Neuroscience

Cortical Plasticity is associated with Blood-Brain-Barrier Modulation

Evyatar Swissa, Uri Monsonego, Lynn T. Yang, Lior Schori, Lyna Kamintsky, Sheida Mirloo, Itamar Burger, Sarit Uzzan, Rishi Patel, Peter H Sudmant, Ofer Prager, Daniela Kaufer, Alon Friedman

Department of Brain and Cognitive Sciences, The School of Brain Sciences and Cognition, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel • Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel • Department of Integrative Biology, University of California, Berkeley, CA 94720, USA • Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720, USA • Department of Medical Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada • Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2
February 13, 2024 (this version)

Reviewed preprint version 1
September 12, 2023

Sent for peer review
June 21, 2023

Posted to preprint server
April 24, 2023

Abstract

Summary

Brain microvessels possess the unique properties of a blood-brain barrier (BBB), tightly regulating the passage of molecules from the blood to the brain neuropil and vice versa. In models of brain injury, BBB dysfunction and the associated leakage of serum albumin to the neuropil have been shown to induce pathological plasticity, neuronal hyper-excitability, and seizures. The effect of neuronal activity on BBB function and whether it plays a role in plasticity in the healthy brain remain unclear. Here we show that neuronal activity induces modulation of microvascular permeability in the healthy brain and that it has a role in local network reorganization. Combining simultaneous electrophysiological recording and vascular imaging with transcriptomic analysis in rats, and functional and BBB-mapping MRI in human subjects we show that prolonged stimulation of the limb induces a focal increase in BBB permeability in the corresponding somatosensory cortex that is associated with long-term synaptic plasticity. We further show that the increased microvascular permeability depends on neuronal activity and involves caveolae-mediated transcytosis and transforming growth factor beta signaling. Our results reveal a role of BBB modulation in cortical plasticity in the healthy brain, highlighting the importance of neurovascular interactions for sensory experience and learning.

eLife assessment

This study used prolonged stimulation of a limb to examine possible plasticity in somatosensory evoked potentials and the role of the blood brain barrier (BBB). The significance is **important** because thus far BBB modulation of plasticity is mostly in the context of pathology. The revisions greatly improved the paper and the strength of evidence is **convincing**.

Introduction

The intricate components comprising the blood-brain barrier (BBB) regulate the bi-directional transport of molecules between the brain and the circulatory system and maintain the extracellular environment needed for normal function of the neurovascular unit (NVU) (Chow and Gu, 2015). Dysfunction of the BBB is common in many neurological disorders and is associated with impaired cross-BBB influx and efflux (Obermeier et al., 2013). For example, seizures were shown to increase cross-BBB influx of serum albumin – in a process partially mediated by excessive release of neuronal glutamate and subsequent activation of N-methyl-D-aspartate (NMDA) receptors (Vazana et al., 2016).

Emerging evidence suggests that cross-BBB influx/efflux may also change in response to physiological neuronal activity in the healthy brain: (1) whisker stimulation (1 hour, 2 Hz) was shown to increase BBB influx of insulin-like growth factor-I in rats (Nishijima et al., 2010); (2) physiological neuronal activity was shown to regulate BBB efflux transport in mice (Pulido et al., 2020); and (3) circadian rhythms were shown to alter BBB efflux in Drosophila (Zhang et al., 2018) and mice (Pulido et al., 2020). However, the mechanisms and the role of these physiological changes in BBB properties are not known.

Here, we sought to test whether physiological BBB modulation may involve mechanisms previously linked to pathological BBB changes, e.g., transcytosis of serum albumin (Ivens et al., 2007; Seiffert et al., 2004) and transforming growth factor β (TGF-β) signaling (David et al., 2009; Heinemann et al., 2012; Ivens et al., 2007; Weissberg et al., 2015). As TGF-β signaling has been linked to synaptic plasticity (Dahlmanns et al., 2022; Gradari et al., 2021; Patel and Weaver, 2021; Weissberg et al., 2015), we also explored the hypothesis that physiological BBB modulation may be associated with neuroplasticity. Lastly, we sought to provide the first evidence for physiological BBB modulation in humans.

Results

Stimulation increases BBB permeability

To test for changes in BBB permeability in response to stimulation we combined electrophysiological recordings with BBB imaging using intravital microscopy (**Figure 1**, see timeline of stimuli in **Figure 1a**). First, we localized vascular response to sensory stimulation using wide-field imaging of arterial diameter and monitoring change in total hemoglobin (HbT) signal during a 1 min stimulation of the limb (**Figure 1b-d**) (Bouchard et al., 2009). Fluorescent angiography before and immediately after 30 min of limb stimulation showed local extravasation of the tracer sodium fluorescein (NaFlu) around the responding blood vessels, indicative of BBB leakage (**Figure 1f-g**). Histological analysis confirmed the presence of NaFlu in the extravascular space around small vessels in the somatosensory cortex, contralateral (and not ipsilateral) to the stimulated limb (**Figure 1m-n**). Next, we intravenously injected a separate cohort of rats with either the albumin-binding dye Evans blue (EB) or albumin conjugated with Alexa Fluor 488 (Alexa488-Alb) without performing craniotomy. Brain extravasation of both dyes was found in the contralateral hemisphere, indicating the presence of serum albumin in the neuropil (**Figure 1h-k**). ELISA performed in cortical tissue at different time points after stimulation confirmed a higher concentration of albumin in the stimulated sensorimotor cortex early after stimulation (30 min) compared to non-stimulated sham-controls, that declined 4- and 24-hours post-stimulation (**Figure 1l**). As a positive control, brain tissues were also prepared from rats subjected to photothrombosis (PT)-induced ischemia (see methods). Albumin

concentrations in the peri-ischemic cortex 24 hours following PT were significantly higher compared to sham and all time-points after stimulation (Figure S1d). These results suggest that the focal modulation of BBB permeability following stimulation is distinct from the extensive BBB dysfunction observed in injury models.

BBB permeability is associated with synaptic potentiation

To study changes in brain activity during repetitive stimulation of the limb, we measured somatosensory evoked potentials (SEP) by averaging neuronal response to a 1 min test stimulation before and after the 30 min stimulation train (6 Hz, 2 mA). SEP amplitudes and area under the curve were increased in stimulated, but not in control cortices (**Figure 2a-e**). Potentiation lasted until the end of each experiment (**Figure 2f**) and was therefore indicative of long-term potentiation (LTP).

To test whether stimulation-induced albumin extravasation has a role in LTP, we recorded local field potentials (LFP) before and after exposure of the cortex to 0.1 mM albumin (corresponding to 25% of serum concentration) (Ivens et al., 2007). Albumin application increased the amplitude and frequency of spontaneous activity (**Figure 2g,k**). Neuronal response to the stimulation showed synaptic potentiation in the presence of albumin compared to that recorded under aCSF (**Figure 2h-j**), indicating that cortical exposure to serum albumin results in synaptic potentiation.

BBB modulation is activity-dependent

To test the neurotransmission pathways involved in regulation of BBB permeability during stimulation we exposed the cortical window to selective antagonists of ionotropic glutamate receptors (**Figure 3a**). The AMPA/kainate receptor blocker CNQX prevented the stimulation-induced BBB modulation, whereas the NMDAR blocker AP5 did not (**Figure 3b-c**). CNQX also reversibly blocked synaptic activity and prevented the vascular response to neuronal activation, indicated by the absence of vasodilation (AKA “neurovascular coupling”; **Figure 3d-e**, Figure S2a, S3a,c). These results indicate that stimulation-induced BBB modulation was mediated by neuronal activation. In contrast, AP5 did not affect baseline SEP (**Figure 3d-e**), nor the vascular response (Figure S2a, S3b). Synaptic potentiation was blocked under both CNQX or AP5 (**Figure 3d-e, g-h**, Figure S2a), consistent with the crucial role of AMPA and NMDA receptors in synaptic plasticity. Next, we tested for molecular correlates of synaptic strength by comparing postsynaptic density 95 (PSD-95) expression levels in the cortex at different time points. PSD-95 is known to increase following LTP and AMPAR trafficking (El-Husseini et al., 2000; Holtmaat and Svoboda, 2009). Western blot analysis showed significantly higher levels of PSD-95 expression in stimulated rats as compared to sham, at both 30 min and 24 h after stimulation (Figure S2c-d).

Albumin extravasation and LTP require caveolae-mediated transcytosis

In endothelial cells, albumin is transported mainly via caveolae-mediated transcytosis (CMT) through binding to the gp60 receptor (Tiruppathi et al., 1997). To test whether CMT mediates stimulation-induced transcytosis of albumin (John et al., 2003) we perfused the exposed cortex with methyl-β-cyclodextrin (mβCD), which inhibits albumin uptake by disrupting caveolae (Schnitzer and Oh, 1994; Skotland et al., 2020). Cortical application of mβCD prevented stimulus-induced BBB opening (**Figure 3c**), without affecting vascular response to stimulation (Figure S3d). mβCD also prevented stimulus-induced LTP (**Figure 3f-h**), further supporting the role of CMT-mediated albumin extravasation in activity-dependent plasticity in vivo.

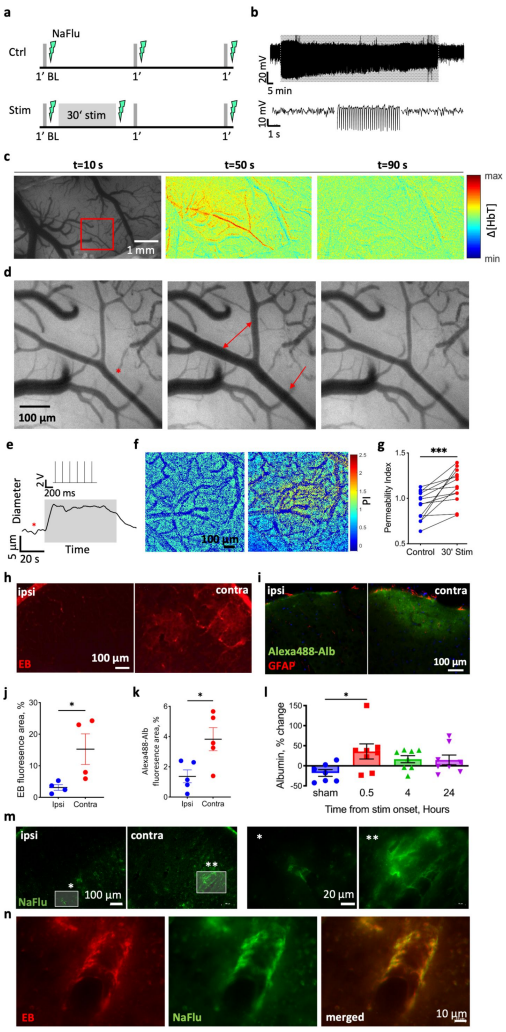

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Figure 1

Limb stimulation modulates blood-brain barrier permeability.

a. The experimental paradigm in control animals (Ctrl, top panel) and animals that underwent a 30 min stimulation (Stim, bottom panel). Gray lines indicate 1 min test stimulations, lightning indicates the timing of BBB imaging (using injection of sodium fluorescein, NaFlu). **b.** Top: local field potential (LFP) trace from the corresponding region in L2/3 sensorimotor cortex before during (greyed area) and after 30 min limb stimulation. Bottom: 5 s excerpts of the above trace (left-to-right: before during and after stimulation). **c.** Image of the cortical window over the rat sensorimotor cortex, followed by total hemoglobin (HbT) concentration maps showing the evolution of the hemodynamic response during and after stimulation. Red rectangle marks the responding region magnified in d. **d.** Images of the responding arteriole (marked with red arrows) in the rat's cortex before (left) during (middle) and after stimulation (right). **e.** The diameter of the responding arteriole during 1 min stimulation. Gray area corresponds to time of stimulation. **f.** NaFlu permeability maps before (left) and after 30 min stimulation (right) showing tracer accumulation around a responding arteriole. **g.** Permeability index is higher after stimulation compared to baseline (n=13 rats, mean ± SEM, Wilcoxon, p<0.001). **h-i.** Fluorescence images of cortical sections of stimulated rats injected with the albumin-binding dye Evans blue (h) and Alexa-488-Alb (i). **j-k.** Total fluorescence of EB (j) and Alexa488-Alb (k) after stimulation in the contralateral hemisphere compared to the ipsilateral hemisphere. (EB n=4 rats, 32 sections, nested t-test, p=0.0296; Alexa488-Alb n=5 rats, 20 sections, nested t-test, p=0.0229; mean ± SEM). **l.** Albumin concentration in the contralateral hemisphere relative to the ipsilateral in three different timepoints after stimulation, compared to sham stimulation. (0.5 and 4 h post-stimulation n=8, sham n=7, mean ± SEM, Kruskal-Wallis with FDR correction, p=0.0242, q=0.0406). **m.** Cortical sections of the area of limb representation from both hemispheres of a stimulated rat (left), and higher magnification images (right) **n.** Fluorescence image of a cortical small vessel in the stimulated hemisphere showing extravascular accumulation of EB and NaFlu.

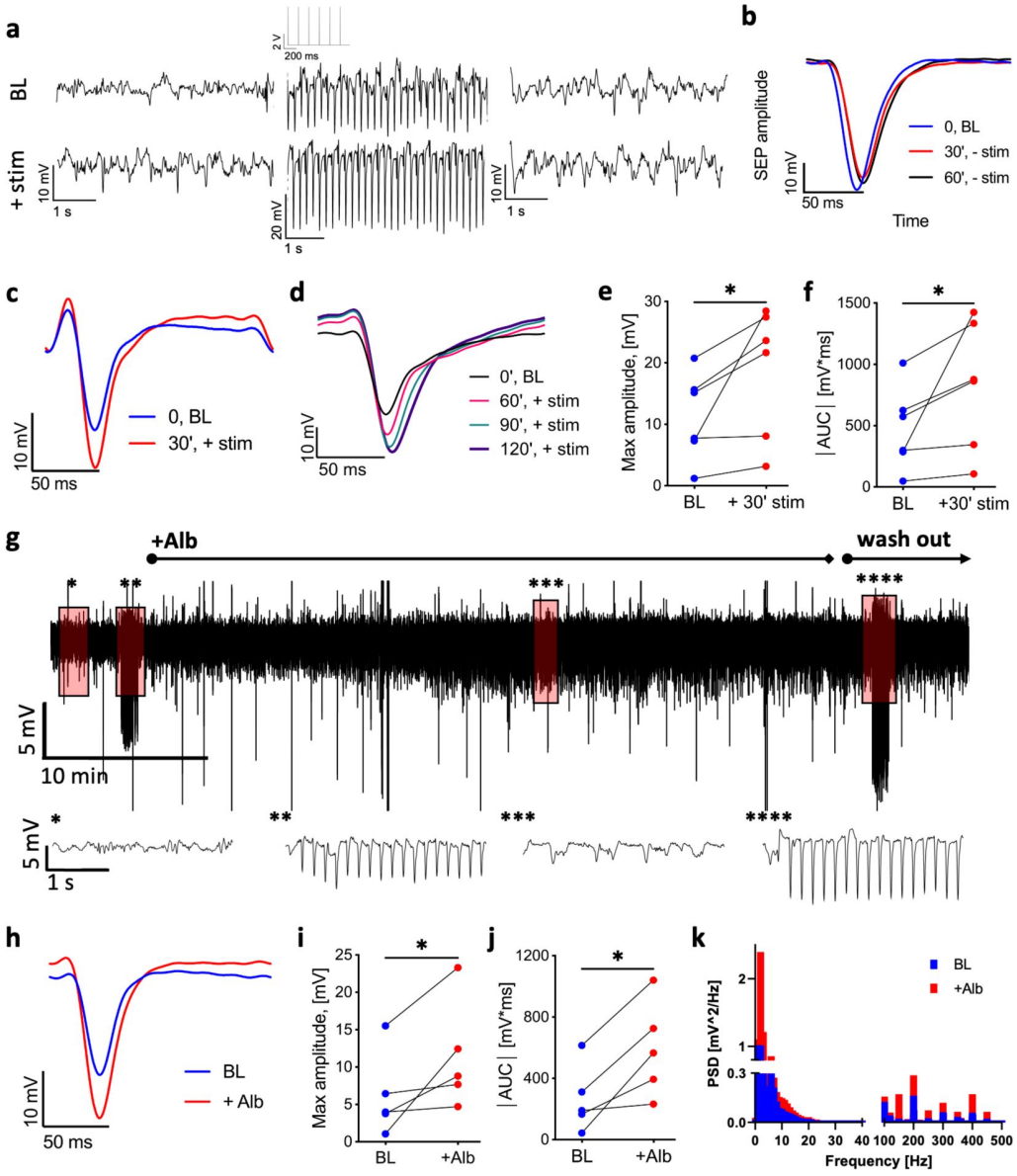

Figure 2

Stimulation and cortical perfusion of serum albumin induce LTP.

a. Top: LFP trace from the rat L2/3 sensorimotor cortex before (left) during (middle) and after (right) test stimulation (1 min, 6 Hz, 2 mA). Bottom: LFP trace from the same rat before (left) during (middle) and after (right) test stimulation (1 min, 6 Hz, 2 mA), administered following a 30 min stimulation (6 Hz, 2 mA). **b.** The somatosensory evoked potential (SEP) of test stimulation at baseline (0 min, BL) and after 30 and 60 minutes (blue, red, black, respectively, each averaged over 360 stimuli). **c.** SEP in response to test stimulation at baseline (blue) and following a 30 min stimulation (red). **d.** SEP in response to test stimulation at baseline (blue) and three time points following a 30 min stimulation (60 min, red; 90 min, green; 120 min, purple). **e.** Maximum amplitude of the SEP (absolute values) following a 30 min stimulation compared to baseline (n=6 rats, mean ± SEM, Wilcoxon, p=0.0312). **f.** Area under curve (AUC) of the SEP following a 30 min stimulation compared to baseline. (n=6 rats, mean ± SEM, Wilcoxon, p=0.0312). **g.** Top: 1-hour LFP trace from a representative rat. Bottom: 5 s magnifications of the above trace at selected time points (noted by asterisks). Left to right: baseline activity; during test stimulation; following cortical application of 0.1 mM albumin (Alb); during test stimulation post-Alb. **h.** SEP amplitude during test stimulation at baseline (normal aCSF, blue) and following 0.1 mM Alb (red). **i.** Maximum amplitude of the SEP during test stimulation post-Alb compared to baseline (n=5 rats, mean ± SEM, Wilcoxon, p=0.0312). **j.** AUC of the SEP post-Alb compared to baseline. (n=5 rats, mean ± SEM, Wilcoxon, p=0.0312). **k.** Power spectrum density of 10 min spontaneous activity before (blue) and post-Alb (red).

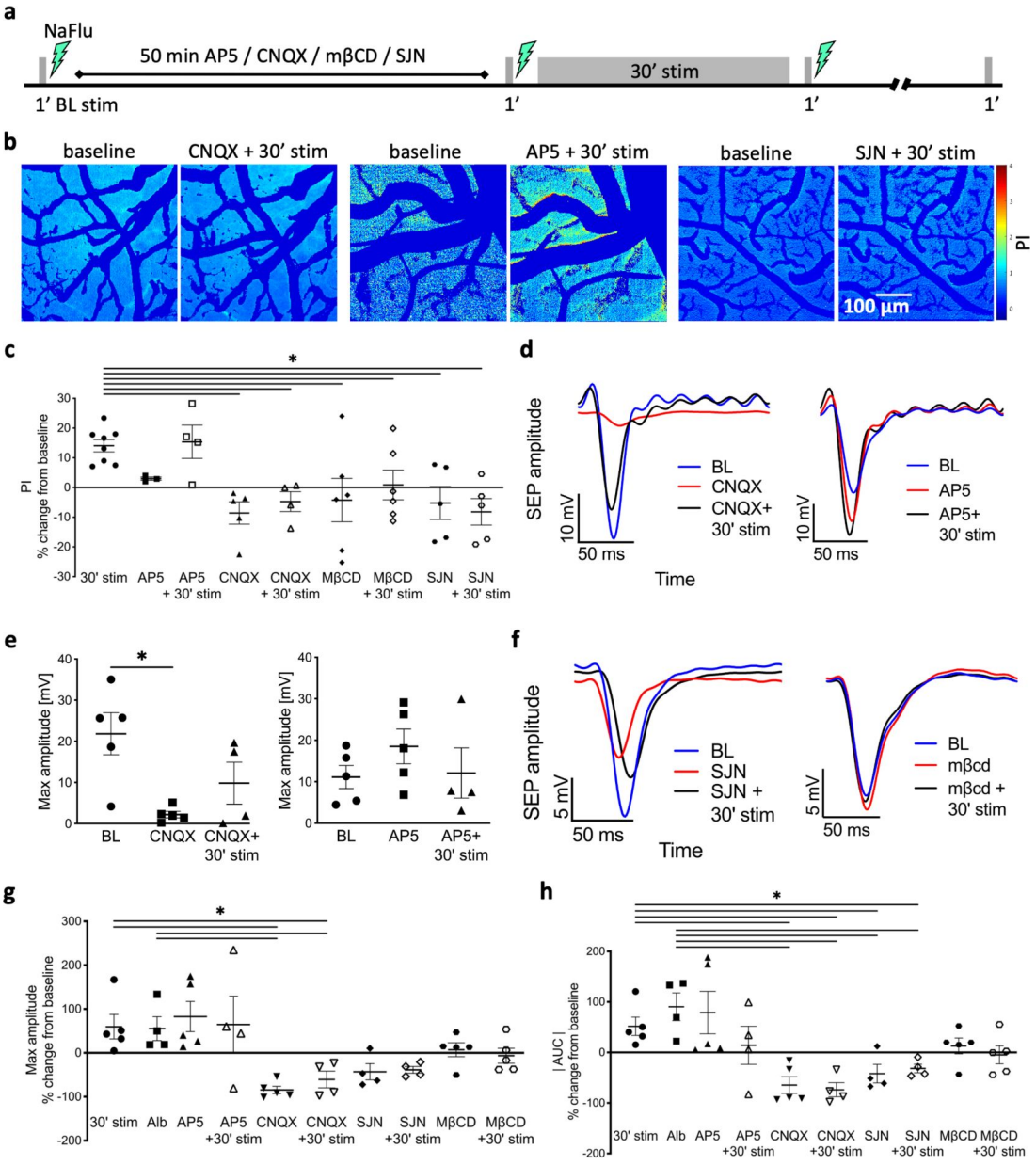

Figure 3

Stimulation-induced plasticity is associated with BBB modulation.

a. Timeline of the experimental protocol for stimulations and imaging with blockers application (CNQX/AP5 (50 µM)/mβCD (10 µM)/SJN (0.3 mM). **b.** NaFlu permeability maps of the cortical window before (control, left), and after CNQX/AP5/SJN + 30 min stimulation (right). **c.** PI following blockers application before and after stimulation compared to a 30 min stimulation. (% change from baseline, 30 min stim n=6, AP5 n=4, CNQX n=5, mβCD n=6, SJN n=5; mean ± SEM, Kruskal-Wallis with FDR correction, *p<0.05). **d.** SEP amplitude in response to test stimulation. baseline (blue); following application of CNQX (left, red) or AP5 (right, red); following CNQX + 30 min stimulation (left, black); and following AP5 + 30 min stimulation (right, black); **e.** Left: Max SEP amplitude to test stimulation following CNQX compared to baseline. (mean ± SEM, n=5, paired t-test, p=0.0176). Right: Max SEP amplitude following AP5 compared to baseline. **f.** SEP amplitude in response to test stimulation. baseline (blue); following SJN (black); following SJN + 30 min stimulation (red); and following mβCD (right). **g.** Max SEP amplitude following 30 min stimulation or albumin application compared to blockers, and blockers + 30 min stimulation. **h.** AUC of the SEP for test stimulation following 30 min stimulation or albumin application compared to blockers and blockers + 30 min stimulation. (**g**-**h**: % change from baseline, mean ± SEM, 30 min stim n=5, Alb n=4, AP5 n=5, CNQX n=5, SJN n=4, mβCD n=5; Kruskal-Wallis with FDR correction, *p<0.05).

BBB modulation and LTP involve TGF-β signaling

Under pathological conditions, synaptogenesis and pathological plasticity that follow BBB dysfunction are mediated by the activation of TGF-βR1 signaling in astrocytes (Weissberg et al., 2015). TGF-β1 was also shown to directly induce BBB opening in endothelial cell cultures (McMillin et al., 2015), and to regulate hippocampal synaptic plasticity (Dahlmanns et al., 2022; Gradari et al., 2021). We therefore tested the effect of stimulation in the presence of the specific TGF-βR1 blocker SJN2511 (SJN, 0.3 mM (Gellibert et al., 2004; Weissberg et al., 2015)). Cortical exposure to SJN prevented stimulation-induced BBB opening (**Figure 3b-c**) with no effect on neurovascular coupling (Figure S3e). SJN also prevented stimulation-induced LTP (**Figure 3f-h**). These results demonstrate that TGF-β signaling is required for activity-dependent BBB modulation, and the consequent activity-dependent plasticity, but not for neurovascular coupling.

Regulation of BBB transport and plasticity genes

To explore gene-expression changes related to our stimulation protocol, we conducted bulk RNA-sequencing transcriptome analysis of tissue dissected from the sensorimotor area of the cortex contralateral and ipsilateral to stimulated limb. Tissue harvested 1 and 24 h post-stimulation (30 min) showed differentially expressed genes (DEGs) in the contralateral ("stimulated") compared to the ipsilateral ("non-stimulated") cortex (13.2% and 7.3% 24 and 1 h, respectively, **Figure 4a**, S4a). To assess the variability between paired samples of stimulated and non-stimulated cortex of each animal at two time points following stimulation (24 h vs 1 h) we used the Jensen–Shannon divergence metric (JSD) (Sudmant et al., 2015). JSD calculations using normalized counts indicated that the RNA expression 24 h after stimulation was significantly different from that of rats 1 h after stimulation (Figure S4b). Gene Ontology (GO) pathway enrichment analysis of DEGs from stimulated rats indicated DEGs were primarily involved in synaptic plasticity processes (**Figure 4b**). A significantly higher number of DEGs related to synaptic plasticity was found in the contralateral cortex of stimulated rats compared to ipsilateral ($p<0.001$, **Figure 4d**). No significant differences were found in BBB or inflammation related genes between the hemispheres, suggesting that the stimulation protocol is not associated with the transcriptional change observed in BBB dysfunction under pathological conditions (Cacheaux et al., 2009; Kim et al., 2017) (**Figure 4d**). In contrast, a larger amount of transporter genes such as solute carrier transporters (Slc) and ATP-binding cassette (ABCs) families were differentially expressed in the stimulated compared to the non-stimulated cortex (Figure S4c-d). Both DEG and enrichment analysis were consistent with activation of TGF-β signaling pathway (**Figure 4d**). Analysis of vascular cell-specific DEGs (Vanlandewijck et al., 2018) showed significantly more arterial smooth muscle cells specific genes in the stimulated cortex (**Figure 4c**, Figure S4e). These results support the roles of stimulation-induced modulation of BBB transcellular transport and TGF-β signaling in activity-dependent plasticity (Figure S5).

Physiological BBB modulation in the human brain

Finally, we tested for neuronal activity-induced BBB modulation in healthy human volunteers by measuring the blood-oxygenation-level dependent (BOLD) response to a 30 min stress-ball squeeze task and blood-to-brain transport using dynamic contrast-enhanced (DCE) MRI (**Figure 5a-b**). As expected, BOLD response was found in the pre- and post-central gyrus, corresponding with the primary motor and sensory cortices, respectively, of the hemisphere contralateral to the squeezing hand (**Figure 5c-d**). DCE-MRI showed higher BBB permeability to the gadolinium-based contrast agent gadoterate meglumine (Dotarem) in sensory/motor-related areas of subjects performing the task, as compared to controls (**Figure 5e-g**). Non activated cortical regions such as the medial frontal cortex and middle occipital gyrus did not differ in BBB permeability between task and controls (**Figure 5g**). Comparison of the spatial distribution of functional and permeability changes revealed a higher percentage of activated regions with modulated BBB in task performers than in control subjects (**Figure 5e-g**).

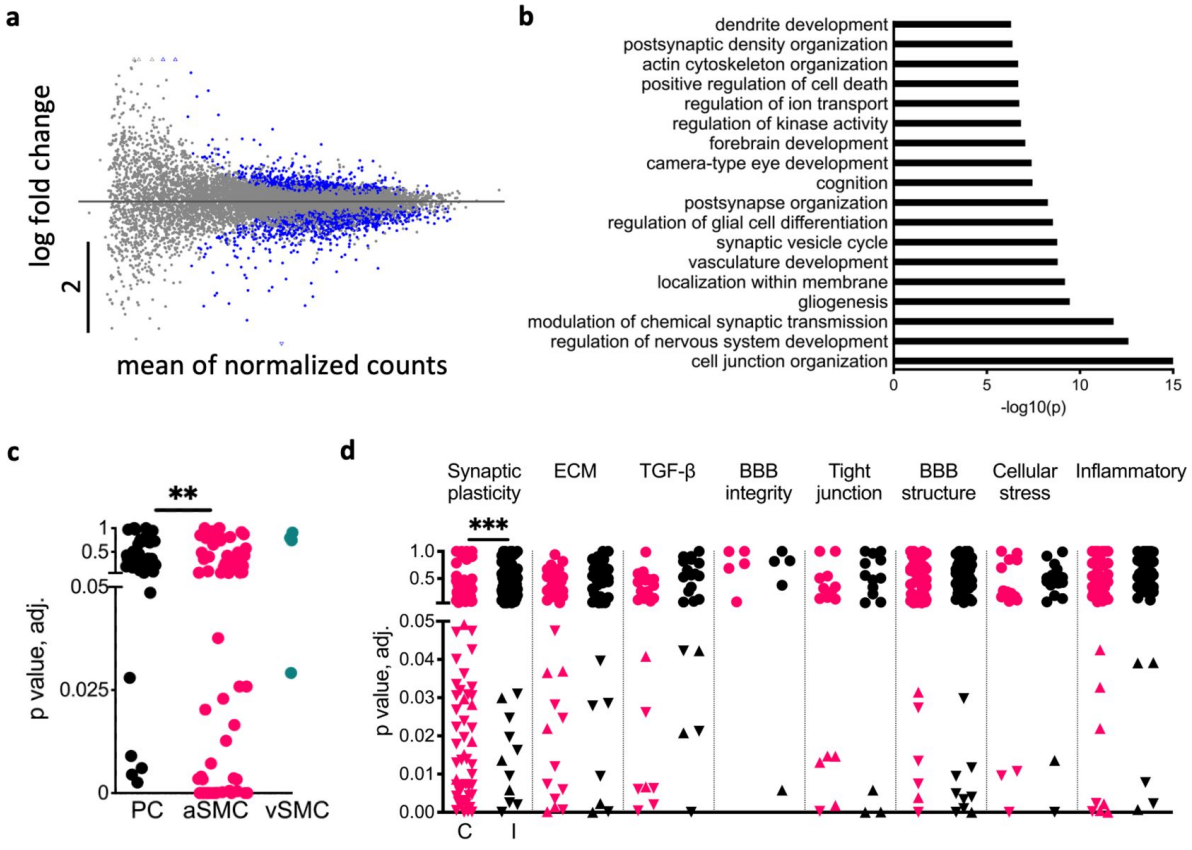

Figure 4

Neuronal activity regulates BBB transport and synaptic plasticity genes.

a. Scatter plot of gene expression from RNA-seq in the contralateral cortex 24 vs. 1 h after 30 min stimulation. The x axis represents the log fold change, and the y axis represents the mean expression levels. Blue dots indicate statistically significant differentially expressed genes (DEGs) by Wald Test (n=8 rats per group). **b.** Top Gene Ontologies (GO) enriched terms in the contralateral cortices of rats 24 vs. 1 h after stimulation. **c.** Vascular cell specific DEGs to pericytes (PC), arterial smooth muscle cells (aSMC), and venous smooth muscle cells (vSMC). (PC n=42, aSMC n=70, p=0.0017, Chi-square). **d.** Scatter plot of DEGs divided by groups of interest: BBB properties, NVU properties, Synaptic plasticity, and inflammatory related genes in the contralateral (red) vs. ipsilateral (black) cortices of stimulated rats. Circles represent genes with no significant differences between 1 and 24 h post-stimulation. Upward and downward triangles indicate significantly up- and down-regulated genes, respectively (synaptic plasticity n=53, ***p<0.001, Chi-square).

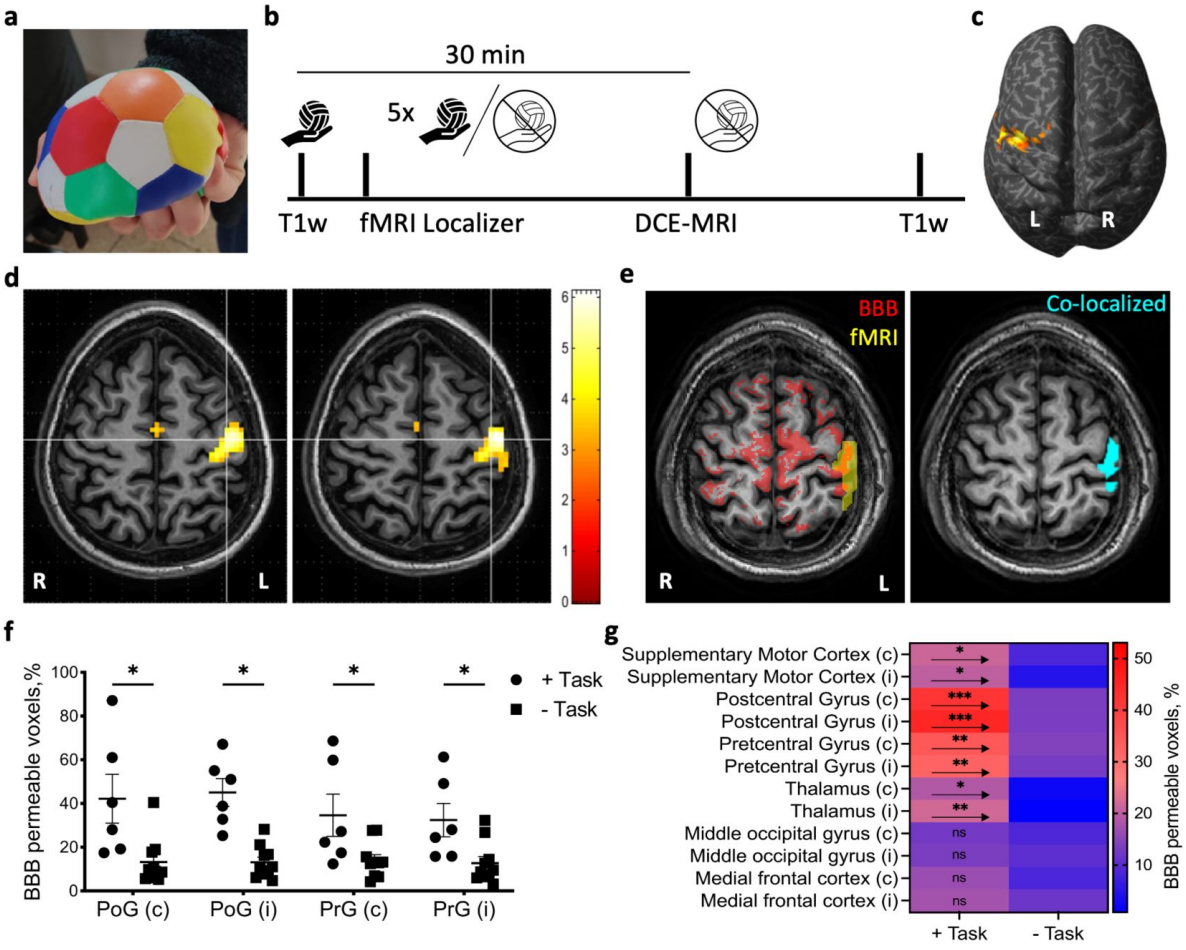

Figure 5

Cortical activation in fMRI co-localizes with BBB modulation.

a. Subjects were given an elastic stress-ball to squeeze continuously for the length of the session (30 min). **b.** Timeline of the experimental protocol for task performance and MRI sequences. **c.** Activation map for the localizer task displayed over the inflated brain of an exemplary subject. (p<0.05, Family wise error (FWE) corrected, neurologic convention). **d.** Activation maps for the localizer task displayed over anatomical axial slices of an exemplary subject. (p<0.05, FWE corrected, radiologic convention). **e.** Left: Superimposed masks of BBB modulated voxels (red) and fMRI activation (yellow). Right: Co-localized voxels map on the same slice (cyan). **f.** BBB modulation in the primary motor cortex (M1, precentral gyrus, PrG) and primary somatosensory cortex (S1, postcentral gyrus, PoG) for subjects performing the task compared to controls (% of voxels in each region with BBB leakage). (Task n=6, controls n=10, i–ipsi, c-contra, mean ± SEM, *p<0.05, two-way ANOVA with FDR correction). **g.** Heatmap of BBB modulated voxels percentage in motor/sensory related areas and some non-activated cortical regions of task vs. controls (+Task n=6, -Task n=10, i–ipsi, c-contra, mean ± SEM, *p<0.05, **p<0.001, ***p<0.0001, ns – non significant, two-way ANOVA with FDR correction).

Discussion

This study demonstrates that prolonged physiological stimulation modulates BBB permeability in the rat and human cortex. In rats, we show that prolonged paw stimulation results in increased cross-BBB extravasation of both low- and high-molecular weight tracers. We also show that this extravasation is: (1) focal and localized to the cortical area corresponding to the stimulation; (2) mediated by AMPA neurotransmission, TGF-β signaling, and caveolae transcytosis; and (3) associated with synaptic potentiation and increased postsynaptic density. In humans, we present the first evidence for increased BBB permeability in response to prolonged limb activity. Together, our results suggest that stimulation-induced BBB modulation may play a role in synaptic potentiation.

Physiological changes in cross-BBB transport have been previously reported in response to whisker stimulation, circadian rhythms, and age (Nishijima et al., 2010; Pulido et al., 2020; Yang et al., 2020). Here, we examined whether the mechanisms involved in this process may include those identified in our studies of pathological BBB modulation. Previous studies from our group showed that brain injury is associated with albumin transcytosis, albumin binding to TGF-βR in astrocytes, and pathological TGF-β-mediated plasticity (Salar et al., 2016). Here, we show that prolonged physiological sensory-motor activation is also associated with albumin extravasation (although at a significantly lower concentration than under pathological conditions, **Figure 1I** and Figure S1d) and TGF-βR signaling. We further show that blocking caveolae-mediated albumin transcytosis or TGF-βR signaling inhibits stimulation-evoked increase in BBB permeability and synaptic potentiation (without affecting the physiological hemodynamic response). These results suggest that caveolae albumin transcytosis and TGF-β signaling are key mediators of activity-dependent BBB modulation and subsequent synaptic plasticity. These findings are supported by reports linking TGF-β signaling to: synaptic plasticity induced by environmental enrichment (Dahlmanns et al., 2022); enhanced spine and synapse formation (Patel and Weaver, 2021); and hippocampal neuroplasticity (Gradari et al., 2021). Moreover, our finding of caveolae-mediated albumin transcytosis in pial arterioles is in line with previous studies showing: caveolae-dependent transcytosis of albumin (Tecedor et al., 2013); caveolae abundance in arteriolar ECs and not capillary ECs (Chow et al., 2020); and the contribution of caveolae transcytosis to neurovascular coupling (Andreone et al., 2017; Chow et al., 2020). Notably, in our experiments, caveolae inhibition prevented BBB modulation yet did not significantly affect neurovascular coupling, likely due to the contribution of other vasodilatory pathways such as endothelial nitric oxide synthase.

As mentioned above, albumin is a known activator of TGF-β signaling, and TGF-β has a well-established role in neuroplasticity. Interestingly, emerging evidence suggests that TGF-β also increases cross-BBB transcytosis (Betterton et al., 2022; Kaplan et al., 2020; McMillin et al., 2015; Schumacher et al., 2023) Hence, we propose the following two-part hypothesis for the TGF-β/BBB-mediated synaptic potentiation observed in our experiments: (1) prolonged stimulation triggers TGF-β signaling and increased caveolae-mediated transcytosis of albumin; and (2) extravasated albumin induces further TGF-β signaling, leading to synaptogenesis and additional cross-BBB transport – in a self-reinforcing positive feedback loop. Future research is needed to examine the validity of this hypothesis.

Our study also shows that physiological BBB modulation requires AMPAR glutamate signaling and not NMDA. This finding is consistent with studies showing the dominant role of AMPAR in electrophysiological and hemodynamic responses to somatosensory stimulation (Graves et al., 2021; Gsell et al., 2006; Zhang et al., 2015). Moreover, the well-established role of AMPAR in neuroplasticity, long-term potentiation (LTP), and experience-dependent sensory cortical plasticity (Daw et al., 1993; Feldman, 2009; Watt et al., 2004) further supports the plasticity-related role of activity-dependent BBB modulation.

Our animal experiments show that a 30 min limb stimulation (at 6Hz and 2mA) increases cross-BBB influx, while a 1 min stimulation (of similar frequency and magnitude) does not. We believe that both types of stimulations fall within the physiological range because our continuous electrophysiological recordings showed no signs of epileptiform or otherwise pathological activity. Moreover, the recorded SEP levels were similar to those reported in previous physiological LTP studies in rats (Eckert and Abraham, 2010; Han et al., 2015; Mégevand et al., 2009) and humans (McGregor et al., 2016). Future studies are needed to explore the BBB modulating effects of additional stimulation protocols – with varying durations, frequencies, and magnitudes. Such studies may also elucidate the temporal and ultrastructural characteristics that differentiate between physiological and pathological BBB modulation.

In humans, skill acquisition often involves motor training sessions that last ≥30 minutes (Bengtsson et al., 2005; Classen et al., 1998) and result in physiological plasticity of sensory and motor systems (Classen et al., 1998; Draganski et al., 2004; Sagi et al., 2012). We, therefore, believe that the experimental task of our human study (30 minutes of repetitive squeezing of an elastic stress-ball) represents physiological activity, with neuronal activation in primarily motor and sensory areas (Halder et al., 2005). We further propose that the activity-related increase in BBB permeability we observed may be associated with learning.

A key limitation of our animal experiments is the fact they were performed under anesthesia, due to the complex nature of the experimental setup (i.e., simultaneous cortical imaging and electrophysiological recordings). Anesthetic agents can potentially alter neuronal activity, SEPs, CBF, and vascular responses (Aksenov et al., 2015; Lindauer et al., 1993; Masamoto and Kanno, 2012). To minimize these effects, we used ketamine-xylazine anesthesia, which unlike other anesthetics, was shown to maintain robust BOLD and SEP responses to neuronal activation (Franceschini et al., 2010; Shim et al., 2018). Another limitation of our animal study is the potentially non-specific effect of mβCD – an agent that disrupts caveola transport but may also lead to cholesterol depletion (Keller and Simons, 1998). To mitigate this issue, we used a very low mβCD concentration (10μM), orders of magnitude below the concentration reported to deplete cholesterol (Koudinov and Koudinova, 2001). Lastly, our animal study is limited by the inclusion of solely male rats. While our findings in humans did not point to sex-related differences in stimulation-evoked BBB modulation, larger animals and human studies are needed to examine this question.

To conclude, our study suggests that BBB modulation in response to physiological neuronal activity involves mechanisms previously identified in pathology-induced BBB changes (despite the significantly smaller increase in cross-BBB influx under physiological conditions). Our findings further suggest that physiological modulation of cross-BBB influx may play a role in synaptic plasticity, calling for future research into the dynamics of BBB function in health and disease.

Methods

Animal preparation and surgical procedures

All experimental procedures were approved by the Animal Care and Use Ethical Committee at the Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Adult Sprague Dawley male rats (300-350 g; Harlan Laboratories) were kept under a 12:12 h light and dark regimen and supplied with drinking water and food ad libitum. Surgical procedures were performed as reported previously(Prager et al., 2010). Rats were deeply anesthetized by intraperitoneal administration of ketamine (100 mg/ml, 0.08 ml/100 g) and xylazine (20 mg/ml, 0.06 ml/100 g). The tail vein was cannulated, and animals were placed in a stereotactic frame under a SteREO Lumar V12 fluorescence microscope (Zeiss). Body temperature was continuously

monitored and kept stable at 37 ± 0.5°C using a feedback-controlled heating pad (Physitemp). Heart rate, breath rate, and oxygen saturation levels were continuously monitored using MouseOx (STARR Life Sciences). A cranial section (4 mm caudal, 2 mm frontal, 5 mm lateral to bregma) was removed over the right sensorimotor cortex. The dura and arachnoid layers were removed, and the exposed cortex was continuously perfused with artificial CSF (aCSF (Prager et al., 2010), containing (in mM): 124 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2, 3 KCl, and 10 glucose, pH 7.4).

Stimulation protocol

The left forelimb or hind limb of the rat was stimulated using Isolated Stimulator device (AD Instruments) attached with two subdermal needle electrodes (0.1 ms square pulses, 2-3 mA) at 6 Hz frequency. Test stimulation consisted of 360 pulses (60 s) and delivered before (as baseline) and after long-duration stimulation (30 min, referred throughout the text as 'stimulation'). In control and albumin rats, only short-duration stimulations were performed. Under sham stimulation, electrodes were placed without delivering current.

Electrophysiology

In-vivo recordings

Stimulus-evoked potentials were recorded in the somatosensory cortex. Local field- and somatosensory evoked potentials (LFP, SEP) were recorded using a glass microelectrode (1.5 × 1.1 mm, 1-2 µm tip, 2-10 MΩ) filled with aCSF, which was inserted with an isolated, chlorinated silver wire connected to a headstage and amplifier (EXT-02B, npi electronic, Germany). The electrode was attached to a digital micromanipulator (MP-225, Sutter Instruments, CA, USA) and was carefully placed over the identified area of the sensorimotor response to the stimulation.

Analysis of electrophysiological recordings

Data acquisition was performed using PowerLab and LabChart (AD Instruments), for stimulation, and extracellular recordings. Signals were digitized (1 kHz) and filtered (high-pass 1 Hz, low pass 45 or 300 Hz). Analyses were performed using in house MATLAB scripts. Two measures were used to quantify synaptic plasticity: the maximal amplitude of the dominant negative peak and the absolute area under curve (AUC) of the SEP waveform (i.e., all positive and negative peaks between 10–160 ms post-stimulus period). For power analysis in albumin experiments, 10 min segments recorded before starting and after terminating albumin perfusion were used.

Drug Application

In some experiments the following blockers were added to the aCSF and continuously perfused to the cortical window for 50 min: D-(-)-2-Amino-5-phosphonopentanoic acid (AP5, 50 µM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 µM), methyl-β-cyclodextrin (MβCD, 10 µM), or SJN2511 (SJN, 0.3 mM (Weissberg et al., 2015)). To simulate BBB opening, bovine serum albumin (Alb, 0.1 mM (Seiffert et al., 2004)) was added to the aCSF and continuously perfused to the cortical window for 30 min.

In-vivo imaging

Dynamic imaging of regional cerebral blood flow, optical imaging of cortical oxygenation, neurovascular coupling (NVC), and vessel diameter measurements were carried out as previously described (Bouchard et al., 2009; Levi et al., 2012; Prager et al., 2010). Before, during, and after electrical limb stimulation, full-resolution (2560 × 2160 px) images were obtained for regional localization of the hemodynamic response, followed by acquisition of localized (512 × 512 px)

images of cortical surface vessels (2 frames/s, EMCCD camera, Neo DC-152Q-COO-FI; Andor Technology) under 525/50 band pass filter (38 HE, Zeiss). Images were analyzed offline using in-house MATLAB (MathWorks) scripts.

Fluorescent angiography and BBB permeability measurements and analyses were performed as described previously (Prager et al., 2010; Vazana et al., 2016). The non-BBB permeable fluorescent dye sodium fluorescein (NaFlu) was injected intravenously (0.2 ml, 1 mg/ml). Images of cortical surface vessels were obtained before, during, and after tracer injection at 5 frames per second. Across all experiments, acquired images were the same size (512 × 512 pixel, ~1×1 mm), centered above the responding arteriole. Images were analyzed offline using MATLAB as described (Vazana et al., 2016). Briefly, image registration and segmentation were performed to produce a binary image, separating blood vessels from extravascular regions. For each extravascular pixel, a time curve of signal intensity over time was constructed. To determine whether an extravascular pixel had tracer accumulation over time (due to BBB permeability), the pixel's intensity curve was divided by that of the responding artery (i.e., the arterial input function, AIF, representing tracer input). This ratio was termed the BBB permeability index (PI), and extravascular pixels with PI > 1 were identified as pixels with tracer accumulation due to BBB permeability.

Histology and immunohistochemistry

Albumin extravasation was confirmed histologically in separate cohorts of rats that were anesthetized and stimulated without craniotomy surgery. Assessment of albumin extravasation was performed using a well-established approach that involves peripheral injection of either labeled-albumin (bovine serum albumin conjugated to Alexa Flour 488, Alexa488-Alb) or albumin-labeling dye (Evans blue, EB – a dye that binds to endogenous albumin and forms a fluorescent complex), followed by histological analysis of brain tissue (Ivens et al., 2007; Obermeier et al., 2013 ; Lapilover et al., 2012; Veksler et al., 2020, Ahishali et al., 2020). Since extravasated albumin is taken up by astrocytes (Ivens et al., 2007; Obermeier et al., 2013 and others), it can be visualized in the brain neuropil after brain removal and fixation (Ivens et al., 2007; Lapilover et al., 2012; Veksler et al., 2020, Ahishali et al., 2020). Five rats were injected with Alexa488-Alb (1.7 mg/ml) and five with EB (2%, 20 mg/ml, n=5). The injections were administered via the tail vein. Following injection, rats were transcardially perfused with cold phosphate-buffered saline (PBS), followed by PBS containing 4% paraformaldehyde (PFA). Brains were then removed, fixed overnight (4% PFA, 4°C), cryoprotected with sucrose gradient (10% followed by 20 and 30% sucrose in PBS) and frozen in optimal cutting temperature (OCT) compound. Coronal sections (40-µm thick) were obtained using a freezing microtome (Leica Biosystems) and imaged for dye extravasation using a fluorescence microscope (Axioskop 2; Zeiss) equipped with a CCD digital camera (AxioCam MRc 5; Zeiss).

For immunostaining, brain slices were incubated in blocking solution (5% donkey serum in 0.1% Triton X-100/tris-buffered saline (TBS)), then incubated in primary antibody, followed by secondary antibody. Finally, slices were mounted and stained for DAPI (DAPI-Fluoromount-G, Invitrogen) to label nuclei. Staining was performed against glial fibrillary acidic protein (mouse anti GFAP−Cy3™, Sigma-Aldrich, C9205), Secondary antibody was anti-mouse Alexa Fluor 488 (Thermo-Fisher, A21206).

Immunoassays

Enzyme-linked immunosorbent assay (ELISA) for albumin extravasation

To assess the degree of albumin extravasation following stimulation, forty adult Sprague Dawley male rats (200-300g) were randomly divided into five groups. Rats were anesthetized (ketamine (75 mg/kg) and xylazine (5 mg/kg)) and underwent either a 30 min stimulation, sham stimulation

(electrodes placed without current delivery), or photothrombosis stroke (PT) as previously described (Lippmann et al., 2017; Schoknecht et al., 2014). Briefly, Rose Bengal was administered intravenously (20 mg/kg) and a halogen light beam was directed for 15 min onto the intact exposed skull over the right somatosensory cortex. Then, rats were deeply anaesthetized and transcardially perfused with cold PBS, and brains were extracted at three time points following stimulation (immediately (real and sham), 4 and 24 h post stimulation) and 24 h post stroke. The ipsi- and contra lateral somatosensory cortices were dissected, weighed, and snap-frozen on dry ice. Fresh frozen samples were then homogenized in PBS + 1% Triton X-100 (v/v). Tissue homogenates were centrifuged at 13000 g for 10 min and supernatants were stored at -80°C until further analysis. Albumin levels were determined in diluted samples, using the Rat Albumin ELISA kit (Bethyl Laboratories, TX, USA) according to the manufacturer's instructions.

Western Blotting

Brain tissues were collected from stimulated and control rats as described above. Protein lysates were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE, Bio-Rad) and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were washed with TBS + 0.1% Tween20 (TBST, 3 × 5 min), then blocked with 5% BSA in TBS for 1 h. Membranes were then incubated overnight at 4°C with primary antibodies, followed by 2 h incubation with secondary antibodies. Protein bands were visualized with the enhanced chemiluminescent HRP substrate Crescendo Immobilon (Millipore). Analysis was performed in ImageJ, and band density levels were expressed as fold-changes to controls after normalization to β-actin and controls within the same blot. Primary antibodies used were mouse anti-PSD-95 (1:1500, Thermo Fisher Scientific, MA1-045), anti-β-actin (Loading control, 1:2000, Abcam, ab8226). Secondary antibodies were Goat anti-mouse (1:4000, Abcam, ab205719) and Goat anti-rabbit (1:4000, Abcam, ab97051).

RNA Extraction & Purification

Total RNA was extracted from 100-150 mg of flash-frozen rat somatosensory cortex tissue using TRIzol reagent (ThermoFisher Scientific #15596026) as previously described (Rio et al., 2010). Glycoblue (Invitrogen #AM9515) was added to assist with RNA pellet isolation. Pellets were washed with 75% ethanol and allowed to dry before being dissolved in 20 µL of RNase-DNase free water. 2 µL of 10x DNase I Buffer (New England Biolabs, #m0303s), 1 µL of rDNase I (ThermoFisher Scientific, AM2235), and DNase Inactivation Reagent (ThermoFisher Scientific, AM1907) were added to each RNA sample for purification.

RNA Sequencing & Bioinformatics

Library preparation was performed by the QB3-Berkeley Functional Genomics Laboratory at UC Berkeley. mRNA selection was done with the oligo-dT beads. Both total RNA and oligo dT bead selected mRNA quality was assessed on an Agilent 2100 Bioanalyzer. Libraries were prepared using the KAPA RNA Hyper Prep kit (Roche KK8540). Truncated universal stub adapters were ligated to cDNA fragments, which were then extended via PCR using unique dual indexing primers into full length Illumina adapters. Library quality was checked on an AATI (now Agilent) Fragment Analyzer. Libraries were then transferred to the QB3-Berkeley Vincent J. Coates Genomics Sequencing Laboratory, also at UC Berkeley. Library molarity was measured via quantitative PCR with the KAPA Library Quantification Kit (Roche KK4824) on a BioRad CFX Connect thermal cycler. Libraries were then pooled by molarity and sequenced on an Illumina NovaSeq 6000 S4 flowcell for 2 × 150 cycles, targeting at least 25M reads per sample. Fastq files were generated and demultiplexed using Illumina bcl2fastq2 v2.20. No other processing, trimming, or filtering was conducted. RNA-seq reads were aligned using STAR v2.9.1a against UCSC rat genome rn6. Downstream analysis was performed using FastQC v0.11.9, MultiQC v1.11, and featureCounts. R studio was used for data analysis, R v4.0.2. with the packages tidyverse v1.3.1, IHW, DESeq2. Gene ontology (GO) analysis of enriched genes in ipsi- and contra-lateral somatosensory cortices of stimulated rats was performed using Metascape (Zhou et al., 2019) (*http://metascape.org/*).

Analysis of cell-specific and vascular zonation genes was performed as described (Vanlandewijck et al., 2018), using the database provided in (*http://betsholtzlab.org/VascularSingleCells/database.html*).

Magnetic Resonance Imaging

Participants

Male and female healthy individuals, aged 18-35, with no known neurological or psychiatric disorders were recruited to undergo MRI scanning while performing a motor task (n=6; 3 males and 3 females). MRI scans of 10 sex- and age-matched individuals (with no known neurological or psychiatric disorders) who did not perform the task were used as control data (n=10; 5 males and 5 females).

All procedures were approved by the Soroka University Medical Center Institutional Review Board. All participants gave their written informed consent before participation.

Data acquisition

MRI scans were performed in the imaging center at the Soroka Medical Center using a 3T Philips Ingenia MRI scanner. A high-resolution T1-weighted anatomical scan (3D gradient echo, TE/TR 3.7/8.2 ms, voxel size of 1 mm^3, 256 × 256 acquisition matrix), followed by a T2-weighted scan (TE/TR = 90/3000 ms, voxel size 0.45 × 0.45 × 4 mm). Functional data were collected by using a gradient echo EPI, with voxel size of 3 × 3 × 3 mm, TE/TR = 35/2000 ms, 83 × 80 acquisition matrix, flip angle of 90°, and FOV 109 mm. For calculation of pre-contrast longitudinal relaxation times, variable flip angle method was used (3D T1w-FFE, TE/TR = 2/10 ms, acquisition matrix: 256 × 256, voxel size: 0.89 × 0.89 × 6 mm, flip angles: 5, 15, 20 and 25°). Dynamic contrast-enhanced (DCE) sequence was then acquired (Axial, 3D T1w-FFE, TE/TR = 2/4 ms, acquisition matrix: 192 × 187 (reconstructed to 256 × 256), voxel size: 0.9 × 0.9 × 6 mm, flip angle: 20°, ýt = 10 s, temporal repetitions: 100, total scan length: 16.7 min). An intravenous bolus injection (0.1 mmol/kg, 0.5 M) of the gadolinium-based contrast agent gadoterate meglumine (Dotarem, Guerbet, France) was administered using an automatic injector after 5 dynamic scans at a rate of 1.5 ml/s.

Preprocessing of functional data

fMRI data preprocessing was performed using the Statistical Parametric Mapping package (SPM12; Wellcome Trust Centre for Neuroimaging, London, UK) for MATLAB. The first ten functional images of each run series were discarded to allow stabilization of the magnet. Functional images were realigned and co-registered to the T1-weighted structural image, followed by segmentation and normalization to the Montreal Neurological Institute (MNI) space. Spatial smoothing using Gaussian kernel (FWHM of 6 mm) was performed.

fMRI Localizer Motor Task

fMRI was used to localize the brain area activated in response to the experimental motor task. During the fMRI scan, participants were asked to squeeze the stress-ball on cue (with green screen indicating Go and red screen indicating Stop) for a total of 5 experimental blocks. Task-related activation was evaluated for each cue, and voxels with statistically significant activation (t-statistic) were used to construct an 'activation map'. The statistical threshold for clusters was set at $p<0.05$ and corrected for multiple comparisons using family-wise error (FWE) over the whole brain (uncorrected threshold $p<0.001$) to minimize detection of false positives (type I error).

BBB permeability quantification

Analysis was performed as reported (Serlin et al., 2019; Veksler et al., 2020). Briefly, preprocessing included image registration and normalization to MNI space using SPM12. BBB permeability was calculated using in-house MATLAB script. A linear fit was applied to the later stage of the scan and the slope of contrast agent concentration changes over time was calculated for each voxel. Positive slopes reflected contrast agent accumulation due to BBB modulation. To compensate for physiological (e.g., heart rate, blood flow) and technical (e.g., injection rate) variability, slopes were normalized to the slope of each subject's transverse sinus. Values were considered to reflect modulated BBB when exceeding the 95th percentile of the corresponding mean cumulative distribution function from healthy young controls. Voxels that reflect modulated BBB values were color-coded and superimposed on the anatomical scans to illustrate the kinetics, localization, and extent of BBB pathology.

Statistical analysis

All statistical tests were performed using Prism9 (GraphPad Software) and are indicated in the figure legends. Tests were two-tailed and corrected for multiple comparisons with two-stage linear step-up false discovery rate (FDR) procedure of Benjamini, Krieger and Yekutieli whenever applicable. Paired tests were used for inter-hemispheric comparisons. Non-parametric tests were used for data which was not normally distributed. Differences in fluorescence in **Figure 1j-k** were tested using a nested t-test. Differences in BBB permeability index, area under the curve and maximum amplitude of the somatosensory evoked potential before and after stimulation, were tested using the Wilcoxon matched-pairs signed rank test. Differences between groups in ELISA, area under the curve and maximum amplitude of the SEP were analyzed using Kruskal-Wallis test with FDR correction. Differences in the amount of DEGs in **Figures 4c-d**, S4c-e were analyzed using chi-square contingency test. Differences in BBB permeability across regions in the human brain were analyzed using two-way ANOVA with FDR correction. $P \leq 0.05$ was defined as the statistical significance level.

Acknowledgements

This study was supported by the Israel Science Foundation (Awards No. 717/15 and 2254/20), Canadian Institutes of Health Research (CIHR Award No. PJT 148896), and the European Research Area Network Neuron Award (Award No. NDD 168164). We thank QB3 Genomics Sequencing Laboratory, UC Berkeley, Berkeley, CA, RRID:SCR_022170 for using their facilities.

Author Contributions

E.S., A.F., and O.P. conceived and designed the project; E.S., U.M., O.P., L.T.Y., L.S., S.U., and I.B. performed experiments; E.S, L.K., and S.M performed magnetic resonance imaging analysis; L.T.Y. performed bioinformatic experiments; L.T.Y., P.S., R.P., and E.S. analyzed the bioinformatic data; E.S. and A.F. wrote the manuscript with substantial input from L.K., D.K. and O.P. All authors reviewed the manuscript.

Declaration of Interest

The authors declare no competing interests.

Data and Code Availability

Source Data for quantification described in the text or shown in Figs. **1**–**4** and Extended Data Figure 1–4 are available with the paper. Additional data are available upon reasonable requests. Code and custom MATLAB scripts for analyses used in this study is also available upon reasonable request from the corresponding authors.

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Article and author information

Evyatar Swissa
Department of Brain and Cognitive Sciences, The School of Brain Sciences and Cognition, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel
ORCID iD: 0000-0002-6644-7230

Uri Monsonego
Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Lynn T. Yang
Department of Integrative Biology, University of California, Berkeley, CA 94720, USA, Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720, USA

Lior Schori
Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Lyna Kamintsky
Department of Medical Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada

Sheida Mirloo
Department of Medical Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada

Itamar Burger
Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Sarit Uzzan
Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Rishi Patel
Department of Integrative Biology, University of California, Berkeley, CA 94720, USA

Peter H Sudmant
Department of Integrative Biology, University of California, Berkeley, CA 94720, USA
ORCID iD: 0000-0002-9573-8248

Ofer Prager
Department of Brain and Cognitive Sciences, The School of Brain Sciences and Cognition, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel, Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Daniela Kaufer
Department of Integrative Biology, University of California, Berkeley, CA 94720, USA, Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720, USA

Alon Friedman
Department of Brain and Cognitive Sciences, The School of Brain Sciences and Cognition, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel, Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel, Department of Medical Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada
For correspondence: alonf@bgu.ac.il

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Reviewer #1 (Public Review):

The goal of the current study was to evaluate the effect of neuronal activity on blood-brain barrier permeability in the healthy brain, and to determine whether changes in BBB dynamics play a role in cortical plasticity. The authors used a variety of well-validated approaches to first demonstrate that limb stimulation increases BBB permeability. Using in vivo-electrophysiology and pharmacological approaches, the authors demonstrate that albumin is sufficient to induce cortical potentiation and that BBB transporters are necessary for stimulus-induced potentiation. The authors include a transcriptional analysis and differential expression of genes associated with plasticity, TGF-beta signaling, and extracellular matrix were observed following stimulation. Overall, the results obtained in rodents are compelling and support the authors' conclusions that neuronal activity modulates the BBB in the healthy brain and that mechanisms downstream of BBB permeability changes play a role in stimulus-evoked plasticity. These findings were further supported with fMRI and BBB permeability measurements performed in healthy human subjects performing a simple sensorimotor task. There is literature to suggest that there are sex differences in BBB dysfunction in pathophysiological conditions and the authors have acknowledged the use of only males as a minor limitation of the study that should be addressed in the future. Future studies should also test whether the upregulation of OAT3 plays a role in cortical plasticity observed following stimulation. Overall, this study provides novel insights into how neurovascular coupling, BBB permeability, and plasticity interact in the healthy brain.

https://doi.org/10.7554/eLife.89611.2.sa2

Reviewer #2 (Public Review):

Summary:
This study builds upon previous work that demonstrated that brain injury results in leakage of albumin across the blood brain barrier, resulting in activation of TGF-beta in astrocytes. Consequently, this leads to decreased glutamate uptake, reduced buffering of extracellular potassium and hyperexcitability. This study asks whether such a process can play a physiological role in cortical plasticity. They first show that stimulation of a forelimb for 30 minutes in a rat results in leakage of the blood brain barrier and extravasation of albumin on the contralateral but not ipsilateral cortex. The authors propose that the leakage is dependent upon neuronal excitability and is associated with an enhancement of excitatory transmission. Inhibiting the transport of albumin or the activation of TGF-beta prevents the enhancement of excitatory transmission. In addition, gene expression associated with TGF-beta activation, synaptic plasticity and extracellular matrix are enhanced on the "stimulated" hemisphere. That this may translate to humans is demonstrated by a break down in the blood brain barrier following activation of brain areas through a motor task.

Strengths:
This study is novel and the results are potentially important as they demonstrate an unexpected break down of the blood brain barrier with physiological activity and this may serve a physiological purpose, affecting synaptic plasticity.

The strengths of the study are:

1. The use of an in vivo model with multiple methods to investigate the blood brain barrier response to a forelimb stimulation.
2. The determination of a potential functional role for the observed leakage of the blood brain barrier from both a genetic and electrophysiological view point
3. The demonstration that inhibiting different points in the putative pathway from activation of the cortex to transport of albumin and activation of the TGF-beta pathway, the effect on synaptic enhancement could be prevented.
4. Preliminary experiments demonstrating a similar observation of activity dependent break down of the blood brain barrier in humans.

Weaknesses:
The authors adequately addressed most of my points. A few remain:

1. Although the reviewers have addressed the possible effects of anaesthesia on neuro-vascular coupling. They have not mentioned or addressed the possible effects of ketamine (an NMDA receptor antagonist) on synaptic plasticity. Indeed, the low percentage of SEP increase following potentiation (10-20%) could perhaps be explained by partial block of NMDA receptors by ketamine.
2. The experimental paradigms remain unclear to me. Now, it appears that drugs are applied for 50 minutes and that the stimulation occurs during the "washout period". The more conventional approach would be to have the drug application during the stimulation period to determine if the drugs occlude or enhance the effects of stimulation and then washout the drugs. The problem is that drugs variably washout at different rates depending upon their lipid solubility.
3. It is still not clear to what extent the experimenters and those doing the analysis were blinded to group. If one or both were blind to group, then please put this in the methods.

https://doi.org/10.7554/eLife.89611.2.sa1

Reviewer #3 (Public Review):

Summary:

This study used prolonged stimulation of a limb to examine possible plasticity in somatosensory evoked potentials induced by the stimulation. They also studied the extent that the blood brain barrier (BBB) was opened by the prolonged stimulation and whether that played a role in the plasticity. They found that there was potentiation of the amplitude and area under the curve of the evoked potential after prolonged stimulation and this was long-lasting (>5 hrs). They also implicated extravasation of serum albumin, caveolae-mediated transcytosis, and TGFb signalling, as well as neuronal activity and upregulation of PSD95. Transcriptomics was done and implicated plasticity related genes in the changes after prolonged stimulation, but not proteins associated with the BBB or inflammation. Next, they address the application to humans using a squeeze ball task. They imaged the brain and suggest that the hand activity led to an increased permeability of the vessels, suggesting modulation of the BBB.

Strengths:

The strengths of the paper are the novelty of the idea that stimulation of the limb can induce cortical plasticity in a normal condition, and it involves opening of the BBB with albumin entry. In addition, there are many datasets and both rat and human data.

Weaknesses:

The conclusions are not compelling however because of a lack of explanation of methods. The explanation of why prolonged stimulation in the rat was considered relevant to normal conditions should be as clear in the paper as it is in the rebuttal. The authors need to ensure other aspects of the rebuttal are as clear in the paper as in the rebuttal too. The only remaining concern that is significant is that it is hard to understand the figures.

https://doi.org/10.7554/eLife.89611.2.sa0

Author Response

> ***Reviewer #1 (Public Review):***
>
> *The goal of the current study was to evaluate the effect of neuronal activity on blood-brain barrier permeability in the healthy brain, and to determine whether changes in BBB dynamics play a role in cortical plasticity. The authors used a variety of well-validated approaches to first demonstrate that limb stimulation increases BBB permeability. Using in vivo-electrophysiology and pharmacological approaches, the authors demonstrate that albumin is sufficient to induce cortical potentiation and that BBB transporters are necessary for stimulus-induced potentiation. The authors include a transcriptional analysis and differential expression of genes associated with plasticity, TGF-beta signaling, and extracellular matrix were observed following stimulation. Overall, the results obtained in rodents are compelling and support the authors' conclusions that neuronal activity modulates the BBB in the healthy brain and that mechanisms downstream of BBB permeability changes play a role in stimulus-evoked plasticity. These findings were further supported with fMRI and BBB permeability measurements performed in healthy human subjects performing a simple sensorimotor task. While there are many strengths in this study, there is literature to suggest that there are sex differences in BBB dysfunction in pathophysiological conditions. The authors only used males in this study and do not discuss whether they would also expect to sex differences in stimulation-evoked BBB changes in the healthy brain. Another minor*

> *limitation is the authors did not address the potential impact of anesthesia which can impact neurovascular coupling in rodent studies. The authors could have also better integrated the RNAseq findings into mechanistic experiments, including testing whether the upregulation of OAT3 plays a role in cortical plasticity observed following stimulation. Overall, this study provides novel insights into how neurovascular coupling, BBB permeability, and plasticity interact in the healthy brain.*
>
> *While there are many strengths in this study, there is literature to suggest that there are sex differences in BBB dysfunction in pathophysiological conditions. The authors only used males in this study and do not discuss whether they would also expect to sex differences in stimulation-evoked BBB changes in the healthy brain.*

We agree with the reviewer regarding the importance of examining sex differences on stimulation-evoked BBB changes. To address this issue we have: (1) clarified in the methods section that the human study involved both males and females; (2) added a section to the discussion highlighting the male bias as a key limitation of our animal experiments; and (3) stated that future work should examine whether stimulation-evoked BBB changes differ between makes and females.

> *Another minor limitation is the authors did not address the potential impact of anesthesia which can impact neurovascular coupling in rodent studies.*

We are grateful for this comment and agree with the reviewer that the potential effects of anesthesia should be discussed. We have added the following discussion paragraph:

“A key limitation of our animal experiments is the fact they were performed under anesthesia, due to the complex nature of the experimental setup (i.e., simultaneous cortical imaging and electrophysiological recordings). Anesthetic agents can affect various receptors within the NVU, potentially altering neuronal activity, SEPs, CBF, and vascular responses (Aksenov et al., 2015; Lindauer et al., 1993; Masamoto & Kanno, 2012). To minimize these effects, we used ketamine-xylazine anesthesia, which unlike other anesthetics, was shown to generate robust BOLD and SEP responses to neuronal activation (Franceschini et al., 2010; Shim et al., 2018).”

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *This study builds upon previous work that demonstrated that brain injury results in leakage of albumin across the bloodbrain barrier, resulting in activation of TGF-beta in astrocytes. Consequently, this leads to decreased glutamate uptake, reduced buffering of extracellular potassium, and hyperexcitability. This study asks whether such a process can play a physiological role in cortical plasticity. They first show that stimulation of a forelimb for 30 minutes in a rat results in leakage of the blood-brain barrier and extravasation of albumin on the contralateral but not ipsilateral cortex. The authors propose that the leakage is dependent upon neuronal excitability and is associated with an enhancement of excitatory transmission. Inhibiting the transport of albumin or the activation of TGF-beta prevents the enhancement of excitatory transmission. In addition, gene expression associated with TGF-beta activation, synaptic plasticity, and extracellular matrix are enhanced on the "stimulated" hemisphere. That this may translate to humans is demonstrated by a breakdown in the blood-brain barrier following activation of brain areas through a motor task.*
>
> *Strengths:*

This study is novel and the results are potentially important as they demonstrate an unexpected breakdown of the blood-brain barrier with physiological activity and this may serve a physiological purpose, affecting synaptic plasticity.

The strengths of the study are:

1. *The use of an in vivo model with multiple methods to investigate the blood-brain barrier response to a forelimb stimulation.*

1. *The determination of a potential functional role for the observed leakage of the blood-brain barrier from both a genetic and electrophysiological viewpoint.*

1. *The demonstration that inhibiting different points in the putative pathway from activation of the cortex to transport of albumin and activation of the TGF-beta pathway, the effect on synaptic enhancement could be prevented.*

1. *Preliminary experiments demonstrating a similar observation of activity-dependent breakdown of the blood-brain barrier in humans.*

Weaknesses:

There are both conceptual and experimental weaknesses.

1. *The stimulation is in an animal anesthetized with ketamine, which can affect critical receptors (ie NMDA receptors) in synaptic plasticity.*

We agree that the potential effects of anesthesia should be considered. The Discussion was revised to address this point: “A key limitation of our animal experiments is the fact they were performed under anesthesia, due to the complex nature of the experimental setup (i.e., simultaneous cortical imaging and electrophysiological recordings). Anesthetic agents can affect various receptors within the NVU, potentially altering neuronal activity, SEPs, CBF, and vascular responses (Aksenov et al., 2015; Lindauer et al., 1993; Masamoto & Kanno, 2012). To minimize these effects, we used ketamine-xylazine anesthesia, which unlike other anesthetics, was shown to generate robust BOLD and SEP responses to neuronal activation (Franceschini et al., 2010; Shim et al., 2018)”

1. *The stimulation protocol is prolonged and it would be helpful to know if briefer stimulations have the same effect or if longer stimulations have a greater effect ie does the leakage give a "readout" of the stimulation intensity/length.*

Thank you for this important comment. We are also very curious about the potential relationship between stimulation magnitude/duration and subsequent leakage and have added the following statement to the discussion:

“Future studies should also explore the effects of stimulation magnitude/duration on BBB modulation, as well as the stimulation threshold between physiological and pathological increase in BBB permeability.”

Our current findings indicate that a one-minute stimulation does not affect vascular permeability or SEP and we aim to test additional stimulation paradigms in future studies.

> 1. *For some of the experiments (see below), the numbers of animals are low and the statistical tests used may not be the most appropriate, making the results less clear cut.*

We appreciate this comment and have revised the statistical analysis of Figure 1J,K. We now use a nested t-test to test for differences between rats (as opposed to sections). The differences remain significant (EB, p=0.0296; Alexa, p=0.0229). The text was modified accordingly.

> 1. *The experimental paradigms are not entirely clear, especially the length of time of drug application and the authors seem to try to detect enhancement of a blocked SEP.*

Thank you for pointing this out. Figures 2&3 were revised for clarification and a 'Drug Application' subsection was added to the methods section.

> 1. *It is not clear how long the enhancement lasts. There is a remark that it lasts longer than 5 hours but there is no presentation of data to support this.*

Thank you for this comment. As the length of experiments differed between animals, the exact length could not be specifically stated. To clarify this point, we revised the text to indicate that LTP was recorded until the end of each experiment (between 1.5-5 hours, depending on the condition the animal was in). We also added a panel to figure 2 (Figure 2d) with exemplary data showing potentiation 60, 90, and 120 min post stimulation.

> 1. *The spatial and temporal specificity of this effect is unclear (other than hemispheric in rats) and even less clear in humans.*

Our animal experiments (using both in vivo imaging and histological analysis) showed no evidence of BBB modulation outside the cortical somatosensory area corresponding to the limbs. We looked at the entirety of the coronal section of the brain and found enhancement solely in the somatosensory area corresponding to limb. The right side of panels h and i in Figure 1 show an x20 magnification of the section, focusing on the enhanced area. The whole section was not shown, as no fluorescence was found outside the magnified area. Moreover, our quantification showed that the enhancement was specific to the contralateral and not ipsilateral somatosensory cortex (Figure 1 j-k).

We agree that temporal specificity needs to be further explored, and we have now stated that in the discussion: "Future studies are needed to explore the BBB modulating effects of additional stimulation protocols – with varying durations, frequencies, and magnitudes. Such studies may also elucidate the temporal and ultrastructural characteristics that may differentiate between physiological and pathological BBB modulation."

We also agree that larger studies are needed to better understand the specificity of the observed effect in humans, and to account for potential inter-human variability in vascular integrity and brain function due to different schedules, diets, exercise habits, etc.

> 1. *The experimenters rightly use separate controls for most of the experiments but this is not always the case, also raising the possibility that the application of drugs was not done randomly or interleaved, but possibly performed in blocks of animals, which can also affect results.*

Thank you for pointing out this lack of clarity. We have now highlighted that drug application was done randomly.

> 1. *Methyl-beta-cyclodextrin clears cholesterol so the effect on albumin transport is not specific, it could be mediating its effect through some other pathway.*

We agree that the effect of mβCD may not be specific. To mitigate this issue, we used a very low mβCD concentration (10uM). Notably, this is markedly lower than the concentrations reported by Koudinov et al, showing that cholesterol depletion is observed at 5mM mβCD and not at 2.5mM/5mM (Koudinov & Koudinova, 2001). This point was added to the discussion.

> 1. *Since the breakdown of the blood-brain barrier can be inhibited by a TGF-beta inhibitor, then this implies that TGFbeta is necessary for the breakdown of the blood-brain barrier. This does not sit well with the hypothesis that TGF-beta activation depends upon blood-brain barrier leakage.*

Thank you for pointing out this lack of clarity. We have added a discussion paragraph that clarifies our hypothesis: "As mentioned above, albumin is a known activator of TGF-β signaling, and TGF-β has a well-established role in neuroplasticity. Interestingly, emerging evidence suggests that TGF-β also increases cross-BBB transcytosis (Betterton et al., 2022; Kaplan et al., 2020; McMillin et al., 2015; Schumacher et al., 2023). Hence, we propose the following two-part hypothesis for the TGF-β/BBB-mediated synaptic potentiation observed in our experiments: (1) prolonged stimulation triggers TGF-β signaling and increased caveolae-mediated transcytosis of albumin; and (2) extravasated albumin induces further TGF-β signaling, leading to synaptogenesis and additional cross-BBB transport – in a self-reinforcing positive feedback loop. Future research is needed to examine the validity of this hypothesis.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *This study used prolonged stimulation of a limb to examine possible plasticity in somatosensory evoked potentials induced by the stimulation. They also studied the extent that the blood-brain barrier (BBB) was opened by prolonged stimulation and whether that played a role in the plasticity. They found that there was potentiation of the amplitude and area under the curve of the evoked potential after prolonged stimulation and this was long-lasting (>5 hrs). They also implicated extravasation of serum albumin, caveolae-mediated transcytosis, and TGFb signalling, as well as neuronal activity and upregulation of PSD95. Transcriptomics was done and implicated plasticity-related genes in the changes after prolonged stimulation, but not proteins associated with the BBB or inflammation. Next, they address the application to humans using a squeeze ball task. They imaged the brain and suggested that the hand activity led to an increased permeability of the vessels, suggesting modulation of the BBB.*
>
> *Strengths:*
>
> *The strengths of the paper are the novelty of the idea that stimulation of the limb can induce cortical plasticity in a normal condition, and it involves the opening of the BBB with albumin entry. In addition, there are many datasets and both rat and human data.*
>
> *Weaknesses:*
>
> *The conclusions are not compelling however because of a lack of explanation of methods and quantification. It also is not clear whether the prolonged stimulation in the rat was normal conditions. To their credit, the authors recorded the neuronal activity during stimulation, but it seemed excessive excitation. Since seizures open the BBB this result calls into question one of the conclusions. that the results reflect a normal brain. The*

> *authors could either conduct studies with stimulation that is more physiological or discuss the caveats of using a supraphysiological stimulus to infer healthy brain function.*
>
> *The conclusions are not compelling however because of a lack of explanation of methods and quantification.*

Thank you for this comment. In the revised paper, we expanded the Methods section to better describe the procedures and approaches we used for data analysis.

> *It also is not clear whether the prolonged stimulation in the rat was normal conditions.*

We believe that the used stimulation protocol is within the physiological range (and relevant to plasticity, learning and memory) for the following reasons:

1. In our continuous electrophysiological recordings, we did not observe any form of epileptiform or otherwise pathological activity.
2. Memory/training/skill acquisition experiments in humans often involve similar training duration or longer (Bengtsson et al., 2005), e.g., a 30 min thumb training session performed by (Classen et al., 1998).
3. The levels of SEP potentiation we observed are similar to those reported in:

a) Rats following a 10-minute whisker stimulation (one hour post stimulation, (Mégevand et al., 2009)).

b) Humans following a 15 min task (McGregor et al., 2016).

This important point is now presented in the discussion.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *The discussion would benefit from additional discussion of the potential impacts of sex and anesthesia in their findings.*

We agree with the reviewer and have added the following paragraph to the discussion:

“A key limitation of our animal experiments is the fact they were performed under anesthesia, due to the complex nature of the experimental setup (i.e., simultaneous cortical imaging and electrophysiological recordings). Anesthetic agents can potentially alter neuronal activity, SEPs, CBF, and vascular responses (Aksenov et al., 2015; Lindauer et al., 1993; Masamoto & Kanno, 2012). To minimize these effects, we used ketaminexylazine anesthesia, which unlike other anesthetics, was shown to maintain robust BOLD and SEP responses to neuronal activation (Franceschini et al., 2010; Shim et al., 2018). Another limitation of our animal study is the potentially non-specific effect of mβCD – an agent that disrupts caveola transport but may also lead to cholesterol depletion (Keller & Simons, 1998). To mitigate this issue, we used a very low mβCD concentration (10uM), orders of magnitude below the concentration reported to deplete cholesterol (Koudinov et al). Lastly, our animal study is limited by the inclusion of solely male rats. While our findings in humans did not point to sex-related differences in stimulation-evoked BBB modulation, larger animals and human studies are needed to examine this question.”

> *The figure text is quite small.*

Thank you for pointing this out, we revised all figures and increased font size for clarity.

> *Including pharmacological concentrations within the figure legends would improve the readability of the manuscript.*

Thank you for this suggestion, the figure legends were modified accordingly.

> *In methods for immunoassays the 5 groups could be more clear by stating that there are 3 timepoints for stimulation experiments. There is a typo in this section where the 24-hour post is stated twice in the same sentence.*

Thank you for pointing this out, the text was modified accordingly.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> 1. *In Figure 1, J and K seem to indicate that in these experiments the statisitics were done per slice and not per animal. This is not a reasonable approach, a repeat measure ANOVA or averaging for each animal are more appropriate statistical approaches.*

We thank the reviewer for pointing this out. The statistical analysis for Figure 1j,k was modified. We now use a nested ttest to test for differences between rats and not sections. The differences are still significant (EB, p=0.0296; Alexa, p=0.0229). The manuscript was modified accordingly.

> 1. *In Figure 2, the protocol does not seem to give much idea about time course. There was a stimulation test for 1 minute before and then 1 minute after the 30-minute stimulation train. How was potentiation assessed for the next 5 hours and where are the data?*

Potentiation was assessed by repeating 1min test stim every 30 min for the duration of the experiment, we added a panel to show late potentiation, see response above.

> 1. *In Figure 2, there is a notable lack of controls eg the effect of sham stimulation and application of saline. These are important as the drift of response magnitude can be a problem in long experiments.*

We did test for the potential presence of response drift, by examining whether SEPs of non-stimulated animals change over time (at baseline, 30 or 60 minutes of recording; n=6). No statistical differences were found. Our analysis focused on using each animal as its own control (i.e., comparing baseline SEP to SEP post albumin perfusion), because SEP studies highlight the importance of comparing each animal to its own baseline, due to the large inter-animal variability (All et al., 2010; Mégevand et al., 2009; Zandieh et al., 2003).

> 1. *Figure 3 a is not clear – were the drugs applied throughout?*

Thank you for pointing this out. We have revised Figure 3 a to show that the drugs were applied for 50 min before the stimulation.

> 1. *In Figure 3 panel d is repeated in panel j. This needs correcting*

Thank you. This mistake was fixed.

> 1. *In LTP-type experiments usually the antagonist is applied during the stimulation and then washed out. This avoids the problem in this figure in which CNQX effectively blocks transmission and so it is not possible to detect any enhancement if it were there. Eg in panel e, CNQX block transmission, and then the assessment is performed when the AMPA receptors are blocked after 30 minutes of stimulation. If receptors are blocked no enhancement will be detectable. Moreover, surely the question is the ratio of the effect of 30-minute stimulation on the SEP in the presence of CNQX and so the statistics should be done on the fold change in the SEP following 30-minute stimulation in the presence of CNQX.*

Thank you. The protocol might have been misrepresented in the original figure. We modified Fig 3a to clarify that the antagonists were indeed washed out upon stimulation start to make sure the receptors are not blocked during the test stimulation following the 30 min stimulation. In addition, we tested for the difference in fold change between 30 min stim, and 30 min stimulation following antagonists wash-in (Fig 3f and Fig S2a).

> 1. *Interesting in Figure f, stimulation, albumin, and AP5 all seem to have the same enhancement of the SEP. Is the lack of effect of 30-minute stimulation in the presence of AP5, a ceiling effect ie AP5 has enhanced the SEP, and no further enhancement from stimulation is possible.*

This is a very interesting point that will require further research.

> 1. *SJN seems to block neurotransmission. What is the mechanism? The same analysis as for CNQX should be performed ie what is the fold change not compared to baseline but in the presence of SJN.*

Our quantification showed that SJN did not significantly reduce the SEP max amplitude, and we therefore did not include this graph in the figure.

> 1. *Please acknowledge that the effect of mbetaCD is non-specific. There is a large literature on the effects of cholesterol depletion on LTP.*

We agree that the effect of mβCD may not be specific. To mitigate this issue, we used a very low mβCD concentration (10μM). Notably, this is markedly lower than the concentrations reported by Koudinov et al, showing that cholesterol depletion is only observed at a concentration of 5mM (Koudinov & Koudinova, 2001). This point is now discussed under the discussion paragraph describing the study's limitations.

> 1. *k&l seem to have used the same control in which case they should not be analysed separately (they are all part of the same experiment).*

We agree with the reviewer and have revised the figure accordingly.

> 1. *The difference in gene expression in Figure 4 would be more convincing if it could be prevented by for example a TGFbeta inhibitor.*

We agree and acknowledge the impact such experiments could provide. We plan to incorporate these experiments into our future studies.

> 1. *Figure 5 seems to indicate bilateral and widespread BBB modulation arguing that this may be a non-specific effect. Panel g should look at other neocortical regions eg occipital cortex.*

We agree and thank the reviewer for this comment. We revised the figure to include other cortical areas, such as the frontal and occipital cortices (Figure 5g)

> *Minor comments*
>
> 1. *Paired data eg in Fig 2D are better represented by pairing the dots usually with a line.*
>
> 1. *Please correct the %fold baseline in axes in graphs which show % change for baseline.*
>
> 1. *Figure 4 is not correctly referred to in the text.*

We agree with all the points raised by the reviewer and revised the figures and text accordingly.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The conclusions are not compelling however because of a lack of explanation of methods and quantification. It also is not clear whether the prolonged stimulation in the rat was normal conditions. To their credit, the authors recorded the neuronal activity during stimulation, but it seemed excessive excitation. Since seizures open the BBB this result calls into question one of the conclusions. that the results reflect a normal brain. The authors could either conduct studies with stimulation that is more physiological or discuss the caveats of using a supraphysiological stimulus to infer healthy brain function.*
>
> *Major concerns:*
>
> *Methods need more explanation. Rationales need more justification. Examples are provided below.*
>
> *Throughout many sections of the paper, sample sizes and stats are often missing. For stats, please provide p-values and other information (tcrit, U statistic, F, etc.)*

Thank you, we added the relevant information where it was missing throughout the manuscript.

> *For transcriptomics, they might have found changes in BBB-related genes if they assayed vessels but they assayed the cortex.*

We agree with the reviewer that this would be a very interesting future direction. The present study could not include this kind of analysis due to lack of access to vasculature isolation methods or single-cell RNA seq.

> *What were the inclusion/exclusion criteria for the subjects?*

Thank you for pointing out this lack of clarity. The methods section (under 'Magnetic Resonance Imaging' – 'Participants') was expanded to include the following:

"Male and female healthy individuals, aged 18-35, with no known neurological or psychiatric disorders were recruited to undergo MRI scanning while performing a motor task (n=6; 3

males and 3 females). MRI scans of 10 sex- and age- matched individuals (with no known neurological or psychiatric disorders) who did not perform the task were used as control data (n=10; 5 males and 5 females.

> *Were they age and sex-matched?*

They were, indeed, age and sex-matched. This was now clarified in the relevant Methods section.

> *Were there other factors that could have influenced the results?*

Certainly. Human subjects are difficult to control for due to different schedules, diets, exercise habits, and other factors that may impact vascular integrity and brain function. Larger multimodal studies are needed to better understand the observed phenomenon.

> *Fig. 1. Images are very dim. Text here and in other figures is often too small to see. Some parts of the figures are not explained.*

Our apologies. Figures and legends were revised accordingly.

> *Fig 2a, f. I don't see much difference here- do the authors think there was?*

We agree that the difference may not be visually obvious. The quantification of trace parameters (amplitude and area under curve) does, however, reveal a significant SEP difference in response to both stimulation (panels X and y) and albumin (panels z and q).

> *Fig 3 d and j seem the same.*

We thank the reviewer for noticing. This was a copy mistake that was now rectified.

> *Lesser concerns and examples of text that need explana9on:*
>
> *Introduction*
>
> *Insulin-like growth factor is transported. From where to where?*

The text was edited to clarify that this was cross-BBB influx of insulin-like growth factor-I.

> *RMT that underlies the transport of plasma proteins was induced by physiological or non-physiological stimulation.*

This was shown without stimulation, in normal physiology of young and aged healthy mice. The text was edited to clarify this point.

> *What was the circadian modulation that was shown to implicate BBB in brain function?*

The text was edited for clarity.

> *Results*
>
> *When the word stimulation is used please be specific if whiskers are moved by an experimenter, an electrode is used to apply current, etc.*

We have now moved the ‘Stimulation protocol’ section closer to beginning of the Methods and emphasized that we administered electrical stimulation to the forepaw or hindlimb using

subdermal needle electrodes.

> *Please explain how the authors are convinced they localized the vascular response.*

The vascular response was localized via: (1) visual detection of arterioles that dilated in response to stimulation (due to functional hyperemia / neurovascular coupling) [figure 1 d]; and (2) quantitative mapping of increased hemoglobin concentration (Bouchard et al., 2009) [Figure 1 b]. This is now mentioned in the methods (under 'In vivo imaging') and results (under the 'Stimulation increases BBB permeability').

> *"30 min of limb stimulation" means what exactly? 6 Hz 2mA for 30 min?*

Thank you. The text was revised for clarity (Methods under 'Stimulation protocol'):

"The left forelimb or hind limb of the rat was stimulated using Isolated Scmulator device (AD Instruments) attached with two subdermal needle electrodes (0.1 ms square pulses, 2-3 mA) at 6 Hz frequency. Test stimulation consisted of 360 pulses (60 s) and delivered before (as baseline) and after long-duration stimulation (30 min, referred throughout the text as 'stimulation'). In control and albumin rats, only short-duration stimulations were performed. Under sham stimulation, electrodes were placed without delivering current."

> *Histology that was performed to confirm extravasation needs clarification because if tissue was removed from the brain, and fixed in order to do histology, what is outside the vessels would seem likely to wash away.*

Thank you for pointing out the need to clarify this point. The Histology description in the Methods section was revised in the following manner:

"Albumin extravasacon was confirmed histologically in separate cohorts of rats that were anesthetized and stimulated without craniotomy surgery. Assessment of albumin extravasacon was performed using a well-established approach that involves peripheral injection of either labeled-albumin (bovine serum albumin conjugated to Alexa Flour 488, Alexa488-Alb) or albumin-labeling dye (Evans blue, EB – a dye that binds to endogenous albumin and forms a fluorescent complex), followed by histological analysis of brain tissue (Ahishali & Kaya, 2020; Ivens et al., 2007; Lapilover et al., 2012; Obermeier et al., 2013; Veksler et al., 2020). Since extravasated albumin is taken up by astrocytes (Ivens et al., 2007; Obermeier et al., 2013), it can be visualized in the brain neuropil after brain removal and fixation (Ahishali & Kaya, 2020; Ivens et al., 2007; Lapilover et al., 2012; Veksler et al., 2020). Five rats were injected with Alexa488-Alb (1.7 mg/ml) and five with EB (2%, 20 mg/ml, n=5). The injections were administered via the tail vein. Following injection, rats were transcardially perfused with…"

> *It is not clear why there was extravasacon contralateral but not ipsilateral if there are cortical-cortical connections.*

Interpersonally, we also did not observe ipsilateral SEP in response to limb stimulation, with evidence of SEP and BBB permeability only in the contralateral sensorimotor region. This finding is consistent with electrophysiological and fMRI studies showing that peripheral stimulation results in predominantly contralateral potentials (Allison et al., 2000; Goff et al., 1962).

> *After injection of Evans blue or Alexa-Alb, how was it shown that there was extravasacon?*

Extravasalon in cortical sections was visualized using a fluorescent microscope (Figure 1 h-i). Since extravasated albumin is taken up by astrocytes, fluorescent imaging can be used for

visualizing and quantifying labeled albumin (Ahishali & Kaya, 2020; Ivens et al., 2007; Knowland et al., 2014). Here is the relevant methods excerpt:

"Coronal sections (40-µm thick) were obtained using a freezing microtome (Leica Biosystems) and imaged for dye extravasacon using a fluorescence microscope (Axioskop 2; Zeiss) equipped with a CCD digital camera (AxioCam MRc 5; Zeiss)."

> *How is a sham control not stimulated - what is the sham procedure?*

In the sham stimulation protocol electrodes were placed, but current was not delivered. A section titled 'Stimulation protocol' was added to the methods to clarify this point.

> *What was the method for photothrombosis-induced ischemia?*

The procedure for photothrombosis-induced ischemia is described under the Methods section 'Immunoassays' – 'Enzyme-linked immunosorbent assay (ELISA) for albumin extravasalon':

"Rats were anesthetilzed and underwent … photothrombosis stroke (PT) as previously described (Lippmann et al., 2017; Schoknecht et al., 2014). Briefly, Rose Bengal was administered intravenously (20 mg/kg) and a halogen light beam was directed for 15 min onto the intact exposed skull over the right somatosensory cortex."

> *Fig 1d. All parts of d are not explained.*

Thank you for pointing this out. In the revised manuscript, the panels of this figure were slightly reordered, and we made sure all panels are explained in the legend.

> *e. Is the LFP a seizure? How physiological is this- it does not seem very physiological.*

Thank you for your comment. We believe that this activity is not a seizure because it lacks the typical slow activity that corresponds to the "depolarizalon shir" observed during seizures (Ivens et al., 2007; Milikovsky et al., 2019; Zelig et al., 2022).

> *f. Permeability index needs explanation. How was the area chosen for each rat? Randomly? Was it the same across rats?*

We have now revised the Methods section to provide a clearer description of the permeability index calculation and the choice of the imaging area:

"Across all experiments, acquired images were the same size (512 × 512 pixel, ~1x1 mm), centered above the responding arteriole. Images were analyzed offline using MATLAB as described (Vazana et al., 2016). Briefly, image registration and segmentation were performed to produce a binary image, separating blood vessels from extravascular regions. For each extravascular pixel, a time curve of signal intensity over time was constructed. To determine whether an extravascular pixel had tracer accumulation over time (due to BBB permeability), the pixel's intensity curve was divided by that of the responding artery (i.e., the arterial input function, AIF, representing tracer input). This ratio was termed the BBB permeability index (PI), and extravascular pixels with PI > 1 were identified as pixels with tracer accumulation due to BBB permeability."

> *g. For Evans blue and Alexa-Alb was the sample size rats or sections?*

Thank you for this question. We revised the statistical analysis for Figure 1j,k to appropriately asses the differences between rats. We used a nested t-test to test for differences between rats

(and not sections). The differences remained significant (EB, p=0.0296; Alexa, p=0.0229) and the text was modified accordingly.

> *h, i, j need more contrast and/or brightness to appreciate the images. Arrows would help. The text is too small to read.*

Thank you. This issue was addressed in the revised paper.

> *To induce potentiation, 6 Hz 2 mA stimuli were used for 30 min. Please justify this as physiological.*

Thank you for the comment. We believe that the used stimulation protocol is within the physiological range (and relevant to plasticity, learning and memory) for the following reasons:

1. In our continuous electrophysiological recordings, we did not observe any form of epileptiform or otherwise pathological activity.
2. Memory/training/skill acquisition experiments in humans often involve similar training duration or longer (Bengtsson et al., 2005), e.g., a 30 min thumb training session performed by (Classen et al., 1998).
3. The levels of SEP potentiation we observed are similar to those reported in:

a. Rats following a 10-minute whisker stimulation (one hour post stimulation, (Mégevand et al., 2009)).

b. Humans following a 15 min task (McGregor et al., 2016).

We have revised the Discussion of the paper to clarify this important point.

> *The test stimulus to evoke somatosensory evoked potentials was 1 min. Was this 6 Hz 2 mA for 1 min? Please justify.*

Yes. We chose these parameters as these ranges were shown to induce the largest changes in blood flow (with laserdoppler flowmetry) and summated SEP (Ngai et al., 1999), corresponding with our findings. We also show that these stimulation parameters do not induce changes in BBB permeability nor synaptic potentiation, therefore served as test control.

> *How long after the 30 min was the test stimulus triggered- immediately? 30 sec afterwards?*

The test stimulus was applied 5 min afterwards to allow for BBB imaging protocol (now explained in the Methods section).

> *How were amplitude and AUC measured? Baseline to peak? For AUC is it the sum of the upward and downward deflections comprising the LFP?*

Yes, and yes. This is now clarified in the 'Analysis of electrophysiological recordings' section in the Methods.

> *How was the same site in the somatosensory cortex recorded for each animal? Potentiation was said to last >5 hrs. How often was it measured? Was potentiation the same for the amplitude and the AUC?*

The location of the cranial window over the somatosensory cortex was the same in all rats. The location of the specific responding arteriole may change between animals, but the recording electrode was places around the responding arteriole in the same approaching angle and depth for all animals.

As the length of experiments differed between animals, the exact length could not be specifically stated. We therefore revised the text to clarify that LTP was recorded until the end of each experiment (depending on the animal condition, between 1.5-5 hours) and added a panel to figure 2 (Figure 2f) with exemplary data showing potentiation 120 min (2hr) post stimulation.

> *Why was 25% of the serum level of albumin selected- does the brain ever get exposed to that much? Was albumin dissolved in aCSF or was aCSF chosen as a control for another reason?*

Yes, albumin was dissolved in aCSF and the solution was allowed to diffuse through the brain. The relatively high concentration of albumin was chosen to account for factors that lower its effective tissue concentration:

1. The low diffusion rate of albumin (Tao & Nicholson, 1996).
2. The likelihood of albumin to encounter a degradation site or a cross-BBB efflux transporter (Tao & Nicholson, 1996; Zhang & Pardridge, 2001).

> *Figure 2.*
>
> *a. Please show baseline, the stimulus, and aftier the stimulus.*
>
> *Please point out when there was stimulacon.*
>
> *What is the inset at the top?*

The inset on top is the example trace of the stimulus waveform, the legend of the figure was modified for clarity.

> *b. Please show when the stimulus artifact occurred. The end of the 1-minute test stimulus period is fine. Why are the SEPs different morphologies? It suggests the different locations in the cortex were recorded.*

What is shown is the averaged SEP response over 1min test stimulus, each SEP is time locked to each stimulus. Regarding SEP waveform, it does indeed show different morphology between animals, as sometimes different arterioles respond to the stimulation, and we localize the recording to the responding vessel in each rat. However, in each rat the recording is only from one location. Once the electrode was positioned near the responding arteriole it was not moved.

> *d, e. What are the stats?*
>
> *h, i. Add stats. Are all comparisons Wilcoxon? Please provide p values.*

The comparisons were performed with the Wilcoxon test. We now state that and provide the exact p values.

> *j. What was selected from the baseline and what was selected during Albumin and how long of a record was selected?*

> *What program was used to create the spectrogram?*
>
> *What is meant by changes at frequencies above 200 Hz, the frequencies of HFOs?*

The Method section (under 'Electrophysiology – Data acquisition and analyses') has been revised for clarification. Spectrogram was created with MATLAB and graphed with Prism. For analysis, we selected a 10 min recorded segment before starting albumin perfusion, and 10 min after terminating albumin perfusion.

> *When the cortial window was exposed to drugs, what were concentrations used that were selective for their receptor? How long was the exposure?*
>
> *Was the vehicle tested?*

We have revised the Methods section (under 'Animal preparation and surgical procedures - Drug application') to clarify the duration and concentration used and justification. All blockers were exposed for 50 min. The vehicle was an artificial cerebrospinal fluid solution (aCSF).

> *For PSD-95, what was the area of the cortex that was tested?*
>
> *Were animals acutely euthanized and the brain dissected, frozen, etc?*

We have revised the Methods section (under 'Immunoassays') for clarity.

> *What is mbetaCD?*

The full term was added to the results section. It is also mentioned in the Methods.

> *Is SJN specific at the concentration that was chosen? Did it inhibit the SEP?*

In the concentration used in our experiments, SJN is a selective TGF-β type I receptor ALK5 inhibitor (see (Gellibert et al., 2004)).

> *Fig. 3b. It looks like CNQX increased the width of the vessels quite a bit. Please explain.*
>
> *For AP5, very large vessels were imaged, making it hard to compare to the other data.*

The vascular dilation in response to the stimulation under CNQX was similar to that seen under "normal" conditions (i.e. aCSF). As for AP5, in some experiments the responding arteriole was in close proximity to a large venule that cannot be avoidable while imaging. For quantification we always measured arterioles within the same diameter range.

> *e. Sometimes CNQX did not block the response after 30 min stimulation. Why?*

CNQX is washed out before the 30 min stimulation starts, so it is not expected to block the response to stimulation. However, in some cases the response to stimulation was lower in amplitude, likely due to residual CNQX that did not wash out completely.

> *Regarding DEGs, on the top of p 10 what are the percentages of?*

In this analysis we tested in each hemisphere how many genes expressed differentially between 1 and 24 hours post stimulation (either up- or down- regulated). The results were presented as the percentages of differentially expressed genes in each hemisphere (13.2% contralateral, and 7.3% ipsilateral). The text was rephrased for clarity.

> *Please add a ref for the use of the JSD metric methods and support for its use as the appropriate method. Other methods need explanation/references.*

References were added to the text to clarify. The Jensen-Shannon Divergence metric is commonly used to calculate the statistical pairwise distance among two distributions (Sudmant et al., 2015). From comparing a few different distance metric calculations including JSD, our results were similar irrespective of the distance metric applied. Therefore, we demonstrate the variability between paired samples of stimulated and non-stimulated cortex of each animal at two time points following stimulation (24 h vs. 1 h) using JSD.

> *What synaptic plasticity genes were selected for assay and what were not?*
>
> *What does "largely unaffected" mean? Some of the genes may change a small amount but have big functional effects.*

The selected genes of interest were taken from a large list compiled from previous publications (see (Cacheaux et al., 2009; Kim et al., 2017)) and are well documented in gene ontology databases and tools (e.g., Metascape, (Zhou et al., 2019)).

We agree that the term 'largely unaffected' is suboptimal, and we rephrased this section of the results to indicate that "No significant differences were found in BBB or inflammation related genes between the hemispheres". We also agree that a small number of genes can have big functional effects. Future studies are needed to better understand the genes underlying the observed BBB modulation.

> *Please note that Slc and ABCs are not only involved in the BBB.*

Thank you. We modified the text to no longer specify that these are BBB-specific transporters.

> *Please explain the choice of the stress ball squeeze task, and DCE.*

DCE is a well-established method for BBB imaging in living humans, and it is cited throughout the manuscript. The ball squeeze task was chosen as it is presumed to involve primarily sensory motor areas, without high-level processing (Halder et al., 2005). This is now stated in the discussion.

> *What is Gd-DOTA?*

Gd-DOTA is a gadolinium-based contrast agent (gadoterate meglumine, AKA Dotarem). Text was revised for clarity. Please see the Methods section under 'Magnetic Resonance Imaging' - 'Data Acquisition'.

> *What does a higher percentage of activated regions mean- how was activacon defined and how were regions counted?*

Higher percentage of activated regions refers to regions in which voxels showed significant BOLD changes due to the motor task preformed. The statistical approaches and analyses are detailed in the Methods section under 'Magnetic Resonance Imaging - Preprocessing of functional data, and fMRI Localizer Motor Task'.

> *Figure. 4*
>
> *Was stimulation 1 min or 30 min.?*

30 min, Text has been revised for clarity.

> *What is the Wald test and how were p values adjusted-please add to the Stats section.*

The Methods section under ‘Statistical analysis’ was revised to clarify this point.

> *Is there a reason why p values are sometimes circles and otherwise triangles?*

The legend was revised to explain that ”Circles represent genes with no significant differences between 1 and 24 h poststimulation. Upward and downward triangles indicate significantly up- and down- regulated genes, respectively.”

> *How can a p-value be zero? Please explain abbreviations.*

The p-value is very low (~10-10) and therefore appears to be zero due to the scale of the y-axis.

> *Fig. 5b.*
>
> *There are unexplained abbreviations.*
>
> *The x on the ball and hand is not clear relative to the black ball and hand.*

Thank you for noticing. We revised the figure for clarity.

> *c. What was the method used to make an activator map and what is meant by localizer task?*

The explanation of the “fMRI Localizer Motor Task” section in the methods was revised for added clarity.

> *f. What is the measurement "% area" that indicates " BBB modulation"?*
>
> *Is it in f, the BBB permeable vessels (%)? f. Please explain: "Heatmap of BBB modulated voxels percentage in motor/sensory-related areas of task vs. controls."*

The %area measurement indicates the percentage of voxels within a specific brain region that have a leaky BBB. See Methods.

> *Is Task - the control?*

Yes.

> *Supplemental Fig. 2.*
>
> *Why is AUC measured, not amplitude?*

The amplitude, and now also the AUC are shown in Figure 3.

> *b. There is no comparison to baseline. The arrowhead points to the start of stimulation but there is no arrowhead marking the end.*

In the revised paper we added a grey shade over the stimulation period to better visualize the difference to baseline. In this panel we wanted to show that NMDA receptor antagonist did not block the SEP, while AMPA receptor antagonist did.

> *c. In the blot there are two bands for PSD95- which is the one that is PSD95? There is no increase in PSD95 uncl 24 hrs but in the graph in d there is. In the blot, there is a strong expression of PSD95 ipsilateral compared to contralateral in the sham-why?*
>
> *What is the percent change fold?*

The PSD-95 is the top and larger band. The lower band was disregarded in the analysis. The example we show may not fully reflect the group statistics presented in panel d. Upon quantification of 8 animals, PSD-95 is significantly higher 30 min and 24 hours post stimulation in the contralateral hemisphere. No significant changes were found in sham animals. The % change fold refers to the AUC change compared to baseline. This panel was now incorporated in Figure 3 (panel h), and the title was corrected to "|AUC|, % change from baseline".

> *Supplemental Fig. 4.*
>
> *a. If ipsilateral and contralateral showed many changes why do the authors think the effects were only contralateral?*

Our gene analysis was designed to complement our in vivo and histological findings, by assessing the magnitude of change in differentially expressed genes (DEGs). This analysis showed that: (1) the hemisphere contralateral to the stimulus has significantly more DEGs than the ipsilateral hemisphere; and (2) the DEGs were related to synaptic plasticity and TGF-b signaling. These findings strengthen the hypothesis raised by our in vivo and histological experiments.

> *Supplemental Fig. 5 includes many processes not in the results. Examples include dorsal cuneate and VPL, dynamin, Kir, mGluR, etc. The top right has numbers that are not mentioned. If the drawings are from other papers they should be cited.*

The drawings of Figure 5 are original and were not published before. This hypothesis figure points to mechanisms that may drive the phenomena described in the paper. The legend of the figure was revised to include references to mechanisms that were not tested in this study.

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