Molecular Neurobiology (2019) 56:3552–3562
https://doi.org/10.1007/s12035-018-1317-0

Chloroquine Restores Ganglioside Homeostasis and Improves Pathological and Behavioral Outcomes Post-stroke in the Rat

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Received: 7 May 2018 / Accepted: 13 August 2018 / Published online: 25 August 2018

Abstract

Perturbations of ganglioside homeostasis have been observed following stroke whereby toxic simple gangliosides GM2 and GM3 accumulate, while protective complex species GM1 and GD1 are reduced. Thus, there is a need for therapeutic interventions which can prevent ganglioside dysregulation after stroke. A pharmacological intervention using chloroquine was selected for its transient lysosomotropic properties which disrupt the activity of catabolic ganglioside enzymes. Chloroquine was administered both in vitro (0.1 μM), to primary cortical neurons exposed to GM3 toxicity, and in vivo (45 mg/kg i.p.), to 3-month-old male Wistar rats that underwent a severe stroke injury. Chloroquine was administered for seven consecutive days beginning 3 days prior to the stroke injury. Gangliosides were examined using MALDI imaging mass spectrometry at 3 and 21 days after the injury, and motor deficits were examined using the ladder task. Chloroquine treatment prevented ganglioside dysregulation 3 days post-stroke and partially prevented complex ganglioside depletion 21 days post-stroke. Exogenous GM3 was found to be toxic to primary cortical neurons which was protected by chloroquine treatment. Motor deficits were prevented in the forelimbs of stroke-injured rats with chloroquine treatment and was associated with decreased inflammation, neurodegeneration, and an increase in cell survival at the site of injury. Chloroquine administration prevents ganglioside dysregulation acutely, protects against GM3 toxicity in neurons, and is associated with long-term functional and pathological improvements after stroke in the rat. Therefore, targeting lipid dysregulation using lysosomotropic agents such as chloroquine may represent a novel therapeutic avenue for stroke injuries.

Keywords Brain lipids · Gangliosides · Lipid metabolism · Mass spectrometry · Molecular imaging · Neurons · Fluorescence microscopy · Chloroquine · Neurodegeneration · Stroke

Background

Strokes are the most frequent cause of permanent disability in individuals aged 60 years and older with the global burden of stroke care expected to increase in line with the aging population [1, 2]. However, there is currently no clinically effective approach to prevent the damage caused by stroke with the exception of mechanical thrombectomy, and tissue plasminogen activator, which has a very narrow therapeutic window of effectiveness [3]. Therefore, the development of

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new preventative approaches and therapeutic interventions which can improve recovery after stroke injuries is imperative. Membrane lipid dysregulation is observed after neurodegenerative injuries in the brain and is thought to play a key mechanistic role in the propagation of neurodegeneration [4]; thus, preventing this dysregulation may provide a novel avenue of intervention for stroke injuries.

Gangliosides are a major component of lipid rafts and play an important role in protein-lipid interactions on the cell surface leading to various signal transduction events. Structurally, simple minor gangliosides GM2 and GM3, while crucial during neurodevelopment, have been linked to neurodegeneration in the adult brain. GM3 in particular has been shown to upregulate pro-apoptotic signaling pathways [5] and inhibit angiogenesis [6, 7] as well as cell proliferation [8], leading to toxicity when accumulated in neural cells [9, 10]. Perturbations in ganglioside homeostasis resulting in the depletion of the more abundant protective complex species, GM1 and GD1, along with the accumulation of toxic simple species have been observed in response to neurodegenerative insults such as stroke and traumatic brain injuries (TBI) [4, 11, 12]. Additionally, previous work from our lab has demonstrated that the degree of ganglioside dysregulation is correlated with the severity of the neurodegenerative insult, with more severe stroke insults resulting in an earlier and prolonged accumulation of toxic simple ganglioside species [4]. Altogether, this suggests that ganglioside dysregulation may be playing an important role in the propagation of neurodegeneration following stroke.

Therapies targeting the restoration of depleted GM1 in the brain through exogenous administration have demonstrated a strong protective effect in preclinical models of stroke [3, 13–16] and have been found to prevent behavioral deficits in mice following TBI [17]. GM1 is thought to exert its protective effects through modulation of neurotrophin release and the maintenance of ion balance [3, 18–20]. However, clinically, GM1 administration alone has produced mixed results and has often failed to confer adequate protection following stroke [21–23]. One potential explanation for this finding may be that the restoration of complex gangliosides alone does not prevent those species from being enzymatically degraded to simple gangliosides during the ischemic cascade; thus, the resulting toxicity is not prevented. Therefore, there is a need for targeted therapies which can prevent the catabolism of complex gangliosides as well as the resulting accumulation of toxic simple species in order to better protect cells from the damage associated with ganglioside dysregulation after a neurodegenerative injury.

Chloroquine (CQ) is best known as an anti-malarial drug but has also used been used as an anti-rheumatic agent due to its anti-inflammatory properties [24]. It has been shown to reduce the accumulation of reactive oxygen species after glutamate-induced toxicity in hippocampal HT22 cells [25] and block the activity of Toll-like receptor 3-mediated inflammatory pathway following stroke in rats [26]. In addition to its widely acknowledged role in the inhibition of autophagy, low doses of CQ have also been shown to have beneficial effects on the vascular system through vessel normalization [27] and protection of the blood brain barrier [25]. CQ freely diffuses across lipid bilayers as it is uncharged at neutral pH but is a weak base and becomes protonated upon entering acidic compartments such as lysosomes, causing it to accumulate in acidic organelles and raise their pH above their acidic optimum [28]. This disrupts the normal function of acidic lysosomal enzymes such as β-galactosidase and β-hexosaminidase, the enzymes responsible for the catabolism of complex ganglioside GM1 to simple species GM2 and GM3 [29]. CQ has been used to inhibit the catabolism of gangliosides in vitro [29–31], making it a promising candidate for the prevention ganglioside dysregulation after stroke.

In the current study, we used several experimental approaches to examine the effects of CQ treatment in the brain after stroke in the rat. Our previous work in this area demonstrated that co-administration of endothelin-1 (ET-1) into the striatum to produce focal ischemia, alongside with bilateral intracerebral-ventricular injections of toxic Aβ (25–35) in Wistar rats, produces a more severe neurodegenerative response as well as increased ganglioside dysregulation than either ET-1 stroke or Aβ toxicity alone [4, 32]. Therefore, this particular model was chosen for the present study as it is the ideal platform to assess the effects of CQ treatment on ganglioside homeostasis as well as pathological and functional recovery after a severe stroke. In order to visualize and quantify the abundance of gangliosides in the rat brain, matrix-assisted desorption ionization imaging mass spectrometry (MALDI-IMS) was performed at 3 and 21 days post-stroke. For the first time, we further demonstrate the toxicity of GM3 accumulation on primary cortical neurons and show the neuroprotective effect of CQ treatment in neurons. Finally, we show the restoration of behavioral deficits and decreased pathology following CQ treatment in rats post-stroke.

Methods

All procedures involving live animals were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Committee (Protocol 2014–2016).

Stroke Model

All animal handling and surgical procedures were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Subcommittee. Three-month-old male Wistar rats weighing 300–400 g (Charles River Laboratories, Quebec, Canada) were housed in pairs under standard conditions (12:12 light/dark cycle) and provided food and water ad libitum. Rats were randomly assigned to experimental groups and were housed individually following surgery. To induce the severe stroke injury, rats received both a bilateral intracerebral ventricular injection of the toxic amyloid beta (Aβ) fragment (25–35) followed by a unilateral striatal injection of ET-1, a potent vasoconstrictor as previously described [4]. This enhanced stroke model has previously been shown to exacerbate the pathological response (inflammation, neurodegeneration, and infarct size) [32, 33] and more severely perturb ganglioside homeostasis than either injury alone [4], providing the ideal platform to test a therapeutic agent aimed at restoring ganglioside homeostasis. Control surgery rats underwent identical surgical procedures with all injections being replaced with saline. A total of 78 rats were used for the current study with 5 rats per group used for MALDI-IMS analysis at 3 and 21 days, 6 rats per group for IHC at 3 and 21 days, and 8 rats per group used for behavioral analysis. There was a mortality rate of roughly 20% associated with the surgical procedures (10 rats total).

Chloroquine Administration

Chloroquine (CQ, Sigma-Aldrich, St. Louis, USA) was prepared daily at a concentration of 45 mg/mL in 0.9% saline. Animals were weighed immediately prior to injection and were administered 45 mg/kg of CQ by intraperitoneal (i.p.) injection for seven consecutive days beginning 3 days prior surgery and ending 3 days after surgery.

Immunohistochemistry and Fluoro Jade B

Immunohistochemistry, standard histochemistry, and Fluoro Jade B staining were performed as previously described [4].

Primary Cortical Neuron Cultures

Primary cortical neurons were extracted from E18 Wistar rat embryos and plated on coverslips in 24-well plates as previously described [34]. Half of the media was exchanged with fresh neurobasal media (NBM) every 2 days until 14 days in vitro (DIV). Each coverslip represented a single biological replicate with eight biological replicates in each experimental group. Experiments were repeated on three separate cultures. On DIV 13, each well in the 24-well plates were assigned as either control cells, CQ control cells (0.1 μM of chloroquine dissolved in NBM), 1 μM of GM3 (50% d18:1, d20:1; Avanti Polar Lipids, Alabaster, Alabama, USA), 100 μM of GM3, 1 μM GM3 with CQ, or 100 μM GM3 with CQ and were fixed with 4% paraformaldehyde for 15 min and then washed 3× with 10 mM PBS either 1, 6, 24, or 48 h after administration of CQ and/or GM3. Propidium iodide (PI; Sigma-Aldrich, St. Louis, USA) was administered (1 mg/mL) 45 min before cell fixation in order to assess cell viability via fluoresce microscopy. Cells were also fluorescently stained with TUNEL using a cell death apoptosis kit (Sigma, St. Louis, MO, USA) after fixation to assess apoptotic cell death and mounted onto microscope glass slides with Fluoroshield (Sigma, Sigma-Aldrich, St. Louis, USA) containing DAPI (Sigma, Sigma-Aldrich, St. Louis, USA).

Fluorescence Microscopy

Neurons were imaged and DAPI (blue), TUNEL (green), and PI (red) and quantified using a Nikon Eclipse Ni (Nikon Instruments Inc., Melville, NY, USA) microscope and Nikon Analysis software. Microscope settings for each fluorescent filter were kept consistent for all analysis. Nikon analysis software allows for cell counting based on staining intensity and cell shape. This feature was used to perform all quantification of DAPI-, TUNEL-, and PI-positive cell staining. Positive TUNEL and PI staining was presented as a ratio to total DAPI-positive cells.

MALDI Imaging Mass Spectrometry

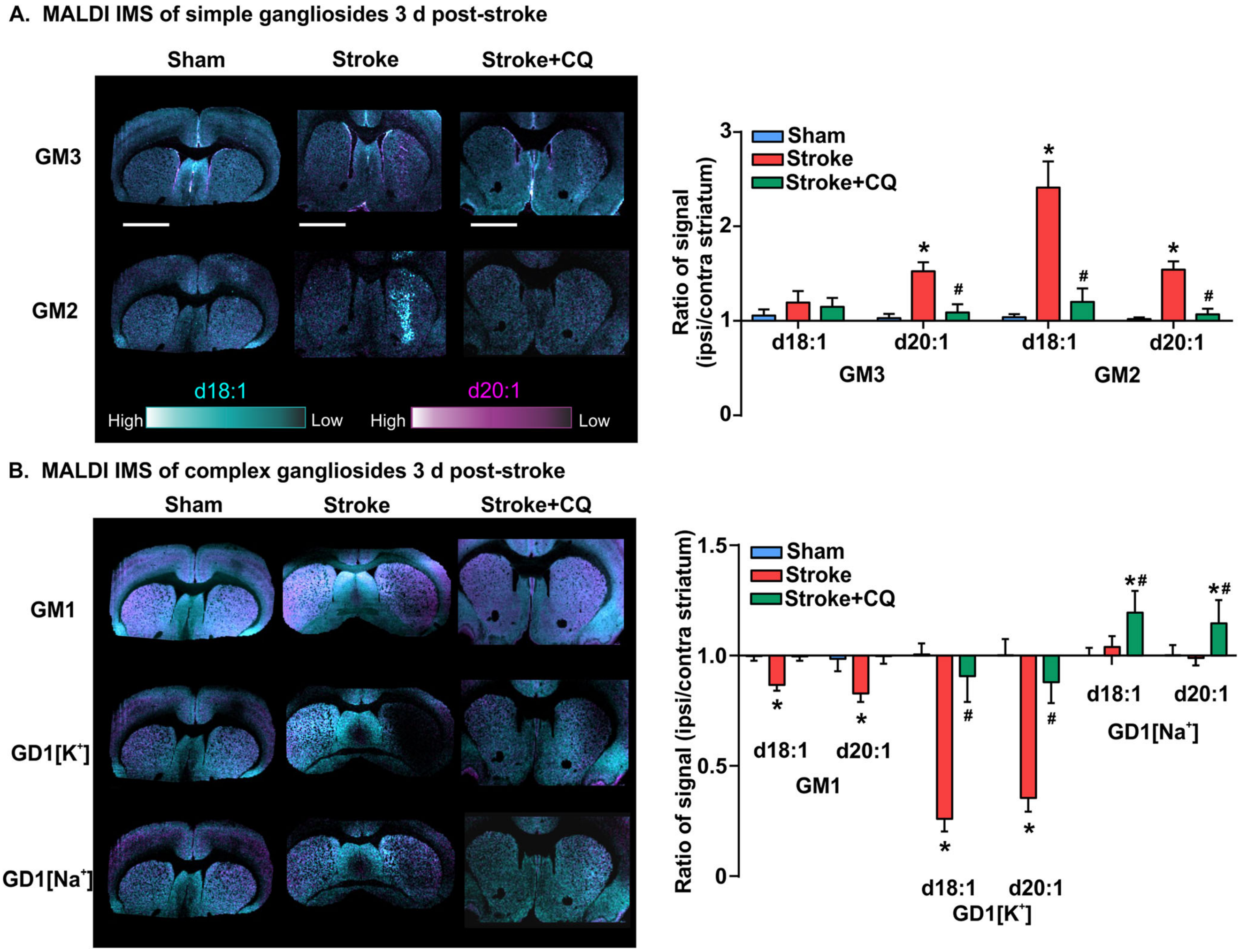
Rats from each experimental group ($n = 5$) were sacrificed at either 3 or 21 days post-surgery for MALDI-IMS analysis. Rat brains were removed via fresh frozen extraction and prepared for MALDI-IMS following the protocol published in Caughlin et al. [35]. Images were acquired in negative mode with a 70-μM laser step size. Data was exported to Tissue View (Sciex, Toronto, Canada) to visualize ganglioside distribution. Representative MALDI images were exported from Tissue View to be pseudo-colored and overlaid in ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). MALDI data was acquired in Tissue View by isolating a region of interest (ROI) of equal size at the site of stroke within the striatum and in the corresponding contralateral uninjured striatum (comparable ROIs were used in control animals when no stroke was present). The numerical data from each A-series ganglioside species was then imported into Prism statistical software to determine the area under the curve (AUC) corresponding to the three largest isotopic peaks of each species. This AUC measurement was then divided by the total signal as a means of normalizing the data for between scan comparisons. The AUC of the individual species relative to total signal was then compared between the ipsilateral hemisphere and contralateral hemisphere to the stroke.

Ladder Task—Motor Assessment

The ladder test was used to assess motor function and determine if CQ treatment could rescue motor impairments induced by the stroke. The ladder task consists of two long pieces of plexi glass 20 cm apart with metal pegs placed at irregular intervals along the bottom. The ladder is raised 30 cm above the ground and connects an empty cage to the rat's home cage. The rat is placed at one end of the ladder (with the empty cage) and must cross the ladder to reach its home cage on the opposite side. The home cage and empty cage are switched sides after three successful runs of the ladder in one direction. A total of six runs (three from each direction) were used to calculate the average number of correct paw placements, errors in paw placement, and peg misses with each limb. Metz et al. described correct paw placement and total misses along with five different types of errors in paw placement that can occur in the ladder test [36]. For the purpose of the current study, the four variations of errors described by Metz et al. were grouped together and simply referred to as "errors."

Data Analysis

All histological, cell count, and MALDI-IMS analysis was performed by investigators that were blinded to the experimental group identify. Data are represented as mean ± the standard error of the mean. Statistical analysis was performed using a two-way ANOVA with a Tukey's post hoc test. *N* values are described in each figure legend.



A. MALDI IMS of simple gangliosides 3 d post-stroke

B. MALDI IMS of complex gangliosides 3 d post-stroke

Fig. 1 Ganglioside homeostasis restored at 3 days in stroke-injured rats treated with CQ. MALDI-IMS overlaid images (left) and quantified data (right) depicting the ratio of abundance of gangliosides in the ipsilateral versus contralateral striatum 3 days following either control, stroke, or stroke + CQ treatment. Gangliosides GM3 d20:1, GM2 d18:1, and GM2 d20:1 were all significantly elevated in response to the stroke which was prevented with CQ treatment (**A**—right). Both GM1 and GD1[K^+] were significantly depleted in response to the stroke, whereas GD1[Na^+] remained unchanged. CQ treatment prevented the depletion of GM1 and GD1[K^+] while increasing the abundance of GD1[Na^+] at the site of the stroke (**B**—right). Scale bar is equal to 4 mm. Asterisk indicates statistical change in ganglioside expression from control rats. Number sign indicates statistical change in ganglioside between stroke and stroke + CQ groups, $p < 0.05$, two-way ANOVA, Tukey post hoc test, $n = 5$ per experimental group

Availability of Data and Materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Pathological Shift in Ganglioside Homeostasis Prevented with CQ Treatment 3 Days Post-stroke

Using MALDI-IMS, we examined the ability of CQ (45 mg/kg i.p.) to prevent the dysregulation of ganglioside homeostasis after a severe stroke injury. Sham control rats displayed no significant differences in ganglioside abundance between the ipsilateral and contralateral sides of the brain in response to the surgical procedures (Fig. 1A, B). Confirming previous findings from our lab, the stroke-injured rats showed a significant increase in simple gangliosides 3 days after stroke. The d18:1 and d20:1 species of GM2 showed the most significant increase in abundance in response to the stroke, while only the d20:1 species of GM3 was significantly increased at the site of stroke (Fig. 1A). CQ treatment resulted in a significant reduction in the abundance of simple ganglioside species at the site of injury compared to the untreated stroke group, showing no significant differences from controls (Fig. 1A).

In addition to examining the ability of CQ to prevent the accumulation of simple gangliosides, we examined the abundance of protective complex gangliosides GM1 and GD1 to

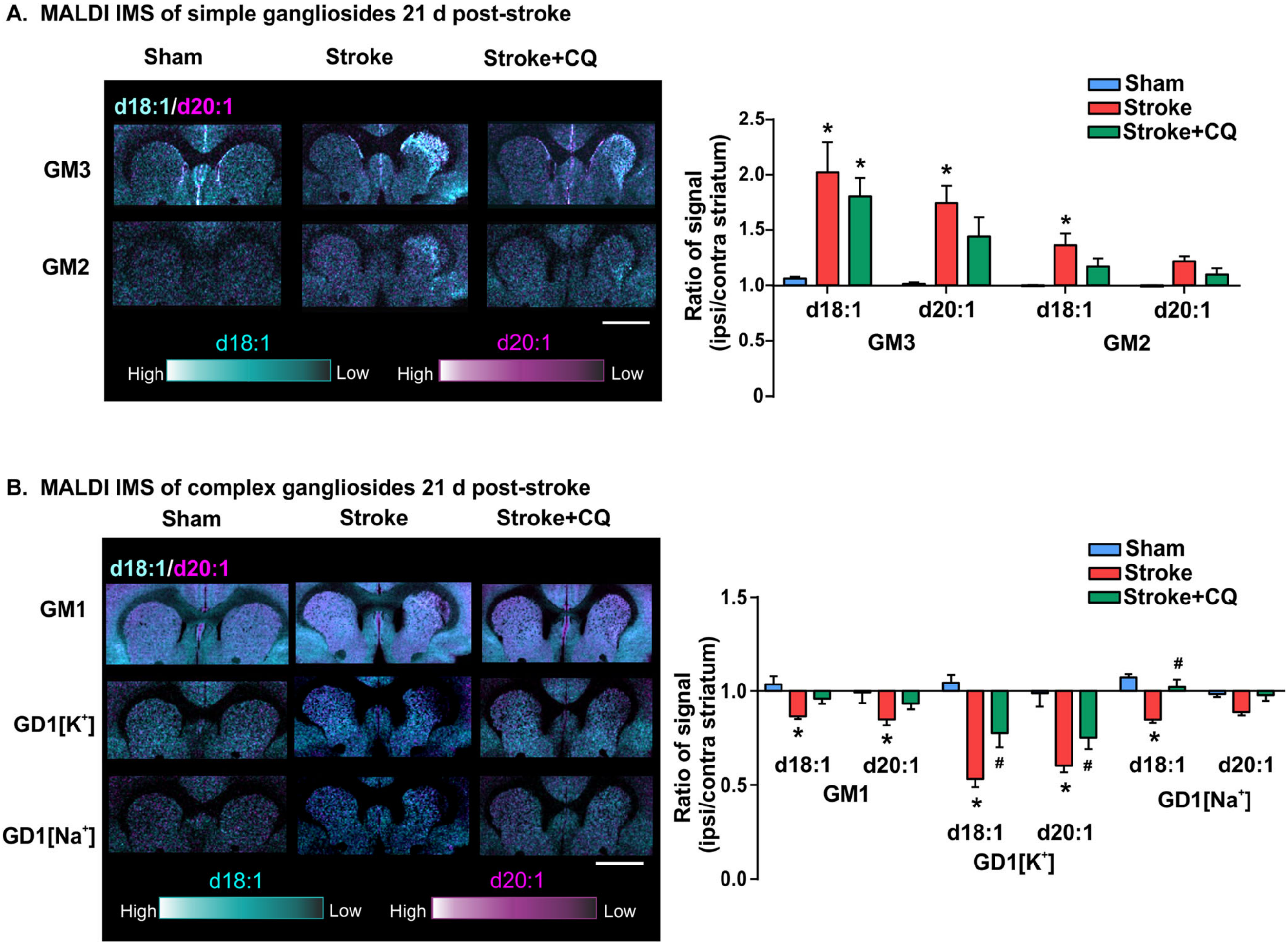


Fig. 2 Partial restoration of complex ganglioside homeostasis observed at 21 days via MALDI-IMS. MALDI-IMS overlaid images (left) and quantified data (right) depicting the ratio of abundance of gangliosides in the ipsilateral versus contralateral striatum 21 days following either control, stroke, or stroke + CQ treatment. GM3 d18:1, GM3 d20:1, and GM2 d18:1 were all significantly elevated over controls at within the striatum 21 days post-stroke. This increase in simple gangliosides was not significantly reduced through CQ treatment; however, GM3 d20:1 and GM2 d18:1 were not significantly elevated over controls either (**A**—right). All complex gangliosides examined, with the exception of GD1[Na$^+$] d20:1, were significantly reduced at the site of injury 21 days after surgery. GM1 and GD1[Na$^+$] levels were not significantly different from controls in CQ-treated rats, and GD1[K$^+$] abundance was significantly increased compared to untreated injured rats. Scale bar is equal to 4 mm. Number sign indicates statistical change in ganglioside between stroke and stroke + CQ groups, $p < 0.05$, two-way ANOVA, Tukey post hoc test, $n = 5$ per experimental group. **B** All complex gangliosides examined, with the exception of GD1[Na+] d20:1, were significantly reduced at the site of injury 21 days after surgery. GM1 and GD1[Na+] levels in CQ-treated rats were not significantly different from those of the controls, and GD1[K+] abundance was significantly increased compared to untreated injured rats

determine if CQ treatment can prevent their depletion at the site of the stroke. Ganglioside GD1 attaches to salt adducts [K$^+$] and [Na$^+$] when analyzed via MALDI-IMS. These two adducts of GD1 have previously been observed to have unique responses following stroke [4]; therefore, we included both adducts in all of the following analyses. As predicted, both the d18:1 and d20:1 species of ganglioside GM1 were significantly lower at the site of injury compared to controls 3 days post-surgery (Fig. 1B). Similar to what was previously reported for the [K$^+$] and [Na$^+$] adducts of GD1, they indeed showed unique responses following stroke such that the [K$^+$] adduct of GD1 was significantly depleted at the site of injury, while the [Na$^+$] adduct remained unchanged. Treatment with CQ prevented the depletion of both the d18:1 and d20:1 species of ganglioside GM1 and the [K$^+$] adduct of GD1 while significantly increasing the abundance of d18:1 GD1 [Na$^+$] at the site of injury compared to untreated injured rats.

CQ Partially Restores Ganglioside Homeostasis 21 Days Post-stroke

We next evaluated the effects of CQ treatment on ganglioside homeostasis during the later stages of stroke recovery. Simple ganglioside GM3 showed a significant increase in abundance for both the d18:1 and d20:1 species 21 days following stroke (Fig. 2A). GM2 levels decreased from their initial upregulation in the early stages of recovery, but the d18:1 species of GM2 remained significantly higher than controls (Fig. 2A). This response to the stroke at 21 days is consistent with what has been reported by our group [4]. CQ treatment was not able to significantly reduce the increased abundance of either GM3 or GM2 at the site of injury 21 days post-stroke, although the d20:1 species of GM3 and both GM2 species were not significantly different from controls either.

Complex gangliosides, on the other hand, were still subject to the protective action of CQ 21 days following stroke. Injured rats continued to show a significant depletion of complex gangliosides GM1 and GD1 at the site of injury, indicating that these species may have continued to undergo degradation 21 days after stroke. CQ treatment prevented this drop in GM1 and significantly increased the abundance of GD1 species at the site of injury, although GD1 [K$^+$] levels in the CQ-treated injured rats were still significantly lower than controls (Fig. 2B).

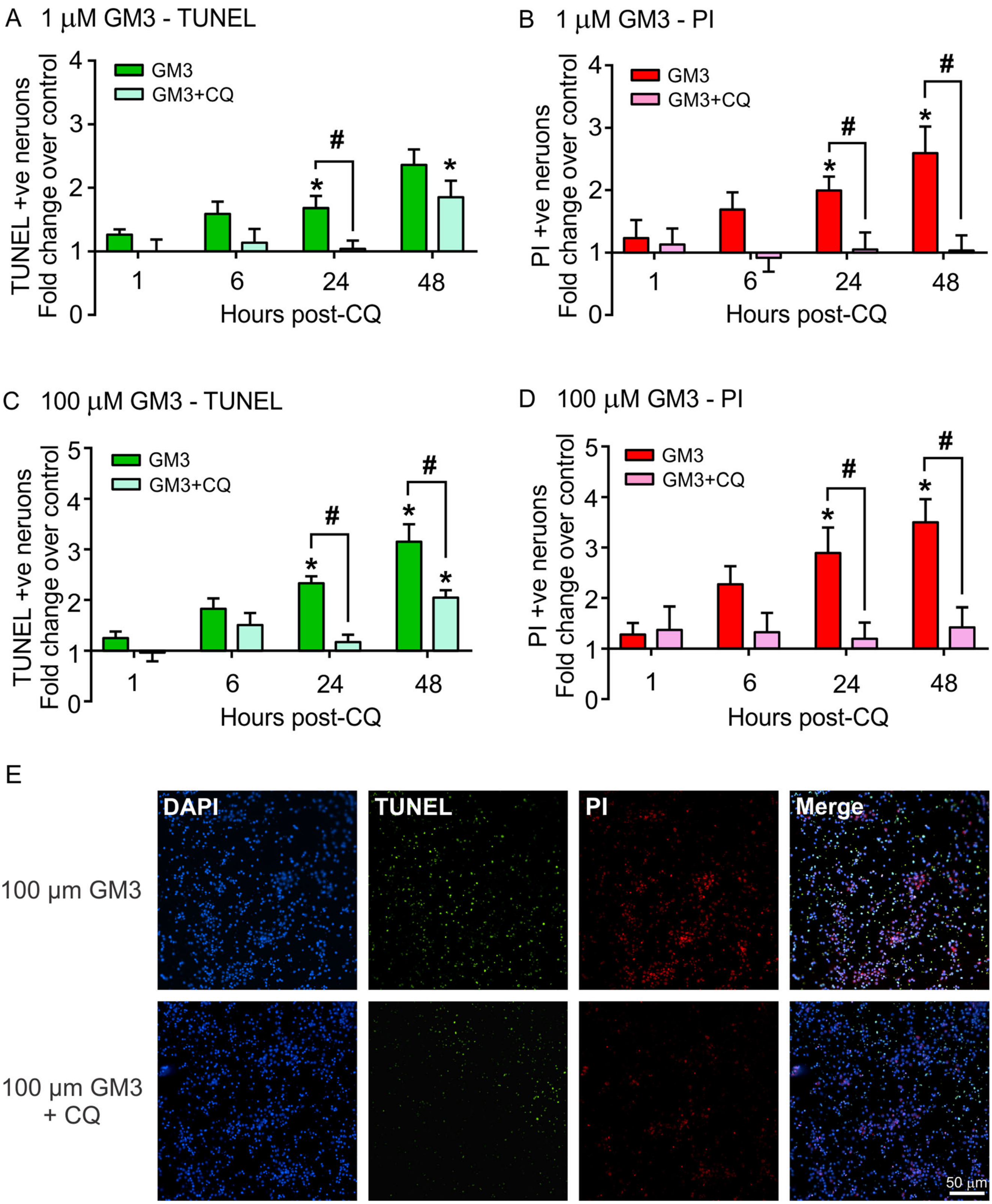
Exogenous GM3 Results in Toxicity in Primary Cortical Neurons Which Is Protected by CQ Treatment

Given the finding that GM3 was increased post-stroke, and that CQ can acutely reduce this increase in GM3, we sought to determine if GM3 could directly cause cell death to neurons. To determine if CQ could protect against GM3-induced toxicity, neurons were also co-administered 0.1 μM CQ along with either the low or high dose of GM3. A low dose of GM3 (1 μM) was found to significantly increase TUNEL-positive apoptotic death at 24 and 48 h post-exposure (Fig. 3A). Co-treatment with CQ protected neurons from apoptosis at 24 h post-exposure (Fig. 3A). This same dose of GM3 resulted in significant PI uptake in neurons at 24 and 48 h post-exposure which was prevented by CQ co-administration (Fig. 3B). In the high-dose (100 μM) GM3-exposed neurons, an increase in apoptosis was seen at both 24 and 48 h post-exposure, which was reduced by co-administration with CQ (Fig. 3C, E). When measuring cell death using PI, the same effects were observed (Fig. 3D, E).

Fig. 3 CQ increases cell viability and decreases apoptosis in response to toxic low- and high-dose GM3 administration. **A, B** Quantification of TUNEL- and PI-positive labelling in primary cortical neurons administered with a low-dose (1 μM) GM3 with and without co-administration of CQ (1 μM). GM3 resulted in increased TUNEL signal 24 and 48 h post-exposure. Co-administration of CQ reduced TUNEL signal at the 24 h time point only. Low-dose GM3 exposure resulted in increased PI signal at 24 and 48 h which was reduced by CQ co-administration. **C, D** Quantification of TUNEL- and PI-positive labelling in primary cortical neurons administered with a high-dose (100 μM) GM3 with and without co-administered of CQ (1 μM). High-dose GM3 resulted in increased TUNEL and PI signal 24 and 48 h post-exposure, which was reduced by co-administration with CQ. **E** Representative photomicrographs of primary cortical neurons exposed to 100 μM GM3 (top panels) and 100 μM GM3 with 1 μM CQ. Asterisk indicates statistical significant increase in signal over controls; number sign indicates statistical protection by co-administration with CQ, $p < 0.05$ via two-way ANOVA, Tukey post hoc test, $n = 8$ per experimental time point ▶

Stroke-Induced Pathology and Motor Impairments at 21 Days Reduced by CQ Treatment

In order to assess motor function in our rats, we used the ladder task which examines both the rat's forelimb and hindlimb activity. Stroke-injured rats displayed significantly more errors in limb placement during the ladder task than the control group for all four limbs (Fig. 4A). Interestingly, the forelimbs of the injured rats showed a higher number of limb placement errors than the hindlimbs. The left forelimb, contralateral to the right hemisphere striatal stroke, displayed the highest number of limb placement errors. CQ-treated rats showed a significant reduction in forelimb placement errors compared to the untreated stroke group, with no significant differences in performance from the control group (Fig. 4A). However, this protective effect of CQ treatment was not observed in the hindlimbs of the CQ-treated rats, which showed a significant increase in limb placement errors compared to controls, similar to the stroke group.



Immunohistochemistry was performed to assess cell survival, neurodegeneration, and inflammation at the site of injury within each experimental group at 3 and 21 days post-stroke in order to evaluate the efficacy of CQ treatment on pathology at the site of injury. Stroke-injured rats treated with CQ had significantly more NeuN-positive neurons at the site of injury compared to untreated stroke rats at both 3 and 21 days post-stroke (Fig. 4B). FJB was used as a marker of neurodegeneration and was significantly reduced in CQ-treated rats at 21 days compared to untreated stroke rats (Fig. 4C). Stroke injury led to a significant increase in OX-6-positive activated microglia staining at the site of injury at 21 days (Fig. 4D). CQ treatment in stroke-injured rats prevented the significant increase in OX-6-positive activated microglia staining observed in untreated stroke rats 21 days post-stroke.

Fig. 4 Reduced forelimb motor impairments and brain pathology in stroke rats treated with CQ. **A** Quantification of limb placement errors on the ladder motor assessment task. Stroke injured rats showed a significant increase in limb placement errors compared to controls in all four limbs. The number of limb placement errors was significantly reduced in the forelimbs of rats treated with CQ. CQ treatment did not significantly reduce the number of limb placement errors in the hindlimbs of stroke injured rats. Asterisk indicates statistical significance from control rats; number sign indicates statistical significance between stroke and stroke + CQ groups, $p < 0.05$, two-way ANOVA, Tukey post hoc test, $n = 8$ per experimental group. **B**–**D** Photomicrographs (21-day survival time point) and quantification of NeuN (**B**), Fluoro jade B (**C**), and OX-6 (**D**) staining at the site of injury at 3 and 21 days post-stroke. **B** CQ treatment significantly increased positive NeuN staining at the site of injury at both 3 and 21 days post-stroke compared to the untreated stroke group. **C** FJB staining was significantly reduced at the site of stroke injury at 21 days in CQ-treated rats. **D** CQ treatment prevented the significant increase in OX-6 staining observed in the stroke group at 21 days. Asterisk indicates statistical effect of surgical time point; number sign indicates statistical effect of CQ treatment, $p < 0.05$, two-way ANOVA, Tukey post hoc test, $n = 5$ per experimental group

Discussion

In the current study, we used MALDI-IMS, pathological assessments, primary cortical neurons, and a behavioral task to examine the effect of CQ treatment on neurodegeneration. The combination of these techniques allows for the most in-depth examination of ganglioside dysregulation after stroke to date and provides novel insight into the benefits of pharmacological interventions targeting ganglioside metabolism for neurodegeneration.

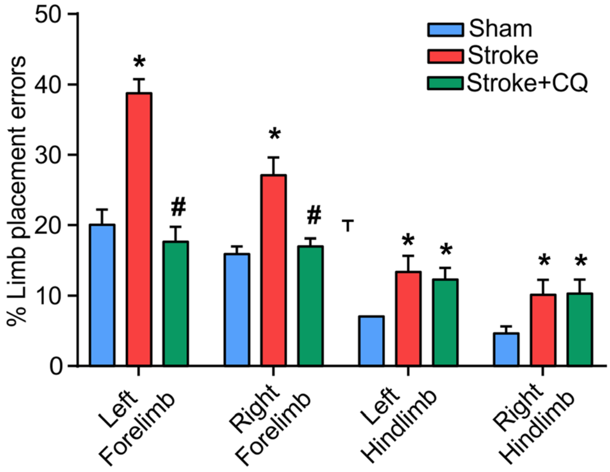
CQ treatment successfully prevented ganglioside dysregulation of both complex and simple species 3 days post-stroke. These findings confirm our hypothesis that CQ can be used to prevent the accumulation of simple gangliosides and the depletion of complex gangliosides acutely after stroke. Ganglioside dysregulation was partially prevented 21 days post-stroke by CQ treatment and was more effective at preventing complex ganglioside degradation than simple ganglioside accumulation. Complex gangliosides contribute to long-term axon stability and axon myelin interactions at the nodes of Ranvier [37, 38]. Although CQ preserved complex gangliosides in the present study, CQ's broad lysomotropic activity makes it difficult to demonstrate its clear selectivity towards preserving complex ganglioside levels. Future studies aimed as using B4galnt1-null mice (for review see [39]) in which complex gangliosides are absent would allow for a more direct confirmation on a role for complex gangliosides post-stroke.

CQ treatment prior to stroke was also associated with a significant decrease in inflammation, neurodegeneration and cell death at the site of injury, and significantly reduced motor impairments in the forelimbs of stroke-injured rats. It is possible that preventing ganglioside dysregulation in the early stages of recovery was sufficient to confer long-term protection during recovery from stroke. In the case of ischemic reperfusion injuries, neuroprotection strategies in the acute stage are crucial in mediating damage to penumbral regions and ultimately the severity of long-term impairments [1, 40]. It is possible that the pre-treatment phase of the CQ paradigm was able to confer protection to neurons prior to the stroke, leading to increased membrane lipid integrity and a less severe acute injury response. The pre-treatment paradigm in this study possesses a potential limitation, as one cannot predict when a stroke will occur. However, the current study points to a potential preventative intervention that may be neuroprotective to individuals at high risk of developing stroke injuries or recurring strokes. The timing of CQ administration may also be an important factor when considering its therapeutic window [27]; thus, it is possible that altering the timing of the treatments would provide a more effective long-term therapeutic response.

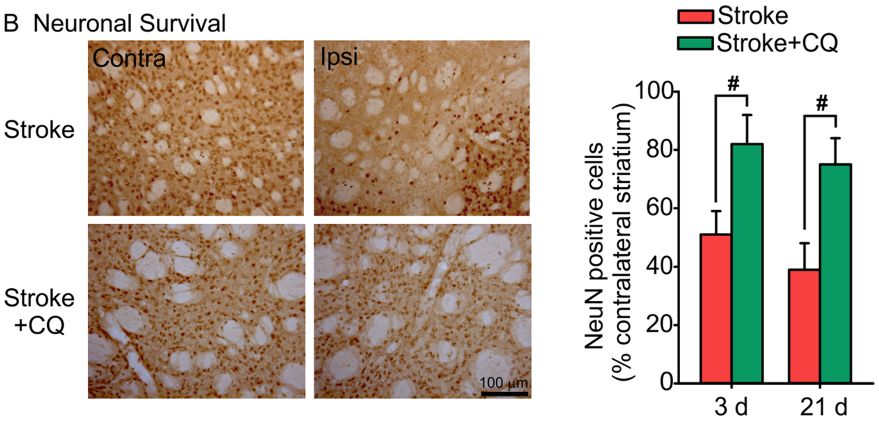
Additionally, we showed, for the first time, that the exogenous administration of GM3 to primary cortical neurons was toxic to primary cortical neurons, supporting the hypothesis that the accumulation of this simple ganglioside at the site of injury after a stroke may be playing a mechanistic role in the propagation of neurodegeneration. Interestingly, CQ administration effectively reduced apoptosis and increased cell viability in primary cortical neurons exposed to exogenous GM3. Although the mechanisms in which CQ was able to protect primary cortical neurons from GM3 toxicity remain unclear, it is possible that CQ was able to block GM3 from binding and/or incorporating into cells to effect cell signaling and alter membrane composition. It was also interesting that CQ was much more effective at reducing cell death indicated by PI-positive staining than TUNEL-labeled apoptosis. The mechanism for this observation is not clear, and future work should assess how CQ protects differentially based on the type of cell death following exposure to GM3. Regardless, these finding points to the possibility that CQ may not only be preventing the breakdown of complex gangliosides in response to the stroke through its lysosomotropic properties, but may also confer protection against external sources of GM3 present in the extracellular environment during the ischemic cascade.

Rubovitch et al. suggest that the restoration of GM1 shortly after the injury may be preventing the cascade of events that lead to neurodegeneration after stroke [17]. Mechanisms such as excitatory amino acids, nitric oxide, inflammatory mediators, reactive oxygen species, immune modulation, withdrawal of trophic

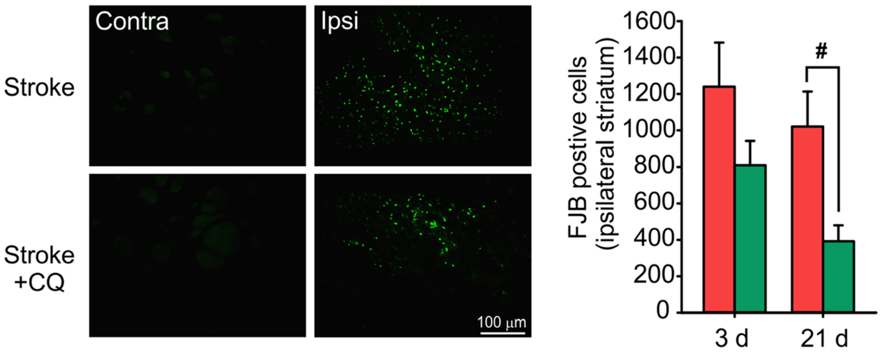
A Limb Motor Assessment



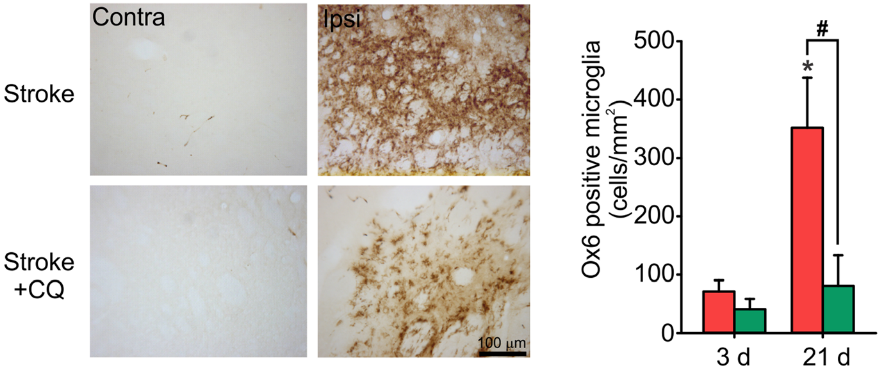
B Neuronal Survival



C Neurodegeneration



D Activated Microglia



factors, and apoptosis are all thought to be involved in the development of the ischemic cascade [3]. Interestingly, GM1, like CQ, has been found to inhibit autophagy after a stroke injury [16] and can upregulate brain-derived neurotrophic factor [19], promoting cell survival. GM1 has also been associated with the attenuation of secondary neuronal damage when administered in the acute stages of stroke by blocking of EEAs-related neurotoxicity [37]. One of the protective effects of CQ administration observed in the current study was the restoration of depleted GM1 at the site of injury. It is, therefore, possible that the decreased pathological response and motor impairments observed in the treated rats at the later time point were associated with both the attenuation of simple ganglioside accumulation in the acute stages of recovery along with the maintenance of GM1 levels throughout.

Summary/Conclusion

Chloroquine treatment can be used to restore ganglioside homeostasis after a severe ischemic stroke injury and is associated with long-term pathological and functional improvements in rats. Interventions targeting membrane lipid dysregulation may be a crucial component of effective multifactorial approaches to stroke prevention and treatment and are worthy of further investigation.

Acknowledgements We are grateful to Dilani Logan and Justin Kim for their assistance in the quantification of MALDI data and behavioral analyses.

Author Contributions SC conceptualized experiments, performed all MALDI-IMS, primary cortical neuron, and behavior experiments, analyzed the data, and wrote the manuscript. LW performed cell extraction and plating of all primary cortical neurons. JH performed and analyzed all immunohistochemistry experiments. DFC and KY conceptualized experiments and edited the manuscript. SNW conceptualized experiments and edited the manuscript and figures.

Sources of Funding This work was funded by NSERC, CIHR, CCNA operating grants and a CFI equipment grant to SNW, an NSERC operating grant to KK-CY, a CIHR emerging team grant to DFC and SNW, and an NSERC PGS-D award to SC.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to disclose.

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