Exogenous inter-alpha inhibitor proteins prevent cell death and improve ischemic stroke outcomes in mice

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Materials and Methods:

Physiological studies: A separate cohort of mice were used to evaluate changes in physiological variables, blood samples were obtained from femoral artery 1h after occlusion as detailed previously (31), drug/vehicle is administered at onset of stroke. Cerebral blood flow measurements by laser Doppler flowmetry (DRT 4/Moor Instruments Ltd, Devon, UK) confirmed ischemic occlusion during MCAO and restoration of blood flow during reperfusion.

Blood collection for mRNA analysis: Blood was collected via cheek bleed (~4 drops) from a subset of these mice at 6h after stroke to confirm individual systemic C5aR1 levels prior to injecting them with IAIP. Once IAIP was injected mice were returned to their home cage and 6h following injection, blood was collected from these mice again to assess C5aR1 levels following treatment.

Permanent Distal MCAO: Mice were subjected to distal stroke as previously described (34). Briefly, mice were anesthetized with 4% isoflurane, and adequate sedation was confirmed by tail pinch. Surgery was performed under 1-2% continuous isoflurane, and body temperature maintained at ~37°C. The skin and underlying temporalis muscle were incised, and a 2 mm burr hole was drilled to expose the right MCA, which was then cauterized to induce ischemia. After successful cauterization, the burr hole was closed with dental cement, the temporalis muscle repaired with Vetbond and the skin incision closed with Vicryl suture. Mice were randomly assigned to receive either IAIP or vehicle

treatment (i.p.), and functional recovery was accessed at regular intervals for 30 days. Two mice died during this follow-up, 1 from the IAIP group and 1 from the vehicle treated groups; these were excluded from the analysis.

Thromboembolic Clot Model in Mice: Mice were anesthetized with 4% isoflurane for induction, and anesthesia was maintained with 1.0–1.5% isoflurane using a face mask. Rectal temperature was maintained at 36.5 ± 0.5°C with a feedback-controlled heating pad during surgery. The common carotid artery and external carotid artery (ECA) were exposed through a 2-cm midline neck incision. A PE-8 catheter filled with 2 IU alpha-thrombin (Sigma) was inserted into the ECA to draw blood to make a clot (37). The catheter with the blood was withdrawn from the artery, briefly exposing the catheter to air. The catheter was then re-introduced through the ECA and gently advanced to allow for intravascular injection of thrombin at the MCA bifurcation, resulting in formation of an autologous thromboembolic clot. Occlusion was confirmed by ~80% reduction in LDF readings. After 30 min of occlusion, mice were then administered a bolus dose of 10 mg/kg t-PA, followed by a 1 mg/kg infusion over 30 minutes to complete reperfusion. A total of 5 mice died, 2 in the IAIP group and 3 from the vehicle treated groups and were excluded from the analysis.

Tail Bleed Assay: To assess if IAIP treatment induced changes in anticoagulant activity, bleeding time was measured in a separate group of mice using tail bleeds. Mice were anesthetized with isoflurane and placed on a heating pad. t-PA was injected via the carotid as in the thromboembolic clot model, but without occlusion. Five hours after the t-PA administration, mice were then randomly administered vehicle or IAIP i.p. which was allowed to circulate for 30 minutes. After 30 min of treatment, the tail tips were cut 1 cm

from the base and were immediately placed into a tube filled with saline at 37 °C to measure the bleeding time and bleeding volume. The bleeding time was recorded until the bleeding completely stopped for at least 5 min. Total bleeding time was recorded using a stop clock.

Immunohistochemical analysis: For immunohistochemistry, we used 30 micron sections obtained at 24h of reperfusion. Brain sections were mounted on slides and incubated in blocking solution followed by microwave irradiation for 5 min in a 0.1M, pH 6 citrate buffer solution. Sections were incubated overnight with primary antibodies (anti-IAIP, R22C, 1:100; anti-Fibrinogen (1:200; NBP2-80414, Novus Biologicals, Centennial, CO); Preconjugated-Lectin (1:500; DL-1178, Vector labs, Burlingame, CA); anti-GFAP (1:1000; ab53554, Abcam, Cambridge, MA)) in TBS + 0.25% Triton-X 100 overnight at 4°C, the sections were washed with PBS three times for 5 min. Then anti-rabbit 488 (1:1000; 406416, BioLegend), anti-rabbit 594 (1:1000; A11012; Invitrogen, Carlsbad, CA) and anti-goat 594 (1:1000; A11012 , Invitrogen, Carlsbad, CA) were used as secondary antibodies (incubated for 60 min). The slides were then dipped in DAPI solution (1:1000; 62248, Thermo Scientific, Waltham, MA) for five minutes or mounting medium with Dapi (H-1200, Vector Laboratories, Burlingame, CA) was used to adhere the coverslip. Sections were visualized utilizing an inverted Leica confocal microscope (Leica, Heerbrugg, Switzerland) (69). All slides were analyzed using ImageJ (Colocalization colormap) by an investigator blinded to treatment conditions.

Novel Object Recognition Test: The novel object recognition test was performed as described earlier (31). In brief, animals were placed individually in a plexiglass chamber

containing two identical objects and allowed to habituate with the objects for ten minutes 29 days after stroke. Mice were returned to their home cages and were reintroduced one hour later for a five-minute exploration test, at this time one of the objects was replaced with a novel object. Data was recorded by an investigator blinded to treatment condition. The time spent exploring the novel object was expressed as a percentage.

Barnes Maze Test: The Barnes maze test was performed on a brightly illuminated elevated circular platform with 20 equally spaced holes around its perimeter. One of the holes had a platform underneath that contains an escape box, so that the mouse can escape and hide in the darkness. During training and testing, the maze is illuminated with bright overhead lights to serve as an aversive stimulus and to encourage the mouse to enter the escape hole. Mice were trained twice a day for four consecutive days (days 22-25) and tested for memory retention on day 27 after stroke. During the first day of training mice were placed in a clear container in the center of the Barnes maze for 30 seconds to allow visualization of their surroundings. Once the 30 seconds had elapsed, mice were allowed to explore the arena and holes for 3 minutes. Mice were directed gently to the escape hole at the end of 3 minutes if they failed to find the escape hole. Testing was performed on day 27 after stroke, 48 hours after the final day of training. Data was recorded and analyzed by EthoVision software (Noldus, Virginia)

DigiGait: Gait disturbance ranging from mild hemiparesis to severe loss of limb function is commonly seen in patients after stroke. Such abnormalities are also well established in animal models of stroke (39). Mouse gait dynamics were obtained using a motorized DigiGait® treadmill (with a transparent belt and digital video camera mounted underneath) by ventral plane videography and analyzed with DigiGait® software (Mouse Specifics,

Inc. Quincy, MA). Mice were tested pre-stroke for individual baseline performance and at 7 days after stroke for gait symmetry. Gait analysis was performed on a flat platform at a speed of 8 cm/s, with an average of 5 s of video analyzed for each mouse. Subjects underwent three trials, with 5-min rest periods. Gait symmetry is expressed as the percentage of ratio of right vs left paw area curves to baseline.

Hang Wire Test: Motor strength was assessed using the hanging wire test before and after stroke as detailed previously (36). Mice were placed in the center of a wire cage top that was slowly inverted and placed at a height of 24 inches above a cage containing regular bedding. Latency to fall from the wire cage top from inversion was measured and plotted as a percentage time compared to its baseline.

Adhesive-tape removal test: The adhesive-tape removal test is used to measure somatosensory dysfunction and stroke recovery (35). Adhesive-backed tape (30x40 mm) was placed on the distal–radial region of the left-wrist and the mean time to remove the tape was recorded (35). Animals were trained for five days once a day prior to stroke and the latency to remove the adhesive tape was recorded on the fifth day of training (baseline/pre-stroke) and on days 7, 14, 21 and 28 after distal stroke in aged mice.

Supplemental Table 1. No significant differences in physiological parameters were seen between vehicle and IAIP treated mice after stroke

Table 1. Physiological measurements in vehicle- and drug-treated mice

Group	pH	pO_2 *mmHg*	pCO_2 *mmHg*	Glucose *mg/dl*	MABP *mmHg*	LDF(%) *Baseline*	LDF(%) Ischemia
Vehicle	7.3 ± 0.05	116 ± 5.6	31.3 ± 4.1	171 ± 10.3	71 ± 3.9	100 ± 14%	14± 6
Drug	7.3 ± 0.04	120 ± 6.1	29.9 ± 5.1	169 ± 13.8	73 ± 3.2	100 ± 16%	13 ± 4

Analysis was performed on blood samples collected from the femoral artery at the onset of MCAO or 60 min after the onset of MCAO. LDF did not detect any significant differences in CBF between the groups. Data are expressed as Mean±SEM. T-test was used to compare the difference between vehicle and IAIP treated groups.

Supplemental Table 2. Demographics of control subjects and ischemic stroke patients for human plasma samples

Variable	Controls (n=39)	Ischemic Stroke (n = 51)
Age, years (mean ± SD)	63.15 ± 13.9	73.5 ± 12.6
Male, n	25	26
Female, n	14	25
Admission NIHSS (mean)		12
MRI positive, n		49
CT positive, n		3

Supplemental Table 3. Demographic and characteristics of human brain samples

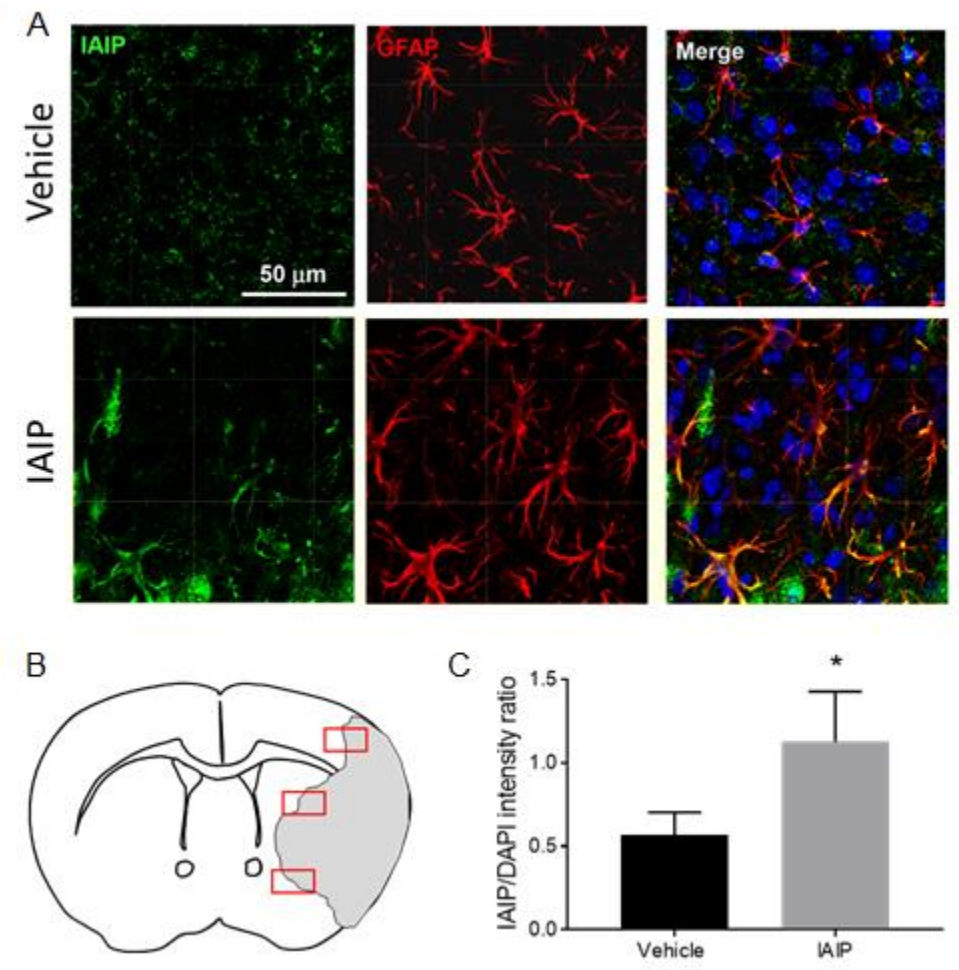
	Control N=8	Stroke N=13	p value
Sex, male (%)	8 (100%)	13 (100%)	1[a]
Mean of age, years (mean±SEM	75.4±3.8	80.8±2.6	0.25[a]
PMT, hours (mean±SEM)	11.5±2.4	8.8±2.0	0.25[a]
Infarct age			
acute		3	
subacute		3	
old		7	

S.E.M, Standard error of the mean; PMT, Post mortem time; [a], Mann-Whitney Test

Table 4. Gene primer sequences used for qPCR.

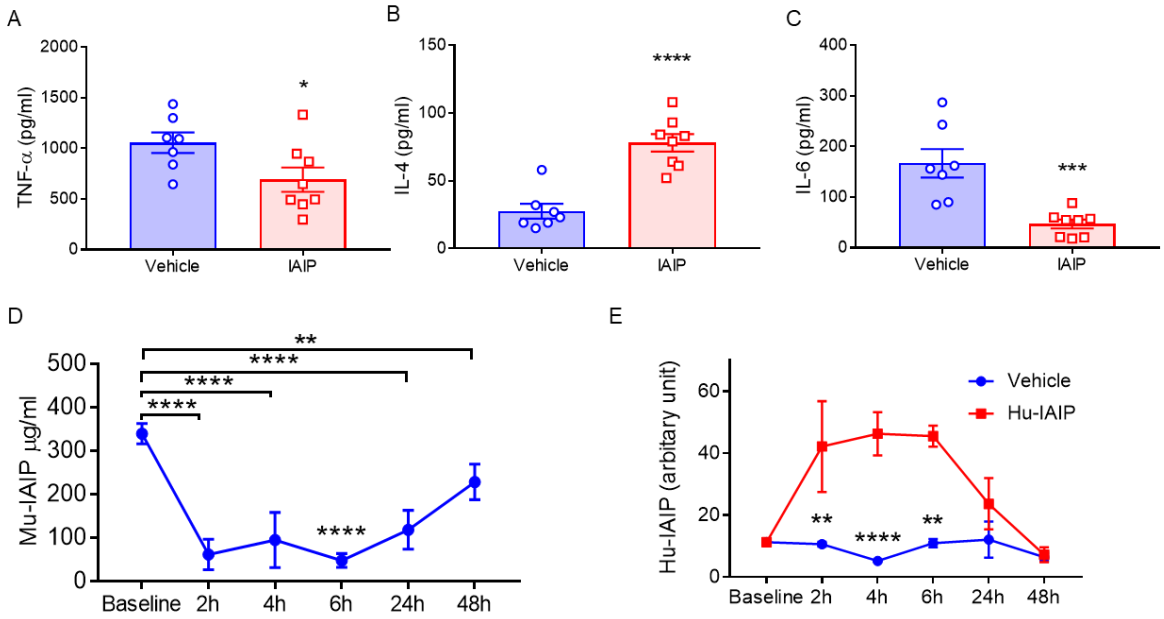
	Forward (5' → 3')	Reverse (5' → 3')
β-actin	GAT CTG GCA CCA CAC CTT CT	GGG GTG TTG AAG GTC TCA AA
C1q	CAA GGA CTG AAG GGC GTG AA	CAA GCG TCA TTG GGT TCT GC
C3	CAC CGC CAA GAA TCG CTA C	GAT CAG GTG TTT CAG CCG C
C5	GGA TTC AAG CGC ATA ATA GCA	ACC CGG ATG TTG ACT CCT C
C5aR1	GTC ACC GCC ATC TGG TTT CT	ACG GTC GGC ACT AAT GGT AG
CFB	CTC CTC TGG AGG TGT GAG CG	GGT CGT GGG CAG CGT ATT G

Figure S1. IAIP levels in the brain are increased 24h after administration of exogenous IAIP



(A) Immunohistochemistry on brains analyzed at 24h after stroke and IAIP treatment shows that IAIP are expressed in the mouse brain in multiple cells (co-labelling with GFAP shown for reference). **(B)** Representative section illustrates the location of immunostained images obtained from for analysis. **(C)** Fluorescent intensity data revels IAIP expression is higher in the brains of IAIP treated mice compared to vehicle treated mice [mean ± SEM; *p=0.0316, t test].

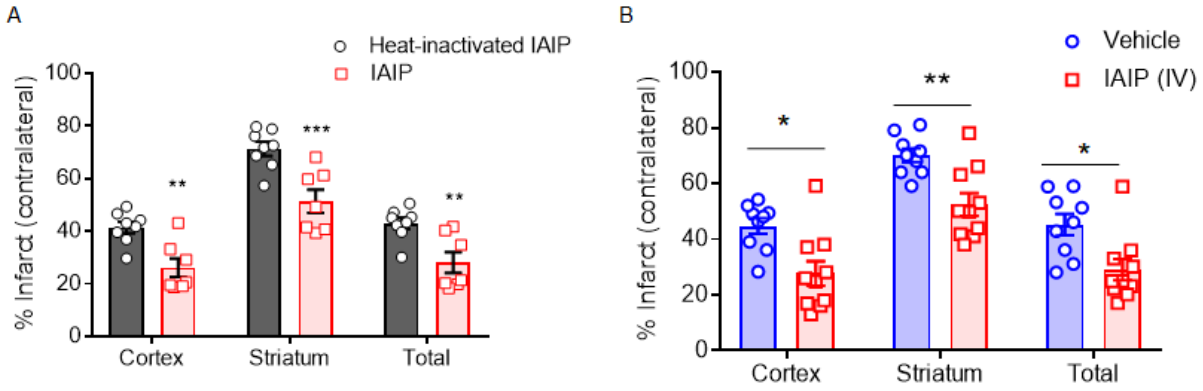
Figure S2*. IAIP treatment reduced inflammation in mice after experimental stroke.*



(A) Plasma cytokines in young mice at 24h after stroke, IAIP treatment significantly reduced TNF-alpha levels compared to vehicle treatment by t-test [*p=0.0399]. **(B)** Mice treated with IAIP had elevated levels of the anti-inflammatory cytokine IL-4 24h after stroke compared to vehicle-treated mice by t-test [****p<0.0001] **(C)** IL-6 levels in IAIP-treated mice were significantly lower compared to vehicle treated mice, t-test [***p=0.0009]. Data are presented as mean ± SEM. **(D)** Stroke mice have a significant reduction in circulating IAIP (Mu-IAIP) levels compared to naïve (baseline) at 2h [****p<0.0001]; 4h [****p<0.0001]; 6h [****P<0.0001]; 24h [****p<0.0001]; and 48h [**p=0.0019], analyzed by mixed model adjusted for multiple testing. **(E)** Mice that received a single dose of Human IAIP (Hu-IAIP) i.p. had detectable IAIP levels in the circulation even at 24h after stroke, and a significant increase was seen at 2h [**p=0.003];

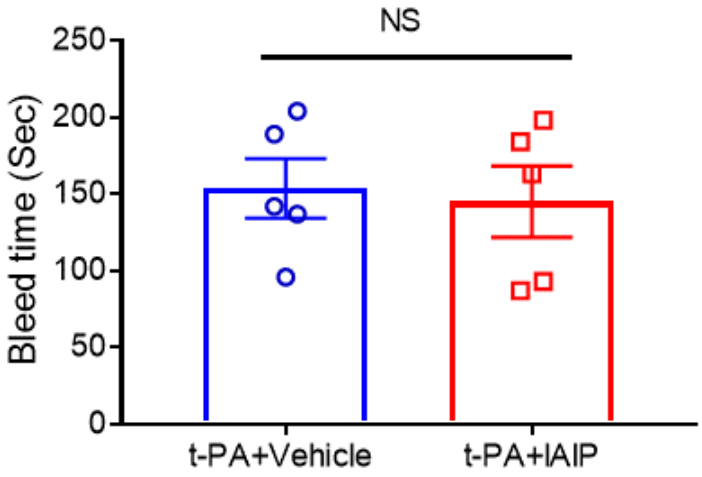
4h [****p<0.0001]; 6h [**p=0.0048] with mixed model adjusted for multiple testing (bottom panel).

Figure S3. ***IAIP treatment is protective compared to heat-inactivated IAIP administered immediately after stroke (A) and when given by an intravenous (IV) injection (B).***



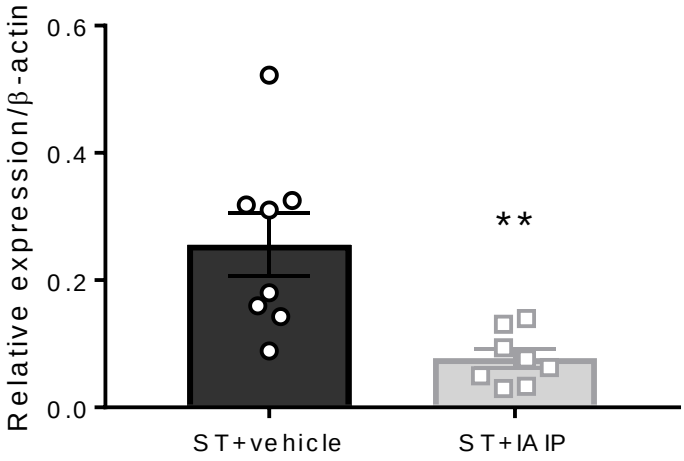
(A) Young male (2-3m old) mice treated with active IAIP had significantly reduced infarct size compared to mice treated with heat-inactivated IAIP after stroke (90 min MCAO, 48h reperfusion), IAIP treatment was administered immediately after onset of stroke, infarcts were quantified at 48h of reperfusion. Infarct volume analysis shows a significant reduction in cortical [**p=0.0045], striatal [***p=0.0002] and total hemisphere [**p=0.0057] infarct at 48h after stroke [Heat-inactivated IAIP vs IAIP 30 mg/kg Mean±SEM; two-sample t test adjusted for multiple testing]. **(B)** Delayed intravenous (IV) administration of IAIP (30 mg/kg, not heat-inactivated) in mice starting 6h after the onset of stroke led to significant reduction in infarct. Infarct volume analysis showed a significant reduction in cortical [*p=0.0107], striatal [**p=0.0063] and total hemispheric infarcts [*p=0.0111] 48h after stroke [vehicle vs IAIP 30 mg/kg via intravenous (IV) injection. Mean±SEM; two-sample t test adjusted for multiple testing].

Figure S4. IAIP treatment had no significant effects on coagulation time in a tail bleed assay in t-PA treated mice.



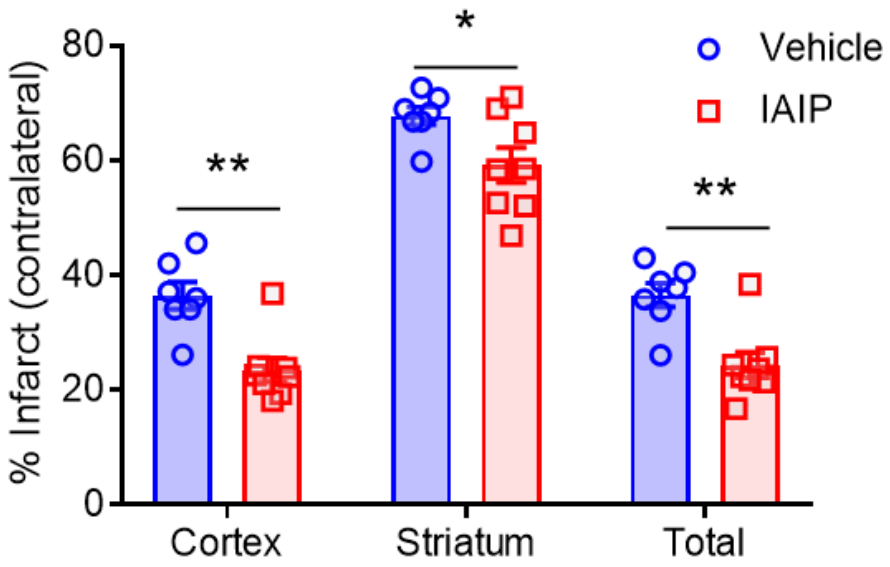
Time for complete coagulation, using a tail bleed assay, showed no significant differences between vehicle and IAIP treated animals in male mice 30 min after administration of vehicle and IAIP. Total bleeding time was measured and reported as total bleed duration for each group, by t-test [p=0.7831], data is presented as mean ± SEM.

Figure S5. Stroke induced C5ar1 expression is significantly reduced with IAIP treatment



In mice subjected to stroke, circulating levels of C5ar1 were analyzed on blood collected from a cheek bleed 6h after stroke and before administration of IAIP. Mice were euthanized at 12h after stroke, 6h post-IAIP treatment, C5ar1 expression was analyzed before and after treatment of IAIP. IAIP treatment significantly reduced the stroke induced C5ar1 expression, analyzed by t-test [**p=0.0064]**.** Data are presented as mean ± SEM.

Figure S6. Neuroprotective effects of IAIP in BalB/c mice given delayed exogenous IAIP after stroke.



Infarct analysis at 72h after onset of stroke, revealed neuroprotective effects with IAIP treatment in wild type BalB/c mice compared to vehicle treated mice; cortex [**p=0.0032]; striatum [*p=0.0316]; and total hemisphere [**p=0.0032] by two-sample t test adjusted for multiple testing. Data are presented as mean ± SEM.