

RESEARCH ARTICLE

Ischemic stroke triggers brain-wide synaptic remodeling within four hours

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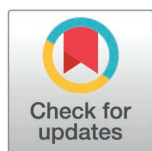
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Abstract

Physiological mechanisms of the key hyperacute (0–24 hours) stage of stroke are poorly understood, hampering the development of new therapies. Synaptic plasticity has been strongly implicated in early stages of neurodegenerative and neurodevelopmental disorders, yet its relevance in early stroke remains unclear. Here, we describe the emergence of distinct region-specific forms of synaptic remodeling following middle cerebral artery occlusion in rats, arising within the critical 4-hour period. Synapses within the severely ischemic core region were rapidly lost, while those in the mildly ischemic penumbra, albeit largely structurally intact, were functionally diminished. In contrast, the contralateral cortex exhibited increased synaptic staining and synaptic vesicle cycling. Systemic pharmacological blockade of NMDA-type glutamate receptors abolished contralateral synaptic increase and exacerbated synaptic decline in the penumbra. Proteomic and transcriptomic analyses showed that cross-brain synaptic plasticity is independent of local gene expression and revealed metabolic rearrangement and synaptic downregulation in the penumbra. These findings identify brain-wide synaptic rebalancing as a potential mechanism for rapid functional compensation in hyperacute stroke, highlighting the extent of brain response to acute perturbation.

Introduction

As the second and third leading cause of mortality and disability, respectively, ischemic stroke represents a significant burden on public health, with rising global

Data availability statement: All relevant data are contained within the paper and its [Supporting information](#) files, or freely available online. Proteomics data were analyzed by Oe Biotech using a proprietary pipeline. Mass spectrometry proteomics data is available at ProteomeXchange (<https://proteomecentral.proteomexchange.org>, dataset identifier PXD058834). Custom code for gene expression analysis has been uploaded to Zenodo (<https://zenodo.org/records/17987265>). RNAseq data is available at GEO (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE283465). Numerical data is presented in [S1 Table](#).

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Abbreviations: BSN, Bassoon; CCA, common carotid artery; ECA, external carotid artery; FC, fold change; GO, gene ontology; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; NMDARs, NMDA-type glutamate receptors; OGD, oxygen-glucose deprivation; PBS, phosphate-buffered saline; PCA, principal component analysis; PFA, paraformaldehyde; PSD, postsynaptic density; ROI, region of interest; RT, room temperature; SV, synaptic vesicle.

incidence [1–3]. The initial 24-hour hyperacute stage of stroke [4] is of particular clinical relevance, and urgent therapeutic intervention within this time period is key for long-term recovery, as reflected in its denomination as “the golden hour” of stroke [5–7]. Nevertheless, current therapy options for hyperacute stroke remain limited, focusing exclusively on blood flow restoration, while clinical criteria exclude a significant proportion of patients from treatment [7–9]. Better understanding of molecular, cellular, and physiological events during the “golden hour” is therefore sorely needed for future development of new stroke therapeutics.

A major factor underlying central nervous system pathology is dysregulation of synaptic function. Synaptic plasticity is extensively implicated in the early etiology of neurodevelopmental and neurodegenerative disorders, becoming a major topic for basic and translational research [10–12]. In stark contrast, investigation of the synapse in early stroke has seen little progress beyond demonstration of broad synaptic failure in *ex vivo* ischemia models more than 2 decades ago [13–17]. Despite some *in vivo* evidence for rapid dendritic spine dynamics in ischemia [18,19], detailed investigation of synaptic plasticity in stroke has been mainly concerned with mid-to-long-term functional adaptation and rehabilitation [20–25], while early synaptic remodeling processes remain largely unknown.

To fill this critical gap in knowledge, we endeavored to chart in detail the earliest stages of synaptic plasticity during ischemic stroke, using immunohistochemistry, proteomics, and transcriptomics. In doing so, we compared 3 key brain regions relevant to the stroke context: the **ischemic core** directly affected by blood flow disruption, the surrounding **penumbra** subjected to mild ischemia, and the **contralateral** cortex. Our findings demonstrate the emergence of distinct area-specific synaptic plasticity processes within 4 hours of stroke onset, revealing a hitherto unappreciated rapid-response brain mechanism.

Results

Ischemia triggers a rapid localized decline of synapses in neuronal cultures and *in vivo*

We first investigated the effect of oxygen-glucose deprivation (OGD), a canonical cell-based model of ischemia [26,27], on synapses in cultured neurons. Our previous work has demonstrated that OGD induces significant cell death within 2 hours [28], consistent with the rapid advent of ischemia [29]. Immunostaining showed loss of punctate labeling for an excitatory postsynaptic density (PSD) marker protein Homer within 45 min of OGD compared to the control medium (Fig 1A and 1B). Similar decrease of punctate staining was observed for a presynaptic active zone scaffold protein Bassoon (BSN) (Fig 1A and 1C) and for an inhibitory PSD protein Gephyrin (Fig 1D and 1E). The ratio of Gephyrin to Homer was significantly decreased by OGD, indicating a stronger effect of OGD on inhibitory synapses compared to their excitatory counterparts (Fig 1F). After 2 hours of OGD, some of the synaptic markers manifested a decrease in the number of puncta and an increase in nearest neighbor distance between the puncta (S1A–S1F Fig), reflecting synaptic loss. Thus, ischemia leads to rapid disassembly of synapses in cultured neurons.

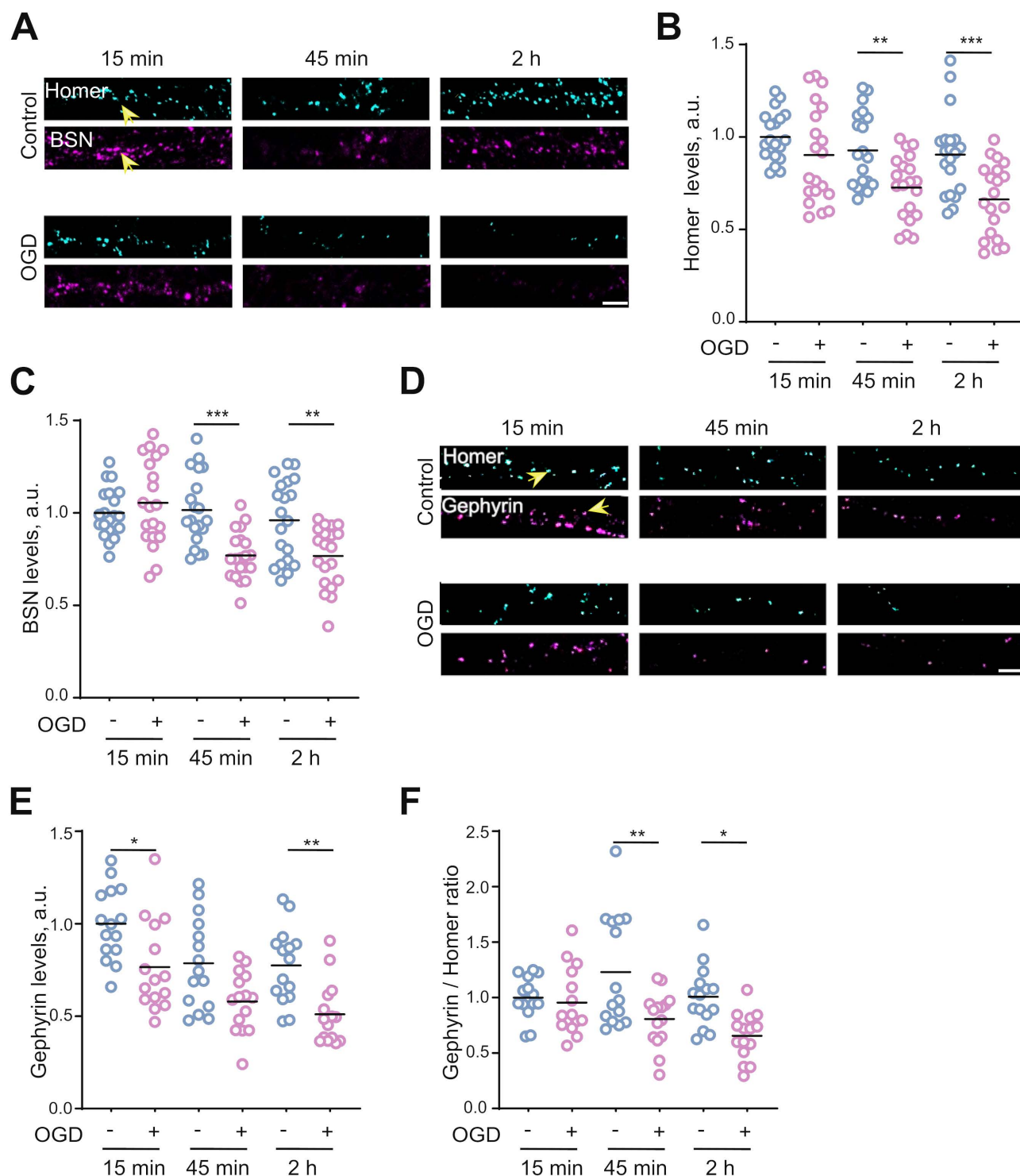


Fig 1. OGD induces rapid structural decline of excitatory and inhibitory synapses in cultured neurons. (A) Representative immunostaining images for Homer and BSN in neuronal cultures after OGD treatment. (B) Quantification of Homer in Homer (+) puncta. $N=20$ cells from 4 experiments. (C) Quantification of BSN in BSN (+) puncta. $N=20$ cells from 4 experiments. (D) Representative immunostaining images for Homer and Gephyrin in neuronal cultures after OGD treatment. (E) Quantification of Gephyrin in Gephyrin (+) puncta. $N=15$ cells from 3 experiments. (F) Quantification of Homer/Gephyrin ratio during OGD treatment at different time points. $N=15$ cells from 3 experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$, Kruskal–Wallis test with Dunn's *post hoc* test and one-way ANOVA with Šidák's *post hoc* test. Scale bar, 5 μm . The original data for this figure can be found in [S1 Table](#).

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Although OGD offers a convenient model to study cell effects of ischemia, it cannot fully recapitulate higher-order aspects of stroke pathophysiology, such as excitotoxicity, damage to the blood-brain barrier, or glial response [30]. Therefore, we leveraged the permanent middle cerebral artery occlusion (MCAO) model in rats, which is widely considered to faithfully reproduce the morphology and physiology of human stroke, notably featuring well-defined core and penumbra areas [30–35]. As expected, MCAO reliably produced large necrotic lesions in the cortex, indicating major ischemic stroke [36] (S2A Fig). In the ischemic core area, all three synaptic markers exhibited significant decline within 4 hours of MCAO (Figs 2A–2E and S2B–S2E), concordant with observations in the OGD model and indicative of rapid ischemia-associated synaptic disruption of both excitatory and inhibitory synapses. Puncta of Homer were also decreased in the penumbra, suggesting some impact on PSD (Fig 2A–2E and S2B–S2E). In line with published evidence [37], MCAO reduced synaptic levels of polymerized F-actin in both ischemic core and the penumbra, supporting the notion of rapid synaptic remodeling (S2F Fig). Taken together, these findings provide evidence that stroke rapidly causes profound synaptic loss in the ischemic core and some synaptic decline in the penumbra.

Stroke induces long-range synaptic remodeling in vivo

Mid- to long-term stroke recovery is known to involve reshaping of functional connectivity across the brain [21,25,38–40]. To investigate the long-range effects of hyperacute stroke on synaptic plasticity, we measured the effect of stroke on synaptic markers in the contralateral cortex, at a considerable distance (0.5–1 cm) from the ischemic core. Remarkably, contralateral punctate staining for both Homer and BSN was significantly increased after as little as 1 hour following MCAO (Fig 2A and 2B). There was no concomitant increase in Gephyrin puncta (Fig 2D and 2E), indicating that structural synaptic enlargement was likely restricted to excitatory synapses. The numbers of Homer- and BSN-positive puncta were not significantly altered, consistent with enlargement of existing synapses rather than generation of new ones (S2B–S2E Fig).

Region-specific synaptic reshaping by ischemia

To get a deeper insight into rapid synaptic remodeling during stroke, we performed immunostaining for several key classes of synaptic proteins. For postsynaptic receptors, we imaged distributions of an AMPA-type glutamate receptor subunit GluA2 and a GABA receptor subunit GABRA1. OGD resulted in rapid loss of GluA2 and GABRA1 puncta within 15 and 45 min respectively (S3A–S3D Fig), in agreement with previously reported effects of OGD [41]. Similar results were obtained in the MCAO model, where a decrease in GluA2 and GABRA1 was observed at the core within 1 and 4 hours respectively; no significant changes were observed in the contralateral cortex or in the penumbra (Fig 3A–3D). These data indicated that local ischemia induced rapid decrease in postsynaptic function.

To further investigate the effects of stroke on presynaptic structure and function, brain sections were immunolabeled for P/Q-type voltage-gated Ca²⁺ channel (VGCC) Ca_v2.1, and a synaptic vesicle (SV) marker protein vesicular glutamate transporter vGlut1. We found that MCAO did not affect Ca_v2.1 staining (S3E Fig), while vGlut1 was increased in ischemic core after 4 hours (S3F Fig). For direct measurement of presynaptic function, we prepared acute brain sections following MCAO and performed live-labeling with an antibody directed against the luminal/extracellular domain of the SV marker protein Synaptotagmin 1 (anti-Syt1) [42,43]. Anti-Syt1 labeling was significantly decreased in both the core and the penumbra (Fig 3E and 3F). Taken together, these findings indicated that ischemia rapidly downregulates local pre- and postsynaptic function.

NMDAR signaling regulates rapid synaptic remodeling

We then aimed to identify the signaling mechanism underlying long-distance synaptic plasticity in hyperacute stroke. NMDA-type glutamate receptors (NMDARs) are key regulators of synaptic plasticity in neurological disease, including stroke [44–50]. On the basis of previous evidence for NMDAR expression during ischemia [51], we imaged synaptic

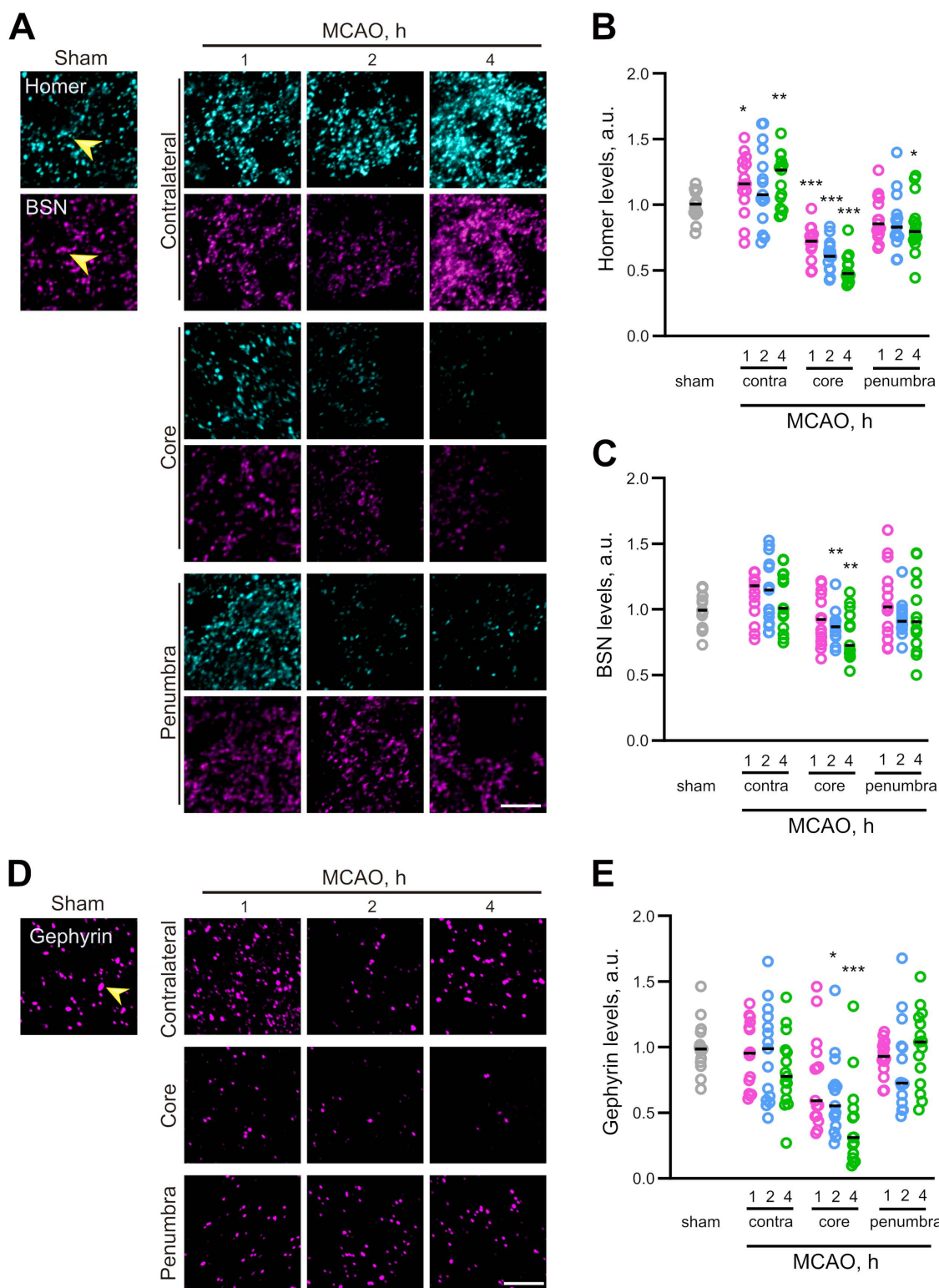


Fig 2. MCAO triggers rapid alteration of synaptic structure across the brain. (A) Representative immunostaining images for Homer and BSN immunostaining in brain sections following MCAO. (B) Quantification of Homer in Homer (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. (C) Quantification of BSN in BSN (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. (D) Representative immunostaining images for Gephyrin immunostaining in brain sections following MCAO. (E) Quantification of Gephyrin in Gephyrin (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point.

animals, 15 MCAO animals per time point. (D) Representative immunostaining images for Gephyrin in brain sections following MCAO. (E) Quantification of Gephyrin in Gephyrin (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$, mixed model analysis followed by Šidák's *post hoc* test. Scale bar, 5 μm . The original data for this figure can be found in [S1 Table](#).

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NMDAR distribution following OGD and MCAO. OGD had no significant effect on synaptic levels of the essential NMDAR subunit GluN1 ([Fig 4A](#) and [4B](#)), and there was no significant change in GluN1 levels across the brain regions during MCAO ([Fig 4C](#) and [4D](#)); similar result was obtained for another NMDAR subunit GluN2B implicated in stroke and neuroprotection [[46,47](#)] ([S3G Fig](#)), suggesting that the timescale of hyperacute stroke was not associated with synaptic NMDAR recruitment.

To investigate the functional relevance of NMDAR signaling for synaptic remodeling, we injected animals with an NMDAR antagonist MK801, which has a neuroprotective effect in this model [[50](#)], performed MCAO, and stained for Homer and BSN ([Fig 4E](#)). Considering the previous reports of MK801-induced hyperthermia [[52,53](#)] or hypothermia [[54,55](#)], we sought to determine whether MK801 affected body temperature in our experiments, and found no effect ([S3H Fig](#)). MK801 injection prior to MCAO resulted in a decrease of punctate Homer staining both in the penumbra and in the contralateral hemisphere, effectively reversing the long-range increase in the latter ([Fig 4E](#) and [4F](#)); similar results were observed for BSN staining ([Fig 4E](#) and [4G](#)). This evidence suggests that NMDAR signaling orchestrates rapid synaptic rearrangement across the brain during hyperacute stroke in a region-specific manner.

Proteomic analysis of synaptosomes confirms long-range synaptic remodeling

To gain a broader insight into synaptic dynamics during hyperacute stroke, we leveraged quantitative proteomics to identify differentially expressed proteins in biochemically purified brain synaptosomes following 4 hours of MCAO ([Fig 5](#) and [S2 Table](#)). Principal component analysis (PCA) showed separation between ischemic core and penumbra on one side and control and contralateral cortex on the other, suggesting that stroke effected profound changes in synaptic composition in regions directly affected by ischemia ([Fig 5A](#)), notwithstanding substantial variability between samples ([Fig 5B](#)). Synapses from the core region and penumbra exhibited considerably more downregulated than upregulated proteins (580 *versus* 210 and 373 *versus* 146, respectively), consistent with a general synaptic decline in ischemia, while in contralateral synapses the numbers of upregulated and downregulated proteins were similar (88 *versus* 93), in line with the notion of synaptic remodeling. There was a degree of overlap between the regional expression profiles, suggesting the emergence of brain-wide as well as localized responses ([Fig 5C](#)).

We focused on contralateral synaptic reorganization in more detail ([Fig 5D](#)). Gene ontology (GO) analysis manifested enrichment in terms related to biological processes and molecular functions relevant to synaptic plasticity, while top cellular component terms were associated with synaptic location ([Fig 5E](#)). These results were confirmed by gene set enrichment analysis (GSEA), returning terms associated with presynaptic membrane trafficking ([S4A–S4C Fig](#)). In contrast, GO terms for downregulated proteins were more diverse, generally pertaining to metabolism and membrane trafficking, with notable enrichment for nucleoplasm in the cellular component category, suggesting a non-synaptic response likely located in the nucleus ([S4D Fig](#)). Among the 88 upregulated proteins, 35 were previously associated with synapses as indicated by their GO terms including postsynaptic function, presynaptic release, ion transport, and extracellular adhesion ([Fig 5F](#)), with a particular increase in voltage-gated K^+ channels, key regulators of neuronal excitability and synaptic plasticity [[56](#)]. The most increased protein was β -Synuclein, previously suggested as a biomarker for stroke [[57,58](#)]. Other noteworthy upregulated proteins include vimentin, the main protein of intermediate filaments, and 9 proteins associated with endocannabinoid signaling, including MgII and Dagla, two enzymes responsible for metabolism of 2-arachidonoylglycerol ([Fig 5F](#) and [S2 Table](#)). Taken together, this evidence confirms synaptic upregulation in the contralateral hemisphere during hyperacute stroke.

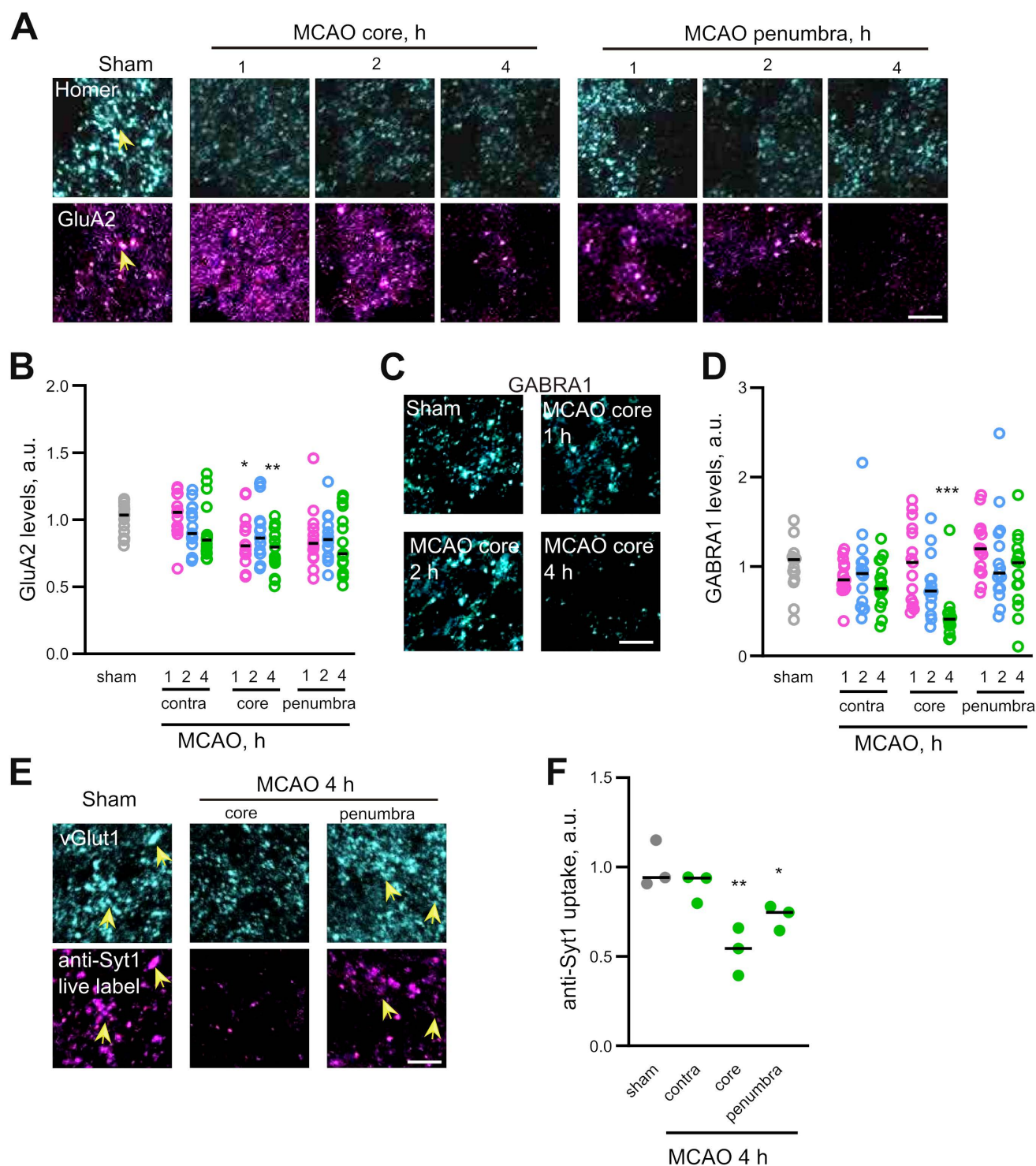


Fig 3. Region-specific presynaptic and postsynaptic changes following MCAO. (A) Representative immunostaining images for Homer and GluA2 in brain sections following MCAO. (B) Quantification of GluA2 in Homer (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. (C) Representative immunostaining images for GABRA1 in brain sections following MCAO. (D) Quantification of GABRA1 in Gephyrin (+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. (E) Representative immunostaining images of acute brain slices live-labeled with anti-Syt1 antibody and stained with vGlut1 post-fixation following MCAO. (F) Quantification of anti-Syt1 uptake into vGlut1(+) puncta in acute brain slices following 4 h of MCAO. $N=3$ sham control animals, 3 MCAO animals. $*P<0.05$, $**P<0.01$,

*** $P < 0.001$, mixed model with Šidák's *post hoc* test (B, D), one-way ANOVA with Dunnett's *post hoc* test (F). Scale bar, 5 μm . The original data for this figure can be found in [S1 Table](#).

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Conversely, no synaptic increase was observed in the ischemic core and penumbra ([Table S2](#) and [S5 Fig](#)). In core samples, upregulated GO terms were enriched for plasma membrane localization, while downregulated terms were nucleus and cytoplasm, possibly indicating contamination by disintegrated cell compartments due to necrosis induced by ischemia ([S5C](#) and [S5D Fig](#)). GO analysis in penumbra suggested upregulation in beta-oxidation of fatty acids in mitochondria and translational downregulation in the cytosol ([S5E](#) and [S5F Fig](#)).

To investigate presynaptic function in the contralateral cortex, we applied the live-labeling anti-Syt1 assay to synaptosomes. Given our previous evidence showing no increase in constitutive SV cycling ([Fig 3E](#) and [3F](#)), we resorted to depolarization by high K^+ to measure evoked SV cycling [[59](#)]. Surface labeling of synaptosomes with anti-Syt1 showed no differences between sham control and MCAO, indicating that surface levels of Syt1 were unchanged, however treatment with 30 mM KCl resulted in a significantly higher signal in synaptosomes purified from the contralateral MCAO cortex, indicating an increase in evoked SV cycling ([Fig 5G–5I](#)). These data confirm the long-distance effect of stroke on presynaptic function across the brain.

Region-specific gene expression programs in synapses during hyperacute stroke

Our data revealed a pattern of region-specific synaptic reorganization in response to hyperacute stroke. To uncover associated gene expression dynamics, we isolated, sequenced and analyzed total RNA from the whole brain tissue and from synaptosomes ([S3 Table](#)). PCA of the data from whole brain tissue exhibited substantial scatter, consistent with inter-individual variability of brain gene expression [[60](#)] ([S6A Fig](#)). Conversely, PCA of synaptosome samples returned clear separation between control, ischemic core and penumbra, suggesting emergence of large-scale, distinct, region-specific gene expression programs at the synapse triggered by stroke, while control and contralateral samples overlapped, suggesting little difference in gene expression profiles ([Fig 6A](#)).

We first performed gene set variation analysis (GSVA) in synaptosomes, using previously published synaptic gene sets [[61](#)] ([Fig 6B](#)). No changes were observed in samples from the contralateral hemisphere, suggesting that synaptic remodeling in the contralateral hemisphere was likely not driven by local gene expression. Conversely, samples from the ischemic core manifested specific downregulation of postsynaptic sets, while the majority of synaptic sets were downregulated in synaptosomes from penumbra, indicating an effect on local synaptic gene expression. The effect on synaptic gene sets was also evident in the whole-brain samples from penumbra ([S6B Fig](#)).

For further insight into biological processes regulated by gene expression in early stroke, we used GO analysis using the terms from Gene Ontology (GO:BP, GO:MF and GO:CC) and KEGG. In the penumbra, upregulated genes were involved in regulation of various biological processes, mainly related to development and immune response, highlighting broad reorganization in response to stroke, while downregulated terms included synapse and cell-cell communication, in line with GSVA results ([Fig 6C–6E](#) and [S6C–S6E](#)). In the contralateral hemisphere, synaptosomes and whole brain tissue featured upregulation of genes pertaining to metabolic processes and complement system, respectively ([S7 Fig](#)). In the ischemic core, downregulated genes from whole brain tissues were enriched for transcription-related terms, while downregulated genes in synaptosomes were enriched in terms pertaining to synapses and cell adhesion ([S8 Fig](#)). Taken together, our data indicate a wide-ranging programme of region-specific gene expression induced by hyperacute stroke.

Discussion

To date, investigation of synaptic plasticity in stroke has mainly focused either on rapid synaptic decline in ischemia [[19,62](#)] or on the recovery stage [[4,21,22,25](#)], spanning minutes and weeks/months respectively. Our study fills the critical

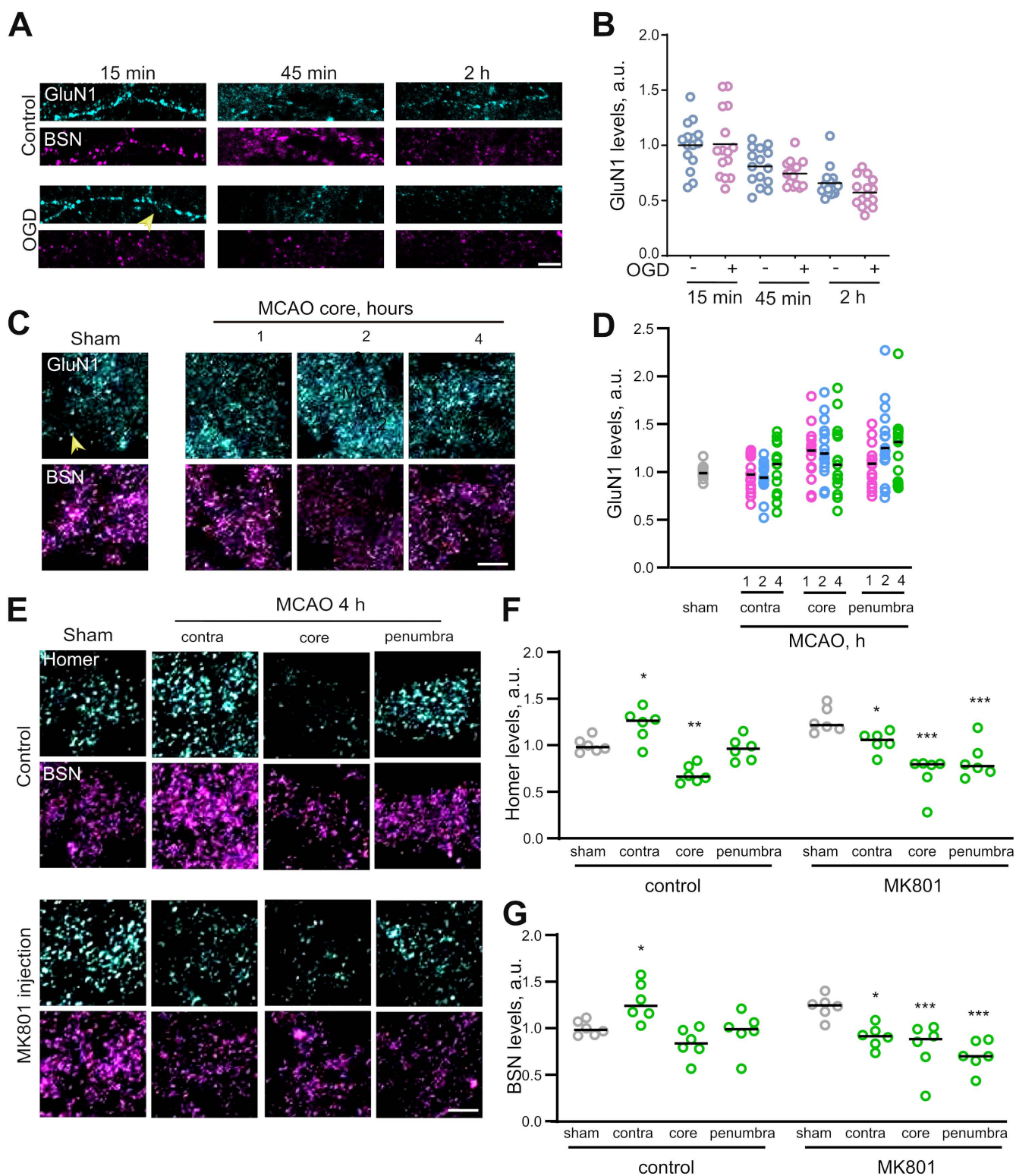


Fig 4. NMDAR signaling regulates rapid synaptic remodeling in the contralateral hemisphere and the penumbra following MCAO. (A) Representative immunostaining images for GluN1 and BSN in neuronal cultures following OGD treatment on DIV14. (B) Quantification of GluN1 in BSN(+) puncta in neuronal cultures following OGD. $N=15$ cells from 3 experiments. (C) Representative immunostaining images for GluN1 and BSN in brain

sections following MCAO. **(D)** Quantification of GluN1 in BSN(+) puncta in brain sections following MCAO. $N=15$ sham control animals, 15 MCAO animals per time point. **(E)** Representative immunostaining images for Homer and BSN in brain sections, taken 1 hour after MK801 injection followed by 4 hours of MCAO. **(F)** Quantification of Homer levels in Homer (+) puncta in brain sections, 1 hour after MK801 injection followed by 4 hours of MCAO. $N=6$ sham control animals, 6 sham control animals preinjected with MK801, 6 MCAO animals, 5 MCAO animals preinjected with MK801. **(G)** Quantification of BSN levels in BSN (+) puncta in brain sections, 1 hour after MK801 injection followed by 4 hours of MCAO. $N=6$ sham control animals, 6 sham control animals injected with MK801, 6 MCAO animals, 5 MCAO animals injected with MK801. $*P<0.05$, $**P<0.01$, $***P<0.001$, Kruskal–Wallis test with Dunn's *post hoc* test **(B)**, mixed model analysis with Šidák's *post hoc* test **(D)**, 2-way ANOVA with Šidák's *post hoc* test **(F, G)**. Scale bar, 5 μm . The original data for this figure can be found in [S1 Table](#).

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gap in between the above timescales, demonstrating brain-wide synaptic rearrangement processes operating within the key 4-hour therapeutic window of stroke. These findings reveal region-specific synaptic dynamics as a novel feature of rapid response to ischemic insult, establishing a new mechanism of interest for stroke therapy.

Rapid contralateral synaptic remodeling in stroke

The key finding of our study is the effect of stroke on excitatory synapses in the contralateral hemisphere, arising within 4 hours of stroke onset. Previously published evidence for contralateral functional alterations beyond 2 days is inconclusive [63–68], suggesting that contralateral enhancement may be a transient feature specifically associated with the hyperacute phase of stroke. Reorienting synaptic function away from the core/penumbra may represent an early-stage response mechanism to safeguard brain-wide functionality (Fig 6F). On a longer timescale, an increase in distal brain activity may provide stimulation to promote recovery of circuits in the stroke region [69,70], consistent with downscaling of inhibitory neurotransmission resulting in an excitatory/inhibitory balance shift that may promote functional recovery [71–73]. The diffuse widespread nature of contralateral upscaling is reminiscent of a homeostatic mechanism on a timescale similar to that of ketamine- and lithium-induced plasticity [74] acting in advance of slower long-range neuronal plasticity in stroke, e.g., functional connectivity, and neurogenesis [39,75,76].

Our proteomic and transcriptomic analyses highlight specific functionalities associated with long-range synaptic plasticity, including membrane potential, cannabinoid signaling and presynaptic function, the latter directly confirmed experimentally (Fig 5G–5I). Considering the limited timescale of our investigation, the 88 contralaterally upregulated proteins identified in our survey may be mere harbingers of subsequent broader changes. Limited gene expression changes observed in the contralateral hemisphere imply that synaptic remodeling is likely effected in a cross-brain manner from the ischemic core and the penumbra, where we find a transcriptional/translational response centered around metabolic reorganization and synaptic downregulation (Fig 6B–6E and S5). A similar metabolic shift towards reduced protein biosynthesis and increased lipid metabolism has been previously demonstrated in the heart, suggesting broad commonalities in metabolic adaptations to ischemia [77–80]. Given the substantial energy demands of synaptic transmission [81], it is tempting to speculate that regional synaptic remodeling and metabolic switches may act in concert to balance functionality with tissue viability in stroke (Fig 6F).

Intriguingly, our findings of long-range synaptic effect evoke the notion of *diaschisis*, referring to functional changes in remote brain regions resulting from a localized brain injury. Although this concept was introduced as early as 1914, experimental evidence remains limited [82]. Within this framework, synaptic plasticity can be interpreted as a substrate of diaschisis, suggesting a new mechanism underlying brain-wide effects of stroke. It remains to be seen whether long-range synaptic response may play a similar role in other forms of brain injury due to trauma, tumor, or infection.

Signaling mechanisms of brain-wide synaptic reorganization

We show that systemic administration of a NMDAR antagonist MK801 disrupts MCAO-induced synaptic remodeling, highlighting the key role of NMDAR in orchestrating brain-wide compensatory synaptic response in early stroke (Fig 4) and revealing a new facet of the complex role played by NMDARs in brain disease [44]. In line with the rapid increase in

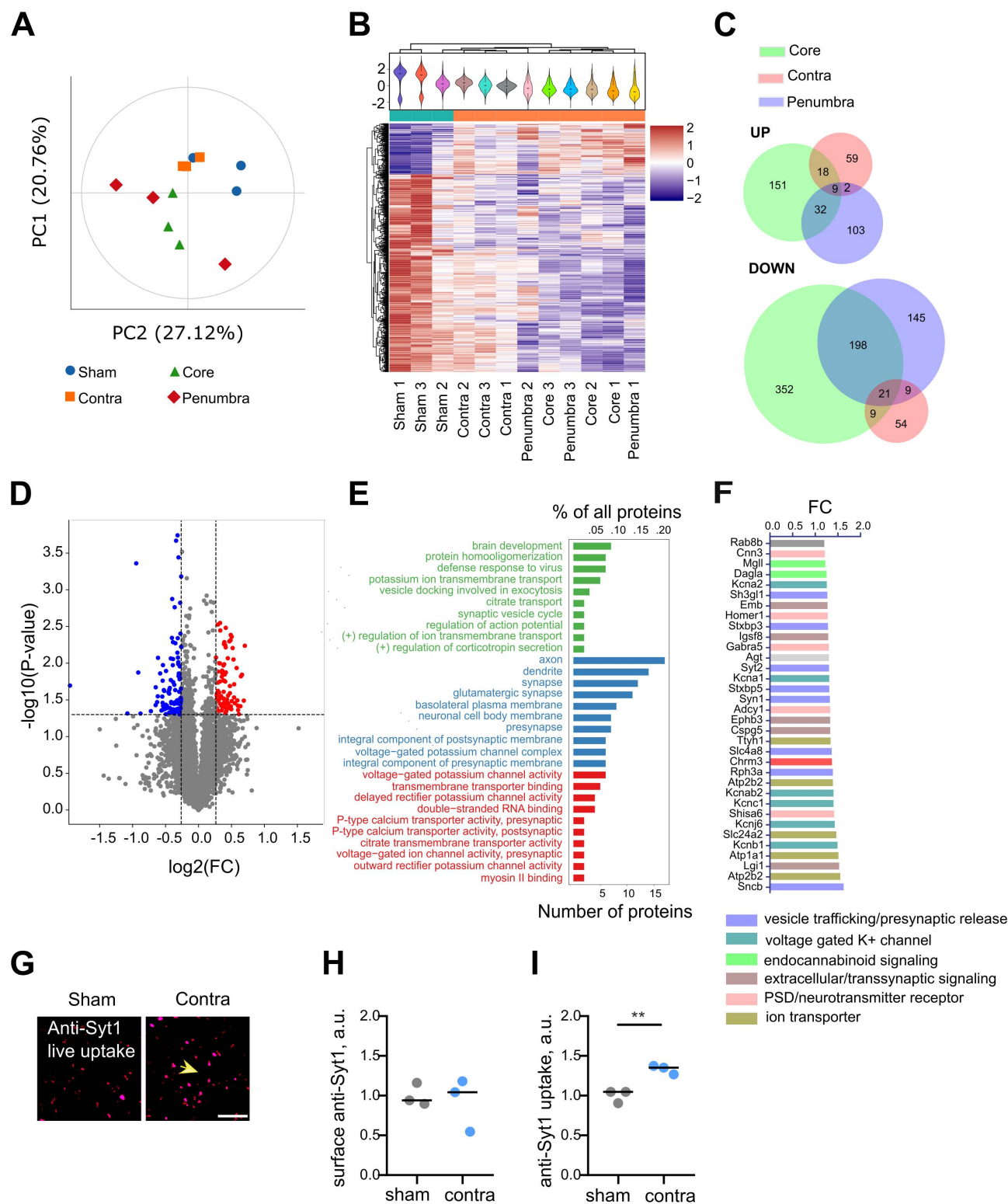


Fig 5. Proteomic analysis of purified synaptosomes confirms cross-brain presynaptic remodeling following MCAO. (A) PCA of protein levels for all samples. $N=3$ sham animals and 3 MCAO animals. (B) Heatmap and clustering analysis for all samples. (C) Venn diagram showing numbers and overlap in upregulated (top) and downregulated (bottom) proteins between regions. (D) Volcano plot for differentially expressed proteins, contralateral

hemisphere vs. sham control. **(E)** Top GO terms for upregulated proteins, contralateral hemisphere vs. sham control. Red, biological processes; blue, cellular components; red, molecular function. **(F)** Enrichment (FC, fold change) of selected synapse-associated proteins upregulated in the control hemisphere vs. sham control; colors highlight different functional attributions. **(G)** Representative immunostaining images showing KCL-induced uptake of anti-Syt1 antibody in synaptosomes from the contralateral hemisphere vs. sham control. **(H)** Quantification of surface anti-Syt1 labeling in synaptosomes from the contralateral hemisphere vs. sham control. **(I)** Quantification of KCL-induced anti-Syt1 uptake in synaptosomes from the contralateral hemisphere vs. sham control. $**P < 0.01$, 2-tailed t test, $N = 3$ sham animals and 3 MCAO animals. Scale bar, 5 μm . The original data for this figure can be found at ProteomeXchange (<https://proteomecentral.proteomexchange.org>, dataset identifier PXD058834) and in [S1 Table](#).

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extracellular glutamate concentration within the core and penumbra during MCAO [83], NMDAR activation is likely localized within the ischemic region. Intriguingly, our data shows that MCAO during NMDAR blockade results in a decrease in contralateral synapses (Fig 4), suggesting that NMDAR signaling in stroke may not only engage a long-range pathway for synaptic **strengthening** but also suppress another mechanism for synaptic **weakening**. Regarding the lack of effect of MK801 on body temperature (S3H Fig), the increase in synaptic staining observed in MK801-treated sham controls (Fig 4F and 4G) is consistent with rapid potentiation triggered by NMDAR blockade [84,85] rather than pyrexia-induced synaptic strengthening [86]. Owing to the broad expression profile of NMDARs in the brain, our evidence does not allow to separately address contributions of synaptic, extrasynaptic [87], or indeed non-neuronal NMDARs [45], which may be differentially involved in stroke [48]; the relevance of NMDARs for this long-distance relay therefore warrants further investigation.

Our proteomic analysis hints at a possible role for another signaling pathway in synaptic rebalancing, namely cannabinoid signaling. Increased synaptic expression of cannabinoid signaling components in the contralateral hemisphere may regulate synaptic dynamics through various forms of plasticity [88], while increased levels of cannabinoid receptor 1 in the penumbral synapses (S2 Table) are likely to facilitate activation of pro-survival pathways previously implicated in stroke [89]. Broad synaptic remodeling may act alongside local mechanisms underlying neuroprotective properties of cannabinoids [90], promoting the brain's general resilience to stroke.

Study limitations

The main caveat of our study relates to use of a specific stroke model. The permanent MCAO model, although recommended by the Stroke Treatment Academic Industry Roundtable [35], cannot account for multiple key features of human stroke, most notably its pathophysiological heterogeneity [91], as well as the complex and poorly understood interplay between ischemic and endothelial damage processes [92]. The findings of our study therefore justify further investigation in other animal models of stroke and eventually in human patients, taking particular notice of factors such as age and sex [93].

Owing to the experimental design considerations, our study focused on the time points of 1, 2 and 4 hours, in line with the generally accepted clinical definition of hyperacute stroke [5,6]. Consequently, we did not consider faster or slower forms of synaptic dynamics, e.g., by Hebbian plasticity [94] or engulfment by microglia [95]. An expanded investigation encompassing a wider range of timescales may provide a more detailed view of synaptic dynamics in stroke; on this note, interrogation of immediate-early synaptic remodeling may be amenable to live imaging approaches [18,19] for a closer look into the clinically important 1st hour of stroke [5].

Evidence of enhanced SV cycling in contralateral presynaptic terminals calls for a more detailed functional assessment of the observed effect. While SV assays represent a workable proxy for general synaptic function, the nature of synaptic role in brain disease can be complex, with different functional aspects differentially affected [10]. Furthermore, it remains unclear to what extent long-range synaptic remodeling may constitute a compensatory mechanism *versus* a sequela of stroke. A dedicated systemic investigation of brain-wide synaptic function in hyperacute stroke will be necessary to address these issues.

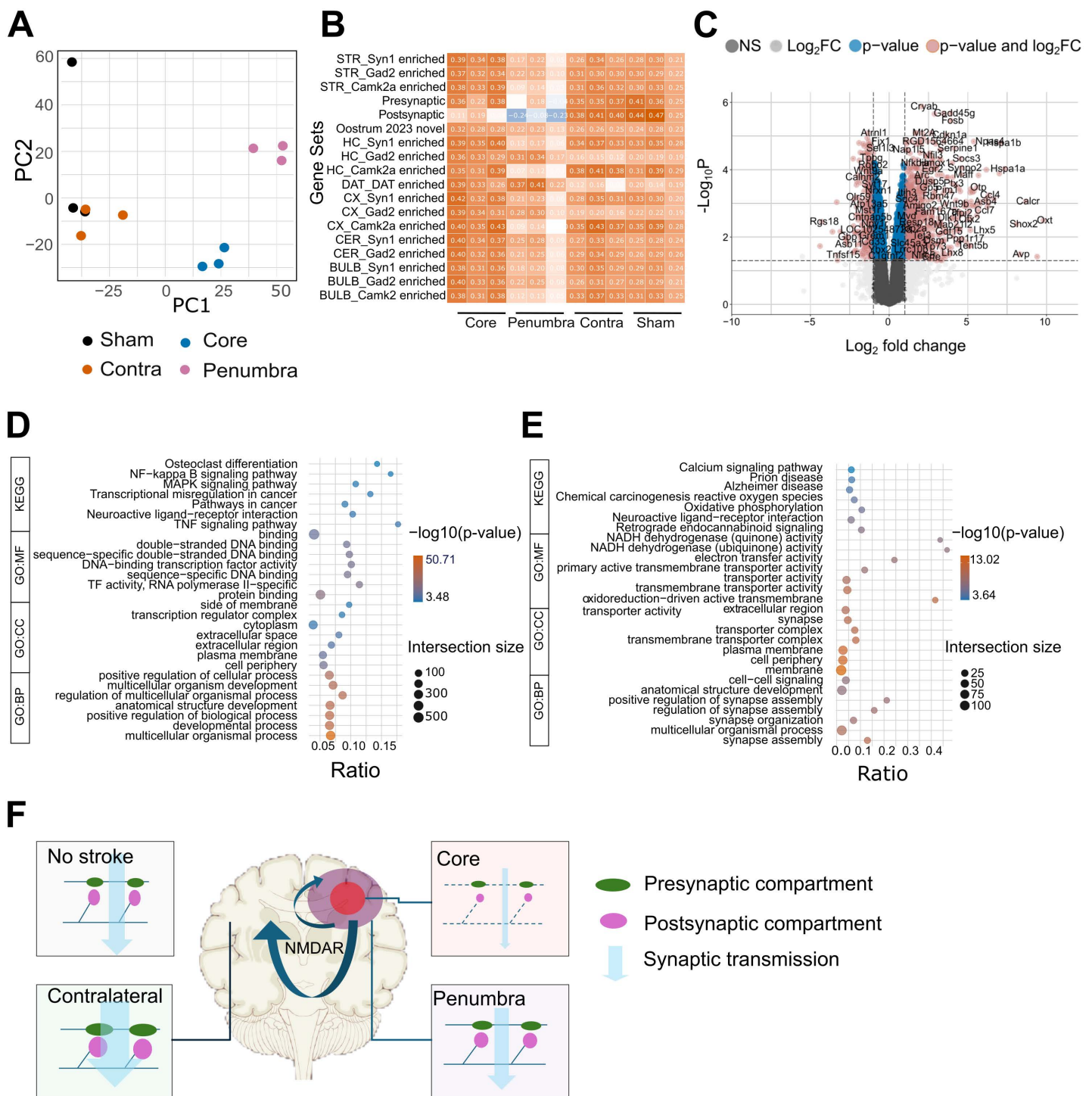


Fig 6. RNAseq reveals rapid induction of region-specific synaptic gene expression programs following MCAO. (A) PCA of gene expression for synaptosome samples. CA – control animals, 4z – 4 hours contralateral, 4p – 4 hours penumbra, 4c – 4 hours (ischemic) core. $N=3$ sham animals and 3 MCAO animals. (B) GSVA enrichment scores for synaptic gene sets in synaptosomal fractions. (C) Volcano plot for differentially expressed genes in synaptosomes, penumbra vs. sham control. (D) Functional enrichment analysis for top upregulated genes in synaptosomes, penumbra vs. sham control. (E) Functional enrichment analysis for top downregulated genes in synaptosomes, penumbra vs. sham control. (F) Schematic model for region-specific synaptic remodeling during hyperacute stroke. In the **ischemic core**, severe ischemia results in cell death and synaptic collapse. In the surrounding

penumbra, mild ischemia triggers a response to preserve synaptic structure in an NMDAR-dependent manner, while energy-intensive synaptic function is scaled down. Across the brain, in the **contralateral hemisphere**, a long-distance relay mechanism dependent on NMDAR signaling induces homeostatic synaptic increase. Based on a Wikimedia Commons image (https://commons.wikimedia.org/wiki/File:Brain_human_coronal_section.svg) by Patrick J. Lynch, medical illustrator, and C. Carl Jaffe, MD, cardiologist. The original data can be found at GEO (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE283465).

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Future directions

The findings of our study raise a number of questions concerning synaptic plasticity in early stroke. How is metabolic response coupled with synaptic reorganization? How is stroke-induced synaptic dynamics affected by aging [96]? Which aspects or rapid synaptic remodeling contribute to the brain's response to stroke, and which represent its unwelcome consequences? In the wider context, the notion of synaptic involvement in early stroke converges with the established discourse on other brain pathologies [10,11] to provide an impetus for further investigation of synaptic plasticity in cerebrovascular disorders, including acute neurological conditions such as traumatic brain injury, transient ischemic attack, and hemorrhagic stroke.

The clinical relevance of our findings remains to be determined, given the lack of evidence on synaptic dynamics in early human stroke. While direct investigation of human brain synapses in the clinical setting remains experimentally challenging, recently published electroencephalographic evidence of contralateral functional enhancement in stroke suggests that similar mechanisms may operate in the human brain [68]. Following further investigation, in the longer-term targeting synaptic plasticity may provide a much-needed new therapeutic modality for improving clinical outcomes during the “golden hour” of stroke and beyond, paralleling emerging synaptic therapies for early treatment of neurodegenerative and neurodevelopmental disease [12,97–99].

Methods

Experimental models

Cell culture. Cultures of primary cortical neurons were derived from embryos at 16–18 days of gestation, which were isolated from pregnant rats anesthetized with isoflurane. Cerebral cortices were isolated, washed, and centrifuged at 900 rpm at room temperature. Brain tissue was digested with trypsin for 20 min in 37 °C, and digestion was terminated by the addition of DMEM containing serum, filtered through a strainer and broken down into cells by careful pipetting. Cell suspension was pelleted by gentle centrifugation, washed, and resuspended in culture medium (Neurobasal medium, 2% B27 supplement, 1% glutamine). Cells were plated into 24-well plates at a density of approximately 40,000 cells per well. Medium was exchanged within 24 hours of plating, and half-changed every three days thereafter.

Animals. The adult male Sprague-Dawley rats (weight 230–250 g) were purchased from Jinan Peng Yue Laboratory Animal Breeding Co. Ltd and were kept under a 12-hour light/dark cycle in the same colony room with appropriate temperature. All animal experiments were conducted in compliance with National Institutes of Health Guidelines and were approved by the institutional animal care and use committee of Qingdao University under protocol number QDU-AEC-2024436.

Method details

OGD. OGD was induced in 14 DIV cortical neuronal cultures with a HEPES-buffered solution (25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM sucrose or supplemented with 10 mM glucose for control). Neuron culture plates were washed twice and incubated with OGD-HEPES solution in an anaerobic workstation (Ruskin Concept 400, United Kingdom) at 37 °C, 95% N₂ and 5% CO₂ prior to being harvested or fixed at specific time

points (15 min, 45 min and 1 hour.). Control neurons were incubated at 37 °C, 5% CO₂ with control HEPES solution and harvested or fixed at the same time points as those subjected to OGD.

MCAO procedure. A heating pad was used to maintain the body temperature at 37°C during the procedure. The skin was cut with scissors after disinfection in the middle of the neck, and the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated with forceps. And all were ligated with surgical sutures, a small incision was cut at the distal end of the ECA, wherein a filament was inserted. The sutures of the ICA were then undone and the inserted filament was pushed from the right CCA into the right ICA, resulting in occlusion of the origin of the right middle cerebral artery (MCA). Following the procedure, animals were sacrificed after 1, 2, and 4 hours. For sham control, the procedure was followed as described above, except no filament was inserted.

Animals were assessed on the Longa 5-point scale [34], with those exhibiting severe functional deficits and death (4–5 points) and those without any neurological impairments (0 points) excluded from further analysis; the percentage of excluded animals was below 30% of the total. The ischemic core and the penumbra were prepared according to the established protocols for MCAO in rats [100–103] and mice [102,104,105]. Briefly, ischemia core and penumbra were separated by a transverse diagonal cut made at approximately the “2 o’clock” position.

MK801 treatment. MK801 (1 mg/kg) was injected intraperitoneally 1 hour before MCAO, and animals were sacrificed following 4 hours of MCAO. To investigate the effect of MK801 on body temperature, 6 animals were injected with MK801 and 6 with saline, and rectal temperature was measured every hour for 5 hours.

Proteomics. Three animals were subjected to MCAO, while 3 animals were used as sham controls. Animals were sacrificed 4 hours after surgery. From MCAO animals, brain tissue was taken from each of the following regions: the ischemic core, the penumbra, and the contralateral hemisphere. From sham control animals, brain tissue was excised from the corresponding area of the cortex. The total number of samples was therefore 12, with 3 samples each in: sham control, ischemic core, penumbra, and contralateral hemisphere.

Three hundred milligrams of the brain tissue from each sample was homogenized in 10 volumes of the Syn-PER Reagent following manufacturer’s instructions, with 10 up-and-down even strokes using a Dounce tissue homogenizer. Processed tissue was then centrifuged at 1,200g for 10 min to remove the debris, and the resulting supernatant was centrifuged at 15,000g for 20 min. The pellets, containing synaptosomes, were gently resuspended in the reagent.

Tandem Mass Tagging proteomic analysis of the synaptosomal fraction was performed and analyzed by Oe Bio-tech (Shanghai, China) as briefly described below. One part of the synaptosomes was used for protein concentration determination and SDS-PAGE detection, and the other part was trypsinized and labeled. An equal amount of each labeled sample was then mixed and separated by chromatography to be analyzed by LC–MS/MS, providing protein abundance data.

Live anti-Syt1 uptake assay. For anti-Syt1 uptake in acute brain sections, 3 animals were subjected to MCAO, while 3 animals were used as sham controls. Animals were sacrificed 4 hours after surgery. Brain tissue was sectioned from the regions as described above. Sections of 200 μm in thickness were prepared using vibratome and allowed to recover for 30 mins following sectioning. Sections were live-labeled with anti-Syt1 antibody (1/200) for 30 min at 37 °C. Section were then washed, fixed for 20 mins in 4% paraformaldehyde (PFA), permeabilized and blocked for 1.5 hours, incubated with anti-vGlut1 primary antibody overnight at 4 °C. Next day, slices were rewarmed to room temperature (RT) for 75 mins, washed for 5 mins 6 times, then incubated in the mix of secondary antibodies for 2 hours at RT. Slices were then washed for 5 mins 6 times and mounted onto slides to be stored at 4 °C.

For anti-Syt1 uptake in synaptosomes, the latter were isolated as described above. Aliquoted synaptosomes were resuspended and live-labeled with 1/200 anti-Syt1 at 4 °C for 20 min. Synaptosomes were either fixed immediately to measure surface Syt1 or incubated with 30 mM KCL for 15 min at 37 °C to measure depolarization-evoked SV cycling. Synaptosomes were pelleted by centrifuging for 5 min at 3,700 rpm and washed twice with phosphate-buffered saline (PBS), then fixed with 4% PFA in PBS, followed by centrifugation for 20 min at 3,700 rpm. Fixed synaptosomes were

washed for 5 min 4 times, permeabilized for 30 min, then labeled with secondary antibodies for 1 hour, washed as above, and mounted in DAPI-containing medium onto microscopy slides.

RNA sequencing and analysis. Three animals were subjected to MCAO, while 3 animals were used as sham controls. Animals were sacrificed 4 hours after surgery. Brain tissue was sectioned from the regions as described above. Total RNA was extracted by TRIzol Reagent. RNA sequencing and quality control were provided by Beijing Novogene (Beijing, China), providing expression profiling data.

Immunostaining. For immunocytochemistry, after treatment cells were fixed with 4% PFA in PBS for 20 min at RT and washed 5 min (3×) before being blocked in 0.3% Triton-X100 in PBS supplemented with 5% goat serum for 30 min. Blocked coverslips were incubated with appropriate primary antibodies for 60 min at RT, then washed 5 min (4×) in PBS and incubated with secondary antibodies (1:500 AlexaFluor-488 and AlexaFluor-594) each for 60 min at RT. Coverslips were washed for 5 min (4×) before being mounted on microscope slides with mounting media (containing DAPI).

Brain tissue was sectioned from the regions as described above. Animals were anesthetized with isoflurane and transcardially perfused with PBS (pH 7.4). The brains were dissected out and kept in −80 °C. Brain sectioning was performed in the coronal plane at −20 °C using a Cryostat (Leica CM1950), at a thickness of 25 μm. Brain sections were attached to positively-charged microscope slides and fixed with 4% PFA in PBS for 20 mins at RT, followed by 3 × 10 min (3×) in PBS. Subsequent sections were collected and stored at −80 °C for future use. Sections were incubated in blocking buffer (5% goat serum, 0.3% Triton-X 100 in PBS) for 1 hour at RT, and incubated for 36–48 h at 4 °C with primary antibodies diluted in blocking buffer. Sections were subsequently washed for 10 min (3×) in PBS, followed by incubating with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies for 75 mins at RT, and finally washed for 10 min (3×) with PBS. Then all sections were mounted with DAPI-containing mount media.

Microscopy imaging. Confocal imaging was carried out using a Nikon Eclipse Ti2 microscope. Serial confocal z-stack images (0.5 μm step for 2 μm at 512 × 512 or 1,024 × 1,024 pixels, zoom 2 or zoom 1) were acquired with a 100×/1.45 Oil objective (Plan APO λ). The pinhole size was 1 Airy unit. Excitation laser wavelengths were 488 and 561 nm. Bandpass filters were set at 500–550 nm and 570–620 nm for imaging Alexa Fluor 488 and 570–620 nm Alexa Fluor 594, respectively. Other settings such as gain value were optimized to ensure appropriate dynamic range, low background and sufficient signal/noise ratio. Image acquisition settings were kept consistent within the experiment.

The following parameters were assessed: synaptic size; levels of synaptic marker proteins inside synapses; nearest neighbor distance between the synapses; number of synapses in an image; ratio of synaptic marker proteins; intensity of live-labeling with an anti-Syt1 antibody.

Blinding, randomization, confounders. All the experiments were carried out by WY and HC. Immunostaining was done by WY, HC, and FD. Image analysis was carried out by WY, HC, FD, ZF, and OOG. Due to the overt presentation of MCAO and the necessity of correct identification of the brain regions, it was not feasible to blind the experimenter to the nature of the treatment up to the point of image acquisition. Further, owing to the semi-automated routine for image analysis in ImageJ, specifically considering region of interest (ROI) selection for analysis of synaptic structure, blinding of the experimenter to the sample was deemed unnecessary and was therefore not conducted. No specific randomization procedures were applied to selection of animals into experimental groups. Given that all the measurements were conducted *post mortem*, no additional confounders were identified.

Quantification and statistical analysis

Sample size calculations. G*Power 3.1.9.7 was used to estimate sample size. Sample size calculations were not carried out for experiments with cell cultures (Fig 1) proteomics (Fig 5), and transcriptomics (Fig 6). Based on the cell culture data, sample size for the time course MCAO experiment (Figs 2, 3, S2, and S3) was estimated using the following parameters: effect size 0.5, 4 groups, alpha error probability 0.05, power 0.8. Sample size for the 4 hour MK801 experiment (Fig 4) was estimated using the following parameters: effect size 2, alpha error probability 0.05, power 0.8.

Image analysis. ImageJ was used for image analysis. For analysis of individual synaptic puncta, images were binarized by using the “Moments” setting, and puncta were identified automatically using the “Analyze Particles” command across the whole image. In double staining experiments, Homer, BSN, Gephyrin, and vGlut1 staining was used to identify synaptic puncta as specified in the Figure Legends. Fluorescence signal intensities were quantified for each synaptic punctum using the ROI Manager function of ImageJ. To avoid rare overlap of multiple synapses, only ROIs with areas ranging from 0.1 to 2 μm^2 were included in further analysis. All values of circularity were included in analysis. Individual ROIs within the image were merged into one compound ROI using the “Combine” and “Add” functions of the ROI Manager interface, whereupon quantification of mean signal intensity in each channel was performed using the “Measure” function. Since background fluorescence intensity was typically less than 1% of the median fluorescence in each channel within a ROI, background subtraction did not significantly affect the measurements and was not performed.

Statistical analysis of microscopy data. All quantitative data are presented as scatter plots. For OGD experiments, one datapoint represents one image containing one neuron and the associated synapses; experiments were repeated in 3 independently prepared cultures as biological replicates. For MCAO experiments, three or more images per brain section per animal were analyzed, and the averaged value for each animal was treated as one data point; the number of animals used is indicated in the Figure Legends as appropriate.

GraphPad Prism 8.0 software was used for statistical analysis of microscopy data. The results of the experiments were presented as scatter graphs, showing individual data points, with lines denoting median values. Distributions were assessed for normality using d’Agostino and Pearson omnibus normality tests. For normally distributed datasets, 1-way ANOVA and Holm-Šidák’s *post hoc* tests were used to assess statistical significance as appropriate; for non-Gaussian datasets, Kruskal–Wallis test with Dunn’s *post hoc* test was used.

Analysis of synaptic structure in different brain regions during MCAO was carried out in two ways. For fixed time analysis, 2-way ANOVA with Dunnett’s *post hoc* test was used for multiple comparisons, with each treatment considered as a family. For time-resolved analysis of MCAO effects, a mixed model with Geisser-Greenhouse correction was employed, with negative or zero random effects removed; Šidák’s *post hoc* test was used for multiple comparisons, with all experimental groups conservatively regarded as one family.

Statistic analysis of proteomic and transcriptomic data. Proteins were considered differentially expressed when the following conditions were met: *p* value <0.05, fold change (FC) <0.8 and >1.2. Overlap in protein expression profiles between different regions was visualized using DeepVenn [106]. GSEA for proteins was carried out using WebGestalt (WEB-based GENE SeT Analysis Toolkit) [107].

Raw RNA-seq data was processed using Salmon [108] with the *Rattus norvegicus* index, followed by differential analysis using linear mixed models implemented in the R packages variancePartition [109] and Dream [110]. Using contrasts, we performed differential gene expression tests between conditions to identify significant upregulated and downregulated genes (*p*-value <0.05 and FC >2). Gene enrichment analysis of the up- and downregulated gene sets was carried out using gProfiler2 [111] with the “GO:BP”, “GO:CC”, “GO:MF”, “KEGG”, “MIRNA” term annotations. Factor analysis using custom genesets [61] was carried out with GSVA [112] using gene-level counts normalized by Dream. Visualizations were done using R packages ggplot2 [113] and enhancedVolcano [114].

Key resources are listed in Table 1.

Supporting information

S1 Fig. Changes in numbers and nearest neighbor distances for different synaptic markers in neuronal cultures after OGD. (A) Quantification of numbers for Homer (+) puncta. (B) Quantification of nearest neighbor distances for Homer (+) puncta. (C) Quantification of numbers for BSN (+) puncta. (D) Quantification of nearest neighbor distances for BSN (+) puncta. (E) Quantification of numbers for Gephyrin (+) puncta. (F) Quantification of nearest neighbor distances

Table 1. Key resources table.

Reagent or resource	Source	Identifier
Antibodies		
Homer 1	Synaptic systems	160002
BSN	Abcam	ab82958
GABRA1	Synaptic systems	224203
GluA2	Sigma-Aldrich	AB1768-I
Gephyrin	Synaptic systems	147011
vGlut1	Synaptic systems	135302
Cav2.1	Synaptic systems	152203
Synaptotagmin1	Synaptic systems	105311
GluN1	Alomone	AGC001
GluN2B	ZenBio	310326
Goat Anti-Rabbit IgG H&L/ AF488 antibody	Bioss	bs-0295G-AF488
Goat Anti-Mouse IgG/Alexa Fluor 594	Solarbio	K1031G-AF594
Chemicals, peptides, and recombinant proteins		
KCl	Sigma Aldrich	
PFA	Sigma Aldrich	
MK801	Sigma Aldrich	
Isoflurane	Sigma Aldrich	
Critical commercial assays		
Syn-PER assay for synaptosome purification	Fisher Scientific	87793
TRIzol assay for RNA extraction	Fisher Scientific	15596026
Experimental models: Cell lines		
Primary rat cortical neurons, mixed culture	E16-E18 embryos	
Experimental models: Organisms/strains		
<i>Rattus norvegicus</i>	Jinan Peng Yue Laboratory Animal Breeding Co	https://www.dnb.com/business-directory/company-profiles.jinan_pengyue_experimental_animal_breeding_co_ltd.0ba8b547d8911a-368fa3a9190a4d42b3.html
Software and algorithms		
ImageJ v1.54g	https://imagej.net/ij/	
Inkscape 1.3.2	https://inkscape.org/	
Salmon	https://combine-lab.github.io/salmon/about/	
DeepVenn	https://www.deepvenn.com/	
ggplot2	https://ggplot2.tidyverse.org/	
WEB-based GENE SeT AnaLysis Toolkit	https://www.webgestalt.org/	

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for Gephyrin (+) puncta. $N = 15$ cells from 3 independent experiments. $*P < 0.05$, Kruskal–Wallis test with Dunn’s *post hoc* test, one-way ANOVA followed by Šidák’s *post hoc* test. The original data for this figure can be found in [S1 Table](#). (TIF)

S2 Fig. Changes in the number and nearest neighbor distance of synapses after MCAO. (A) Example TTC staining of a coronal brain section following 4 hours of MCAO (Longa score 3); discolored area, lesion. (B) Quantification of numbers for Homer (+) puncta in brain sections following MCAO. $N = 15$ sham control animals, 15 MCAO animals per time point. (C) Quantification of nearest neighbor distances for Homer (+) puncta in brain sections following MCAO. $N = 15$

sham control animals, 15 MCAO animals per time point. **(D)** Quantification of numbers for Gephyrin (+) puncta in brain sections following MCAO. $N = 15$ sham control animals, 15 MCAO animals per time point. **(E)** Quantification of nearest neighbor distances for Gephyrin (+) puncta in brain sections following MCAO. $N = 15$ sham control animals, 15 MCAO animals per time point. **(F)** Quantification of phalloidin staining in BSN (+) puncta in brain sections following MCAO. $N = 15$ sham control animals, 15 MCAO animals per time point. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, mixed model analysis followed by Šidák's *post hoc* test. The original data for this figure can be found in [S1 Table](#).
(TIF)

S3 Fig. Further experiments showing synaptic remodeling during OGD and MCAO. **(A)** Representative immunostaining images for Homer and GluA2 in neuronal cultures after OGD treatment on DIV14. **(B)** Quantification of GluA2 in Homer (+) puncta in neuronal cultures following OGD. $N = 15$ cells from 3 independent experiments. **(C)** Representative immunostaining images for GABRA1 in neuronal cultures after OGD treatment on DIV14. **(D)** Quantification of GABRA1 in Gephyrin (+) puncta in neuronal cultures following OGD. $N = 15$ cells from 3 independent experiments. **(E)** Quantification of $\text{Ca}_v2.1$ in BSN (+) puncta in brain sections following MCAO. $N = 9$ sham control animals, 9 MCAO animals per time point. **(F)** Quantification of vGlut1 in vGlut1 (+) puncta in brain sections following MCAO. $N = 12$ sham control animals, 152 MCAO animals per time point. **(G)** Quantification of GluN2B in vGlut1(+) puncta in brain sections following MCAO. $N = 15$ sham control animals, 15 MCAO animals per time point. **(H)** Body temperature measurements in 6 control and 6 MK801-injected animals. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Kruskal–Wallis test with Dunn's *post hoc* test (B, D), mixed model with Šidák's *post hoc* test (E, F) one-way ANOVA with Šidák's *post hoc* test (G), two-way ANOVA (H). Scale bar, 5 μm . The original data for this figure can be found in [S1 Table](#).
(TIF)

S4 Fig. Detailed analysis of differentially expressed proteins in the synaptosomal fraction, contralateral hemisphere vs. sham control. **(A)** Graph showing significantly enriched GO Cellular component terms. **(B)** Graph showing significantly enriched GO Biological function terms. **(C)** Graph showing significantly enriched KEGG terms for signaling pathways. **(D)** Graph showing top downregulated GO terms. The original data for this figure can be found at ProteomeXchange (<https://proteomecentral.proteomexchange.org>, dataset identifier PXD058834).
(TIF)

S5 Fig. Analysis of differentially expressed proteins in the synaptosomal fraction, ischemic core and penumbra vs. sham control. **(A)** Volcano plot for differentially expressed proteins, ischemic core vs. sham control. **(B)** Volcano plot for differentially expressed proteins, penumbra vs. sham control. **(C)** Graph showing top downregulated GO terms, ischemic core vs. sham control. **(D)** Graph showing top upregulated GO terms, ischemic core vs. sham control. **(E)** Graph showing top downregulated GO terms, penumbra vs. sham control. **(F)** Graph showing top upregulated GO terms, penumbra vs. sham control. The original data for this figure can be found at ProteomeXchange (<https://proteomecentral.proteomexchange.org>, dataset identifier PXD058834).
(TIF)

S6 Fig. Analysis of gene expression in whole brain tissue and GO analysis of differentially expressed genes in the whole brain tissue from penumbra. **(A)** PCA of gene expression for the whole brain tissue. CA – sham control, z – contralateral hemisphere, c – ischemic core, p – penumbra. **(B)** GSVA for synaptic sets in the whole brain tissue samples. **(C)** Volcano plot for differentially expressed genes in the whole brain tissue, penumbra vs. sham control. **(D)** Functional terms for upregulated genes in the whole brain tissue, penumbra vs. sham control. **(E)** Functional terms for downregulated genes in the whole brain tissue, penumbra vs. sham control. The original data can be found at GEO (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE283465).
(TIF)

S7 Fig. GO analysis of differentially expressed genes in the contralateral hemisphere. (A) Volcano plot for differentially expressed genes in the synaptosomal fraction, contralateral hemisphere vs. sham control. (B) Functional terms for upregulated genes in the synaptosomal fraction, contralateral hemisphere vs. sham control. (C) Functional terms for downregulated genes in the synaptosomal fraction, contralateral hemisphere vs. sham control. (D) Volcano plot for differentially expressed genes in the whole brain tissue, contralateral hemisphere vs. sham control. (E) Functional terms for upregulated genes in the whole brain tissue, contralateral hemisphere vs. sham control. (F) Functional terms for downregulated genes in the whole brain tissue, contralateral hemisphere vs. sham control. The original data can be found at GEO (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE283465). (TIF)

S8 Fig. GO analysis of differentially expressed genes in the ischemic core. (A) Volcano plot for differentially expressed genes in the synaptosomal fraction, core vs. sham control. (B) Functional terms for upregulated genes in the synaptosomal fraction, core vs. sham control. (C) Functional terms for downregulated genes in the synaptosomal fraction, core vs. sham control. (D) Volcano plot for differentially expressed genes in the whole brain tissue, core vs. sham control. (E) Functional terms for upregulated genes in the whole brain tissue, ischemic core vs. sham control. (F) Functional terms for downregulated genes in the whole brain tissue, ischemic core vs. sham control. The original data can be found at GEO (<https://www.ncbi.nlm.nih.gov/geo/>, accession number GSE283465). (TIF)

S1 Table. Complete numerical data for Figs 1–4, 5H, 5I, and S1–S3. Each sheet corresponds to one figure panel. (XLSX)

S2 Table. Differentially expressed proteins in synaptosomes from contralateral, core, and penumbra regions, relative to the sham controls. (XLSX)

S3 Table. Differentially expressed genes in whole-brain tissue and synaptosomes from contralateral, core, and penumbra regions, relative to the sham controls. (XLSX)

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