SUPPLEMENTAL MATERIAL

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Supplemental Methods:

Animals

Young (8-12 weeks, 21-27g) C57BL/6 male and female mice were purchased from Jackson Laboratories. Aged C57BL/6 mice (18-21 months) of both sexes were obtained from the National Institute on Aging. All mice were housed in a temperature- and humidity-controlled vivarium, 5 per cage (11" L, 6" W, 6" H) with a 12-hour light/dark schedule with ad libitum access to food and water for 4 weeks after arrival before use. Mice in each cage were randomly assigned a number 1-5 by a member of the laboratory who was not involved in the surgical procedure. A random number generator was used to allocate each number to stroke or sham groups. Surgeries were performed in sets of 5 with no more than 10 surgeries being performed in one day. The order of stroke or sham procedure was based on the mouse's individual number and which group it was assigned to as described above. Therefore, the order in which procedures were performed varied on each day of surgery. When measuring breathing parameters, 4 animals could be tested at a time. Prior to any surgical procedure or experimentation, animals were allowed to acclimate to the room in which studies were being performed for at least 1 hour prior to any handling. A total of 111 animals were used in this study. Animals that did not survive to the end point of their respective study were excluded from data analysis. Additionally, if an animal did not display physical or histological evidence of an MCA stroke they were also excluded from the study. No sham animals were excluded. 6 aged female mice were excluded due to premature death. 10 aged male mice were excluded due to premature death. 6 young male mice were excluded due to premature death. No mice were excluded due to lack of cerebral infarction There were no mice excluded from the permanent distal MCAO group.

All experiments were performed according to NIH guidelines for the care and use of animals in research and under protocols approved by the University of Texas Health Science Center Houston Institutional Animal Care and Use Committee.

Middle cerebral artery occlusion

Focal transient ischemia was induced by middle cerebral artery occlusion for 60 minutes under Isoflurane anesthesia followed by reperfusion as described previously.[24,25] Briefly, mice were placed in the supine position on a heating pad, and 0.25% bupivacaine was administered SQ at the surgical site before a midline incision was made into the skin. The carotid artery was ligated to allow for the placement of a silicon filament through the external carotid into the internal carotid, which allowed access to the middle cerebral artery (MCA). An 80% drop in cerebral blood flow confirmed occlusion of the MCA by Laser Doppler (Moor Instruments). After 60 minutes of occlusion the silicone filament was withdrawn, the surgical site was sutured and the animals were returned to home cages and monitored. The same procedure was conducted in sham animals except the silicone filament was not introduced past the internal carotid. Body temperatures were monitored rectally and maintained at approximately 37° C. Mice were followed up for 7 days post-

surgery, weighed daily, and given supplemental fluids and softened food as needed. Mice that exhibit >20% body weight loss, lethargy, or areflexia are euthanized.

Distal middle cerebral artery occlusion

Mice were subjected to permanent distal middle cerebral artery occlusion (pdMCAO) as previously described.[26] Following anesthesia induction, the right dorsolateral cranium was shaved and a 1-cm^2 skin flap was cut over the temporalis muscle, which was then incised with Vannas scissors to expose the temporal bone. A 2 mm burr hole was drilled over the middle cerebral artery, immediately dorsal to the zygomatic arch. Stroke was then induced by cauterization of the exposed middle cerebral artery, and ischemia was confirmed by laser doppler as a greater than 90% drop in blood flow to the cortex distal to the occlusion. Sham surgeries were performed following an identical procedure, except that no cauterization was performed. In the permanent distal MCAO model, craniotomy was not sealed. We are aware that this is a limitation of this method. There is a possibility that CSF leaked out during the first 3 days of recovery and relieved the intracranial hypertension, which is thought to be one factor that leads to Cheyne-Stokes breathing. This might account for the lack of apneas in the young male mice with pdMCAO. However, the craniotomy wasn't sealed in either aged or young mice. If CSF was leaking out, it would have the same effect on both young and aged.

Whole body plethysmography

Respiratory parameters (frequency, tidal volume, minute ventilation, number of apneas) were measured using whole-body plethysmography, a well-established technique for respiratory activity.[27,28] Recordings were done on days: 3, 7 ,14, 21 and 42 post-surgery. Mice were placed individually into a ventilated (1L/min) plexiglass chamber and allowed 1 hour to acclimate. Inspiration and expiration result in decreases or increases in chamber pressure relative to a reference chamber that are detected using a pressure transducer, which was calibrated prior to every experiment. Tidal volume (ml, normalized to body weight and corrected for chamber temperature, pressure, and humidity) and respiratory frequency (breaths/min) was recorded on a breath-to-breath basis and analyzed from periods of relative quiescence during the last 2 minutes of each experimental condition; the product of tidal volume and frequency is minute ventilation (ml/min/g). The frequency of apneas (defined as ≥3 or more missed breaths) was determined for the duration of the recording. We confirmed that the section of data selected for analysis is devoid of motion artifacts and was most representative of each animal's breathing pattern. All animals received baseline assessment of respiratory activity prior to undergoing surgery.

Pulse oximetry

On day 3-post surgery, pulse oximetry measurements (SpO_2) were recorded using MouseOx neck collar (Starr Life Sciences Corp.). Collars were placed around shaved neck of both stroke and sham mice. Mice were returned to their home cages and allowed to acclimate to the collars. Recordings were taken

during periods of time when the animals were at rest (calmly sitting in cage without any signs of distress or motor activity).

Behavioral Tests

All behavior tests were conducted in a quiet temperature-controlled room at the same time each day, by an investigator blinded to the surgical condition of the animals, and NORT, Barnes maze and Fear conditioning data was recorded by an overhead camera system and analyzed by (Noldus Ethovision XT) software.

Novel Object Recognition Test (NORT)

The novel object recognition test (NORT) was conducted on day 28 post-surgery. Mice were individually placed into separate plexiglass rectangular boxes with 2 identical objects, termed familiar objects, equally placed on each side of the testing box. Mice were allowed to freely explore the chamber and objects for 10 minutes. At the end of the training period mice are returned to their home cage for 5 minutes. During this time the chambers are cleaned and one of the objects in each arena was replaced with a novel object of different shape, size and color. At the conclusion of the 5-minute rest period mice were placed into the same chamber as the training trial and again allowed to freely explore for 10 minutes. Following this test trial mice were returned to their home cage and the test was concluded. Time spent with each object was automatically calculated by Ethovision software. Data was scored and analyzed as a ratio between time spent on novel object/time spent on familiar objects.

Barnes Maze

The Barnes maze was conducted on an elevated circular platform (92cm diameter) with 20 evenly spaced holes (5 cm diameter).[29] A randomly chosen hole was designated as the escape hole, which allowed the animal to escape the platform into a dark rectangular box below. During training trials, mice learn the location of the escape hole by spatial clues positioned around the platform. Animals received 3 training trials followed by a test trial 4 hours later on day 21 or 42 post-surgery. During the first training trial, the mouse was placed into the center of the arena and then guided to the escape hole by a clear cylindrical chamber, which it was allowed to explore for 1 minute. During the second and third trials the animal was again placed into the center of the platform and allowed to freely explore the arena for 5 minutes. At the end of each trial if the animal did not find the escape hole it was guided to it using the same clear chamber. The testing period consisted of one 3-minute trial. The trial was terminated when the animal entered the escape hole or at the end of the 3-minute period. Any animal that did not find the escape hole once during any training trial was excluded. Total number of errors made and the latency to enter the escape hole was automatically calculated by Ethovision software.

Contextual Fear Conditioning

For contextual fear conditioning, animals were allowed to acclimate to a square plexiglass container with metal grid floor (Harvard Apparatus) for 2 minutes on day 42 post surgery. Following all acclimation trials animals were placed back

into the plexiglass container for 2 minutes then received a 0.7mA shock through the metal grid floor lasting 2 seconds. Animals were then returned to their home cage. 24 hours later animals were again placed in the chambers and the session was recorded for 3 minutes. Freezing time was automatically calculated by Ethovision, and the percent of freezing time was reported.[30]

Corner Test.

The corner test was carried out on day 3 post-surgery as described.[31] Briefly, a mouse is encouraged to enter a 30-degree corner created by two cardboard pieces. Once in the corner, the boards stimulate both sides of the vibrissae, the mouse then rears forward and up, turning to face the open end. Twenty trials are performed and the percentage of right turns is calculated.

Statistical analysis

Statistics are presented as means ± SEM for all experiments. Statistics were performed using GraphPad Prism 9. Interval power analysis was performed to determine group size. After normality testing using Shapiro-Wilk test, student's t-test or Mann-Whitney test was used when comparing 2 groups and a 2-way ANOVA was performed when comparing multiple groups. If an interaction was statistically significant, then Sidak's post-hoc analysis was used to assess where the interaction occurred. If there was no significant interaction, main effects were reported when significance. Linear regression analysis was used to assess changes in chemosensitivity. A probability value of $p<0.05$ was considered statistically significant. All investigators were blinded to the surgical condition when analyzing data.

Supplemental Tables and Figures

Stroke-induced respiratory dysfunction is associated with cognitive decline

Table S1

	Sham	Stroke
O_2	104.3 ± 4.7	82.3 ± 1.4
$PaCO_2$	33.97±5.1	48.23±0.8
pH	7.359±0.02	7.37±0.01

Table 1. Arterial Blood Gases. Obtained day 3 following surgery from stroke and sham young male mice. Stroke mice have a decrease in PaO_2, 104.3±4.7 vs. 82.3±1.4, $p<0.05$, suggesting a state of hypercapnia and hypoxia. $PaCO_2$ measurements suggest stroke mice retain CO_2 as a result of disordered breathing, 33.97±5.1 vs 48.23±0.8, p=0.052, while arterial pH remained unchanged between the two groups, 7.359±0.02 vs 7.37±0.01, p=0.63. n=3/group.

Figure S1

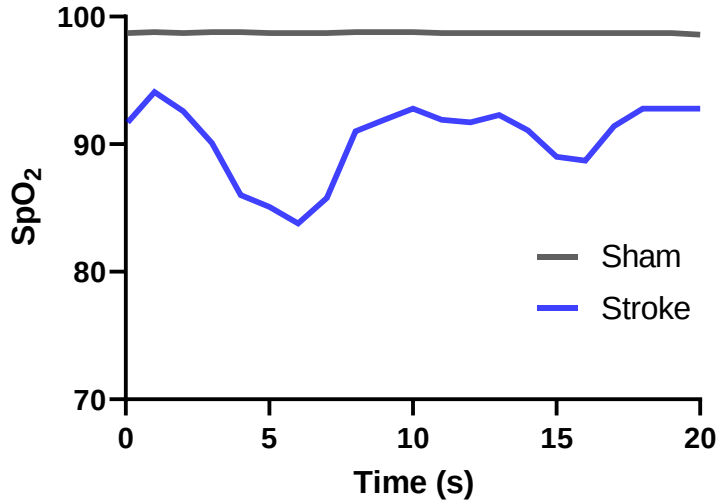

Figure S1. SpO_2 measurement in aged mice with MCAO showed severe and frequent hypoxic events. Oxygen saturation was assessed after MCAO on day 3 post-surgery in aged male mice. SpO_2 levels showed frequent periodic drops, fluctuating between 90-80% in stroke mice, while remaining constant at ~98% in sham mice.

Figure S2

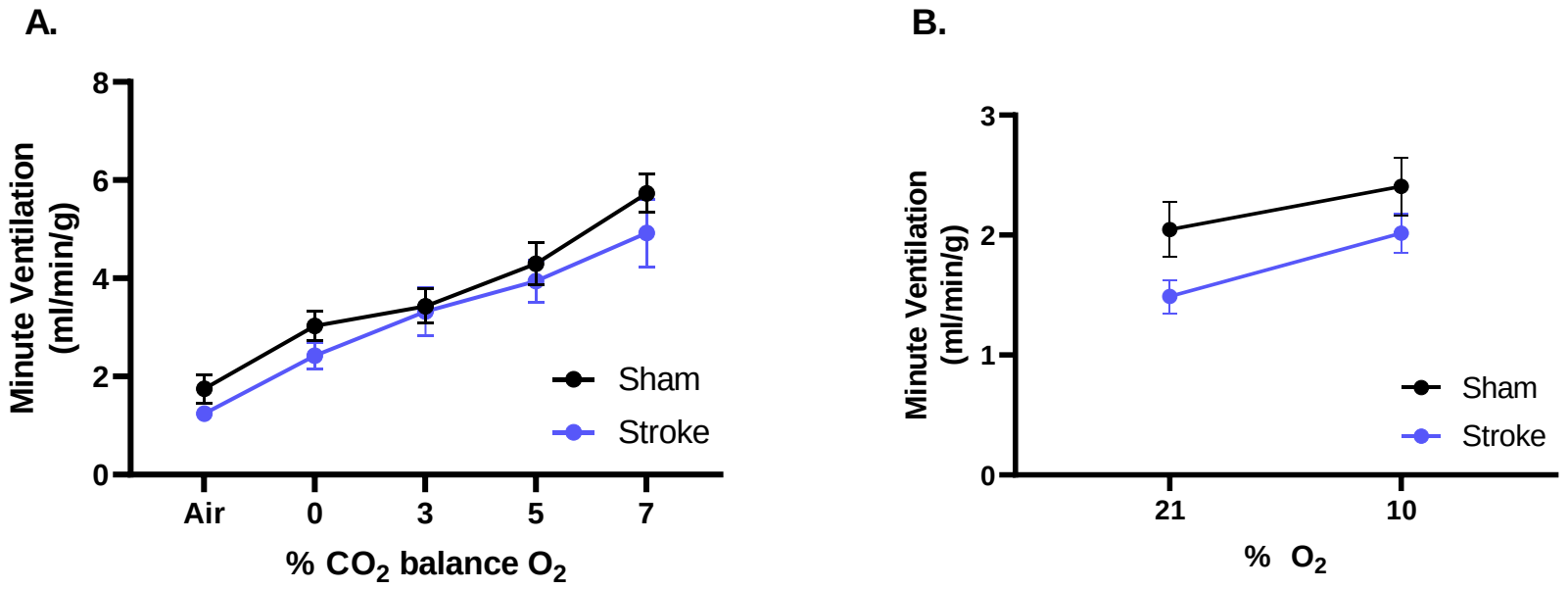

Figure S2. MCAO does not result in a change in chemosensitivity. MCAO does not alter CO2/H+ sensitivity. (Slope: p=0.77) stroke n=9, sham n=14 (A). The chemosensitivity to hypoxia remains unaltered between stroke and sham. Slope: p=0.73. stroke n=19, sham n=7 (B)

Figure S3

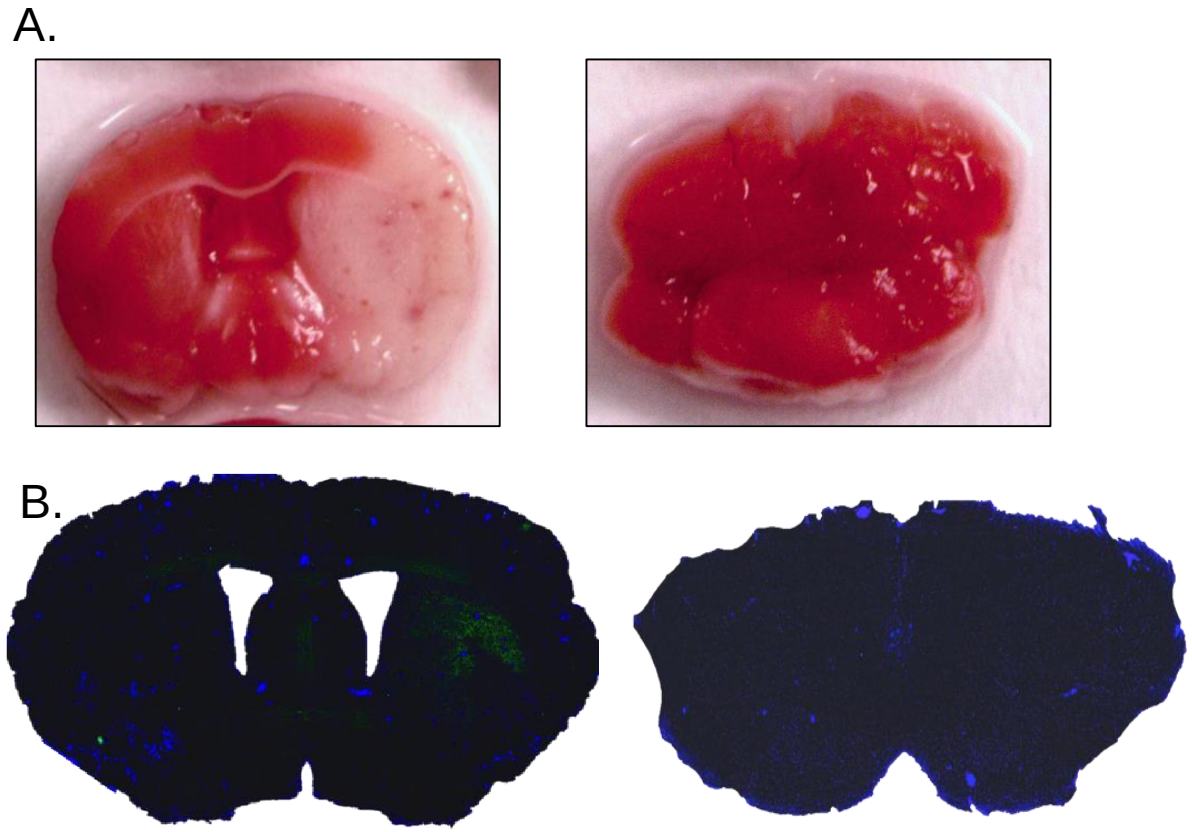

Figure S3. MCAO does not result in direct brainstem cell death. Brainstem cell death was assessed by both TTC (A) and fluorojade staining **(B)** on day 3 following MCAO in young male mice. MCAO produces a large wedge-shaped infarct in the hemisphere indicated by the lack of “red” staining by TTC. The brainstem was unaffected **(A)**. Fluorojade, a marker for degenerating neurons, was positive in the striatum of the right hemisphere in mice that underwent MCAO. The brainstem was negative for any fluorojade + cells, n=3. Fluorojade green, Dapi blue **(B)**.

Figure S4

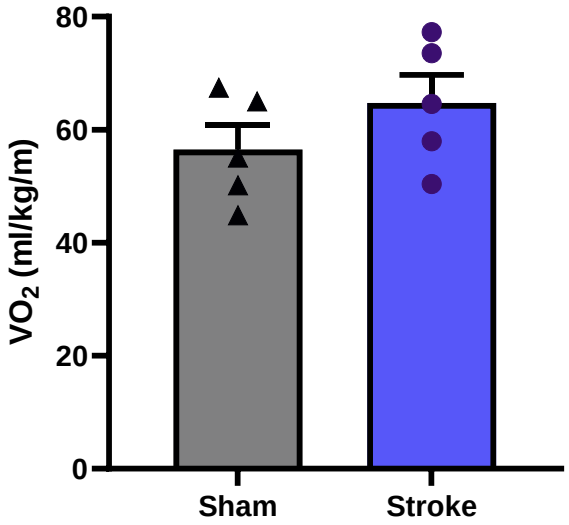

Figure S4. Oxygen consumption as a measurement of metabolic activity. Metabolic activity was assessed on day 3 post-surgery in young male mice. The volume of oxygen consumed was recorded every 10 minutes and averaged per mouse over a 4-hour period during resting hours. MCAO did not alter basal metabolic activity, minimizing the possibility that alterations in metabolic activity contribute to SIRD. p=0.24, n=5/group.

Figure S5

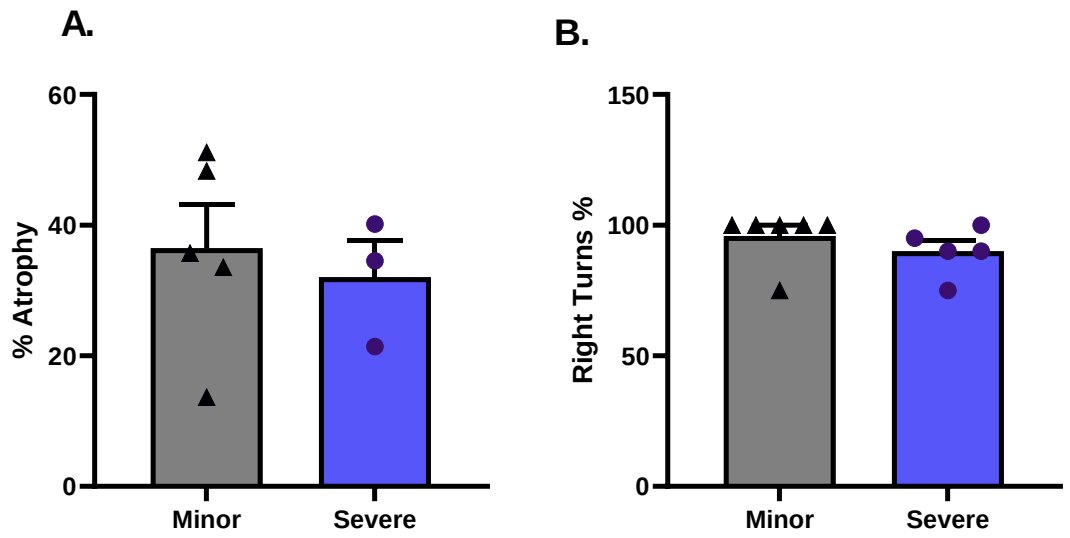

Figure S5. Variations in stroke severity do not define the severity of respiratory dysfunction in young male mice. No differences in the volume of cerebral atrophy were found at day 42 post-stroke between the minor and severe groups of disordered breathing, p=0.66 (**A**). Performance on the Corner test was the same for both groups of mice when they were assessed at day 3 post-stroke. p=0.35 **(B)** N=5-6 minor and 3-5 severe.

Figure S6

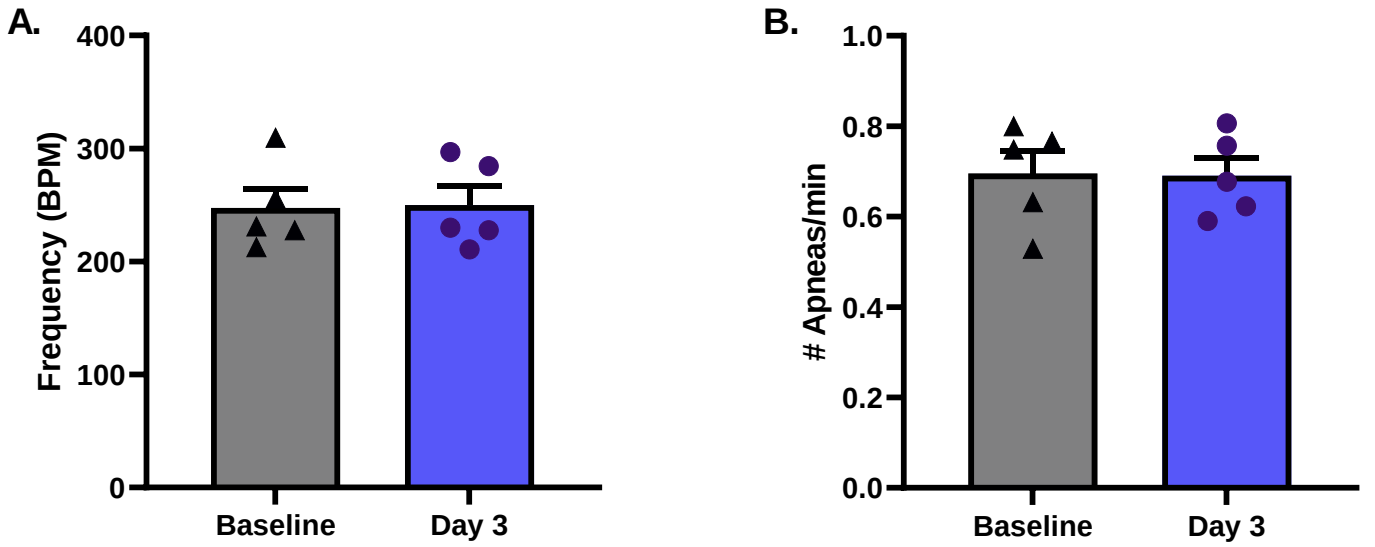

Figure S6. Sham neck surgery for the MCAO filament model concurrently with distal MCAO doesn't produce respiratory disorder in young male mice. To exclude the possibility that the resulting breathing disorder may be due to the damage of the pulmonary afferents in the vagus nerve adjacent to the dissected carotid artery or effect of possible inflammation and pain at neck during MCAO surgery, we performed pdMCAO with sham neck surgery and there was no resulting breathing disorder.

Figure S7

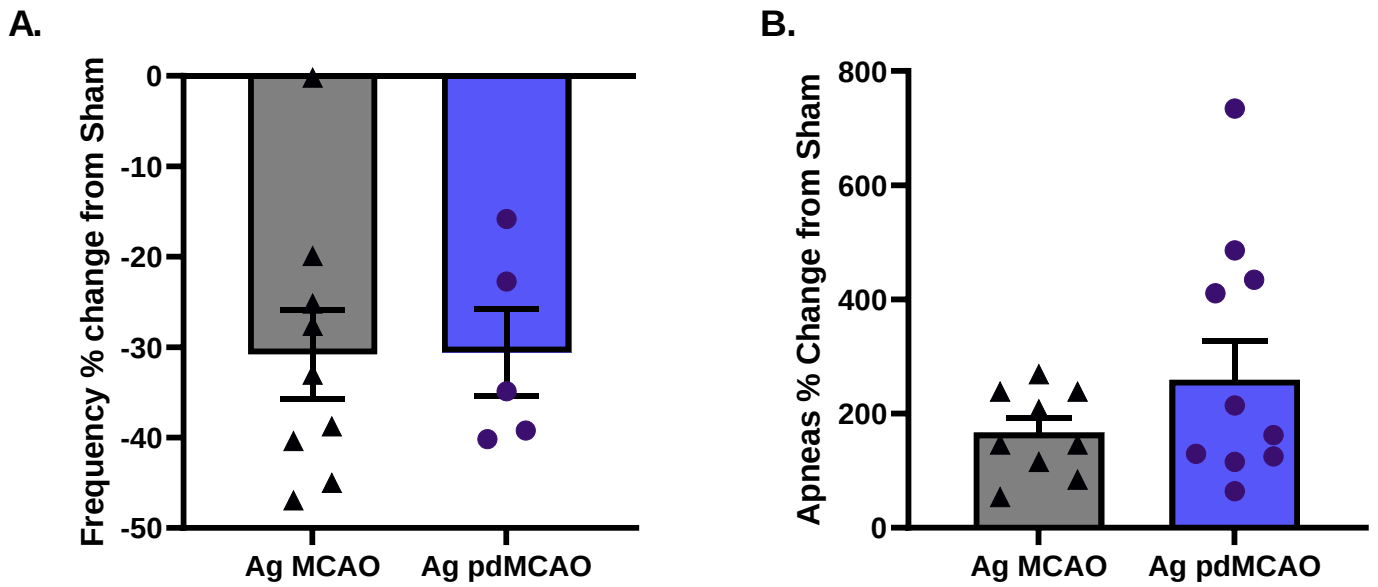

Figure S7. MCAO and pdMCAO produced similar changes from sham in aged male mice. To compare the response to stroke between the two models, a percentage change from sham was performed in each model. No difference in the ratio change from sham was observed between the two models in the frequency (p=0.98), n=9 Ag (aged) MCAO, n=5 Ag pdMCAO (A), or the number of apneas (p=0.26), n=9 Ag MCAO, n=10 Ag pdMCAO (B)