

RESEARCH ARTICLE | *Integrative Cardiovascular Physiology and Pathophysiology*

## Detrimental effects of 2-arachidonoylglycerol on whole blood platelet aggregation and on cerebral blood flow after a focal ischemic insult in rats

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<sup>1</sup>Physiology Department, School of Medicine, National University of Ireland, Galway, Ireland; and <sup>2</sup>Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, United Kingdom

Submitted 1 June 2017; accepted in final form 3 January 2018

**Shearer JA, Coker SJ, Carswell HVO.** Detrimental effects of 2-arachidonoylglycerol on whole blood platelet aggregation and on cerebral blood flow after a focal ischemic insult in rats. *Am J Physiol Heart Circ Physiol* 314: H967–H977, 2018. First published January 5, 2018; doi:10.1152/ajpheart.00299.2017.—2-Arachidonoylglycerol (2-AG) is a major modulator of blood flow and platelet aggregation and a potential neuroprotectant. The present study investigated, for the first time, the effects of 2-AG on cerebral blood flow (CBF) in the first critical hours during middle cerebral artery occlusion (MCAO) and on platelet aggregation in rats. Adult male Sprague-Dawley rats ( $n = 30$ ) underwent permanent MCAO under isoflurane anesthesia and were randomly assigned to receive either 2-AG (6 mg/kg iv), monoacylglycerol lipase inhibitor JZL-184 (10 mg/kg iv), or vehicle ( $n = 6$  rats/group) treatment. CBF and cardiovascular responses were measured, by a blinded investigator, for up to 4 h. In separate experiments, platelet aggregation by 2-AG (19–300  $\mu$ M) was assessed by whole blood aggregometry ( $n = 40$ ). 2-AG and JZL-184 significantly increased the severity of the CBF deficit versus vehicle ( $20.2 \pm 8.8\%$  and  $22.7 \pm 6.4\%$  vs.  $56.4 \pm 12.1\%$  of pre-MCAO baseline, respectively,  $P < 0.05$ ) but had no effect on blood pressure or heart rate. While JZL-184 significantly increased the number of thrombi after MCAO, this did not reach significance by 2-AG. 2-AG induced platelet aggregation in rat whole blood in a similar manner to arachidonic acid and was significantly reduced by the cyclooxygenase inhibitors indomethacin and flurbiprofen and the thromboxane receptor antagonist ICI 192,605 ( $P < 0.05$ ). This is the first study showing that 2-AG increases the severity of the CBF deficit during MCAO, and further interrogation confirmed 2-AG-induced platelet aggregation in rats. These findings are important because 2-AG had previously been shown to exert neuroprotective actions and therefore force us to reevaluate the circumstances under which 2-AG is beneficial.

**NEW & NOTEWORTHY** 2-Arachidonoylglycerol (2-AG) has neuroprotective properties; however, the present study revealed that 2-AG increases the severity of the cerebral blood flow deficit during middle cerebral artery occlusion in rats. Further interrogation showed that 2-AG induces platelet aggregation in rats. These findings force us to reevaluate the circumstances under which 2-AG is beneficial.

2-arachidonoylglycerol; anti-platelet; cerebral blood flow; cerebral ischemia; endocannabinoids; stroke; thrombosis

### INTRODUCTION

Ischemic stroke is caused by a cerebral blood flow (CBF) deficit due to vessel occlusion by thrombi or emboli and is a

major cause of mortality and morbidity in older age. With a paucity of treatments, new information on the role of potential neuroprotective therapies in ischemic stroke is critical.

The endocannabinoid 2-arachidonoylglycerol (2-AG) induces neuronal survival after brain injury such as traumatic brain injury (TBI) (48), spinal cord injury (1), and cerebral ischemic insult (59). 2-AG can act as a full agonist at both cannabinoid (CB)1 and CB2 receptors and can be rapidly metabolized in vivo to arachidonic acid and prostaglandin glycerol ester through enzyme pathways including monoacylglycerol lipase (MAGL) (16) and cyclooxygenase (34). Cyclooxygenase can further metabolize arachidonic acid to PGG<sub>2</sub> and PGH<sub>2</sub>, which in turn may be converted to thromboxane, prostacyclin, and other prostaglandins, all of which have important physiological and pathological actions. 2-AG can be produced by many cells, including endothelial cells and platelets (47). Taken together, endogenous 2-AG carries tremendous implications for disease prevention and progression.

With regard to effects of 2-AG on endothelial cells, 2-AG has potent vasodilatory effects on the rat middle cerebral artery (MCA) (24) and acts as a potent vasorelaxant against endothelin-1 (9), which is a potent vasoconstrictor linked to CBF deficit after ischemic stroke (42). Therefore, 2-AG-induced vasodilatory effects may alleviate the CBF deficit in ischemic stroke, which in turn, at least in part, may be responsible for the neuroprotective effect of 2-AG after MCA occlusion (MCAO) observed by Wang et al. (59). However, no data are available concerning the effects of 2-AG on CBF during MCAO.

Regarding the effects of 2-AG on platelets, 2-AG is the primary endocannabinoid released from activated platelets (41) with potent proaggregatory effects evident in human platelet studies (30, 41). Platelet aggregation is known to exacerbate the severity of the CBF deficit after MCAO; however, despite evidence that drugs that modify platelet aggregation influence the outcome in rat models of cerebral ischemia (27), no studies, to our knowledge, have assessed 2-AG effects on rat platelets.

Therefore, to establish whether 2-AG alleviates or exacerbates the severity of the CBF deficit during a cerebral ischemic insult, we monitored the effect of 2-AG on CBF in the critical hours after MCAO onset and measured the evolution of pathology. JZL-184 is a potent and selective inhibitor of MAGL and has been shown to increase endogenous 2-AG in the brain at 30 min after administration (38). Therefore, we have addressed our objectives using two strategies, namely, 2-AG treatment to examine exogenous 2-AG effects and JZL-184 treatment to examine endogenous 2-AG effects. We report that exogenous and endogenous 2-AG aggravated rather than alle-

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viated the CBF deficit with a subsequent increase in the evolution of neuronal injury but with no effects on blood pressure (BP) or heart rate (HR). Interrogation of rat platelet effects confirmed, for the first time, potent proaggregatory effects of 2-AG in rats as a potential explanation for the increased CBF deficit caused by 2-AG.

## METHODS

### *Ethical Statement*

All animal experiments were carried out in strict accordance with the United Kingdom Home Office Guidelines on the Operation of the Animals (Scientific Procedures) Act of 1986 (Project License Nos. PPL 60/3775 and 60/3665; Personal License No. PIL 60/11356) and were performed under ethical approval of University of Strathclyde Animal Welfare Ethical Review Body. All experimental design, data analysis, and statistical procedures were performed in accordance with Animal Research Reporting In Vivo Experiments guidelines for reporting in vivo experiments (31).

### *Animals*

Male adult Sprague-Dawley rats (stroke study:  $n = 30$  and platelet aggregation study:  $n = 40$ ) were obtained from in-house breeding in the Biological Procedures Unit, University of Strathclyde. Male animals, used to exclude potential estrous cycle effects (13), were group housed under standard conditions at 21°C on a 12:12-h light-dark cycle with environmental enrichment and ad libitum access to water and food. All efforts were made to minimize animal suffering, and the number of animals used,  $n = 6$  rats/group for the MCAO study and  $n = 5-7$  rats/group for the platelet study, was based on a priori power calculations at 80% power and a significance level of 5% (52) and on previous publications (30, 43, 54). In all experiments, terminal anesthesia was induced and maintained by inhalation of isoflurane. Suitable depth of anesthesia was assessed by lack of a pedal withdrawal reflex or corneal reflex, and this depth of anesthesia was maintained throughout the procedure. Body temperature was monitored continuously with a rectal probe throughout the procedure and maintained within 36.5–37.5°C using a homeothermic blanket (Harvard Apparatus, Kent, Edenbridge, UK).

### *Permanent Cerebral Ischemia: MCAO Model*

**Induction of ischemia and measurement of CBF.** Anesthesia was induced in 30 male Sprague-Dawley rats (275–350 g, 8–10 wk old) by inhalation of isoflurane (5% for induction, 2.25–2.5% for maintenance via artificial ventilation) in 30% O<sub>2</sub>-70% N<sub>2</sub>O at 1 l/min. Physiological measurements were monitored with a pulse oximeter/capnograph (Medair Life-Sense), and a femoral artery was cannulated for continuous BP monitoring and arterial blood gas measurement before and at 2 and 4 h after occlusion. A venous cannula was inserted for drug administration. CBF was monitored throughout the procedure using a laser-Doppler probe (Moor Instruments, Axminster, UK) in the cortical region distal to the MCA at approximately anterior/posterior –3 mm, medial/lateral 4 mm. Readings were recorded beginning 60 min before the induction of ischemia, at 15-min intervals, and data were expressed as a percentage of the baseline value. Permanent MCAO was induced by a 5-0 silicon-coated monofilament (50-3033, Docol, Sharon, MA), which was inserted into the left external carotid artery and advanced along the internal carotid artery to occlude the origin of the MCA (39). Sham-operated animals underwent MCAO surgery without insertion of the filament. Animals were maintained for up to 4 h after the onset of MCAO, as neuroprotective effects of 2-AG have been shown to be mediated by a reduction in the early (1–4 h) inflammatory response (48) or early endogenous activation of CB receptors (2). Therefore, the 4 h time point not only allows cardiovascular and cerebrovascular responses to be monitored continuously

during MCAO but also allows insights into the effects of 2-AG on the evolution of neuronal injury and the early microglial response.

A priori exclusion criteria were any rat that exhibited no ischemic injury on histological examination, showed signs of rupture of the internal carotid artery, whose laser-Doppler signal was not reduced to  $\leq 60\%$  of baseline following filament insertion, whose physiological measurements were outside normal parameters based on Flecknell (18), or whose oxygen saturation remained below 90%. On the basis of these criteria, no animals were excluded from the present study.

**Treatment protocol.** Rats were randomly assigned to five groups ( $n = 6$  rats/group). Work was done in six batches. For each batch, the names of the five groups were written on one piece of paper each, crumpled up, and selected out of a beaker by a third party. This process was repeated for each batch of experiments (or  $n$ ) with a different third party each time. In the treatment groups, animals received either 2-AG (1.2 mg/kg iv bolus over 2 min followed by 4-h infusion to give a total dose of 6 mg/kg), the MAGL inhibitor JZL-184 (5 mg/kg iv bolus over 5 min followed by 4-h infusion to give a total dose of 10 mg/kg), or vehicle (PEG-300 iv) starting 15 min before MCAO. In two groups, rats underwent either MCAO surgery without treatment or sham surgery. The dose of 2-AG used in this study was based on Panikashvili et al. (49) and Wang et al. (59), who both used a single dose of 2-AG at 5 mg/kg (ip bolus) to test effects on neuronal damage after traumatic brain injury and cerebral ischemia, respectively. Given that the total dose in the present study was not administered until the end of the occlusion period, an additional 20% of 2-AG and JZL-184 was used.

**Termination and histological processing.** The experimenter was blinded to the experimental group to which the animal belonged (treatment received, stroke, or sham surgery) in all analyses by recoding of animals by an independent investigator. At the end of each experiment, animals were euthanized quickly by exsanguination and decapitation, and the brains were immediately and carefully removed and frozen in isopentane (–42°C) in an attempt to maintain structural integrity and morphology, avoid autolysis, and avoid pressure on the unfixed brain, which might have led to morphological artifacts (29, 61). Tissue was stored at –20°C for histological analysis. For the measurement of injury volume, eight coronal sections (20  $\mu$ m thick) were taken from distinct neuroanatomic regions within the forebrain (45) and stained with hematoxylin and eosin. Hematoxylin and eosin-stained sections were viewed under a light microscope to identify damaged tissue based on neuronal morphology. Irreversibly damaged neurons were pyknotic (shrunken or triangular in shape), and the surrounding neuropil was disrupted and displayed pallor. The injury was transcribed onto line diagrams of the eight coronal levels for quantification as previously described (60). The injury boundary was transcribed based on its relation to anatomic landmarks shown on the line diagrams. The use of line diagrams prevented quantification of injury volume from being influenced by tissue edema. Each line diagram was calibrated before use by confirming that the area of each coronal section was measured accurately. The injury area in each line diagram was then quantified using MCID image analysis software. The injury volume was then calculated from the area under the curve measured at each of the eight sections against the interaural (IA) distance, where  $y$  intersected  $x$ -axes at 12.72 mm IA and 0 mm IA, respectively, and expressed in cubic millimeters.

Immunofluorescence was performed to identify and quantify microglia numbers and activation, based on cell morphology, within the MCA territory at the coronal level of the globus pallidus (2.92 mm IA) and anterior hippocampus (5.88 mm IA). Microglia were labeled using a goat polyclonal antibody for ionized Ca<sup>2+</sup>-binding adaptor molecule 1 (Iba-1; 1:250) and FITC-conjugated rabbit anti-goat secondary antibody (1:200). Cell nuclei were stained with Vectashield mounting medium containing DAPI. Thrombi occluding vessels in the ischemic hemisphere were identified by double labeling with rabbit antibody for CD41 (integrin- $\alpha$ II, 1:250) and mouse antibody for CD62P (P-selectin, 1:100) and appropriate secondary antibodies

(FITC-conjugated goat anti-rabbit and TRITC-conjugated goat anti-mouse antibodies).

Multilayer *z*-stack images (1  $\mu\text{m}$ ) were taken at the cortical injury boundary in the ipsilateral hemisphere and corresponding contralateral regions using a laser scanning confocal microscope (Leica Microsystems, Milton Keynes, UK) and analyzed using Velocity image-analysis software (Perkin-Elmer, Cambridge, UK). Activated microglia cells were clearly identified by a skilled observer on the basis of their morphology, including an enlarged nucleus and shorter, thicker processes. For each image, the number of microglia present was quantified and the number of activated microglia was expressed as a percentage of the total number of microglia present.

#### Whole Blood Aggregometry

Whole blood aggregometry measures changes in impedance between two electrodes as platelets adhere without the removal of other blood constituents that can affect platelet function (21). Forty naïve adult male Sprague-Dawley rats (300–500 g, aged 10–16 wk) were anesthetized with 5% isoflurane in 100%  $\text{O}_2$  at 1 l/min and maintained with 2–2.75% isoflurane in  $\text{O}_2$  delivered via a nose cone. A carotid artery was dissected free of connective tissue and cannulated with polythene tubing containing heparinized saline (10 U/ml). A 9- to 10-ml volume of arterial blood was withdrawn into heparinized syringes (20 U/ml of whole blood), diluted 1:1 with 0.9% (wt/vol) NaCl solution (saline), and stored at room temperature before aggregometry experiments. After blood withdrawal, rats were euthanized with an overdose of 1 ml pentobarbital sodium (Euthatal, 200 mg/ml iv). The diluted blood from each rat was divided into 1-ml aliquots in plastic cuvettes and warmed to 37°C for 5–10 min before any aggregating agents were added. Platelet aggregation was measured using a two-channel Chrono-log 590 whole blood aggregometer (Labmedics, Stockport, UK), which measured changes in impedance across electrodes to which platelets and platelet aggregates adhered (7). After the addition of an aggregatory agent, activated platelets will adhere and impede conductance of the electrical current between the electrodes.

**Treatment protocol.** After an equilibrium period, platelet aggregation was induced with ADP (0.1–30  $\mu\text{M}$ ,  $n = 5$ –6), 2-AG ( $n = 6$ –13), and arachidonic acid ( $n = 4$ –11) at 19–300  $\mu\text{M}$ , and responses were measured at the peak or after 10 min, respectively. In further experiments, aggregation to ADP (1  $\mu\text{M}$ ), 2-AG (150  $\mu\text{M}$ ), and arachidonic acid (75  $\mu\text{M}$ ) was examined in the absence and presence of the cyclooxygenase inhibitors indomethacin (3 and 10  $\mu\text{M}$ ) and flurbiprofen (10  $\mu\text{M}$ ) and the thromboxane receptor (TP) antagonist ICI-192,605 (1  $\mu\text{M}$ ) added 10 min before the aggregating agent ( $n = 6$ –7). Aggregation to 2-AG (150  $\mu\text{M}$ ) and arachidonic acid (75  $\mu\text{M}$ ) was examined in the absence and presence of the fatty acid amide hydrolase (FAAH) inhibitor URB597 (0.3  $\mu\text{M}$ ) and MAGL inhibitor JZL-184 (0.1  $\mu\text{M}$ ) added alone or in combination with ICI-192,605 (1  $\mu\text{M}$ ) 10 min before the aggregating agent ( $n = 6$ ). To confirm the role of MAGL, in an additional set of experiments, JZL-184 (1  $\mu\text{M}$ ) was preincubated with blood for 30 min at 37°C before addition of the aggregating agent ( $n = 5$ –7). Interactions of 2-AG and arachidonic acid with 1  $\mu\text{M}$  ADP were studied using concentrations that produced a small aggregation response when added alone (75  $\mu\text{M}$  2-AG and 38  $\mu\text{M}$  arachidonic acid), added 1 min before ADP, and the response was measured at 10 min ( $n = 12$ ). Interactions of 2-AG and arachidonic acid with ADP were also studied in the presence of cyclooxygenase inhibitors indomethacin and flurbiprofen and the TP antagonist ICI-192,605 added 10 min before the first aggregating agent ( $n = 6$ ).

#### Data and Statistical Analysis

Values are expressed as means  $\pm$  SE of  $n$  rats. In MCAO experiments, multiple treatment groups were compared using one-way ANOVA with Bonferroni correction or a paired *t*-test for ipsilateral versus contralateral hemisphere comparisons. Physiological param-

eters and CBF were recorded at baseline and at 1-h intervals throughout the experimental period and compared using mixed-model repeated-measures ANOVA. In the whole blood aggregometry experiments, aggregation responses in the presence and absence of antagonists or inhibitors and agonist interactions were compared in aliquots of blood taken from a single animal. Since some responses were not normally distributed, as determined by the Shapiro-Wilk test, aggregation responses in the absence and presence of antagonists or inhibitors were compared using Friedman tests. For agonist interactions, area under the curve values were calculated. A probability of  $P < 0.05$  was considered to be statistically significant for all comparisons.

## RESULTS

### 2-AG Increased the CBF Deficit During MCAO With No Effects on BP or HR

To assess the effects of endogenous and exogenous 2-AG on local cortical blood flow during MCAO, we monitored CBF by laser-Doppler flowmetry throughout the experiment. A significant increase in the severity of the CBF deficit was observed during MCAO in 2-AG- and JZL-184-treated animals compared with vehicle treatment (Fig. 1). After placement of the microvascular clip on the left internal carotid artery, blood flow dropped to  $\sim 80\%$  of baseline levels as seen in sham animals [as previously described (43)]. Perfusion was further decreased upon insertion of the filament in vehicle-treated and untreated MCAO groups, to  $\sim 45\%$  of baseline flow, where it remained stable throughout the occlusion period. However, there was a further significant reduction in CBF to  $\sim 20\%$  of baseline levels in 2-AG- and JZL-184-treated animals versus vehicle treatment (Fig. 1).

Given that the potential hypotensive effects of 2-AG (for a review, see Ref. 46) might explain the observed decreased CBF, we monitored BP and HR throughout the experiment. However, we found no significant difference in 2-AG- and JZL-184-treated groups compared with vehicle treatment for BP or HR (Fig. 2, A and B). In addition, blood gases and body

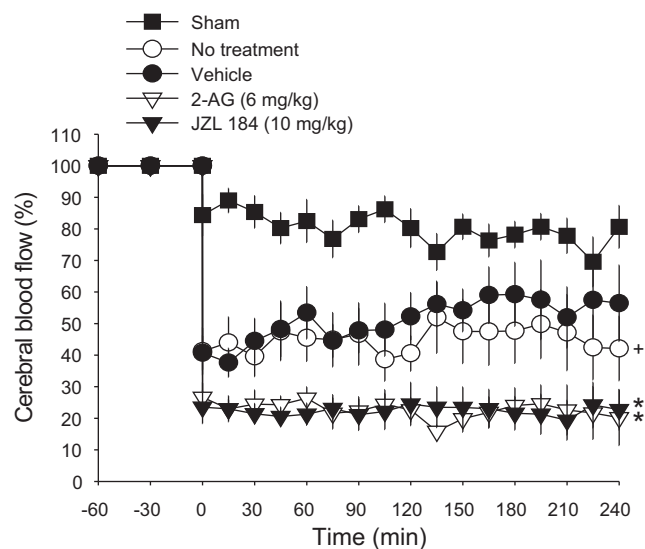


Fig. 1. Cortical cerebral blood flow monitored before and during middle cerebral artery occlusion (means  $\pm$  SE;  $n = 6$  for all groups). 2-AG, 2-arachidonoylglycerol. + $P < 0.05$  for untreated vs. sham groups and \* $P < 0.05$  vs. the vehicle-treated group by mixed-model repeated-measures ANOVA followed by multiple comparisons with Bonferroni correction.

temperature were tightly controlled, with no significant differences among the groups (Table 1).

To determine the effect of this reduction in CBF on the evolution of pathology, we measured early neuronal perikaryal damage and early microglial activation at the termination of the experiment. Neuronal damage in the cortex was significantly increased by ~40% and 50% by 2-AG and JZL-184, respectively, versus vehicle at 4 h after MCAO (Fig. 3, A and C, and Fig. 4). Increased neuronal damage was not evident in subcortical regions, consistent with the MCA branches supplying the striatum, being end arteries with no collateral flow, unlike cortical branches (19). Microglia activation occurs as early as 3–4 h after stroke and is known as a key initial event in the evolution of damage after stroke (50); however, the efficacy of 2-AG to modulate microglia after stroke has not been studied. Therefore, the microglial response was assessed in the present study in brains at 4 h after MCAO. Whereas there was a significant increase in activation in the ipsilateral hemisphere versus contralateral, there were no significant effects of 2-AG or JZL-184 compared with vehicle control (Fig. 3, B and D). 2-AG has previously been shown to induce platelet aggregation in human blood. As such, we also assessed thrombus formation in the ischemic hemisphere in the present study to investigate the potential role of platelet aggregation in mediating the effect of 2-AG. There was a significant increase in the number of thrombi in the ipsilateral hemisphere after treatment with JZL-184 (Fig. 3, E and F). However, this did not reach significance in 2-AG-treated animals. Collectively, these data show that exogenous and endogenous 2-AG aggravated the deficit in CBF with potential pathological consequences, most likely not mediated via changes in BP or HR.

#### 2-AG Induced Platelet Aggregation Via the Cyclooxygenase Pathway and Thromboxane A<sub>2</sub>

To further interrogate mechanisms of 2-AG-induced exacerbation of ischemia, proaggregatory effects of 2-AG, previously shown in human platelets, were studied for the first time in rats. 2-AG induced platelet aggregation over a concentration range of 19–300  $\mu$ M with a concentration response curve comparable to that of arachidonic acid, which were both less potent than ADP with maximum responses of  $12.0 \pm 2.1 \Omega$  ( $n = 6$ ) and  $11.7 \pm 0.5 \Omega$  ( $n = 11$ ) for 2-AG and arachidonic acid, respectively, and  $18.7 \pm 1.5 \Omega$  ( $n = 6$ ) for ADP (Fig.

5A). 2-AG (150  $\mu$ M) produced slowly developing platelet aggregation with the maximum response occurring at the end of the 10-min observation period (Fig. 5B). A similar aggregation response was observed with arachidonic acid (150  $\mu$ M; Fig. 5B). In contrast, ADP (1  $\mu$ M) produced a rapid and reversible response, which peaked at 2 min (Fig. 5B).

2-AG can be metabolized to arachidonic acid with the further production of proaggregatory mediators. Given the similar profiles of 2-AG- and arachidonic acid-induced aggregation, the role of arachidonic acid metabolism and production of proaggregatory mediators in the response to 2-AG were assessed. The cyclooxygenase inhibitors indomethacin (10  $\mu$ M) and flurbiprofen (10  $\mu$ M) and the TP antagonist ICI-192,605 (1  $\mu$ M) significantly abolished aggregation induced by 2-AG and arachidonic acid (Fig. 6, A and B). 2-AG-induced aggregation was also significantly reduced by indomethacin at 3  $\mu$ M (Fig. 6A); however, this concentration did not affect the response to arachidonic acid (Fig. 6B).

To establish whether MAGL is responsible for the release of arachidonic acid in 2-AG-induced aggregation, we used a selective inhibitor of MAGL (JZL-184) as well as FAAH (URB597). 2-AG- and arachidonic acid-induced aggregation were not altered by URB597 (0.3  $\mu$ M) or JZL-184 (0.1  $\mu$ M) (Fig. 7). In addition, attenuation of the 2-AG response by the TP antagonist ICI-192,605 was not prevented by either URB597 or JZL-184. To confirm the role of MAGL in rat platelets, an additional set of experiments was performed in which a higher concentration of JZL-184 (1  $\mu$ M) was preincubated with blood for a longer period (30 min at 37°C) before the addition of the aggregating agent. In these experiments, JZL-184 (1  $\mu$ M) significantly inhibited the aggregation response to both 2-AG ( $n = 5$ ) and arachidonic acid ( $n = 7$ ) (Fig. 7).

2-AG is known to interact with other physiological agonists to enhance aggregation in human platelet-rich plasma (40). We investigated whether 2-AG/ADP-enhanced aggregation involved arachidonic acid metabolism and production of thromboxane A<sub>2</sub>, as previously described by Baldassarri et al. (3), in human platelets. Suboptimal concentrations of 2-AG (75  $\mu$ M) and arachidonic acid (38  $\mu$ M) prolonged and potentiated ADP-induced aggregation, which was significantly reduced by flurbiprofen (Fig. 8, A and B) and partially reduced by indomethacin at 10  $\mu$ M and ICI-192,605 (Fig. 8A). In contrast, indo-

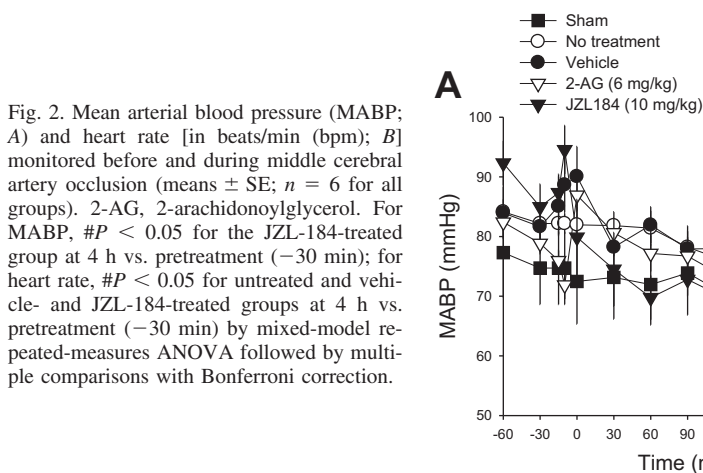


Fig. 2. Mean arterial blood pressure (MABP; A) and heart rate [in beats/min (bpm); B] monitored before and during middle cerebral artery occlusion (means  $\pm$  SE;  $n = 6$  for all groups). 2-AG, 2-arachidonoylglycerol. For MABP,  $\#P < 0.05$  for the JZL-184-treated group at 4 h vs. pretreatment ( $-30$  min); for heart rate,  $\#P < 0.05$  for untreated and vehicle- and JZL-184-treated groups at 4 h vs. pretreatment ( $-30$  min) by mixed-model repeated-measures ANOVA followed by multiple comparisons with Bonferroni correction.

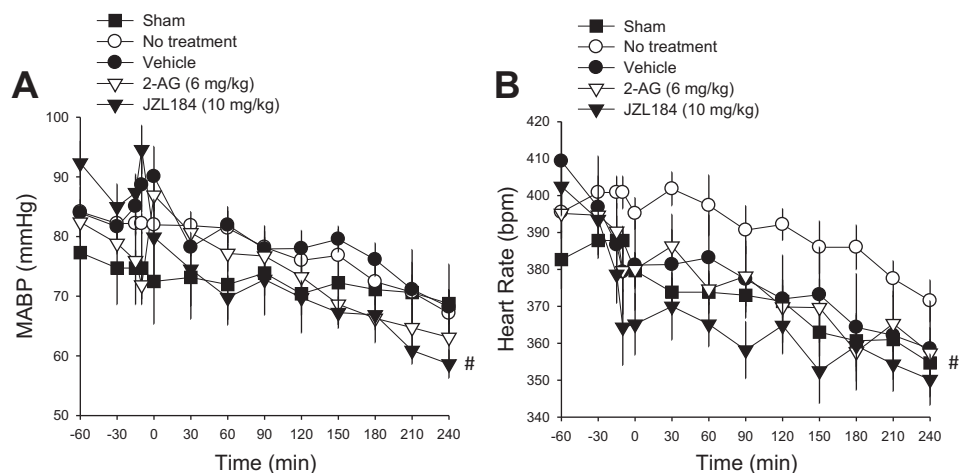


Table 1. Physiological data recorded before (0 h) and at 2 and 4 h after middle cerebral artery occlusion

Parameter	Sham			Untreated			Vehicle			2-Arachidonylglycerol			JZL-184		
	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
pH	7.38 ± 0.02	7.35 ± 0.02	7.34 ± 0.02	7.36 ± 0.02	7.35 ± 0.01	7.32 ± 0.02	7.39 ± 0.02	7.35 ± 0.02	7.34 ± 0.02	7.35 ± 0.02	7.35 ± 0.02	7.32 ± 0.02	7.36 ± 0.02	7.35 ± 0.02	7.33 ± 0.02
Pco <sub>2</sub> , mmHg	31.7 ± 3.0	36.9 ± 1.2	34.6 ± 3.0	37.9 ± 3.3	35.7 ± 2.7	41.2 ± 1.9	30.9 ± 2.7	34.0 ± 1.4	39.4 ± 1.3	34.7 ± 2.2	39.9 ± 4.0	45.3 ± 1.4	33.0 ± 3.0	39.8 ± 1.5	45.1 ± 1.6
Po <sub>2</sub> , mmHg	129.7 ± 10	104.5 ± 5.6	107 ± 8.7	103.5 ± 6.0	94.8 ± 3.1	87.5 ± 3.0	125.7 ± 10.3	105.5 ± 9.8	98.5 ± 10.1	115.8 ± 6.0	99.0 ± 9.1	90.5 ± 4.9	129.7 ± 8.4	98.5 ± 4.3	94.0 ± 3.8
Temperature, °C	37.1 ± 0.1	37.0 ± 0.1	36.9 ± 0.1	36.5 ± 0.2	37.0 ± 0.2	36.7 ± 0.1	36.9 ± 0.2	37.0 ± 0.1	36.8 ± 0.2	36.8 ± 0.1	36.8 ± 0.3	36.9 ± 0.1	37.0 ± 0.1	37.1 ± 0.2	37.1 ± 0.1

Values are expressed as means ± SE; n = 6 for all data.

methacin at 3 μM significantly increased 2-AG potentiation of ADP (n = 6; Fig. 8A).

## DISCUSSION

The major finding in the present study is that 2-AG and JZL-184 aggravated the severity of the CBF deficit in the first few pivotal hours during MCAO compared with vehicle, which led to a different evolution of brain injury.

To our knowledge, this is the first study to investigate the effects of 2-AG and JZL-184 on CBF during stroke. The first few hours after permanent MCAO are critical to the development of neuronal damage, as the fate of vulnerable, but potentially viable, tissue surrounding the core is dependent on residual blood flow through collaterals, and the duration and severity of ischemia (23). The laser-Doppler probe was placed above the region perfused by the MCA to measure collateral blood flow in numerous small vessels supplied by the anterior communicating and anterior cerebral arteries during the first 4 h. After intraluminal filament insertion, there was a sharp reduction in CBF, which stabilized at 45% of the baseline signal in the vehicle-treated and untreated groups, indicating successful occlusion of the MCA (28), as confirmed by the presence of neuronal perikaryal damage. There was a further significant reduction in CBF to ~20% of baseline levels in 2-AG-treated animals versus vehicle treatment. This result was reproduced by pharmacological enhancement of endogenous 2-AG using JZL-184. In addition, the enhanced CBF deficit resulted in a significant increase in early neuronal damage in 2-AG- and JZL-184-treated groups versus the vehicle-treated group. 2-AG and JZL-184 administration were started 15 min before MCAO, but CBF baseline monitoring was started 60 min before MCAO. Therefore, it can be seen from our data that there was no influence of either agent before (independently of) the induction of cerebral ischemia. Taken together, we are confident that the laser-Doppler flow differences observed in the present study were indeed due to 2-AG-induced aggravation of the CBF deficit during MCAO, probably due to reduced blood flow through collaterals, given the placement of the laser-Doppler probe. Although the present results are perhaps surprising, given that 2-AG is a known vasodilator (24), they provide new information about the role of 2-AG in ischemic stroke in rats and provide some insights into the published results from recent clinical studies that have shown that use of cannabis increased stroke risk (56).

If CBF drops below a certain threshold, tissue damage will be extended (25). To assess whether the drop in CBF by 2-AG and JZL-184 affects the evolution of ischemic injury, we assessed early pathology by identifying pyknotic neurons and activated microglia histologically postmortem at 4 h post-MCAO. 2-AG or JZL-184 did not significantly increase microglial activation as detected by Iba-1 immunostaining. Iba-1 is highly and specifically expressed in monocytic cell lines, including peripheral macrophages as well as both resting and activated microglia (27). However, at this early time point, macrophages are unlikely to have infiltrated the brain parenchyma (51). A significant increase in microglial activation in the ipsilateral hemisphere versus contralateral was observed, which is consistent with the literature at 4 h after MCAO (50), indicating that the lack of effect of 2-AG and JZL-184 on microglial activation is likely to be a valid result and not a type

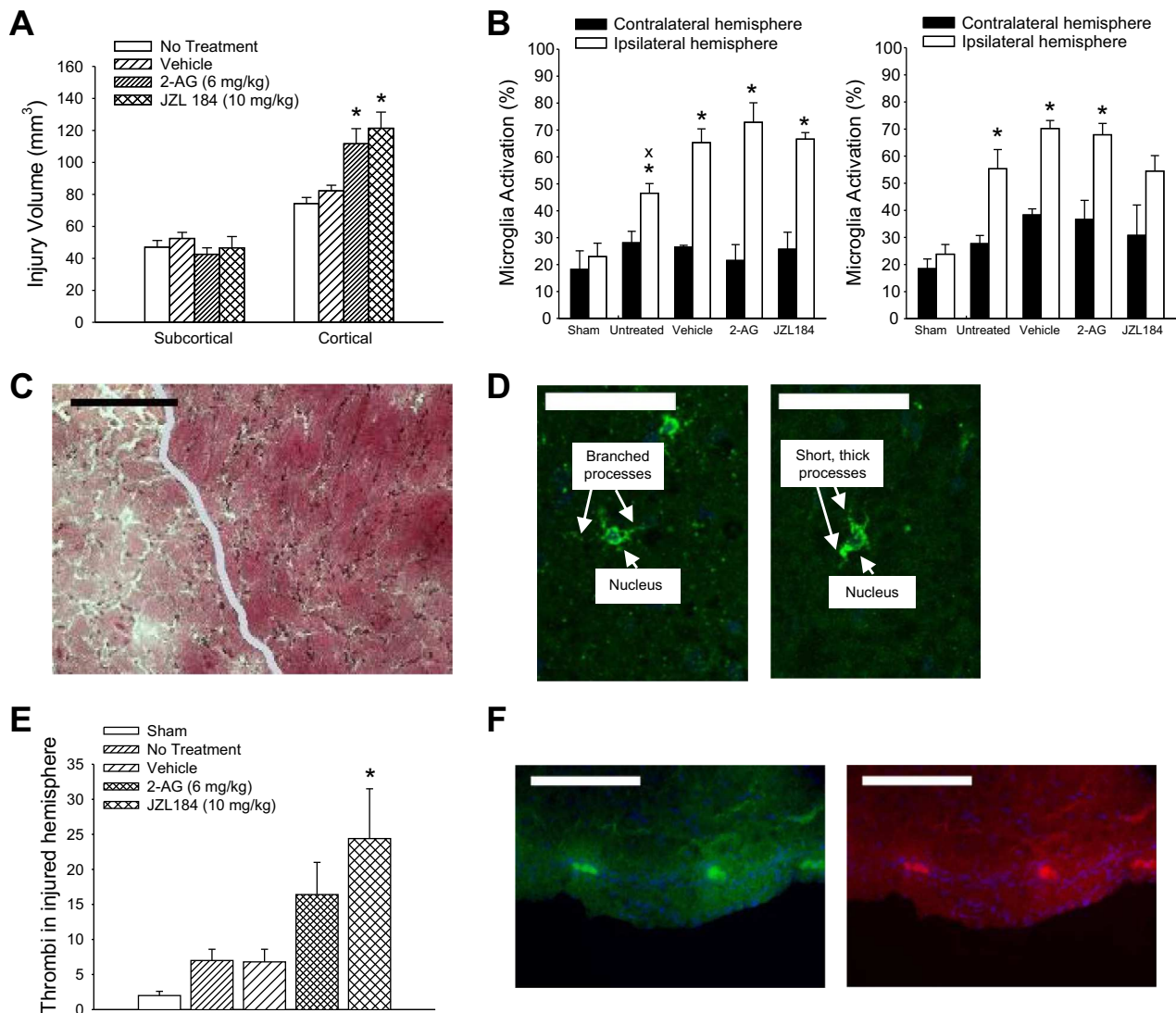


Fig. 3. *A*: injury volume at 4 h after middle cerebral artery occlusion. *B*: microglia activation in the cortex at the level of the globus pallidus (*left*) and anterior hippocampus (*right*) for sham, untreated, vehicle-treated, 2-arachidonoylglycerol (2-AG)-treated, and JZL-184-treated groups. *C*: representative image of a hematoxylin and eosin-stained section showing pyknotic shrunken nuclei and pale neuropil with vacuolated cytoplasm in injured tissue (*left*) vs. large nuclei and strongly stained, undisrupted cytoplasm in viable tissue (*right*) (line represents the boundary for injured vs. viable tissue; magnification:  $\times 200$ ). *D*: representative confocal images ( $\times 400$ , compressed *z*-stack) showing an example of resting (*left*) and activated (*right*) microglia (scale bar = 50  $\mu\text{m}$ ). *E*: thrombus formation in the ischemic hemisphere at 4 h after middle cerebral artery occlusion in sham, untreated, vehicle-treated, 2-AG-treated, and JZL-184-treated groups. *F*: representative image of positive staining for CD41 (green) and CD62P (red) showing thrombus formation in the ipsilateral hemisphere (scale bar = 100  $\mu\text{m}$ ). Values are means  $\pm$  SE;  $n = 6$  for all groups for injury,  $n = 4-6$  for microglia activation, and  $n = 4-5$  for thrombus formation. For injury volume,  $*P < 0.05$  vs. the vehicle-treated group; for microglial activation and thrombus formation,  $*P < 0.05$  vs. the contralateral hemisphere by paired *t*-test.  $*P < 0.05$  vs. the sham group by one-way ANOVA followed by multiple comparisons with Bonferroni correction for treatment group comparisons.

II (false negative) error. Whereas microglia become activated by 4 h after MCAO (50), microglial activation peaks within 2–3 days after MCAO (36). Therefore, further studies at a later time point postocclusion ( $\geq 24$  h) are needed to conclude whether changes in microglial activation across different experimental groups exist. On the other hand, 2-AG and JZL-184 increased the development of neuronal perikaryal damage at 4 h after MCAO in the present study. In the present study, we used fresh-frozen brains for analysis rather than perfused brains to allow examination of intravascular cellular responses (platelets and thrombi). This approach has the potential to generate histological artifacts; therefore, due care was taken to exsanguinate quickly and remove the brains carefully into

$-42^{\circ}\text{C}$  to maintain structural integrity and morphology, avoid autolysis, avoid pressure on the unfixed brain, and avoid dark neuron, vacuole, or pigment artifacts (29, 61). Although it is recognized that early ischemic neuronal changes are histologically detectable at this early 4 h time point (58), the infarction is not fully evolved, and the effects of intravenous 2-AG on ischemic damage needs to be further investigated in future studies with a longer (24–48 h) survival time point to draw conclusions on final infarct size. Wang et al. (59) showed that 2-AG induced neuroprotection 24 h after MCAO, implying that the early increase in injury in the present study was likely to be an acceleration in evolution of damage rather than a definitive exacerbation of infarct. Whether a different dose of

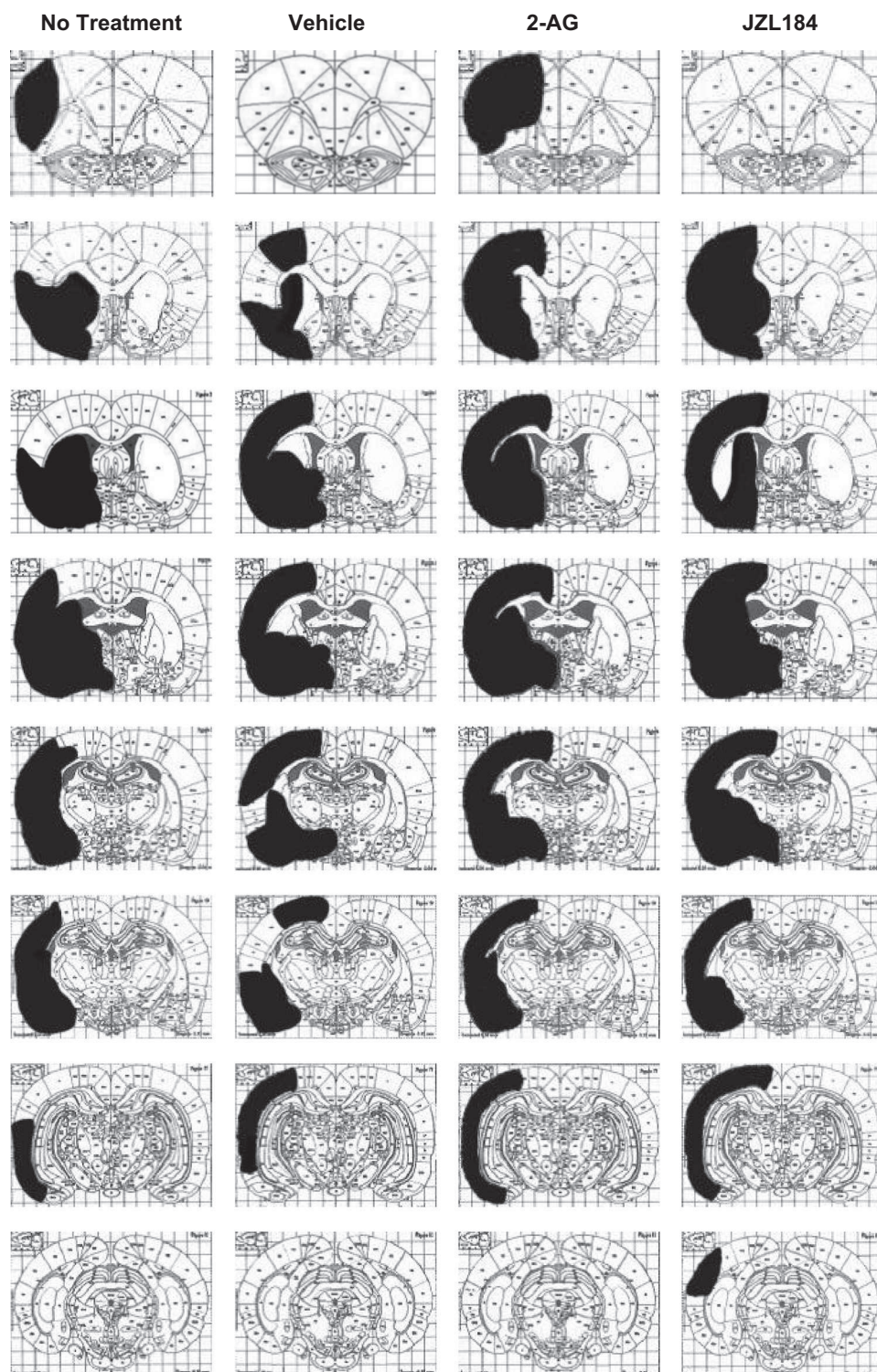


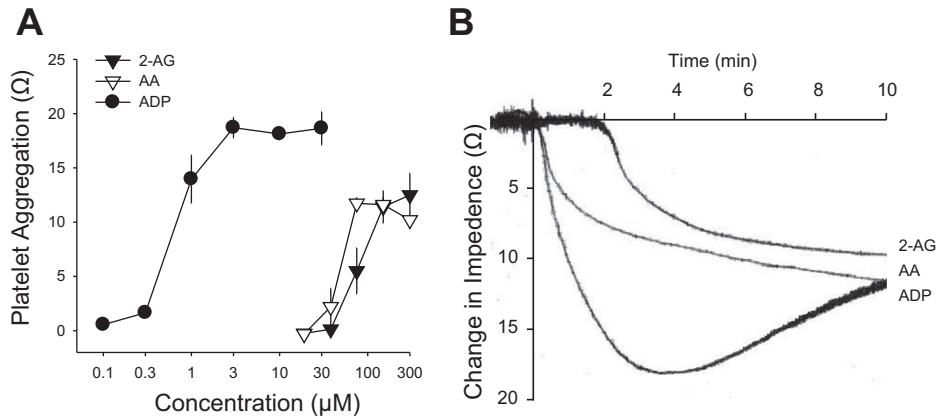
Fig. 4. Representative line diagrams showing the injury topography observed in untreated, vehicle-treated, 2-arachidonoylglycerol (2-AG)-treated, and JZL-184-treated animals.

2-AG in the present study may be beneficial despite the deleterious effects of platelet aggregation is worthy of note; however, Wang et al. used a single dose similar to that in the present study. Also worthy of note is that Wang et al. administered 2-AG by an intraperitoneal bolus rather than intravenous infusion, which was chosen in the present study to increase the likelihood of exogenous 2-AG reaching the ischemic tissue, given its high labile nature in rat plasma (33) and

which may lead to greater platelet effects than an intraperitoneal bolus.

The mechanisms behind the aggravation of the CBF deficit during MCAO in the present study are currently unclear. Parameters such as age, body temperature, BP, HR, and arterial blood gases were similar in 2-AG- and JZL-184-treated animals versus the vehicle control and are unlikely to account for the aggravation of the CBF deficit, implying some other

Fig. 5. *A*: platelet aggregation responses to 2-arachidonoylglycerol (2-AG; 19–300  $\mu$ M,  $n = 6$ –13) and physiological platelet agonists arachidonic acid (AA; 19–300  $\mu$ M,  $n = 4$ –11) and ADP (0.1–30  $\mu$ M;  $n = 5$ –6). *B*: representative aggregation responses to a single concentration of ADP (1  $\mu$ M), 2-AG (150  $\mu$ M), and AA (150  $\mu$ M).



underlying cerebrovascular effects of 2-AG. Vasodilatory action on uncompromised vessels is known to cause redistribution of blood away from compromised regions if auto-regulation is failing, known as the “steal” phenomenon (20). However, the steal phenomenon after cerebrovasodilators still remains controversial (for a review, see Ref. 35), and

whether this explains the increased severity of CBF deficit by 2-AG after stroke needs further investigation.

After MCAO, the cerebral vasculature becomes prothrombotic (10, 14), and platelet aggregation contributes significantly to MCAO-induced focal cerebral ischemia (11). We showed that, whereas JZL-184 induced a significant increase in the number of thrombi in the ipsilateral hemisphere, this significant increase was not apparent in 2-AG-treated animals. Whether this reflects a slightly more potent or local effect of endogenous 2-AG on thrombi is unclear. 2-AG is a known proaggregant in human platelets (30) but has not yet been studied in rat platelets. Therefore, to establish whether proaggregation is a plausible mechanism of exacerbation of the ischemic deficit in our rat model, we confirmed that 2-AG does indeed induce aggregation of rat platelets. Following this line of thought, increased neuronal perikaryal damage was not evident in subcortical regions, consistent with the MCA branches supplying the striatum, being end arteries with no collateral flow (37, 53), which would be expected to be more resistant to the detrimental influence of proaggregatory effects of 2-AG in permanent MCAO models.

In the present study, 2-AG induced aggregation of rat platelets at similar concentrations used in human platelet studies (5, 30, 40), indicating that any species differences in the stability of 2-AG in plasma between rat and humans (33) is unlikely to have influenced the response to 2-AG in the present study. Washed human platelet studies showed a maximal effect of 2-AG at lower concentrations (10  $\mu$ M) (3, 55), probably due to lack of other blood components (blood cells, enzymes, or plasma proteins) that affect the labile nature of 2-AG and/or platelet function (21).

2-AG and arachidonic acid induced similar aggregation responses, which were reduced markedly by cyclooxygenase inhibitors and a TP antagonist, indicating that 2-AG induces aggregation of rat platelets through metabolism of arachidonic acid via the cyclooxygenase pathway and production of the proaggregatory mediator thromboxane A<sub>2</sub>. This mechanism is in agreement with work in human whole blood (5, 30) and washed platelets (3), where 2-AG stimulated the synthesis of thromboxane A<sub>2</sub> in human platelets (3). However, an earlier study (40) in platelet-rich plasma did not show any sensitivity of the 2-AG response to cyclooxygenase inhibition. This discrepancy may again be explained by the different methodologies of platelet analysis (22). In the present study, whole blood

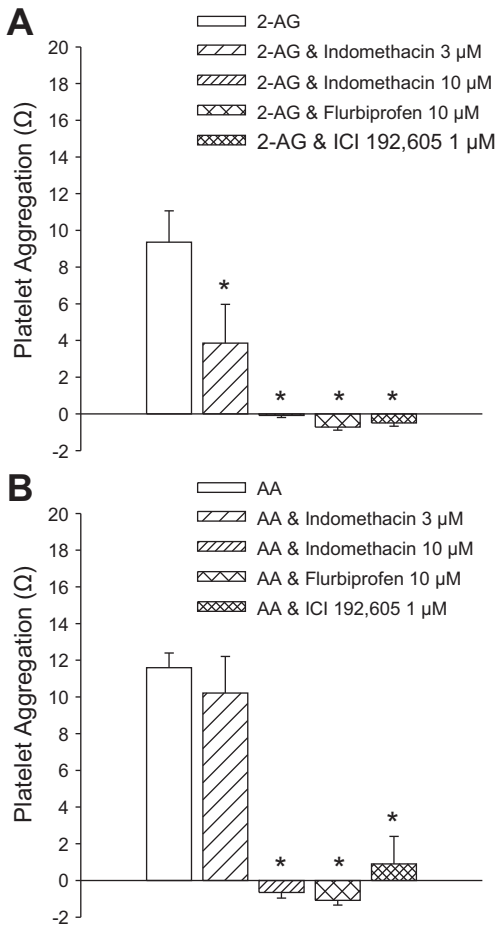


Fig. 6. Effect of the cyclooxygenase inhibitors indomethacin (3 and 10  $\mu$ M) and flurbiprofen (10  $\mu$ M) as well as the thromboxane receptor antagonist ICI-192,605 (1  $\mu$ M) on platelet aggregation induced by 2-arachidonoylglycerol (2-AG; 150  $\mu$ M; *A*) and arachidonic acid (75  $\mu$ M; *B*).  $n = 6$  each group. \* $P < 0.05$  vs. agonist alone by Friedman test.



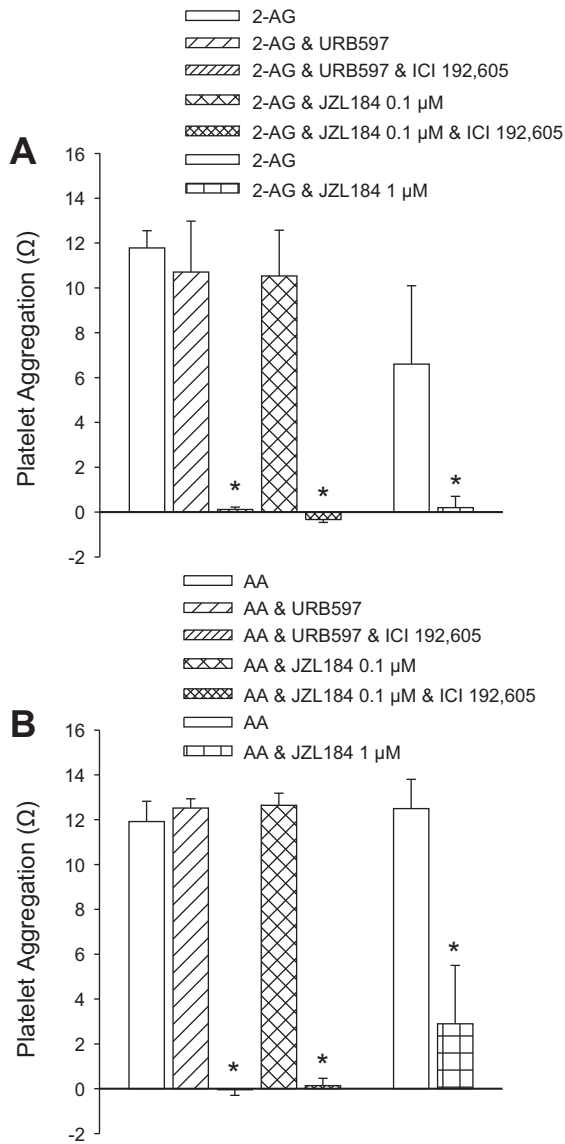


Fig. 7. Effect of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) inhibitors URB597 (0.3  $\mu\text{M}$ ) and JZL-184 (0.1 and 1  $\mu\text{M}$ ), given alone and in combination with the thromboxane receptor antagonist ICI-192,605 (1  $\mu\text{M}$ ), on aggregation induced by 2-arachidonoylglycerol (2-AG; 150  $\mu\text{M}$ ,  $n = 5-6$ ; A) and arachidonic acid (AA; 75  $\mu\text{M}$ ,  $n = 6-7$ ; B). \* $P < 0.05$  vs. agonist alone by Friedman test.

aggregometry was used to allow platelet function to be evaluated in its physiological milieu, in the presence of red blood cells and leukocytes, which can modulate platelet aggregation (17), more akin to the *in vivo* microenvironment.

Our findings that neither the FAAH inhibitor nor the MAGL inhibitor altered the aggregation response to 2-AG or arachidonic acid are in agreement with some human platelet studies (3, 40, 55) but not others (5, 30). The reasons for this discrepancy are unclear. We used incubation periods (10 min) of JZL-184 (0.1  $\mu\text{M}$ ), similar to those in Straiker et al. (57) and Chouinard et al. (12), although JZL-184 is known to produce time-dependent inhibition (maximal at 40 min) (38). However, in the present study, longer incubations of JZL-184 of 1  $\mu\text{M}$  for 30 min (8, 44) inhibited both 2-AG- and arachidonic

acid-induced platelet aggregation, implying a nonselective role of JZL-184 at longer incubations.

Taken together, our data suggest that 2-AG is unlikely to induce platelet aggregation in rat whole blood through metabolism by FAAH or MAGL to form arachidonic acid. Rather, it is possible that 2-AG may induce aggregation via phospholipase  $A_2$ -mediated release of arachidonic acid or via metabolism of 2-AG to arachidonic acid by other enzymes, such as cyclooxygenase-2, or plasma esterases (33), which are present in whole rat blood. In addition, given the reduction in poten-

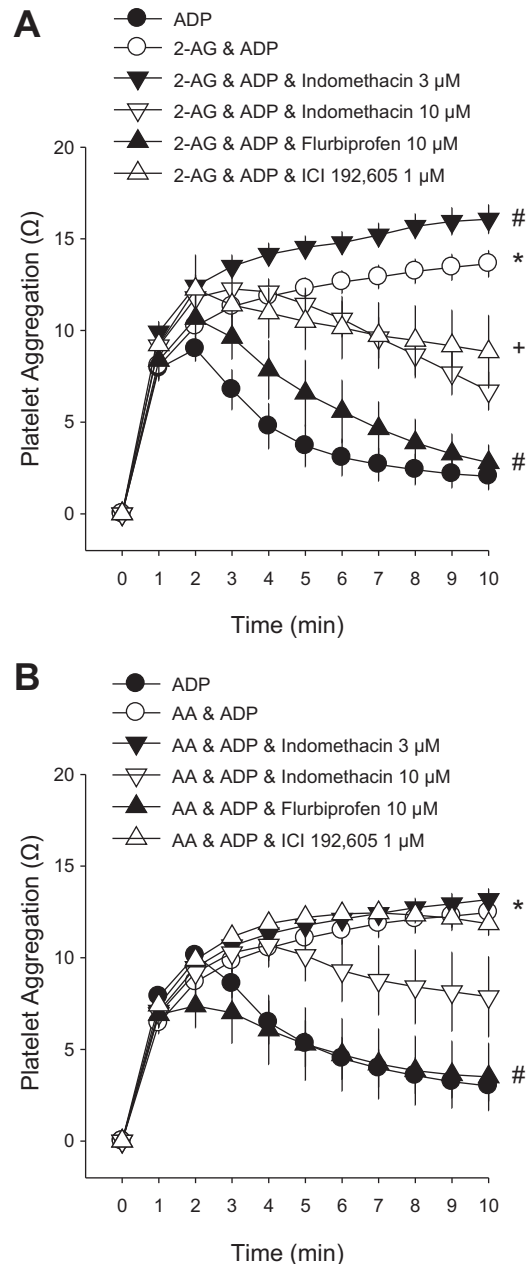


Fig. 8. Effect of the cyclooxygenase inhibitors indomethacin (3 and 10  $\mu\text{M}$ ) and flurbiprofen (10  $\mu\text{M}$ ), and thromboxane receptor antagonist ICI-192,605 (1  $\mu\text{M}$ ) on platelet responses to 2-arachidonoylglycerol (2-AG; 75  $\mu\text{M}$ ,  $n = 6-12$ ; A) or arachidonic acid (AA; 38  $\mu\text{M}$ ,  $n = 6-12$ ; B) added in combination with ADP (1  $\mu\text{M}$ ). \* $P < 0.05$  vs. 2-AG, AA, or ADP alone, # $P < 0.05$  vs. 2-AG or AA and ADP, and + $P = 0.0608$  vs. 2-AG and ADP by Friedman test on areas under the time-response curves.

tiation by ICI-192,605 and flurbiprofen (Fig. 6A), the interaction between 2-AG and ADP in the present study appears also to involve metabolism of arachidonic acid and enhanced secretion of thromboxane A<sub>2</sub>.

In summary, whether 2-AG-induced platelet aggregation is responsible for the increased CBF deficit in our study needs further analysis, given that a significant increase in thrombi was evident only after JZL-184 and not after 2-AG treatment in the present study, and especially given that preventing platelet aggregation had beneficial effect in only some (26) but not all stroke studies (32). However, with millions of people worldwide taking cannabis, and with endocannabinoids recognized as potential therapeutic agents in cerebral and myocardial ischemia (for reviews, see Refs. 4, 6, and 14), it is essential to identify the mechanisms responsible for the detrimental effects of 2-AG in stroke uncovered in this study, so that new endocannabinoid-related therapies can be developed that will selectively mediate the beneficial effects but not the detrimental effects in ischemia. Therefore, further, more indepth studies, outside the scope of the present work, are required to determine whether 2-AG, by inducing platelet aggregation, contributes to the formation of thrombi in the brain vessels that reduce the blood flow after stroke and whether the effect of 2-AG on CBF deficit is reversed by treatment with cyclooxygenase inhibitors or thromboxane receptor antagonists. The present findings force us to reevaluate the circumstances under which 2-AG is beneficial and whether systemic 2-AG treatment may represent an increased risk of thromboembolic-related disorders.

## GRANTS

This work was supported by Capacity Building Award BB/E527071/1 from the Integrative Mammalian Biology Initiative, funded by the Biotechnology and Biological Sciences Research Council, British Pharmacological Society Integrative Pharmacology Fund (donors AstraZeneca, GSK, and Pfizer), the Medical Research Council, the Higher Education Funding Council for England, SFC, and DBIS. The research data in this publication is openly available from the University of Strathclyde Pure, UK Data Service at <http://dx.doi.org/10.15129/f9972309-02f6-4855-a99c-8125f5de31e4>.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

J.A.S. performed experiments; J.A.S. analyzed data; J.A.S., S.J.C., and H.V.O.C. interpreted results of experiments; J.A.S. prepared figures; J.A.S. and S.J.C. drafted manuscript; J.A.S. and H.V.O.C. edited and revised manuscript; S.J.C. and H.V.O.C. conceived and designed research; H.V.O.C. approved final version of manuscript.

## REFERENCES

- Arevalo-Martin A, Garcia-Ovejero D, Molina-Holgado E. The endocannabinoid 2-arachidonoylglycerol reduces lesion expansion and white matter damage after spinal cord injury. *Neurobiol Dis* 38: 304–312, 2010. doi:10.1016/j.nbd.2010.02.002.
- Arevalo-Martin A, Garcia-Ovejero D, Sierra-Palmares Y, Paniagua-Torija B, Gonzalez-Gil I, Ortega-Gutierrez S, Molina-Holgado E. Early endogenous activation of CB1 and CB2 receptors after spinal cord injury is a protective response involved in spontaneous recovery. *PLoS One* 7: e49057, 2012. doi:10.1371/journal.pone.0049057.
- Baldassarri S, Bertoni A, Bagarotti A, Sarasso C, Zanfa M, Catani MV, Avigliano L, Maccarrone M, Torti M, Sinigaglia F. The endocannabinoid 2-arachidonoylglycerol activates human platelets through non-CB1/CB2 receptors. *J Thromb Haemost* 6: 1772–1779, 2008. doi:10.1111/j.1538-7836.2008.03093.x.
- Benyó Z, Ruisanchez É, Leszl-Ishiguro M, Sándor P, Pacher P. Endocannabinoids in cerebrovascular regulation. *Am J Physiol Heart Circ Physiol* 310: H785–H801, 2016. doi:10.1152/ajpheart.00571.2015.
- Brantl SA, Khandoga AL, Siess W. Mechanism of platelet activation induced by endocannabinoids in blood and plasma. *Platelets* 25: 151–161, 2014. doi:10.3109/09537104.2013.803530.
- Capetfani LS, Savergnini SQ, da Silva RF, Stergiopulos N, Santos RA, Mach F, Montecucco F. Update on the role of cannabinoid receptors after ischemic stroke. *Mediators Inflamm* 2012: 824093, 2012. doi:10.1155/2012/824093.
- Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J Pharmacol Methods* 3: 135–158, 1980. doi:10.1016/0160-5402(80)90024-8.
- Carr RL, Borazjani A, Ross MK. Effect of developmental chlorpyrifos exposure, on endocannabinoid metabolizing enzymes, in the brain of juvenile rats. *Toxicol Sci* 122: 112–120, 2011. doi:10.1093/toxsci/kfr081.
- Chen Y, McCarron RM, Ohara Y, Bembry J, Azzam N, Lenz FA, Shohami E, Mechoulam R, Spatz M. A novel functional interaction between 2-arachidonoglycerol, an endocannabinoid, and endothelin-1 in human brain capillary endothelium. *Circ Res* 87: 323–327, 2000. doi:10.1161/01.RES.87.4.323.
- Cherian P, Hankey GJ, Eikelboom JW, Thom J, Baker RI, McQuillan A, Staton J, Yi Q. Endothelial and platelet activation in acute ischemic stroke and its etiological subtypes. *Stroke* 34: 2132–2137, 2003. doi:10.1161/01.STR.0000086466.32421.F4.
- Choudhri TF, Hoh BL, Zerwes HG, Prestigiacomo CJ, Kim SC, Connolly ES Jr, Kottirsch G, Pinsky DJ. Reduced microvascular thrombosis and improved outcome in acute murine stroke by inhibiting GP IIb/IIIa receptor-mediated platelet aggregation. *J Clin Invest* 102: 1301–1310, 1998. doi:10.1172/JCI3338.
- Chouinard F, Lefebvre JS, Navarro P, Bouchard L, Ferland C, Lalancette-Hébert M, Marsolais D, Laviolette M, Flamand N. The endocannabinoid 2-arachidonoyl-glycerol activates human neutrophils: critical role of its hydrolysis and de novo leukotriene B4 biosynthesis. *J Immunol* 186: 3188–3196, 2011. doi:10.4049/jimmunol.1002853.
- Craft RM, Marusich JA, Wiley JL. Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? *Life Sci* 92: 476–481, 2013. doi:10.1016/j.lfs.2012.06.009.
- Cunha P, Romão AM, Mascarenhas-Melo F, Teixeira HM, Reis F. Endocannabinoid system in cardiovascular disorders - new pharmacotherapeutic opportunities. *J Pharm Bioallied Sci* 3: 350–360, 2011. doi:10.4103/0975-7406.84435.
- Dougherty JH Jr, Levy DE, Weksler BB. Platelet activation in acute cerebral ischaemia. Serial measurements of platelet function in cerebrovascular disease. *Lancet* 1: 821–824, 1977. doi:10.1016/S0140-6736(77)92774-X.
- Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci USA* 99: 10819–10824, 2002. doi:10.1073/pnas.152334899.
- Dyszkiewicz-Korpanty AM, Frenkel EP, Sarode R. Approach to the assessment of platelet function: comparison between optical-based platelet-rich plasma and impedance-based whole blood platelet aggregation methods. *Clin Appl Thromb Hemost* 11: 25–35, 2005. doi:10.1177/107602960501100103.
- Flecknell PA. *Laboratory Animal Anaesthesia* (4th ed.). New York: Academic, 2015.
- Fluri F, Schuhmann MK, Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. *Drug Des Devel Ther* 9: 3445–3454, 2015.
- Fürst H, Hartl WH, Janssen I. Patterns of cerebrovascular reactivity in patients with unilateral asymptomatic carotid artery stenosis. *Stroke* 25: 1193–1200, 1994. doi:10.1161/01.STR.25.6.1193.
- Harrison P. Progress in the assessment of platelet function. *Br J Haematol* 111: 733–744, 2000.
- Harrison P, Lordkipanidzé M. Testing platelet function. *Hematol Oncol Clin North Am* 27: 411–441, 2013. doi:10.1016/j.hoc.2013.03.003.
- Heiss WD, Rosner G. Functional recovery of cortical neurons as related to degree and duration of ischemia. *Ann Neurol* 14: 294–301, 1983. doi:10.1002/ana.410140307.
- Hillard CJ, Ho WS, Thompson J, Gauthier KM, Wheelock CE, Huang H, Hammock BD. Inhibition of 2-arachidonoylglycerol catabolism modulates vasoconstriction of rat middle cerebral artery by the

- thromboxane mimetic, U-46619. *Br J Pharmacol* 152: 691–698, 2007. doi:10.1038/sj.bjp.0707468.
25. Hossmann K-A. Viability thresholds and the penumbra of focal ischemia. *Ann Neurol* 36: 557–565, 1994. doi:10.1002/ana.410360404.
  26. Hsiao G, Lin KH, Chang Y, Chen TL, Tzu NH, Chou DS, Sheu JR. Protective mechanisms of inosine in platelet activation and cerebral ischemic damage. *Arterioscler Thromb Vasc Biol* 25: 1998–2004, 2005. doi:10.1161/01.ATV.0000174798.25085.d6.
  27. Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. A novel gene *iba1* in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun* 224: 855–862, 1996. doi:10.1006/bbrc.1996.1112.
  28. Imai H, Masayasu H, Dewar D, Graham DI, Macrae IM. Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia. *Stroke* 32: 2149–2154, 2001. doi:10.1161/hs0901.095725.
  29. Jordan WH, Young JK, Hyten MJ, Hall DG. Preparation and analysis of the central nervous system. *Toxicol Pathol* 39: 58–65, 2011. doi:10.1177/0192623310391480.
  30. Keown OP, Winterburn TJ, Wainwright CL, Macrury SM, Neilson I, Barrett F, Leslie SJ, Megson IL. 2-arachidonoyl glycerol activates platelets via conversion to arachidonic acid and not by direct activation of cannabinoid receptors. *Br J Clin Pharmacol* 70: 180–188, 2010. doi:10.1111/j.1365-2125.2010.03697.x.
  31. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412, 2010.
  32. Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G. Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation* 115: 2323–2330, 2007. doi:10.1161/CIRCULATIONAHA.107.691279.
  33. Kozak KR, Crews BC, Ray JL, Tai HH, Morrow JD, Marnett LJ. Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides *in vitro* and *in vivo*. *J Biol Chem* 276: 36993–36998, 2001. doi:10.1074/jbc.M105854200.
  34. Kozak KR, Prusakiewicz JJ, Marnett LJ. Oxidative metabolism of endocannabinoids by COX-2. *Curr Pharm Des* 10: 659–667, 2004. doi:10.2174/1381612043453081.
  35. Kunz A, Iadecola C. Cerebral vascular dysregulation in the ischemic brain. *Handb Clin Neurol* 92: 283–305, 2009. doi:10.1016/S0072-9752(08)01914-3.
  36. Lalancette-Hébert M, Gowing G, Simard A, Weng YC, Kriz J. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J Neurosci* 27: 2596–2605, 2007. doi:10.1523/JNEUROSCI.5360-06.2007.
  37. Liebeskind DS. Collateral circulation. *Stroke* 34: 2279–2284, 2003. doi:10.1161/01.STR.0000086465.41263.06.
  38. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavón FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH, Cravatt BF. Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* 5: 37–44, 2009. doi:10.1038/nchembio.129.
  39. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20: 84–91, 1989. doi:10.1161/01.STR.20.1.84.
  40. Maccarrone M, Bari M, Menichelli A, Giuliani E, Del Principe D, Finazzi-Agrò A. Human platelets bind and degrade 2-arachidonoylglycerol, which activates these cells through a cannabinoid receptor. *Eur J Biochem* 268: 819–825, 2001. doi:10.1046/j.1432-1327.2001.01942.x.
  41. Maccarrone M, Bari M, Battista N, Finazzi-Agrò A. Estrogen stimulates arachidonoyl ethanolamide release from human endothelial cells and platelet activation. *Blood* 100: 4040–4048, 2002. doi:10.1182/blood-2002-05-1444.
  42. McCarron RM, Chen Y, Tomori T, Strasser A, Mechoulam R, Shohami E, Spatz M. Endothelial-mediated regulation of cerebral microcirculation. *J Physiol Pharmacol* 57, Suppl 11: 133–144, 2006.
  43. McKittrick CM, Lawrence CE, Carswell HV. Mast cells promote blood brain barrier breakdown and neutrophil infiltration in a mouse model of focal cerebral ischemia. *J Cereb Blood Flow Metab* 35: 638–647, 2015. doi:10.1038/jcbfm.2014.239.
  44. Nomura DK, Long JZ, Niessen S, Hoover HS, Ng SW, Cravatt BF. Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. *Cell* 140: 49–61, 2010. doi:10.1016/j.cell.2009.11.027.
  45. Osborne KA, Shigeno T, Balarsky AM, Ford I, McCulloch J, Teasdale GM, Graham DI. Quantitative assessment of early brain damage in a rat model of focal cerebral ischaemia. *J Neurol Neurosurg Psychiatry* 50: 402–410, 1987. doi:10.1136/jnnp.50.4.402.
  46. Pacher P, Bátkai S, Kunos G. Cardiovascular pharmacology of cannabinoids. *Handb Exp Pharmacol* 168: 599–625, 2005. doi:10.1007/3-540-26573-2\_20.
  47. Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* 58: 389–462, 2006. doi:10.1124/pr.58.3.2.
  48. Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, Shohami E. The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis* 22: 257–264, 2006. doi:10.1016/j.nbd.2005.11.004.
  49. Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413: 527–531, 2001. doi:10.1038/35097089.
  50. Patel AR, Ritzel R, McCullough LD, Liu F. Microglia and ischemic stroke: a double-edged sword. *Int J Physiol Pathophysiol Pharmacol* 5: 73–90, 2013.
  51. Schilling M, Besselmann M, Müller M, Strecker JK, Ringelstein EB, Kiefer R. Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. *Exp Neurol* 196: 290–297, 2005. doi:10.1016/j.expneurol.2005.08.004.
  52. Schlattmann P, Dirnagl U. Statistics in experimental cerebrovascular research: comparison of more than two groups with a continuous outcome variable. *J Cereb Blood Flow Metab* 30: 1558–1563, 2010. doi:10.1038/jcbfm.2010.95.
  53. Scremin OU. Cerebral vascular system. In: *The Human Nervous System*, edited by Paxinos G, Mai JK. San Diego, CA: Elsevier Academic, 2004, p. 1325–48. doi:10.1016/B978-012547626-3/50038-7.
  54. Shaw LA, Batey AJ, Coker SJ. Combined administration of 5-HT<sub>2</sub> and thromboxane A<sub>2</sub> antagonists: effects on platelet aggregation and isolated cardiac muscle. *Br J Pharmacol* 121: 875–882, 1997. doi:10.1038/sj.bjp.0701208.
  55. Signorello MG, Giacobbe E, Leoncini G. Activation by 2-arachidonoylglycerol of platelet p38MAPK/cPLA<sub>2</sub> pathway. *J Cell Biochem* 112: 2794–2802, 2011. doi:10.1002/jcb.23194.
  56. Singh NN, Pan Y, Muengtaweponsa S, Geller TJ, Cruz-Flores S. Cannabis-related stroke: case series and review of literature. *J Stroke Cerebrovasc Dis* 21: 555–560, 2012. doi:10.1016/j.jstrokecerebrovasdis.2010.12.010.
  57. Straiker A, Hu SS, Long JZ, Arnold A, Wager-Miller J, Cravatt BF, Mackie K. Monoacylglycerol lipase limits the duration of endocannabinoid-mediated depolarization-induced suppression of excitation in autaptic hippocampal neurons. *Mol Pharmacol* 76: 1220–1227, 2009. doi:10.1124/mol.109.059030.
  58. Sun L, Kuroiwa T, Ishibashi S, Miki K, Li S, Xu H, Endo S, Mizusawa H. Two region-dependent pathways of eosinophilic neuronal death after transient cerebral ischemia. *Neuropathology* 29: 45–54, 2009. doi:10.1111/j.1440-1789.2008.00939.x.
  59. Wang Q, Peng Y, Chen S, Gou X, Hu B, Du J, Lu Y, Xiong L. Pretreatment with electroacupuncture induces rapid tolerance to focal cerebral ischemia through regulation of endocannabinoid system. *Stroke* 40: 2157–2164, 2009. doi:10.1161/STROKEAHA.108.541490.
  60. Yam PS, Patterson J, Graham DI, Takasago T, Dewar D, McCulloch J. Topographical and quantitative assessment of white matter injury following a focal ischaemic lesion in the rat brain. *Brain Res Brain Res Protoc* 2: 315–322, 1998. doi:10.1016/S1385-299X(98)00005-1.
  61. Zille M, Farr TD, Przeszdzing I, Müller J, Sommer C, Dirnagl U, Wunder A. Visualizing cell death in experimental focal cerebral ischemia: promises, problems, and perspectives. *J Cereb Blood Flow Metab* 32: 213–231, 2012. doi:10.1038/jcbfm.2011.150.