Defining the Penumbra in a Preclinical Model of Subarachnoid Hemorrhage

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Abstract

Subarachnoid hemorrhage (SAH) is a form of stroke that most often results from a ruptured cerebral aneurysm that spills blood into the surrounding tissue. In contrast with the well-established and predictable ischemic penumbra regions in ischemic stroke, this crucial therapeutic target has not yet been well-described in SAH. Considering that SAH may cause micro-infarcts and delayed cerebral ischemia far from the aneurysm rupture, and that these disruptions are closely linked to behavioral impairments, it is important to study the progression of penumbras. Notably, behavioral assessments can detect and approximately localize dysfunctional brain regions before permanent damages occur following SAH. Therefore, we hypothesized that the spatiotemporal distribution and progression of the core and penumbra in SAH may be predicted by specific patterns of behavioral impairment. To test this hypothesis, we induced SAH using an endovascular filament perforation model, which is considered a close mimic of ruptured aneurysms in humans, and employed a behavioral battery at multiple time points followed by a histopathological analysis of brain tissue. Our results demonstrate that sensorimotor deficits occur early after SAH and remained static, while impairments in working memory, reference memory, exploration, and anxiety evolved in association with specific histologic lesions. All SAH rats displayed core infarctions in the cerebral cortex, basal ganglia and hypothalamus; whereas penumbras were found in the hippocampus (100%), thalamus (80%), and amygdala (60%). Our study underscores the importance of identifying the penumbra regions following SAH and the utility of neurobehavioral tests for assessing multiple cognitive domains to detect and localize penumbra.

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a devastating disease caused by the sudden rupture of an intracranial aneurysm associated with a high risk of death or severe disability [1, 2]. Survivors of SAH often have a significant neuropsychological impairment, including sensorimotor dysfunction, memory impairment, and language dysfunction [1, 3–5]. Even survivors with good functional outcomes are at risk for disabling impairments such as fatigue, depression, and anxiety [6]. These deficits are caused by early brain injury (EBI) occurring within 1 min after aneurysmal rupture and delayed cerebral ischemia (DCI) occurring 3–14 days after SAH [7, 8]. Despite tremendous efforts, nimodipine is currently the sole FDA-approved treatment for SAH patients and its benefits are marginal at best [9]. No other agents have been shown to significantly improve outcomes for SAH patients [10]. This may be due to the lack of a clear definition of the penumbra, a potentially salvageable brain tissue, after SAH. As we enter the exciting era of effective therapy for SAH, a new conceptual understanding of penumbra after SAH is needed.

The challenge of differentiating between irreversible and reversible ischemia is of critical importance. In ischemic stroke, the concept of core and penumbra has led to advances in reperfusion therapies and has been integrated into routine clinical practice to select patients that can best benefit from therapy [11]. Likewise, improved understanding of salvageable brain tissue following SAH could potentially benefit patients receiving novel or targeted interventions, and hence improve functional outcomes. Since EBI and DCI can affect diffuse, non-contiguous regions of the brain, many cerebral structures are at risk for SAH-
induced ischemia/hypoxia even without classical vascular territory infarction [12]. For example, ischemia causes microcirculatory dysfunction, micro-thrombosis, and microinfarctions, which may cause insidious neuronal dysfunction and progressive cognitive impairment seen following SAH [13–16]. Therefore, interventions that target the penumbra following SAH may represent an opportunity to prevent long-term neuropsychological dysfunction. However, unlike in ischemic stroke, there is no preclinical definition or characterization of core and penumbra in SAH. Furthermore, there is a relative paucity of literature on the evolution of the penumbra after SAH and its contribution to neuropsychological deficits.

During SAH, the core may develop at the time of aneurysm rupture. Severe elevations in intracranial pressure (ICP) overcome cerebral perfusion pressure causing temporary global cerebral ischemia and occasionally permanent injury during bleeding [8, 17]. Furthermore, subarachnoid blood and subsequent hemoglobin degradation may cause irreversibly damaged core regions [18]. The SAH penumbra may develop following spontaneous reperfusion during EBI and DCI. Intriguingly, the penumbra may occur in focal cortical and subcortical regions both near and far from the index bleed and may involve multiple vascular territories [15, 19]. The distribution and evolution of core and penumbra after SAH have not been well explored; however, they have been described in clinical and preclinical studies [20, 21]. Behavioral assessments following SAH have been able to detect deficits correlated with increases in inflammation and cell death in the affected brain regions, suggesting that behavioral tests can have a high degree of sensitivity for detecting dysfunctional regions associated with core and penumbra [22, 23]. In this study, we test the hypothesis that the core and penumbra may be defined in part by a multifaceted evaluation of neurocognitive functions after SAH. There are several studies assessing neurobehavioral deficits following SAH, however, they did not track the long-term evolution of behavioral impairments post-SAH and thus are limited in their ability to define the core and penumbra [24, 25].

To better define the SAH-associated core and penumbra, we employed a comprehensive battery of behavioral assessments followed by histologic analysis. We induced SAH using an endovascular filament perforation model, which is considered the closest mimic to ruptured aneurysms in humans, to explore our hypothesis in the most clinically relevant setting [26]. We propose a definition of core and penumbra utilizing a combination of neurocognitive function and histology and demonstrate that there exists widespread penumbra following SAH. These findings highlight the need for therapeutic investigations to account for the penumbra in SAH.

**Results**

**Evaluation of core and penumbra in the rat SAH model**

The endovascular perforation model of SAH, a procedure simulating rupture of an intracranial aneurysm, leads to acute hemorrhage from the ICA terminus, forming a clot within the subarachnoid space inferior to the piriform cortices, with resulting cerebral injury [27]. In our model, similar hemorrhage was found within the basal cisterns at 72 h after endovascular perforation (Fig. 1A, bottom). At 30 days after SAH, widespread lesions were observed throughout the brain, including the primary motor, secondary motor,
primary somatosensory, secondary somatosensory and piriform cortices, periventricular nuclei, hippocampus, thalamus, hypothalamus, amygdala, corpus callosum and corticofugal tract (Fig. 2).

There are significant differences in regional cerebral morphology between the SAH and sham rats. Specifically, the piriform cortices and hypothalamus displayed a significant degree of neuronal death and architecture disruption in all SAH animals (Fig. 3A, Fig. 6A). The periventricular nuclei and primary motor, secondary motor, primary somatosensory, secondary somatosensory and retrosplenial cortices also contained extensive neuronal death and vacuolations (Fig. 3A, Fig. 7A). Our results also demonstrated damage in the hippocampus (Fig. 4A, Fig. 5A), amygdala, and thalamus (Fig. 6A). These regions had a moderate loss of neurons without extensive destruction of brain parenchyma. Additionally, a loss of normal confluence was appreciated in the corpus callosum (Fig. 7A) and corticofugal tract (Fig. 6A).

Furthermore, our assessment of sectioned brains revealed lesions attributable to the core and penumbra. In piriform cortices, 5/5 (100%) of SAH animals had > 80% neuronal death in all five high-power fields, consistent with our definition of core infarctions (Fig. 1B, Fig. 2C). The primary motor, secondary motor, primary somatosensory, and secondary somatosensory cortices also demonstrated a similar pattern, with 5/5 (100%) of SAH animals showing greater than 80% neuronal loss or pan-necrosis in all fields. The hypothalamus was more variable, with 4/5 (80%) of SAH animals demonstrating greater than 80% neuronal loss in all fields, and one animal with 3/5 fields containing core lesions and 2/5 fields in the 30–80% range consistent with penumbra lesions. The retrosplenial cortex, however, in all five SAH animals had 30–80% neuronal loss in all fields. The hippocampus also qualified as a region with a high density of penumbra lesions, as all five SAH animals had 30–80% neuronal loss in all fields. The thalamus demonstrated penumbra ischemic regions as well on histology, with 4/5 (80%) animals showing penumbra-range neuronal loss and one animal with 3/5 fields in the 30–80% range and 2/5 non-ischemic (less than 30% neuronal loss). The amygdala as variable, with 3/5 (60%) of SAH animals with all fields demonstrating 30–80% neuronal loss, one SAH animal with 4/5 fields in the penumbra range and one non-ischemic, and one SAH animal with no field showing neuron loss greater than 30%. No sham animals demonstrated a degree of neuronal loss greater than 30%.

The results from ROI quantification of core and penumbra regions, however, showed a different pattern of core and penumbra distribution (Fig. 2D). The piriform, primary motor, secondary motor, primary somatosensory, and secondary somatosensory cortices had a mix of core and penumbra that on average favored penumbra (core: 3.37 ± 2.20 mm²; penumbra: 6.34 ± 2.78 mm²). The hypothalamus also had both core and penumbra present, with a predominance of penumbra (core: 0.65 ± 0.59 mm²; penumbra: 0.71 ± 0.65 mm²). The hippocampus had both core and penumbra, but with a greater amount of penumbra than core (core: 0.30 ± 0.62 mm²; penumbra: 1.74 ± 0.390 mm²). Similarly, the thalamus had almost entirely penumbra (core: 0.04 ± 0.07 mm²; penumbra: 1.27 ± 0.69 mm²). The amygdala displayed a small amount of both core and penumbra, with increased penumbra relative to core (core: 0.08 ± 0.18 mm²; penumbra: 0.15 ± 0.34 mm²). The nucleus accumbens region of the basal ganglia also demonstrated a predominance of penumbra (core: 0.07 ± 0.05 mm²; penumbra: 0.15 ± 0.09 mm²). The
distribution of core and penumbra also varied significantly on ROI quantification, with core making up only 0.25% of the thalamus and almost 13% of the hypothalamus. Penumbra similarly ranged from 2.33% in the amygdala to nearly 24% in the nucleus accumbens.

**Sugawara behavioral score**

There were no differences in baseline values for the Sugawara score (Fig. 3B; Sham: 18 ± 0, SAH: 18 ± 0). The Sugawara score remained unchanged for the sham group in the postoperative period. SAH rats showed a significant decrease in the Sugawara scores compared to sham (Fig. 3B; 24H: 11.70 ± 1.16, 72H: 12.30 ± 1.34, 7D: 10.40 ± 1.51, 14D: 11.60 ± 1.58, 21D: 12.20 ± 2.30, 28D: 12.00 ± 1.89; $F = 99.8$, $P = 1.3 \times 10^{-14}$, mixed-effects ANOVA). Post-hoc comparisons revealed that the SAH scores were not statistically different across days ($F = 2.95$, $P = 0.02$) except for D3 vs. D7 ($t = 4.7$, $P = 0.019$, Tukey test). Our results demonstrate that the SAH-induced neurologic impairment occurs as early as 24 h post-bleed and does not improve in the 28 days after SAH.

**Adjusted-neurological severity score**

There were no differences in baseline values for the A-NSS (Fig. 3C; Sham: 0 ± 0, SAH: 0 ± 0). The scores remained at zero following sham surgery. At all postoperative periods, SAH rats demonstrated a significant increase in A-NSS as compared to sham baseline (Fig. 3C; 24H: 8.20 ± 3.12, 72H: 7.20 ± 2.30, 7D: 10.10 ± 3.84, 14D: 9.00 ± 4.57, 21D: 9.70 ± 5.12, 28D: 8.60 ± 4.67; $F = 28.7$, $P = 1.26 \times 10^{-6}$, mixed-effects ANOVA). The A-NSS beam-walk portion, which measures balance and coordination, showed significant increases in the SAH rats when compared to the sham group (Fig. 3D; 24H: 1.20 ± 1.03, 72H: 1.80 ± 0.91, 7D: 1.60 ± 0.84, 14D: 1.40 ± 0.84, 21D: 1.50 ± 0.85, 28D: 1.50 ± 0.53; $F = 21.38$, $P = 1.92 \times 10^{-5}$, mixed-effects ANOVA). There were no statistically significant changes between A-NSS measurements in the SAH group at any of the post-ictal time periods ($F = 0.45$, $P = 0.81$). These results suggest that SAH induces neurological dysfunction that remains static in the acute to chronic periods.

**Rotarod test**

During the rotarod training, the SAH and sham groups displayed similar riding times (Fig. 3E). In the rotarod tests following SAH or sham procedure, there was a statistically significant decrease in riding time in the SAH rats as compared to the sham group (Fig. 3E; 72H: 70.00 ± 24.86 s, 7D: 72.20 ± 21.37 s, 14D: 81.67 ± 21.51 s, 21D: 82.50 ± 27.95 s, 28D: 92.90 ± 16.66 s; $F = 27.68$, $P = 2.52 \times 10^{-6}$, mixed-effects ANOVA). There was no statistically significant variation in riding time across days in the SAH group ($F = 1.69$, $P = 0.17$). These results suggest that SAH impairs motor function and coordination in a long-lasting manner.

**Novel object recognition test**

When recognition memory was assessed after SAH or sham surgery, rats in the SAH group had a statistically significant decrease in the amount of time spent interacting with either the familiar or novel objects (Fig. 4B; 24H: 3.49 ± 2.39 s, 72H: 6.62 ± 5.18 s, 7D: 4.37 ± 5.80 s, 14D: 6.27 ± 5.02 s, 21D: 5.59 ± 6.35 s, 28D: 4.30 ± 2.90 s; $F = 6.2015$, $P = 0.014$, mixed-effects ANOVA for treatment and object). There
was also a statistically significant decrease in the interaction time of the novel object with respect to the familiar object by rats in the SAH group compared to the sham group ($F = 19.78, P = 1.84 \times 10^{-5}$, mixed-effects ANOVA for treatment and object), as well as a statistically significant decrease in discrimination ratio between groups (Fig. 4C; 24H: -0.465 ± 0.500, 72H: -0.212 ± 0.615, 7D: -0.801 ± 0.244, 14D: -0.670 ± 0.335, 21D: -0.784 ± 0.224, 28D: -0.693 ± 0.379; $F = 38.55, P = 4.68 \times 10^{-8}$, mixed-effects ANOVA vs. sham).

There was a significant reduction in the total distance traveled (Fig. 4D; 24H: 9.61 ± 7.24 m, 72H: 9.7 ± 5.57 m, 7D: 5.11 ± 3.31 m, 14D: 8.4 ± 5.35 m, 21D: 5.46 ± 2.44 m, 28D: 8.59 ± 4.44 m, $F = 9.33, P = 0.0033$) which can be observed on representative traces (Fig. 4E). Our findings suggest that SAH induces persistent impairment in recognition memory and motor function.

**Y-maze test**

For the Y-maze test of working spatial memory, the SAH group had a significantly lower level of novel arm entries (Fig. 5B; Sham: 51.9 ± 14.2%; SAH: 14D: 31.3 ± 13.6%, 28D: 26.2 ± 10.8%; $F = 14.1, P = 8.44 \times 10^{-4}$, mixed-effects ANOVA) and a lower percentage of successful complete alternations (Fig. 5C; Sham: 71.6 ± 13.1%; SAH: 14D: 39.8 ± 38.3%, 28D: 33.28 ± 27.03%; $F = 7.1, P = 0.012$, mixed-effects ANOVA). Moreover, SAH rats were impaired in the Y-maze test of reference spatial memory with significantly lower total entries to the previously-closed arm (Fig. 5D; 24H: 5.06 ± 3.31 m, 72H: 4.78 ± 2.19 m, 7D: 4.78 ± 2.19 m, 14D: 3.84 ± 0.09 m, 21D: 3.28 ± 2.18 m; $F = 12.9, P = 0.0013$, mixed-effects ANOVA) as well as significantly less average speed as compared to the sham group (Fig. 5E; Sham: 2.97 ± 0.81 cm/s; SAH: 14D: 1.34 ± 0.9 cm/s, 21D: 1.5 ± 0.75 cm/s, 28D: 1.14 ± 0.76 cm/s; $F = 27.55, P = 3.99 \times 10^{-6}$, mixed-effects ANOVA). These results suggest that SAH induces impairment in working and reference spatial memory in the subacute time period. These deficits seem to be durable throughout the early chronic and chronic phases of SAH-induced neurologic dysfunction.

**Elevated maze test**

In the EM test of anxiety, rats in the SAH group were found to have a statistically significant decrease in the proportion of time spent in the open arms when compared to sham (Fig. 6B; Sham: 17.56 ± 0.87%; SAH: 7D: 0.29 ± 0.87%, 14D: 0.77 ± 1.07%, 21D: 5.06 ± 7.38%, 28D: 3.29 ± 5.18%; $F = 42.37, P = 6.02 \times 10^{-8}$, mixed-effects ANOVA), as well as a statistically significant decrease in the percent of entries to the open arms compared to sham (Fig. 6C; Sham: 47.32 ± 4.21%; SAH: 7D: 13.36 ± 6.56 m, 28D: 9.43 ± 2.92 m, $F = 12.46, P = 0.0015$, mixed-effects ANOVA). Incidentally, we found that SAH rats showed a significant decrease in the total distance traveled over the course of the test (Fig. 6D; Sham: 19.99 ± 2.68 m; SAH: 7D: 13.36 ± 6.56 m, 21D: 13.36 ± 6.56 m, 28D: 9.43 ± 2.92 m; $F = 12.46, P = 0.0015$, mixed-effects ANOVA) as well as significantly less average speed as compared to the sham group (Fig. 6E; Sham: 2.97 ± 0.81 cm/s; SAH: 7D: 1.27 ± 0.47 cm/s, 14D: 1.34 ± 0.9 cm/s, 21D: 1.5 ± 0.75 cm/s, 28D: 1.14 ± 0.76 cm/s; $F = 27.55, P = 3.99 \times 10^{-6}$, mixed-effects ANOVA) which can be observed in the relative decreased movement shown on representative traces (Fig. 6F). These results...
demonstrate that SAH induces anxiety in rats, as well as impairment in locomotor function. These behavioral deficits began within 7 days following SAH and persisted for weeks.

**Porsolt forced swim test**

In the FS test SAH rats demonstrated a significant decrease in the fraction of time they spent highly mobile (Fig. 7B; Sham: 37.65 ± 7.78%; SAH: 7D: 19.3 ± 11.65%, 14D: 20.9 ± 7.69%, 21D: 25.5 ± 7.54%, 28D: 12.7 ± 6.48%; $F = 25.1, P = 9.29 \times 10^{-6}$, mixed-effects ANOVA), as well as a significant increase in the percentage of time they spent immobile when compared to sham (Fig. 7C; Sham: 34.01 ± 8.43%; SAH: 7D: 51.6 ± 21.3%, 14D: 53.7 ± 16.2%, 21D: 46.8 ± 11.1%, 28D: 63.3 ± 18%; $F = 7.28, P = 0.0097$, mixed-effects ANOVA). The SAH group also showed a worsening of depression for the 21D and 28D time points, with a significant decrease in highly mobile percent time ($t = 5.077, P = 0.0075$, Tukey test) as well as a significant increase in immobile percent time ($t = 3.98, P = 0.042$, Tukey test). Our results suggest that depression begins as early as 7 days following SAH and worsens over the chronic timeframes.

**Discussion**

Despite being less common than other forms of stroke, SAH has a large clinical impact with greater than half of SAH patients experiencing death or functional impairment [2]. Even patients with mild SAH show significant neuropsychological dysfunction in the months after SAH [28]. These deficits occur due to a combination of acute (e.g., EBI) and subacute (e.g., DCI) mechanisms following SAH. While EBI and DCI are well established in the literature, the concept of core and penumbra regions has not been fully explored in SAH, although these regions have been documented using a variety of techniques [19, 29, 30]. Furthermore, the pathophysiologic mechanisms underlying SAH penumbra regions are similar to corresponding regions in other cerebral insults including the ischemic penumbra in ischemic stroke as well as peri-contusional secondary injury following traumatic brain injury, suggesting that penumbra regions in SAH are driven by a similar process [31, 32]. To provide better insight into the diagnosis and treatment of SAH, the identification of potential penumbra regions will be very valuable.

Understanding the location of core and penumbra regions, as well as their evolution over time, is of critical importance for predicting brain systems at risk following SAH. The ischemic stroke literature offers several methods that may be used to localize ischemic cores and penumbrae, such as imaging techniques including computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). All these techniques can localize cores and penumbrae with a high degree of accuracy in ischemic strokes [33–35]. While highly accurate, these imaging techniques may fail to detect small core and penumbra regions, especially in the first hours following their onset [36, 37]. Behavioral assessments may reveal signs of cerebral ischemia within one hour of the onset, earlier than many imaging techniques could reliably detect core and penumbra [38]. While less able to localize exact regions of cerebral ischemia, behavioral assessments can detect regions of even mild neuronal death and inflammation [22, 23], suggesting that they are a highly sensitive but less specific diagnostic technique. In
this light, behavioral testing offers the opportunity for early and sensitive identification of ischemic core and penumbra but requires additional methods to verify the exact locations of these pathologies.

We used histologic analysis following behavioral testing to define the location of the core and penumbra after SAH. In our model of SAH, we observed widespread and noncontiguous neuronal damage in the piriform, retrosplenial, primary motor, secondary motor, primary somatosensory cortices, basal ganglia, periventricular nuclei, hypothalamus, thalamus, hippocampus, amygdala and corpus callosum. This is consistent with the described endovascular perforation model of SAH, in which an initial hematoma forms in the basal cortical subarachnoid space, damaging the piriform cortices with concurrent damage to the primary motor, secondary motor, primary somatosensory, and secondary somatosensory cortices secondary to increased ICP. This is followed by widespread DCI-induced lesions in the hippocampus, striatum, thalamus, and white matter tracts in the subacute and chronic phases [18, 27, 39]. Regions with severe neuronal death meeting our definition for core were identified in the piriform, primary motor, secondary motor, primary and secondary somatosensory cortices as well as the hypothalamus. In addition, our SAH group displayed injury to the hippocampus meeting our definition of penumbra. Additionally, penumbras were observed in the retrosplenial cortex, amygdala, and thalamus, with variability in the degree and severity of the affected regions. We found that the location and distribution of penumbras were more variable than core lesions, as would be expected for the diffuse secondary ischemia caused by SAH. Many regions, including the hypothalamus and hippocampus, displayed a mixture of core and penumbra. While macroscopic ROI analysis identified some regions (i.e. the cortices) as containing predominantly penumbra, when examined microscopically our histologic analysis demonstrated that these regions met our definition for core. As SAH is known to induce microthrombosis and microinfarctions [13, 15], it is possible that macroscopic analysis techniques may underestimate the true burden of core in SAH. Our results suggest that a combination of histologic analysis and behavioral deficits can help to define the core and penumbra following SAH, as well as track the evolution of penumbras.

As damage inflicted by SAH can affect a range of cortical and subcortical structures, the extent of cerebral structures at risk following SAH is extensive [17, 40]. To better capture the range of potential neurobehavioral deficits, and thus define evolving core and penumbra, we employed a behavioral battery including tests of sensorimotor function, memory, anxiety, and depression. The Sugawara behavioral score, A-NSS score, and rotarod test were used to assess sensorimotor function, which was similar in both experimental groups prior to SAH induction or sham surgery but showed a significant deficit in the SAH rats. Previous studies using similar scales and methods have also found a significant worsening of sensorimotor function in the acute period post-SAH [41–44]. Our results show that these deficits in sensorimotor function persist into the chronic period, with animals displaying significant impairment at 28 days post-SAH. While fewer studies have investigated persistent sensorimotor dysfunction, impairment of SAH animals in the 14–28-day period has been previously suggested [45]. As sensorimotor impairment was consistent across all three measures, it would be reasonable to assess sensorimotor dysfunction following SAH using rotarod testing due to its relative objectivity, with the Sugawara score as a potential adjunct measure as it was specifically created for SAH [41]. In our results, sensorimotor
dysfunction occurred acutely after SAH and persisted throughout all phases of testing, which is consistent with the pattern of deficits that would be expected from lesions to the regions of core ischemia. In our model, extensive core lesions were observed in the piriform, primary motor, secondary motor, primary somatosensory, secondary somatosensory, and retrosplenial cortices, which may explain the deficits seen in the SAH group. Sensory and motor functions are complex processes in the brain, originating predominantly in the cortex but with the involvement of the basal ganglia, thalamus, white matter tracts, and brainstem [46]. In experimental models, cortical lesions similar to those we observed on histology are visible on MRI as early as 48 h post-SAH, supporting the early appearance of core lesions seen here [47]. Furthermore, penumbra-associated neuroinflammation in the cortex and basal ganglia is associated with sensorimotor deficits in animal models of SAH, even in the absence of large infarctions [13, 14, 16]. We observed penumbra in the retrosplenial cortices, which may have contributed to the observed sensorimotor defects through neuroinflammation, however the severity and burden of existing core infarctions make this difficult to delineate.

As memory is one of the most cited cognitive domains impaired following SAH, memory tests are particularly important in our model [4]. We employed the NOR and Y-maze tests to assess recognition memory, spatial working memory, and spatial reference memory. Our results demonstrate significantly impaired recognition memory in the NOR test that began within 24 h of SAH induction and persisted through the 28-day period. These results are supported by literature demonstrating impaired recognition memory in SAH rats in the chronic period [44]. Our SAH group also had significant impairment in both working and reference spatial memory on the Y-maze tests, in both the subacute and chronic periods. When the NOR test is supplemented with the Y-maze test, a broad assessment of memory function is feasible with only two behavioral assessments. Anatomically, the critical neural substrate for memory function involves the hippocampus and nearby cortical regions [48]. Functional MRI has shown altered function and connectivity in the hippocampus and parahippocampal gyrus following SAH, and diffusion tensor imaging has shown disruption in the fornix and mammillothalamic tract following SAH, with these abnormalities linked to memory deficits [49, 50]. Impaired recognition memory was apparent as early as 24 h post-SAH on NOR testing, and there was a long-term decline in discriminative ability in the SAH rats, suggesting a permanent ischemic penumbra affecting the hippocampal region. Fittingly, we observed prominent penumbrae in the hippocampus on histologic analysis, along with disruption of white matter tracts.

While anxiety and depression are frequently reported in patients who survive SAH, emotional outcomes have been less frequently studied than neurologic outcomes in preclinical models, despite their significant impact on long-term function and quality of life [5, 6]. We found that SAH rats demonstrated anxiety on EM testing at seven days post-SAH, and while the SAH group had increased anxiety at all time points, there was a trend towards decreasing anxiety as time progressed. Work from other groups has shown increased anxiety in SAH animals particularly in the chronic period [44, 51]. Some studies have found that clinical anxiety tends to worsen in the chronic period following SAH [52] whereas others have shown anxiety levels to be stable [53]. Our results also indicated a significant and persistent increase in depressed behavior beginning as early as seven days after SAH, with a significant worsening of
depression in the chronic phase. A similar increase in depressive behavior in SAH animals has been observed, peaking at the 8-week mark [51]. This is also seen in the clinical literature, in which patients tend to have worsening depression in the initial 12 months following SAH [54]. In preclinical models of SAH, damages to the ventromedial prefrontal cortex, perirhinal cortex, and hypothalamus were associated with the development of anxiety [44, 55]. While no studies have directly investigated the anatomic correlates of post-SAH depression, patients with SAH have shown altered functional connectivity in the cingulate cortex, which is also seen in major depressive disorder [56, 57]. Areas of the ventromedial prefrontal cortex containing the rodent correlate of the cingulate cortex are known to be involved in depression in rats, however, this has not been directly studied in SAH models [58]. Our results showed an initial high level of anxiety that displayed a trend towards improvement over time, a pattern consistent with partial resolution of ischemic penumbra regions. Our findings of worsening depression suggest the presence and worsening of penumbra regions in the ventromedial prefrontal cortex. We found that SAH animals had prominent penumbra lesions in the amygdala, thalamus, and ventromedial prefrontal cortex, helping to localize the penumbra regions suggested by the deficits observed on neurobehavioral testing.

The framework of core and penumbra is a new concept in SAH, and therefore several limitations exist with this study. Due to the lack of a standard definition of core and penumbra regions in SAH, we adapted definitions from outside the SAH literature. While we based the definitions on existing physiologic and pathophysiologic data, our definitions require further study and validation before widespread use is feasible. Histopathologic analysis is a terminal procedure, and therefore cannot assess the evolution of core and penumbra over time. However, there are existing definitions for core and penumbra lesions in the ischemic stroke literature using noninvasive and repeatable imaging techniques, including MRI and PET [34, 35]. Given that PET has been used to establish core and penumbra lesions following stroke, it may represent a valuable noninvasive method to identify core and penumbra ischemic areas and would allow for direct visualization of lesion changes over time [19, 59]. In this way, further work may be able to leverage the concept of the penumbra in SAH to characterize, diagnose and treat the neuropsychologic impairments suffered by survivors of SAH.

Conclusions

The post-SAH penumbra is increasingly recognized as a key concept in stroke research, as it represents a brain region that might be salvaged to preserve neurological function and improve functional outcomes after stroke. Our experimental results have delineated a set of penumbra regions after SAH induced by endovascular perforation in a rat model. Moreover, this crucial therapeutic target could be defined with the use of a battery of neurobehavioral assessments and histologic analysis, though limited by spatiotemporal resolution and specificity. Further studies are needed to support the concept of post-SAH penumbra and its spatiotemporal resolution should be confirmed with gold-standard techniques used to validate the penumbra in ischemic stroke and other brain injuries.

Methods
Animals

All experimental protocols involving animals were performed in accordance with the National Institutes of Health guidelines for the use of animals and ARRIVE guidelines for reporting animal research. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, Manhasset, NY (Protocol #2019-018). A total of 30 adult male Sprague-Dawley rats (Taconic Biosciences Inc, Germantown, NY), weighing 300 to 375 g, were housed (three per cage) in temperature-controlled conditions with 12-hour light/dark cycle and ad libitum access to food and water. Cages were lined with Enrich-o’Cobs bedding (The Andersons, OH) and animals were provided with a semi-translucent acrylic tube for enrichment purposes.

SAH model

Animals were assigned to SAH and sham groups at random in a 2:1 ratio to account for experimental mortality. In the SAH group the endovascular perforation model of SAH was performed as previously described [60]. Briefly, rats were placed in a small airtight chamber for induction of anesthesia with 4% isoflurane in medical air. After confirmation of adequate anesthesia with lack of response to a toe pinch, rats were transferred to a temperature-controlled heating pad maintained at 38°C, placed supine and anesthesia maintained with 1.5% isoflurane in medical air delivered via nose cone. The rats were then draped, the surgical field shaved and sterilized with betadine and ethyl alcohol, and a midline incision made just superior to the sternum. The left common carotid artery (CCA) was exposed, and clips were applied to the proximal CCA, distal internal carotid artery (ICA), and left external carotid artery (ECA). The arteries were ligated and cut to form a vascular stump. A sharpened 3 – 0 Prolene suture was inserted into the ECA stump, the ICA clip was removed, and the suture was advanced into the ICA to the point of resistance representing the ICA bifurcation. In the SAH group, the suture was advanced 1mm beyond the ICA bifurcation to induce SAH, as confirmed by an immediate reduction in cerebral blood flow (CBF, measured by laser doppler) (Fig. 1A). At this point, the suture was removed, the ECA stump was ligated and cauterized, the incision closed, and the rat was placed in a clean cage for observation. In the sham group, the same procedure was performed but the suture was withdrawn immediately after meeting resistance.

Experimental groups and timeline

A total of 10 rats underwent sham surgery, and 20 rats underwent the SAH model. In the SAH group, eight rats died in the immediate postoperative period (less than 72 h following SAH induction). One rat in the SAH group died in the late postoperative period (23 days). In the sham group, no rats died during or after the sham procedure. All groups underwent sensorimotor testing before and after surgery, and animals with inconsistent neurobehavioral assessment results in the preoperative period, as well as evidence of failed SAH induction on postoperative sensorimotor testing, were excluded from experimentation and analysis. One rat from the SAH group was excluded due to inconsistent results from the pre-SAH neurobehavioral assessment. No SAH rats were excluded based on postoperative testing results. No sham group animals were excluded. Animals were included in experimentation and analysis if they had
consistent preoperative neurobehavioral testing results, survived the full 30-day course, and underwent successful SAH induction if applicable. A total of 10 SAH rats and 10 sham rats were included in full experimentation and analysis. Animals underwent SAH induction or sham surgery on day zero, and then underwent sensorimotor assessment (Sugawara score, A-NSS score, rotarod assessment) at 24 h (termed 24H henceforth), 72 h (72H), 7 days (7D), 14 days (14D), 21 days (21D), and 28 days (28D). Animals were subjected to the novel object recognition task at the same intervals and the Y-maze task at 13/14 and 27/28 days. Assessments of anxiety (elevated maze task) and depression (Porsolt forced swim task) were performed at 7D, 14D, 21D, and 28D. At 30 days, animals were euthanized and samples were taken for histopathologic analysis. Within this experimental model, the period from day zero to 72 h was defined as the acute period, 72 h to 14 days as the subacute period, 14 to 21 days as early chronic, and 21 + days as chronic (Fig. 1C).

Neurobehavioral Assessments

Detailed procedures for neurobehavioral assessments including Sugawara score, A-NSS score, rotarod assessment, novel object recognition task, Y-maze task, elevated maze, and Porsolt forced swim are available in Supplemental Methods.

Core And Penumbra Region Analysis

Brain tissue preparation

At 30 days following SAH induction or sham surgery, animals were deeply anesthetized with 5% isoflurane in medical air. Once anesthesia depth was confirmed with lack of response to painful stimuli, animals were transcardially perfused with cold phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde (PFA) in PBS as a fixative. The brains were removed and immersed in PFA overnight, then cryopreserved in 10% sucrose solution for 48 h followed by 20% sucrose solution for 48 h, after which they were embedded in a 1:3 mixture of 30% sucrose and Optimal Cutting Temperature Compound (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C. The brains were then serially cryosectioned with a cryotome (Leica Biosystems, Germany), in 14 µm thick coronal sections at 400 µM intervals from caudal to rostral and mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, Waltham, MA) and Polysine glass slides (Thermo Fisher Scientific, Waltham, MA), and stored at -30°C.

Histological analysis

For identification of regions of interest (ROI), samples mounted on Superfrost Plus slides were stained with hematoxylin and eosin (H&E) and imaged using PathScan Enabler (Meyer Instruments) at 2500 dpi. Images were converted from 24-bit RGB to a weighted 8-bit grey scale and processed using ImageJ (ImageJ v153, U. S. National Institutes of Health, Bethesda, Maryland, USA). The Threshold tool was used to assess potential lesion areas. On H&E staining healthy brain tissue typically displays a normal to left-skewed distribution of grey scale pixel intensities, reflecting darker predominantly basophilic staining. In
contrast, lesioned tissue has a right-skewed distribution of pixel intensities due to increased lighter eosinophilic staining. We assessed whether the pixel value distributions were normally distributed before applying a prespecified minimum pixel intensity threshold (165 out of 256), derived from the average intensity of visually identified lesion areas taken from prior sets of training slides. Areas with average intensity lighter than 165 were identified and selected using the Wand tool after which the image was color-inverted for easier ROI visualization. The selections were mapped to the original 24-bit RGB slides to identify the anatomic locations of the ROIs. After ROI identification, the H&E-stained slides were imaged using EVOS M7000 (Thermo Fisher Scientific, USA) at low power (20x = 0.2mm$^2$) and high power (40x = 0.01mm$^2$). ROIs were compared to equivalent locations in sham brain sections using the Waxholm Space atlas for anatomic landmark verification [61]. Five animals were selected pseudo-randomly from each group to undergo morphologic analysis and quantification of the ischemic core and penumbra regions. Five high-power fields were selected at random from within ROI and anatomically corresponding regions in sham animals. Overall neuronal damage and cellular architecture were compared between SAH and sham animal sections, and lesions attributable to core and penumbra ischemia were quantified. Due to the lack of an established definition of core and penumbra regions in SAH, we adapted regions from existing ischemic stroke literature to establish definitions for core and penumbra regions. While selective neuronal loss is an observed histologic correlate of the ischemic penumbra [62], a small degree of selective neuronal loss has been seen following even brief periods of reversible ischemia and is not typically associated with temporary or permanent neurobehavioral deficits [63]. We thus set a level of 30% neuronal loss as the baseline for ischemic penumbra regions based on established CBF thresholds for significant and prolonged ischemia [63]. As brain tissue can tolerate reductions in CBF to 20% of normal perfusion for brief periods of time [63, 64], we defined a core region as one with greater than 80% neuronal loss, or a region with pan-necrosis [65]. High-power fields from SAH and sham animals were compared and classified as non-ischemic (less than 30% neuronal loss), penumbra regions (30–80% neuronal loss), and core regions (> 80% neuronal loss or pan-necrosis). In addition, the distribution and relative quantification of the core and penumbra were performed using ROI analysis. Areas within preidentified ROI were analyzed for pixel intensity, and regions of high intensity were selected using the Wand tool (with tolerance set to zero). Areas of high intensity, corresponding to regions of intense neuronal loss, were defined as core regions. Regions within the identified ROI but with intensity outside the threshold for Wand selection were defined as penumbra as they represented less intense neuronal damage. Core and penumbra areas were quantified as a percentage of the anatomic region affected, as well as the total area affected, and averaged across all five animals.

**Statistical analysis**

Statistical tests were performed using the Statistics-and-Machine-Learning Toolbox in MATLAB 2022a (The MathWorks, Inc. Natick MA) and Origin Pro (version 2022b, Origin Lab Corp, Northampton, MA, USA). As all comparisons were between SAH and sham groups, effect sizes and comparisons were performed using a mixed-effects model with an ANOVA. For all tests, group and day were treated as fixed effects while the subject was treated as a random effect. For object exploration time, the effect of treatment as well as the interaction between treatment and object investigated were tested. Post-hoc analysis was
performed with the Tukey test. Results are presented as mean ± standard deviation (SD). Statistical significance was set to $P<0.05$.

**Declarations**

**Authors’ Contributions** DL and KS interpreted data and wrote the manuscript. PU, KP, SW, and WTA acquired and analyzed data. JS and PTH performed the statistical analysis. HW, DE, and PTH critically revised the manuscript. CL conceived and designed experiments; acquired and analyzed data; and wrote the manuscript.

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**Data Availability** The authors declare that all supporting data are available within this article.

**Code Availability** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

**References**


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**Figures**
Figure 1

Conceptual diagram of experimental protocol and hypothesis. **A:** Subarachnoid hemorrhage is induced via endovascular perforation at the internal cerebral artery (ICA) bifurcation using a sharpened suture. **B:** Hematoma forms in the basal subarachnoid space damaging the piriform cortex and hypothalamus, secondarily increased intracranial pressure (ICP) damages the primary motor, secondary motor, primary somatosensory, secondary somatosensory and retrosplenial cortices and induces predominantly core...
infarctions (red) associated with early brain injury (EBI). Following this, secondary penumbral lesions (blue) develop along with delayed cerebral ischemia (DCI) in the hippocampus, white matter tracts, amygdala, thalamus and periventricular nuclei. C: After a pre-training period before SAH induction or sham surgery, these lesions were assessed with a battery of neurobehavioral assessments, each of which assesses a neurologic or psychologic function that can be attributed to core and penumbra lesions in specific cerebral structures (Created with BioRender.com).
Figure 2

Distribution of core and penumbra regions following SAH. A: Sham rats did not demonstrate significant lesions with hematoxylin and eosin (H&E) staining or region of interest (ROI) analysis. B: SAH rats showed lesions on H&E staining (top, yellow) as well as ROI analysis (bottom, yellow) C: Lesion development, shown on representative diagrams of coronal sections based on histologic analysis demonstrated a pattern consistent with our hypothesized development of core and penumbra lesions, in which SAH initially leads to core lesions (red) in the piriform cortex and hypothalamus, areas in direct contact with the initial blood clot, as well as core and penumbra (blue) lesions in the retrosplenial, primary motor, secondary motor, primary somatosensory and secondary somatosensory cortices resulting from intracranial pressure elevation. Penumbra was also found in the hippocampus, thalamus, amygdala, periventricular cortex and white matter tracts. Black bar = 5mm D: Quantification of the relative distribution of core and penumbra at different brain regions.
SAH induces sensorimotor dysfunction associated with core and penumbra lesions in the cortex and basal ganglia. A: Neuronal death consistent with core lesions (red outline) was observed in the piriform, primary motor, secondary motor, primary somatosensory and secondary somatosensory cortex. Additionally, penumbra lesions were seen in the basal ganglia (blue outline). B: Sugawara score, a measure of general sensorimotor function following SAH, was significantly decreased consistent with
sensorimotor impairment. C: Adjusted neurologic severity score (A-NSS) was elevated indicating worsened sensorimotor function in SAH animals. D: Beam-walk score, a measure of coordination and sensorimotor function, was elevated in rats following SAH indicating impaired sensorimotor function. E: Riding time prior to falling was decreased in the SAH group indicating impaired coordination and sensorimotor ability. SAH rats displayed significantly worsened sensorimotor function at all time points relative to sham baseline. Black bar = 100µm; white bar = 25µm; red arrowheads = dead neurons; blue arrows = examples of live neurons; solid outline = 20x = 0.2mm²; dashed outline = 40x = 0.01mm²; **** P < 0.0001, mixed-effects ANOVA.
SAH induces working reference memory dysfunction on Novel Object Recognition (NOR) assessment associated with penumbra lesions in the hippocampus. **A:** Examination of the dentate gyrus (DG), dentate hilus (DH), CA3, and CA1 regions of the hippocampus reveals presence of penumbra lesions (blue outline). **B:** The amount of time SAH rats spent exploring either object was diminished suggesting impaired sensorimotor and exploratory function. SAH rats also spent less time investigating the novel
object relative to time spent investigating the familiar object. **C:** Discrimination ratio, defined as the amount of time spent investigating the novel object minus the amount of time spent investigating the familiar object divided by the total time investigating either object, was decreased in animals following SAH consistent with impaired reference memory. **D:** Total distance moved during the NOR task was reduced in SAH rats. **E:** Representative traces demonstrating reduced movement and interaction in SAH animals. Square = novel object, circle = familiar object. Black bar = 100µm; white bar = 25µm; red arrowheads = dead neurons; blue arrows = examples of live neurons; solid outline = 20x = 0.2mm²; dashed outline = 40x = 0.01mm²; * $P < 0.05$, **** $P < 0.0001$, mixed-effects ANOVA.
SAH induces working and reference spatial memory dysfunction and decreases exploratory and locomotor behavior on Y-Maze assessment in association with hippocampal penumbra regions. **A:** Penumbra lesions (blue outline) were observed in the hippocampus following SAH, in particular regions CA1 and CA3 which are closely related to formation of spatial memory. Neuronal cell death consistent with penumbra regions was also observed in the dentate hilus (DH), and dentate gyrus (DG) regions. **B:**

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**Figure 5**

SAH induces working and reference spatial memory dysfunction and decreases exploratory and locomotor behavior on Y-Maze assessment in association with hippocampal penumbra regions. **A:** Penumbra lesions (blue outline) were observed in the hippocampus following SAH, in particular regions CA1 and CA3 which are closely related to formation of spatial memory. Neuronal cell death consistent with penumbra regions was also observed in the dentate hilus (DH), and dentate gyrus (DG) regions. **B:**
Decreased entries of the previously closed arm were observed following SAH, consistent with impaired reference spatial memory. 

C: Percentage of complete spontaneous alternations was decreased in SAH animals, indicating impaired working spatial memory. 

D: Rats in the SAH group had fewer total entries into arms, consistent with impaired locomotor and exploratory function. 

E: Total distance travelled during the Y-Maze assessment decreased, indicating exploratory and locomotor function impairment following SAH. 

F: Representative traces illustrating impaired reference and working spatial memory. Black bar = 100µm; white bar = 25µm; red arrowheads = dead neurons; blue arrows = examples of live neurons; solid outline = 20x = 0.2mm²; dashed outline = 40x = 0.01mm²; *P < 0.05, **P < 0.01, ***P < 0.001, mixed-effects ANOVA.
Figure 6

SAH induces anxiety and decreases exploratory and locomotor behavior in the elevated maze (EM) assessment in association with cortical and subcortical penumbra lesions. A: Penumbral lesions (blue outline) were observed in the thalamus and amygdala following SAH induction, with core lesions (red outline) observed in the hypothalamus and loss of confluence in the corticofugal tract (yellow outline). B: SAH rats spent less time in the open arms indicating increased anxiety-related behavior. C: SAH rats
made fewer entries into the open arms, consistent with increased anxiety. **D**: Decreased locomotor function was observed in SAH rats, as shown by decreased total distance travelled. **E**: Velocity of movement was also decreased following SAH, indicating impaired locomotor function. **F**: Representative traces illustrate decreased movement into the open arms by SAH rats. Black bar = 100µm; white bar = 25µm; red arrowheads = dead neurons; blue arrows = examples of live neurons; solid outline = 20x = 0.2mm$^2$; dashed outline = 40x = 0.01mm$^2$; **** $P < 0.0001$, mixed-effects ANOVA.
**Figure 7**

SAH induces situational depression measured via Porsolt Forced Swim (PFS) in association with cortical, subcortical and white matter injury. **A:** Core lesions (**red outline**) were observed following SAH in the hypothalamus, along with penumbra regions (**blue outline**) in the thalamus and ventromedial prefrontal cortex (VMPC) and loss of confluence in the corpus callosum (**yellow outline**). **B:** SAH group animals showed a significant decrease in highly mobile behavior, indicating a more depressed state. **C:** Following SAH, animals displayed a significant increase in immobile behavior consistent with depression. Black bar = 100µm; white bar = 25µm; red arrowheads = dead neurons; blue arrows = examples of live neurons; solid outline = 20x = 0.2mm$^2$; dashed outline = 40x = 0.01mm$^2$; ** $P < 0.01$, **** $P < 0.0001$, mixed-effects ANOVA.

**Supplementary Files**

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- [SupplementalMethods.docx](#)