on the other hand, were feasible, albeit yielding fragile devices).

2.2. Cell Models

Our hiBBB-on-chip is based on endothelial-like cells (hiBMEC) and astrocyte-like cells (hiAstro) cultured in the two respective compartments of our device (Figure 1b). As mentioned in the Introduction, this type of isogenic hiPSC-derived coculture provides clear benefits toward studying multicellular interactions and disorder- or even patient-specific biological function. No cellular model should be applied indiscriminately, however; a caveat with hiPSC-derived ones can be the question of their cellular identity. For hiBMEC, some indications also of epithelial transcriptional signatures have been documented and discussed by ourselves and others.^[1,13,43,44] It is therefore important to evaluate the functional properties of interest, including beyond TEER, e.g., the passive drug permeability, active transporter function, and inflammatory response. On many if not most

of these measures, hiBMEC have shown superior in vivo predictive powers compared to other cell models.^[44] In our view, these documented properties make hiBMEC well suited to a study of RONS stress and prophylaxis. The same applies to our hiAstro, which—besides astrocyte-like mRNA and protein expression—exhibit, e.g., relevant glutathione generation and inflammatory potency.^[10]

2.3. Study Design

We followed the experimental timeline in Figure 1c with $N = 23$ hiBBBs-on-chips, as well as $N = 33$ gold-standard transwell (TW) equivalents, spread out across three (TW: four) independent experimental repeats. Before applying interventions on d3, we verified the formation of intact hiBMEC barriers—and excluded compromised ones from subsequent interventions (cf. Section 2.6.1). In brief, we found that intact hiBBBs show permeability measures in line with literature reports. We then applied a prophylactic dose of NACA to randomly selected intact hiBBBs in the vascular compartments. With a 1.5 h delay, we additionally introduced SIN-1 into all of the perivascular compartments. We assessed biological outcomes on d4 (end-point) using immunocytochemistry (Section 2.4), untargeted metabolomics (Section 2.5), and barrier integrity (Section 2.6). Moreover, we were able to follow the dynamic barrier integrity response using our integrated ECIS sensors (Section 2.6.3).

2.4. Imaging

In fluorescent imaging, we observed positive immunostaining for the tight junction marker ZO-1, showing a well-delineated network of cell borders in the vascular-compartment hiBMEC (Figure 2a,b). The morphology closely resembled our transwell control group (Figure 2c) as well as hiBMEC literature. The one difference we note is that cells are more densely packed in devices. We roughly quantified this in terms of effective (circular) cell radius r' at an interquartile range IQR [4.0; 5.3] μm (vs [5.2; 8.3] μm in transwells; Figure S1a, Supporting Information).

Our hiAstro, distributed throughout the perivascular compartments, exhibited clear expression of astrocytic markers S100B (Figure 2a,b) and CD44 (transwells only; Figure S2, Supporting Information). Cells were sparser and appear to feature a spindlier morphology in devices than in transwells. This is likely due to the presence of media flow, and thus fluid shear—in our case an average of 1.2 mPa. As we require equal flow in both compartments for our metabolomic mass balances (even if differential compartmental flow would be more biomimetic), we chose to globally apply shear more in line with the lower interstitial/perivascular environment (than with Pa-scale vasculature).^[45,46]

2.4.1. Effects of SIN-1 and NACA on hiBMEC

With the application of 4×10^{-3} M SIN-1 (++), two subpopulations emerged for the hiBMEC: One of these populations—accounting for roughly half of the chips, evenly distributed across

the independent experimental replicates—retained fully intact barriers and tight junctions, with indistinguishable morphology from the control* group. In the other half (★), however, we observed significant barrier disruption. In Figure 2a we illustrate the types of disruption across three zones (see also Figure S3, Supporting Information for nuclear stain): i) the tight junction network remains intact as seen by clear ZO-1 boundaries between cells; ii) we observe a partially disrupted network with ZO-1 in disjointed clusters; and iii) the cell layer itself is disrupted, with voids in the barrier (detached cells) surrounded by cells that are condensed or feature clustered ZO-1 expression. The voids (bordered by the other zones) can be quite extensive and are distributed throughout the devices, as seen in Figure 2b (white arrows). Regarding the two apparent subpopulations of hiBMEC response to SIN-1, we refer to further discussion in the context of later sections (and their additional observations) but note here that we can rule out imaging or fixation artifacts on their basis. Regardless, the prophylactic application of 4×10^{-3} M NACA (++) proved highly effective in preventing damage to the hiBMEC, with no apparent differences in cellular network morphology compared to the control* group across devices.

The preventative effect of NACA on nitrosative stress damage was wholly replicated in our transwell hiBMEC (Figure 2c). When subjected to SIN-1 (++) only, no cells remained on the membranes (see also Figure S3, Supporting Information for nuclear stain), indicating widespread death and/or loss of adhesion (the lack of even cell debris is likely a wash-related artifact, which are on balance more vigorous compared to laminar wash buffer perfusion in devices). To enable further analysis, we thus included lower-dose 1×10^{-3} M (+) SIN-1 exposure for transwells. This revealed concentration-dependent behavior, with hiBMEC barriers showing no differences here compared to controls (additional NACA administration did not elicit further changes).

Our observations of barrier damage caused by SIN-1 (where present), in both the hiBBBs-on-chips and transwells, are consistent with literature reports. Severe fragmentation of ZO-1 morphology was documented for peroxynitrite exposure of rat arterial sections.^[47] In another study with primary hBMEC, SIN-1 exposure caused a loss of claudins and occludins (though not ZO-1),^[48] which would result in ZO-1 becoming unmoored inside the cell cytoplasm, something that our images support (Figure 2a). The same study further demonstrated increased matrix metalloproteinase activity leading to ECM degradation, explaining cell detachment. Concentration-wise, existing studies have reported survival at $\approx 70\%$ compared to control, up to 1×10^{-3} M SIN-1, with significant cell death at $> 2 \times 10^{-3}$ M, in line with our transwell results.^[49,50] The trends we observe are also similar to an hBMEC study showing that 2 h prophylactic application of 1×10^{-3} M NACA was successful in reducing or preventing barrier damage from $2.5\text{--}5 \times 10^{-3}$ M methamphetamine, an inducer of oxidative stress.^[51] NACA prophylaxis against direct application of RONS, as we demonstrated here, had however not previously been demonstrated.

2.4.2. Effects of SIN-1 and NACA on hiAstro

Unlike hiBMEC, hiAstro-on-chip responded to SIN-1 consistently with massive cell death. This is expected due to their more direct exposure (being cultured in the compartment SIN-1 is

Figure 2. Confocal micrographs of hiBBB-on-chip and transwell cultures. hiBMEC are stained for tight junction marker ZO-1 (magenta) and hiAstro for astrocytic marker S100B (cyan). a) High-magnification and b) corresponding whole-compartment images under our various experimental conditions. Without intervention (control*; see Section 2.6), hiBMEC form a barrier layer with clear tight junctions in the vascular compartment, whereas hiAstro

administered in) and more sensitive nature (as single cells of brain parenchymal nature, instead of a continuous sheet of vascular-like cells). A differing response may also be linked to inherent differences in cellular uptake, as previously observed between endothelial cells and fibroblasts.^[52] The result matches literature reports of primary neurons exhibiting near-complete apoptosis with >12 h exposure to even 1×10^{-3} M SIN-1.^[36,53] One of these studies further showed how SIN-1 exposure results closely matched those achieved via coculture of activated microglia.^[36] Of the hiAstro that remained in the channels, nearly 100% were fully condensed (Figure 2b). NACA prophylaxis had a less pronounced effect on hiAstro than on hiBMEC, preventing complete cell death and condensation only for a fraction of cells near the outlets. We illustrate this in Figure 2b by circling the area where we identified rescued astrocytes, i.e., those remaining fully attached and spread out. High-magnification images (Figure 2a; also Figure S2, Supporting Information) were taken in the outlet regions to highlight the relevant trends. Higher efficacy near the outlet is consistent with slow NACA permeation across the hiBMEC barrier (see also Section 2.6.1). Near the inlet (in the perivascular space constantly delivering NACA-free, SIN-1 containing media), this prevents a meaningful amount of NACA reaching the hiAstro. Toward the outlet, however, more and more NACA can permeate into the perivascular space (cumulatively across the length of the barrier) to eventually reach a sufficient concentration for partial prophylactic effect on the hiAstro.

These findings largely held in the transwells (though, lacking flow, also lacking localization), with <1% hiAstro survival for SIN-1 (++) (independent of NACA prophylaxis) as estimated from cell coverage (Figure S4, Supporting Information). We observed that NACA increased low-dose SIN-1 (+) cell survival from 53% (CI_{95%} [27; 81]) to 68% (CI_{95%} [68; 92]). This is in line with multiple previous studies where NACA prophylaxis was proven to counteract a range of stressors on neuronal cells.^[54–56] The lack of NACA effect for SIN-1 (++) on hiAstro can be attributed to the longer diffusion distance and less favorable compartment mass balance for transwells compared to devices.

2.5. Metabolomics

Untargeted UPLC/MS (ultrahigh performance liquid chromatography/mass spectrometry) metabolomics provides a wealth of information about the small (<1 kDa) molecules consumed and excreted by the cells. The approach is by nature broadly exploratory, rather than fully definitive. By

separating all our analysis by sampling compartment—vascular, perivascular, and (to account for conditions with disrupted barriers, i.e., non-negligible compartmental mixing) pooled compartments—we can gain insight into their respective contributions. Specifically, we employed two analysis pipelines: one aimed at individual compounds and supported by MS/MS, the other aimed at network-level changes (cf. Experimental section). Depending on the pipeline, we detected around 2700 compounds in the supernatant. It is worth noting that these numbers are on the lower end for this type of study (e.g., $\approx 1/3$ – $1/2$ compared to our prior work with Caco-2 or primary BBB-on-chip).^[6,57] We attribute this to the relatively minimal, protein-poor media we employ here.

2.5.1. Verification of Dosage Levels

While untargeted metabolomics is not suitable for absolute quantification, we can still obtain information about both NACA and SIN-1 (or, more accurately, its stable breakdown product SIN-1C). We were able to assess SIN-1C as qualitative ratios (Figure S5, Supporting Information); importantly, we could infer that concentrations in SIN-1 (++) devices were not significantly different between disrupted and intact barriers, and matched (++) media dose controls. This suggests that the observed stochastic behavior of the endothelial layer was not concentration related. We did find that concentrations in high-dose transwells were higher than even media controls, possibly due to an increased transwell evaporation rate altering intended dilution ratios. For NACA, where we could rely on a calibration curve and much better UPLC/MS signal quality, we were able to establish that vascular concentrations in both devices (IQR [3.8; 4.5] $\times 10^{-3}$ M) and transwells (IQR [3.8; 4.8] $\times 10^{-3}$ M) fell within our target range (Figure S5, Supporting Information).

Based on perivascular concentrations, we further found that NACA successfully permeated all our hiBMEC barriers. We can report the first estimate for its hiBBB $P_{app} = 6 \text{ nm s}^{-1}$ (CI_{95%} [4; 8]; fully overlapping for both devices and transwells). This is broadly in line with our expectations for passive paracellular permeability of a small, hydrophilic molecule (162.2 Da, log $P \approx -1.5$; cf. Section 2.6.1). While the literature does not provide prior in vitro examples for NACA, one study with an epithelial Caco-2 barrier model has reported P_{app} for NAC below their assay detection limit (≈ 5 – 10 nm s^{-1}).^[58] Compared to hiBMEC, Caco-2 are typically leakier (e.g., 13 vs <2.5 nm s^{-1} for sodium fluorescein).^[8,59] Taken together, this may indicate that indeed NACA features higher BBB permeability than

with high S100B expression are distributed across the perivascular channel. SIN-1 (++) treatment results in tight junction breakdown and formation of larger defects in approximately half the cases (★); in the other half, barriers remain intact. The barrier breakdown denoted by zones (a, i–iii) and the white arrows (b) is described in the text. Barriers additionally exposed to NACA (++) remain indistinguishable from controls. Conversely, a majority of hiAstro have detached or condensed (arrows) in devices treated with NACA (++) and/or SIN-1 (++) . A small fraction of hiAstro remain attached and spread out, with a comparable morphology to those in the control group (white circled area). c) Similar trends are seen in transwells, where hiBMEC exhibit intact barriers in all conditions except SIN-1 (++) . hiAstro condense and detach to a large extent when treated with SIN-1 at both 1×10^{-3} M (+) and even more so at 4×10^{-3} M (++) doses (white arrows). NACA prophylaxis for lower-dose SIN-1 (+) results in higher hiAstro survival. With a higher dose of 4×10^{-3} M SIN-1 (++) , rescue with NACA is not successful. Scale bars a,c) are 50 μm , b) 1500 μm . Note that SIN-1 (+) conditions are only present for transwells.

Figure 3. Untargeted metabolomic analysis. a) Principal component analysis (PCA) of untargeted metabolomics, separated by compartment, showing broad changes in metabolic profiles. Symbols indicate devices (filled) and transwells (small, open), as well as intervention condition. The region containing the majority (80%) of media-only controls is indicated with a dotted outline. PCs (or linear combinations thereof) are selected for least variation in media controls; the remaining 2 (out of first 4) PCs can be found in the Supporting Information. b,c) Changes in metabolomic profiles compared to controls on the b) compound and c) network level. The matrices are separated vertically by experimental conditions, and horizontally by pathway/compound. The results matrix is very information-dense, but as with all untargeted metabolomic analysis should be considered more for trends than for any single entry. Each cell contains sub-entries on a per-compartment basis (sketch top middle), indicating both the respective significance levels (from $p = 0$ (purple) to 0.1 (cyan), or uncolored if not measured). Compound symbols indicate generalized-log₂ change in production or consumption (or a change in modality; squares). Network arrows indicate up/downregulation. Compounds were ranked by $\log(p < 0.005) \times (|\log_2\text{-change}| > 1)$ to select the top 11 endogenous hits (↑ indicating tentative ID). We include all pathways (size > 10) with at least one compartment at $p < 0.05$. Refer to the Supporting Information for complete lists of potential pathways/compounds. Note that SIN-1 (+) conditions are only present for transwells.

NAC. However, exemplary drugs with highly effective passive BBB permeability typically rely on a transcellular route and have exhibited P_{app} in the range of 100+ nm s⁻¹ in a similar transwell hiBBB model.^[60] Our results—including a comparison with our later tracer dye results (Section 2.6.1)—thus suggest that NACA transcellular permeability is present but limited, and the less-efficient paracellular pathway remains relevant if not dominant. While the amide modification may thus indeed improve NACA bioavailability compared to NAC, alternative modification or encapsulation strategies may be beneficial for effective brain delivery.

2.5.2. Principal Component Analysis

We consider first the principal component analyses (PCA), comparing hiBBBs-on-chips (and -in-transwells) for the various treatment conditions. PCA can reveal broad changes in metabolic profiles by considering a wide range of compounds, but without any specificity. The first four PCs account for ~50% of the variation; in Figure 3a we show the two PCs with the closest grouping of media controls in each case (see Figure S6, Supporting Information for the other two PCs, capturing largely within-group variation). Under control conditions—where,

unlike with endpoint imaging, we could rely on measurements from intact preintervention hiBBBs-on-chips—we observe that transwells and devices are closely co-located for both compartments, yet distinguishable, indicating similar biological function. The clearest intervention-induced difference emerged in the vascular compartment with NACA prophylaxis, which contrasts with our morphological observations showing little difference between that and controls. In the perivascular compartment, we note opposing trends for our systems: Devices clearly reverted toward media-only, which is in line with the hiAstro death and detachment we observe in imaging and the continuous perfusion of fresh media in the devices (opposed to continuous accumulation in transwells). The pooled-compartment PCA again shows matching trends between systems, with transwells simply offset toward the right. One notable feature of interest is the inclusion of conditions with SIN-1 (++)-disrupted barriers (★ as identified by permeability; cf. Section 2.6.1), which here are indistinguishable from nondisrupted SIN-1 (++) device barriers. This suggests that metabolic function of the surviving cells in either subpopulation remained broadly similar.

2.5.3. Networks and Compounds

Here, we consider the putative metabolic changes in the hiBBBs relative to control conditions both for individual compounds as well as at the network level. In Figure 3b,c, we show the effective changes as well as significance levels for the top 20. These analyses give higher biological meaning than PCA, but inherently consider a smaller fraction of the underlying data. Overall, our potential pool of hits entails 161 annotated compounds (of which 57 are tentative[†]; cf. Tables S1 and S2, Supporting Information) and 24 pathways (Table S3, Supporting Information). Similar to PCA, we find that NACA prophylaxis induces pronounced metabolomic alterations. On the compound level, we also observe a good share of hits with SIN-1 only. Most of these hits are conserved with NACA prophylaxis; we therefore suggest that NACA does not reverse the metabolic effects of SIN-1. Indeed, all our analyses (PCA, compounds, networks) point to that NACA prophylaxis prevents RONS damage not simply by reverting SIN-1-induced changes back towards controls, but by significant stimulation/regulation of separate metabolic processes. This is in agreement with NAC (the parent molecule of NACA) being only poorly reactive with most RONS (including peroxynitrite, superoxide, and nitric oxide).^[61] Its dominant pathway is as a precursor of Cys and glutathione; however, it is believed to affect a wide range of cellular functions through interactions with proteins. Glutathione regulation has also been directly confirmed with NACA in glutamate-stressed neurons.^[54]

For individual compounds (Figure 3b), we differentiate up/downregulation in terms of consumption versus production. The overall patterns we observe in our hiBBBs-on-chips are largely conserved from transwells, though changes are generally smaller. This is to be expected due to the different mass balance in devices, where continuous perfusion of larger media volumes results in less accumulation. A decrease in production, particularly when present largely in perivascular (or pooled)

compartments is likely linked to (particularly hiAstro) cell death, such as for lactate. We ascribe that hypothesis also to 2-ketovaline, which is a Val metabolite readily produced and released by astrocytes,^[62] as well as retinoate, which is produced by astrocytes and metabolized by BMEC.^[63] 5'-methylthioadenosine, on the other hand, shows downregulation also vascularly, more strongly so in the presence of NACA. This may be a result of this metabolite's involvement in antioxidant and particularly Met pathways.^[64] 2-piperidinone, one of the few consistently upregulated compounds (including devices), does not allow for much analysis due to its nature as a small fragmentary metabolite.^[65] Strong upregulation of adenosine production by astrocytes is associated with inflammatory events, and is aimed at modulating inflammatory response.^[66] This would be consistent with our transwell results, where the high levels of adenosine can also pass into the vascular compartment over the experimental duration. Lowered production/increased consumption of carnitine across compartments and conditions in the hiBBBs-on-chips (ameliorated by NACA) is consistent with its function as an endogenous antioxidant (thus reactive with peroxynitrite).^[67] The lack of equivalent transwell response may point to this being a late-stage response, thus more easily picked up in devices. Dimethylarginine is an *in vivo* marker of elevated oxidative stress, in apparent contradiction to the downregulation we observe.^[68] This likely indicates a more systemic nature of this metabolite's RONS regulation, which our systems do not recapitulate. Indole-3-acetate is typically associated with the gut microbiome (possibly indicating a false ID), but it can also occur as part of the endogenous Trp metabolism, and may thus correlate with the Trp network results (see below).^[65] Isobutyrylglycine and S-sulfocysteine, meanwhile, are metabolically not well understood;^[65] their potential involvement in RONS may warrant further study.

The individual compound results are numerous, with some suggestive of interesting future research. To gain an additional view that is both broader than compounds, but more specific than PCA, we finally turn to network-level analysis in Figure 3c. Here, one of the strongest and most well-conserved hits with SIN-1 (conserved with additional NACA) was Met & Cys metabolism. This aligns well with the central role of sulfur-containing amino acids in both pro- and antioxidant interactions.^[69,70] Met & Cys are also highly favored targets for direct reactions with peroxynitrite.^[25] A third directly implicated amino acid is Tyr, but this does not appear to extend to pathway-level changes here. NACA prophylaxis additionally revealed a strong hit—particularly in our hiBBBs-on-chips—on the downstream glutathione pathway, matching *in vivo* findings for the central mechanism behind NAC action.^[61] This is further supported by the hit on the closely linked glutamate pathway. Moreover, we find that Trp is deeply tied to NACA application in both devices and transwells (though in the latter it is also linked to SIN-1, albeit with roughly one order of magnitude lower significance than for NACA). We note one extensive *in vivo* metabolomic study of NAC (using lymphocytes from lupus patients) implicated three pathways—Trp, glutathione, and a mitochondrial pathway too small to be included in our analysis.^[71] The presence of significant CYP450 regulation—indicated in a range of brain disorders—in transwells only may reflect the higher survival of hiAstro in that system (and lack of fresh media

Figure 4. Barrier permeability. Data are separated by condition as per the legend. a,b) Transwell TEER and tracer dye permeability. c) Device tracer dye permeability. d) Preintervention ECIS R averaged over the signal plateau phase (cf. Figure S7 in the Supporting Information). Data in this graph only are displayed as median $\pm$ IQR, and grouped by their corresponding measured $P_{app}^{CB} > 10\text{ nm s}^{-1}$. The */** subgrouping is assigned based on a threshold for the median ($100\text{ }\Omega$) and the relative variability (IQR / median; 10%). e) ECIS R endpoints for TEER comparison. To highlight intervention-induced changes, the data are normalized to respective pre-intervention signals. f) Time-resolved normalized ECIS R , separated by condition. Note that SIN-1 (+) conditions are only present for transwells.

perfusion even after cell death).^[72] Due to the discrepancy also in vascular-side CYP450 response, involvement of this pathway may additionally be early-onset and/or time-limited (which for devices implies significant dilution). For devices, we found additional hits in the Asp & Asn, butanoate, porphyrin, and Tyr pathways. Being each present in only one compartment and condition at $p < 0.05$ calls for more targeted studies to confirm their involvement.

2.6. Barrier Integrity

2.6.1. Pre/Postintervention (Tracer Dye)

As mentioned earlier, we relied on d2–3 tracer dye permeability (Figure 4c) to confirm formation of $n = 13$ intact barriers in our hiBBBs-on-chips (an additional $n = 6$ will constitute the control* group; Section 2.6.2). Specifically, we employed the small and highly hydrophilic Cascade Blue hydrazide (5275 Da, $\log P \approx -7$),^[73] and established a threshold value of 10 nm s^{-1} (based on the first minimum in our data distribution). The

higher device failure compared to transwells (based on d3 TEER; Figure 4a) is to be expected due to continuous flow removing detaching cells. The intact-barrier control $P_{app}^{CB} = 2.4\text{ nm s}^{-1}$ ($\text{CI}_{95\%}$ [1.6; 3.4]) closely resembles the range of the only other hiBBB-on-chip system where comparable data is available ($2\text{--}6\text{ nm s}^{-1}$; lucifer yellow, 457.3 Da, $\log P \approx -5$).^[15] Our observations also fall within the $1\text{--}5\text{ nm s}^{-1}$ range of broader hiBBB literature observations (though many of these utilize fluorescein, which is smaller but also less hydrophilic, thus a less optimal paracellular marker; 332.3 Da, $\log P \approx 0$).^[1,74]

With our own transwell controls, we determined $P_{app}^{CB} = 0.8\text{ nm s}^{-1}$ ($\text{CI}_{95\%}$ [0.4; 1.4]; Figure 4b). Some of the discrepancy compared to devices may arise from conservative estimates of cell area and flow rates in the latter. However, we posit that a large part of this discrepancy arises from two factors, related to our microscopy observations of the hiBMEC shape (cf. Section 2.4), and to the membranes utilized. First, from cell shape analysis (Figure S1a, Supporting Information), we can calculate that cell perimeter per area (and therefore the total length of the tight junction network) increases 1.35-fold in the devices, allowing more opportunities for paracellular permeation.^[75]

Second, as with many other studies of this type, the membranes utilized in transwells and devices feature different pore sizes—known to affect barrier formation in primary BMEC.^[76,77] In independent transwell experiments, we found that this effect was conserved for hiBMEC, with TEER decreasing by 1.6-fold for 1 μm pores (compared to 0.4 μm ; Figure S1b, Supporting Information). We note that the higher device $P_{\text{app}}^{\text{CB}}$ may be considered more in vivo-like, where fluorescein permeability has been measured at 14.6 nm s^{-1} in live rat brain capillaries.^[78] Interestingly, the significant system-based discrepancy here does not match our earlier observation of identical NACA permeability in devices and transwells. For the antioxidant, this substantiates a contribution also of a passive transcellular route (which would remain unaffected by tight junction network length).

When we applied 4×10^{-3} M SIN-1 (++) perivascularly to the intact hiBBBs-on-chips, $P_{\text{app}}^{\text{CB}}$ jumped to 130 nm s^{-1} (CI_{95%} [4.9; 3500]) by d4 (Figure 4c). The data exhibit a clear bimodal distribution, with $n = 3$ remaining practically unchanged compared to controls, whereas $n = 4$ (★) show clear barrier breakdown with $P_{\text{app}}^{\text{CB}}$ in excess of 100 nm s^{-1} . We reiterate that each of our three fully independent experimental replicates contained at least one of either scenario. The intact/disrupted barrier subpopulations here correlate with those from imaging. The on-chip hiBMEC response does, in a sense, closely match the transwells: one subpopulation similar to SIN-1 (++) transwells ($>100 \text{ nm s}^{-1}$), the other—like lower-dose SIN-1 (+) transwells—matching the pre/nointervention controls. However, the response appears stochastic rather than concentration-dependent (discussed further in Section 2.6.3). Importantly, we observe that an additional prophylactic application of 4×10^{-3} M NACA (++) to $n = 6$ devices was wholly effective in preventing significant barrier breakdown, with $P_{\text{app}}^{\text{CB}} = 3.2 \text{ nm s}^{-1}$ (CI_{95%} [1.5; 6.6]) largely overlapping the control confidence interval.

2.6.2. Pre/Postintervention (Electrical)

For the ECIS resistance R from our integrated sensors, we first consider summarized results akin to those obtained with $P_{\text{app}}^{\text{CB}}$ (and thus similar to daily transwell TEER). Through d3, the intact barriers ($n = 13$) plateaued at 490 Ω (CI_{95%} [440; 530]; Figure 4d; see also Figure S7, Supporting Information). The relative confidence interval here is wider than in transwell TEER (Figure 4a), which we attribute to fabrication process variability. Specifically, we suspect that the variable (and difficult-to-control) metallization depth inside the pores is a factor. Still, we find that a drop of ECIS R signals below 100 Ω , or signal fluctuations (measured as IQR/median) in excess of 10%, always corresponded with a visibly disrupted barrier (**). To highlight this, for Figure 4d only, we include also $N' = 7$ devices (all within one experimental repeat, otherwise excluded) where erroneous cell seeding resulted in limited barrier formation, followed by breakdown/delamination within 24–48 h (confirmed by visual bright-field inspection). However, due to the locally sampled nature and somewhat higher inherent variability of ECIS, confirmation of complete continuity by tracer dyes is required (Figure 4c). Those devices with stable ECIS but above-threshold $P_{\text{app}}^{\text{CB}}$ (*; $n = 6$)—i.e., featuring barrier defects away from the electrodes, often not

apparent even on visual inspection as in Figure 2b—were retained as the control* group, considered only for imaging and ECIS.

To reduce the influence of device variability when considering the impact of SIN-1 on barrier integrity, we normalized the ECIS R data for the intervention period (d3–4) with respect to the per-device values recorded immediately prior. The resulting endpoint data (Figure 4e) closely mirror those from $P_{\text{app}}^{\text{CB}}$ (Figure 4c), with the control* group remaining unchanged at 0.99 (95% CI: 0.92–1.05), while SIN-1 (++) caused a drop to 0.43 (95% CI: 0.08–0.78) by d4—with a bimodal signal distribution matching the tracer dye assay. Unlike with $P_{\text{app}}^{\text{CB}}$, however, prophylactic NACA (++) did not fully protect measured barrier integrity, showing instead a small but significant decrease to 0.84 (95% CI: 0.79–0.89). Interestingly, this mismatch between ECIS R and $P_{\text{app}}^{\text{CB}}$ is very similar to that between TEER and $P_{\text{app}}^{\text{CB}}$ for transwells (Figure 4a,b). The measurable change in electrical, but not (tracer dye-) molecular permeability compared to no-intervention controls suggests that the induced RONS damage in the NACA prophylaxis group is largely confined to very small (ion-size) defects. This is akin to a prior study showing some antioxidants fully preventing RONS damage to primary hBMEC in terms of P_{app} , but not TEER.^[48] Overall, not only do our ECIS sensor results reflect independently measured alterations in barrier permeability; their trends also align with gold-standard methods. Based on data from both the pre- and post-treatment phase, we can further calculate that in spite of the aforementioned limitations our sensors achieve 71% sensitivity, and notably 100% specificity, in barrier breakdown detection.

2.6.3. Dynamic Measurements

As mentioned in the Introduction, hiBBB-on-chip studies to date show at best daily sensor readouts similar to what we present above. The advantage of our ECIS sensors here is their continuous measurement capability with <2 min temporal resolution. We want to briefly mention the barrier formation phase (Figure S7, Supporting Information), where we find that the barrier formation time course is different from transwell TEER, with ECIS R leveling off from d1 (rather than d2 for TEER). In part, we attribute this to performing ECIS using a permeable membrane (including partially metallized pores), where a conduction path through the perivascular channel is always available (and can become more pronounced as the barrier tightens). As mentioned in the Introduction, ECIS signals are moreover impacted by cell parameters beyond tight junction formation alone. Although we seek to minimize those parameters by analyzing resistance at a frequency of 6 kHz (in line with similar experiments),^[18] cell spreading does likely contribute to our early signal plateaus. Importantly, our observations of an early plateau match some^[79,80] (though not all; inconsistencies likely due to different electrode geometries)^[18,81] prior studies of ECIS with hiBMEC (using monoculture on commercial, non-permeable substrates). Interestingly, the daily measurements presented by Motallebnejad et al. with their on-chip hiPSC coculture model show a similar pattern to ours, with a plateau from d1 in spite of a TEER-type approach.^[14]

Of larger interest for our present study are the full measurement traces during the intervention period shown in Figure 4f (normalized as in 2.6.2). 72 h marks the 4×10^{-3} M NACA (++) administration into the vascular compartments of our hiBBBs-on-chips (where applicable); timing of the 4×10^{-3} M SIN-1 (++) in the perivascular compartments, with a ≈ 1.5 h delay, is indicated by the arrows. Up to that point, we observe a small down-up oscillation across conditions (including control*), likely reflecting cell response to freshly prepared media. Control* barriers (blue-green) remain very flat after this point—most of them decreasing to ≈ 0.95 by the endpoint, with two slightly increasing. This may be a result of small defects from d3 (which this group is known to include based on P_{app}) being present on top of the electrodes for these two devices, and the hiBMEC gradually closing them up over the course of d4. Already by 75 h, we note a distinct drop in ECIS R signals to ≈ 0.95 for all SIN-1 (++) devices compared to that control* group. Differences from NACA prophylaxis (yellow) only arise around 76 h, with those barriers only showing a very gradual decline to the aforementioned 0.84 endpoint signals. The ECIS R data from SIN-1-only devices (magenta), on the other hand, exhibit more varied characteristics. Three devices remain practically indistinguishable from those that also received NACA (matching P_{app}^{CB} data). The other four devices show ECIS R dropping below 0.2, but with unexpectedly varied time courses—from largely continuous (≈ 20 h) degradation to more sudden (≈ 4 – 6 h) loss of integrity, with onset times varying from 75 to 82 h. Besides the bimodal nature of barrier breakdown already seen in P_{app}^{CB} , the ECIS R data thus further reveals stochastic dynamics of the breakdown process.

As we have previously stated, the equal distribution of intact and disrupted SIN-1 (++) barriers across our three independent experimental replicates indicates these dynamics are not an artifact arising out of any batch effects (e.g., cellular differentiation, device coatings, etc.). Our metabolomics data moreover suggest that SIN-1 (++) concentrations were largely similar between the devices in question (cf. Section 2.5.1), and no clear subgroup distinction regarding SIN-1 barrier breakdown (★) appeared in PCA (cf. Figure 3a and Figure S6, Supporting Information). Based on all the available evidence, we posit a process whereby SIN-1 (as it approaches a certain threshold concentration) causes minor barrier damage which cannot be wholly prevented by NACA. These initial defects are likely ion-sized, based on the decrease in electrical signals (ECIS/TEER; based on ionic currents) while the larger-molecule P_{app}^{CB} remains constant. However, NACA prevents any larger-scale damage. We hypothesize that major hiBMEC disruption happens when the small defects accumulate across the barrier, allowing for random formation of clustered defects where larger-molecule diffusion can occur. This would result in SIN-1 penetration into the vascular compartment, where it can more directly disrupt the hiBMEC across the entire area and, in combination with fluid shear stress, accumulate further damage leading to cascade barrier failure. The concentration of SIN-1 in our devices is not sufficiently above the threshold concentration, however, to guarantee that clusters of defects are consistently formed, leading to bimodal observations. In transwells, on the other hand, SIN-1 (++) is sufficiently above the threshold concentration to ensure breakdown, due to the significantly higher mole-per-area (based

on a much larger perivascular compartment) as well as potentially higher liquid evaporation (cf. Section 2.5.1). Lower-dose SIN-1 (+), meanwhile, remains below the threshold. We have, however, recorded heterogeneous SIN-1 response in prior transwell experiments using TEER, where the SIN-1 concentration was closer to the threshold (Figure S8, Supporting Information; not directly comparable due to different media).

While verification of the biological mechanisms underlying heterogeneity will require further study, this does not diminish the added value provided by continuous ECIS monitoring in our hiBBBs-on-chips. It allowed us to follow the temporal dynamics of barrier alterations (or lack thereof), depending on the applied interventions, with the unprecedented temporal resolution, yielding a rich new dimension to hiBBB studies. Such temporal resolution is especially important for acute biological injuries such as stroke or traumatic brain injury, as well as to study the pharmacokinetics and pharmacodynamics of potential treatments, where 15–30 min timescales are highly relevant.^[82–84]

3. Conclusion

We presented a new type of hiBBB-on-chip system based on OSTE+ microfluidics, which enabled simple integration of both the permeable membrane as well as sensors to monitor barrier function continuously. Our isogenic hiBBB coculture in this microfluidic system exhibited properties similar to both traditional cell culture models and to the few existing hiBBB-on-chip systems. Specifically, we observed formation of continuous tight junction networks and expression of the glial marker S100B in the respective cell types, and verified junction integrity using fluorescent tracer diffusion. When applying perivascular nitrosative stress—akin to microglia activation—using the peroxynitrite generator linsidomine (SIN-1), we observed consistent and widespread hiAstro cell death, and hiBMEC barrier disruption in approximately 50% of cases. Prophylactic vascular administration of the antioxidant NACA increased hiAstro survival fractionally. However, we found that its hiBBB permeability was low compared to drugs classified as BBB-permeable. On the vascular side, NACA proved wholly effective in preventing barrier disruption. The network-level metabolomic response profile we observed moreover aligned with the available literature findings for nitrosative stress and NAC, particularly stimulation of cellular glutathione pathways that help prevent damage caused by RONS exposure. Last but certainly not least, we were able to monitor changes in barrier integrity using the integrated ECIS sensors with (for hiBBB systems) unprecedented temporal resolution. Our study thus revealed for the first time the effects of direct RONS application on the BBB, as well as the effects of NACA prophylaxis, in a microphysiological system.

We recognize that, compared to TEER, our ECIS-based sensor approach samples the barrier integrity locally rather than globally, thus requiring confirmation of the intact-barrier state from a global measure such as tracer dye permeability. Drops in ECIS, notably, were 100% specific with regard to barrier disruption. We also expect that optimization of the fabrication process will allow us to lower signal variability between

devices in the future. Overall, we believe the advantages of a coplanar electrode layout in terms of fabrication complexity and off-chip interfacing (a critical factor in facilitating continuous measurements) outweigh the caveats. In terms of biological functionality, we acknowledge that a lack of pericytes in our hiBBB coculture presents a limiting factor. However, markers for pericyte lineage are significantly less well established than those for endothelial cells or astrocytes.^[1] Research on this front is rapidly evolving, however, and we expect to utilize a hiAstro-hiPeri mixture for the perivascular compartment in the near future. We also emphasize the continued need for optimization and functional characterization even in the better-established models (including hiBMEC) to ensure hiPSC-derived cell identity matches the in-vivo characteristics desired for a given study. Another goal is to introduce microglia to these hiBBB models, where onset of neuroinflammation could be biologically rather than chemically induced. Such a model could help understanding cell-cell interactions in the BBB, e.g., gain insight on the dual effect of microglia on BBB integrity—namely their neuroprotective effect during inflammatory states but also microglia-astrocytic endfeet interactions for example causing barrier disruption.

In conclusion, we have demonstrated a hiPSC-based microphysiological system of the BBB featuring truly continuous monitoring, with RONS barrier disruption and antioxidant prophylaxis as a proof-of-concept. We have validated that OSTE+ is not only suitable for simple fabrication of—and sensor integration in—microphysiological systems, but also for long-term culture of sensitive hiPSC-derived cells and high-quality imaging. Our hiBBB-on-chip can find immediate utility in the study and screening of drugs with barrier-modulating effects, providing unique readouts about temporal pharmacodynamic profiles. Based as it is on isogenic coculture, it is furthermore well suited to disorder-specific studies, such as shedding light on the potential causative involvement of BBB leakage in neurodegenerative and neuropsychiatric disorders.

4. Experimental Section

Detailed methods can be found in the Supporting Information.

In brief, OSTE+ devices were fabricated in a double-molding process that directly integrates track-etched PC cell growth supports. Starting from a CNC-machined positive aluminum master mold, negative PDMS-based molds were created and subsequently clamped around the PC membrane for reaction-injection molding of OSTE+.^[85,86] Prior to integration, interdigitated gold electrodes for ECIS were patterned onto the membranes.^[87] The resulting open-channel OSTE+ structures on either side of the membrane were sealed with commercial PC parts to form the cell culture microfluidics, and disinfected before use. A detailed process flow of the fabrication is shown in Figure S9 (Supporting Information).

On d–1, devices were placed into an incubator alongside a peristaltic pump and media reservoirs, and the electrodes were connected (via a matrix switching unit) to an LCR meter. The vascular and perivascular compartments were coated with fibronectin/collagen and laminin, respectively. To recreate the vascular hiBBB, predifferentiated hiBMEC were thawed and seeded on d0.^[8] To recapitulate the astrocyte–endothelial interaction, predifferentiated hiAstro were concurrently thawed and seeded on the perivascular side of the membrane.^[10] After cell seeding, ECIS *R* was continuously measured and media were continuously perfused at an average flow rate of 1.5 $\mu\text{L min}^{-1}$ in both the vascular and

perivascular channel (corresponding to a shear stress of 1.2 mPa). On d3, media were collected for control-group tracer dye measurements and metabolomics (the latter immediately frozen until their off-site analysis). Subsequently, utilizing the continuous media perfusion, NACA was administered to the vascular channels to serve as a neuroprotective agent, before introducing SIN-1 in the perivascular channel with a 1.5 h delay. On d4, media were again collected for permeability and metabolomics. Immediately afterward, the cells were fixated and stained for ZO-1 and S100B; nuclei were counter-stained with Hoechst. Transwell cocultures were kept in parallel throughout the experiment for comparison of a dynamic vs static model, assaying TEER daily and collecting media for tracer dye permeability and metabolomics on d4. Table S4 (Supporting Information) summarizes the number of hiBBBs across all experimental conditions.

Image analysis focused on maximum intensity projections of the confocal stacks acquired. Besides visual inspection, cell coverage (transwell hiAstro) and cell size (hiBMEC) were further quantified in ImageJ. Tracer dye fluorescence measurements, with the aid of calibration curves, were used to calculate apparent permeability. These data, together with TEER and ECIS, were analyzed in OriginPro. Values were reported in terms of means and their 95% confidence intervals, excepting cases where the distribution width was of greater importance (in which case medians and interquartile ranges were reported). For metabolomics, two parallel workflows were followed, aimed either at annotated compounds using ThermoFisher's proprietary software,^[88] or at network-level results using open-source tools.^[57] Since both SIN-1 and NACA acted not only on the cells, but also reacted with the media itself, media background-subtraction was employed in the workflows for more meaningful comparisons. For metabolomics data only, statistical analyses were conducted in *R* and (by design) needed to rely on *p*-values. Data are available on figshare.^[89]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

T.E.W. and A.H. contributed equally to this work. I.M.: Conceptualization (supporting); Methodology (equal); Investigation (equal); Analysis & Visualization (equal); Writing (equal). D.V. and P.N.: Methodology (supporting); Review & Editing (supporting). T.E.W.: Conceptualization

(equal); Methodology (equal); Investigation (equal); Software; Analysis & Visualization (equal); Writing (equal); Supervision & Administration (supporting). A.H.: Conceptualization (equal); Methodology (supporting); Review & Editing (supporting); Supervision & Administration (lead).

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Metabolomics data are openly available at Figshare: 10.6084/m9.figshare.14528409.

Keywords

continuous monitoring, human-induced pluripotent stem cells, microphysiological systems, neurovascular unit, oxidative and nitrosative stress

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