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MiR-204-5p signalling modulates apoptosis and mitophagy-related changes after intracerebral hemorrhage in adult rats

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Abstract

Background: The survival of neurons around the hematoma is crucial for the recovery of neurological function after spontaneous intracerebral hemorrhage (ICH). Neuronal apoptosis and mitochondrial quality control disturbances are important factors for secondary brain injury after ICH, but their molecular mechanisms have not been fully elucidated. This study investigated the role of miR-204-5p in ICH and analyzed its association with apoptotic and mitophagy-related signalling.

Methods: A collagenase-induced ICH model was established in adult male Sprague-Dawley rats. miR-204-5p expression was assessed in perihaematoma brain tissue and in serum from patients with ICH. Neurological deficits, brain water content and blood-brain barrier permeability were evaluated after intracerebroventricular administration of a miR-204-5p agomir. Apoptosis-associated markers and mitophagy-related proteins were assessed by western blotting, TUNEL staining, immunofluorescence and transmission electron microscopy. Target prediction, luciferase reporter assays and gene knockdown experiments were used to investigate TAOK1 and CAMK1. GATA3 promoter activity and the effects of donepezil pretreatment were also examined.

Results: miR-204-5p was reduced in perihaematoma brain tissue and in serum from patients with ICH, and lower serum levels were associated with higher NIHSS and mRS scores in the available clinical samples. miR-204-5p overexpression improved neurological deficits and reduced apoptosis-associated changes and mitophagy-related molecular and ultrastructural changes after ICH. TAOK1 and CAMK1 were identified as direct post-transcriptional targets of miR-204-5p by bioinformatic prediction, expression analysis and dual-luciferase assays. TAOK1 knockdown reduced TUNEL-

positive signals, whereas CAMK1 knockdown attenuated mitochondrial ultrastructural abnormalities. GATA3 increased miR-204-5p promoter activity, and its expression was reduced after ICH. Donepezil pretreatment increased GATA3 expression and was associated with reduced apoptosis-associated and mitophagy-related changes.

Conclusions: Together, these findings suggest that the GATA3 – miR-204-5p – CAMK1/TAOK1 axis may contribute to neuronal injury after ICH. Mitophagic flux and the causal relationships among these components remain to be established. The clinical association and donepezil findings are preliminary and require further validation.

Keywords: intracerebral hemorrhage, miR-204-5p, mitophagy-related changes, neuronal apoptosis, donepezil

Introduction

Spontaneous intracerebral hemorrhage(ICH) accounts for 10–15% of strokes,with high morbidity,mortality,and disability[1,2]. With an ageing population, the lack of effective therapies to improve prognosis and functional recovery remains an important clinical challenge.

ICH involves primary mechanical compression and secondary injury[3]. Surgical hematoma removal has not consistently improved neurological outcomes, highlighting the importance of secondary injury,though its mechanisms remain complex.Survival of perihematomal neurons is critical for recovery[4].Numerous studies show that secondary injury induces substantial neuronal death around the hematoma[5-7],leading to severe deficits and poor prognosis,yet the specific mechanism is still unclear[8].

ICH induces excessive mitochondrial damage and neuronal apoptosis[9,10], both of which contribute to secondary brain injury. Mitophagy selectively removes damaged mitochondria and is an important component of mitochondrial quality control[11]; however, changes in mitophagy-related markers may reflect either increased mitochondrial turnover or impaired autophagic flux. Proper autophagy clears damaged organelles and proteins and can influence neuronal survival[12,13]. Previous studies have investigated the effects of modulating apoptosis and mitochondrial quality control in stroke and ICH[14]. Identifying molecular pathways that coordinately influence these processes may therefore provide additional insight into secondary brain injury.

MicroRNAs (miRNAs) are noncoding RNAs that regulate gene expression post-transcriptionally. Accumulating evidence links miRNA dysregulation to the pathological process of ICH[15,16]. Our group previously reported roles for miR-181c, miR-21-5p and miR-23a-3p in inflammatory or blood-brain barrier-related responses after ICH[15-17]. The present study extends this line of investigation to miR-204-5p, which showed a marked reduction in our previously generated ICH miRNA profile. Importantly, our data combine neuronal localization of miR-204-5p with two target-related branches involving TAOK1 and CAMK1, linking miR-204-5p to apoptosis-associated and mitochondrial quality-control phenotypes. We therefore investigated whether miR-204-5p contributes to secondary injury after ICH and explored upstream GATA3-associated regulation and the potential pharmacological relevance of donepezil.

Ethical approval

All animal experiments were reviewed and approved by the Ethics Committee of the Affiliated Hospital of Jiujiang University(approval number:20200012).All animal-related experimental procedures were performed in accordance with the animal care guidelines of Jiujiang University.

Experimental animals

A total of 140 male Sprague-Dawley rats (18-week-old, weighing 500–600 g) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. These

77 animals were used as an adult rat model. Because only male adult rats were studied, the
78 generalizability of the findings to females and to aged animals requires further
79 investigation.
80
81 Materials and methods
82 **Injecting agomir or virus and inducing a model of intracerebral hemorrhage**
83 The rats were anaesthetized and fixed in a stereotaxic apparatus,the head skin was
84 prepared and disinfected,and a median incision was made.The coordinates of the lateral
85 ventricle(perpendicular to the bregma,2 mm to the right,1.2 mm to the back,and 4 mm
86 deep)were used to connect the Hamilton microsyringe to the micropump.miR-204-5p
87 agomir or scramble was injected at 0.5 μl/min.One week later,a rat model of
88 intracerebral hemorrhage was established using collagenase according to the method of
89 Park et al.[18].One day later,experiments such as behavioural experiments were
90 performed.Similarly,CAMK1 knockdown lentivirus or TAOK1 knockdown lentivirus
91 and corresponding control virus were injected in situ in the right basal ganglia
92 coordinates(3 mm perpendicular to bregma,0.2 mm anterior,6 mm deep)for 3
93 weeks.After the intracerebral hemorrhage model was induced,the detection experiment
94 was performed one day later.
95 **Western blotting**
96 Tissues or cells were lysed in RIPA buffer(Sigma‐Aldrich),and protein concentrations
97 were determined by BCA assay.Equal amounts of protein(20 μg) were separated by

SDS-PAGE,transferred onto nitrocellulose membranes,and blocked with 3% BSA-TBST for 1 h at room temperature.Membranes were then incubated overnight at 4° C with primary antibodies against β -actin(Abcam,1:200),LC3II(Cell Signaling Technology,1:1000),Parkin(CST,1:1000),PINK1(Abclonal,1:1000),Cleaved caspase-3(CST,1:1000),total caspase-3(CST,1:1000), CAMK1(Abcam,1:5000), TAOK1(CST,1:1000),GATA3(Abcam,1:1000),Cleaved PARP(CST,1:1000),or Bak(CST,1:1000).After three TBST washes(10 min each),membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature in the dark,followed by another three TBST washes.Immunoreactive bands were visualized using an Odyssey imaging system and quantified by densitometry.

RT–qPCR

According to the manufacturer's instructions,the miRNA Isolation Kit(TIANGEN,Beijing,China).Lot:DP 501)extracts total RNA from rat brain tissue,human serum specimens or HEK 293 T cells.Total RNA was reverse transcribed for miRNA using the miRcute Enhanced miRNA First-Strand cDNA Synthesis Kit(TIANGEN,Beijing,China,Lot KR 211).Total RNA was reverse transcribed for mRNA using the QuantiNova™ Reverse transcription Kit(QIAGEN,Germany,ID:205410).The SYBR Green I fluorescent dye method(SYBR® PrimeScript™ RT–PCR Kit(Takara Biotechnology Co.)was used for PCR detection.Amplification of miRNA/mRNA was performed on an Applied Biosystems StepOne™ Real-Time PCR System(Applied Biosystems).The cycling

conditions were according to the manufacturer's instructions.U6 snRNA/GAPDH served as the internal control for RNA quantification.The relative concentration was calculated by the 2-ΔΔCT method.

Brain slice preparation

In short,the rat were perfused with normal saline,fixed with 4% paraformaldehyde and then treated with a gradient of 10%,20%,30%,and 30% sucrose solutions.Tissues were dehydrated by gradient sucrose solutions,embedded in OCT,and then placed in a microtome,and the corresponding parameters were set.Brain slices were stored in PBS-glycerol at 4 °C for subsequent experiments.

Immunofluorescence

The brain slices were rinsed 3 times in PBS at room temperature for 10 min each time;PBS solution of 0.5% Triton X-100 was added,and the membrane was broken by shaking slowly for 15 mins.LC 3II(Cell Signaling Technology,1:2000)was incubated at 4 °C for 24-28 hours and then rinsed with PBS 3 times for 10 min each time.Thereafter,secondary antibody was added and incubated for 2 hours at room temperature in the dark,after which the sections were rinsed with PBS 3 times for 10 min each time .Next,DAPI was added to stain the nuclei for 10 min at room temperature in the dark,then rinsed 3 times with PBS,attached to a glass slide,air-dried naturally in the dark,and mounted with PBS-glycerol.

TUNEL

Brain slices were rinsed 3 times in PBS at room temperature for 10 min each,and 3% BSA was added to a shaker and slowly shaken for 10 mins.Then,the slices were rinsed 3 times with PBS for 5 min followed by 0.1% Triton X-100 containing 0.1% sodium citrate solution,shaken slowly for 8 min on a shaker in cold storage at 4 °C,rinsed with PBS 3 times for 5 min each time,and then attached to a glass slide.TUNEL detection solution was prepared according to the TUNEL kit(Beyotime,C1089)(5μl Td T enzyme +45μl fluorescent labelling solution),TUNEL detection solution was added dropwise,the slides were placed in a wet box,incubated for 1 hour at 37 °C in the dark,and rinsed with PBS 3 times for 5 min each.Thereafter,DAPI was added to stain cell nuclei for 10 min at room temperature in the dark and rinsed 3 times with PBS every 5 min.Brain slices were naturally air-dried under dark conditions and mounted with PBS-glycerol.

Fluorescence in situ hybridization

RNase-free consumables and reagents were used.At room temperature,the brain slice was rinsed in PBS containing 0.1% RNase Remover,a PBS solution of 0.5% Triton X-100 was added,and the shaking table was slowly shaken for 15 min.Then,the sections were digested at 37°C for 1 min followed by fixation with 1% paraformaldehyde containing 0.1% DEPC for 15 min.The brain slices were placed into a wet box,prehybridization solution was added dropwise at 38°C for 4 hours,hybridization solution was added dropwise,and the sections were hybridized overnight at 42°C. After hybridization,the cells were washed twice with 2x SSC at 37°C for 5 min each and

then washed once with 0.5x SSC at 37°C for 15 min and twice with 0.2x SSC at 37 °C for 15 min.The blocking solution was added dropwise at 37 °C for 30 min,and target–protein primary antibodies(GFAP(Abcam 1:1000),Iba-1(Abcam 1:1000),NeuN(Abcam 1:1000),TOM20(Cell Signaling Technology,1:400))were added overnight at 4 °C.Biotinylated mouse anti-digoxigenin was added dropwise at 37 °C for 60 mins,then rinsed 4 times with PBS for 5 min each;1% SABC-FITC-containing PBS solution was added dropwise for 30 min at 37 °C after rinsing with PBS.Next,target protein secondary antibody was added,incubated at 37 °C for 60 min,rinsed with PBS,and then DAPI was added to stain nuclei for 10 min at room temperature in the dark and rinsed with PBS,air-dried,and mounted with PBS- glycerol.

TEM observation

Rats were anaesthetized,and the volume of rice grains was taken from the fresh brain tissue around the foci,quickly placed under an electron microscope for fixation,and fixed at 4 °C for 4 hours.Then,the cells were rinsed with PBS 3 times for 15 mins each,followed by the addition of 1% osmic acid-containing PBS solution at room temperature,and fixed for 2 hours.The cells were rinsed 3 times with PBS for 15 min each.Then,50%-70%-80%-90%-95%-100%-100% alcohol-100% acetone-100% acetone dehydration was added successively,each time for 15 min.Then,1/2 acetone in 812 embedding medium was added for 2-4 hours,infiltrated in 1/3 acetone-containing 812 embedding medium overnight,in pure 812 embedding medium for 5-8 hours,and poured into pure 812 embedding medium.The samples were inserted into the

embedding plate,placed in a 37 °C oven overnight,polymerized at 60 °C for 48 hours and cut into 70 nm ultrathin sections in an ultramicrotome.Then,the sections were double-stained with uranium lead(2% uranyl acetate saturated alcohol solution,lead citrate,stained for 15 min each),dried at room temperature overnight and finally observed under a transmission electron microscope,and images were collected for analysis.

Cell transfection

The cells were cultured in a well plate to 70% density for transfection,and the medium was changed 2 h before transfection.Lipo3000 reagent was diluted with Opti-MEM and mixed ,the plasmid was diluted with Opti-MEM,and P3000 reagent was added and mixed(transfected siRNA)or(miRNA without P3000 reagent).The diluted Lipo3000 was mixed with the plasmid diluent in equal proportions and incubated at room temperature for 10 min.Then,the incubated mixture was added to the cells and cultured for 48 hours before performing subsequent experiments.

Dual luciferase

For the luciferase reporter assay, psiCHECK2 constructs containing the wild-type (WT) or mutant (MUT) 3′UTRs of TAOK1 or CAMK1 were generated based on the predicted miR-204-5p binding sites and transfected into HEK293T cells together with miR-204-5p mimic or negative control. After 48 h, cells were harvested and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA). Each transfection was performed in triplicate.

Corner experiment

Two plates forming a 30° corner with a small gap at the junction were used.Rats placed facing the corner naturally approached it;stimulated by whiskers,they turned to one side.Only complete turns were counted,and the number of right turns out of 10 trials was recorded,with intervals ≤2 min.

Determination of water content in brain tissue

Brain water content was measured by wet-dry weight method.Ipsilateral and contralateral peri-lesional tissues were collected after anesthesia,surface water blotted,and wet weight immediately weighed.Tissues were dried at 100° C for 24h to obtain dry weight.Water content =(wet weight − dry weight) / wet weight × 100%.

Blood–brain barrier permeability test

Evans blue assay was performed.After anesthesia,2% Evans blue(4 ml/kg) was injected via tail vein.Two hours later,rats were perfused with saline to remove intravascular dye.Brain tissues were homogenized in 50% trichloroacetic acid,centrifuged,and supernatant diluted 1:3 in ethanol.Absorbance was measured at 620 nm.

Statistical methods

For statistical analysis, SPSS 22 software (IBM, Armonk, USA) was used, and the results are expressed as the mean ± standard error (mean ± SEM). $P < 0.05$ indicates a significant difference between the two groups of results. When analysing the data of the two groups, if the variance was homogeneous, the test was used to analyse the significant difference, and if the variance was unequal, Mann–Whitney

analysis was used. To analyse multiple groups of sample data, one-way ANOVA or two-way ANOVA was used for significant difference analysis if the variance was homogeneous, and the Kruskal–Wallis method was used to approximate the variance analysis if the variance was unequal.

Results

3.1 Reduction of miR-204-5p is associated with neurological deficits after ICH

We first examined the top 10 downregulated miRNAs identified in our previous ICH miRNA profiling study (Ouyang Y et al., 2019) (Fig. 1A). Four miRNAs—miR-501-3p, miR-409-3p, miR-204-5p and miR-150-5p—were significantly decreased in ICH rats (Fig. 1B). Among these candidates, miR-204-5p showed the largest reduction and was therefore selected for further study (Supplementary Table S1). We then examined miR-204-5p expression and neurological outcomes at different time points after ICH. Neurological impairment, assessed by mNSS and the corner test, was greatest at 24 h and gradually improved from days 1 to 7 (Fig. 1C–D), whereas miR-204-5p was most strongly reduced at 24 h and gradually recovered thereafter (Fig. 1E). Serum miR-204-5p in patients with spontaneous ICH showed a similar temporal pattern (Fig. 1F). In the available clinical samples, serum miR-204-5p was inversely associated with NIHSS and mRS scores at the assessed time point (Fig. 1G–H). These findings support an association between reduced miR-204-5p expression and ICH severity, but do not establish that circulating miR-204-5p originates from perihaematoma neurons.

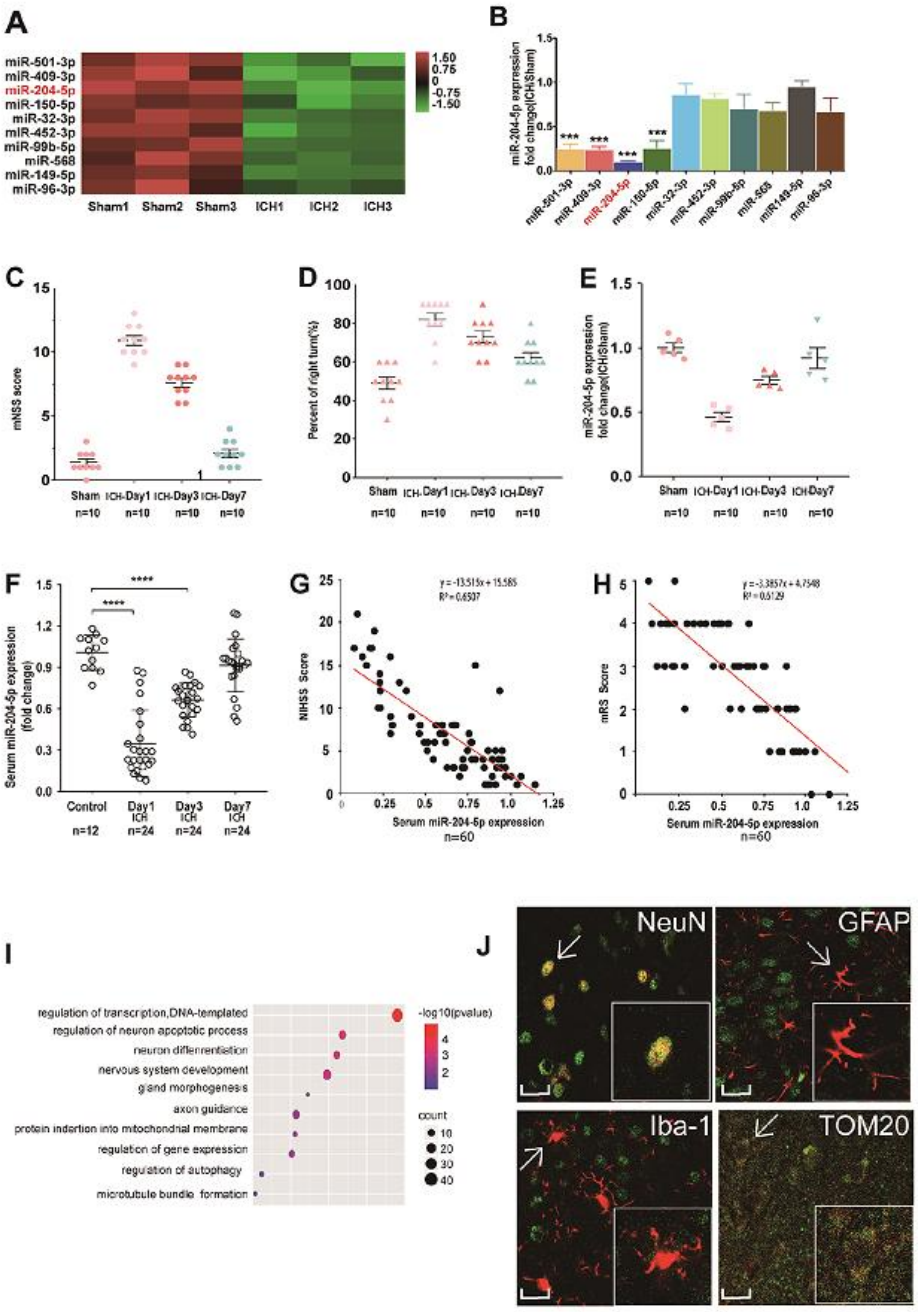
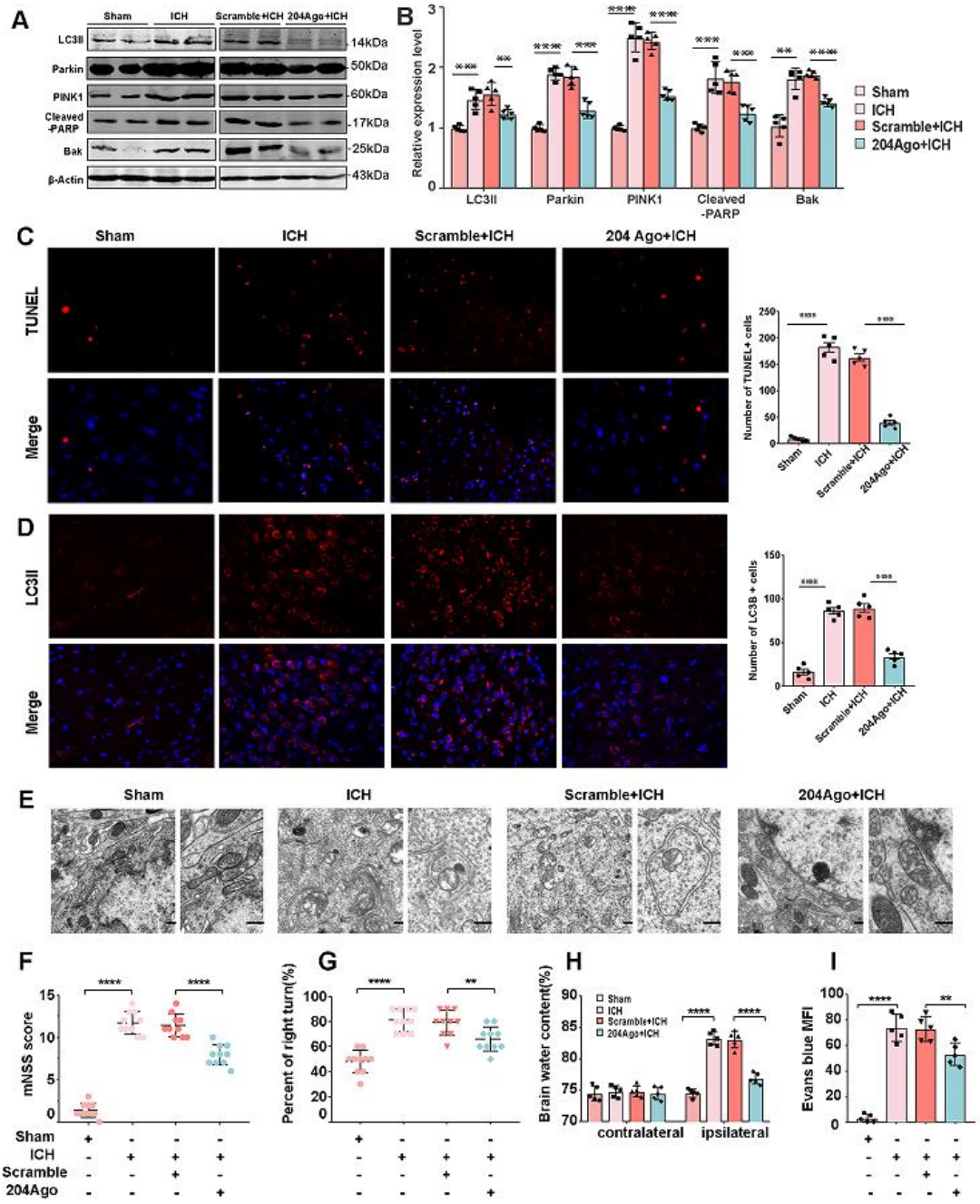


Fig.1

3.2 miR-204-5p overexpression attenuates apoptosis-associated and mitophagy-related changes after ICH

We used TargetScan, miRDB and miRWalk to predict potential targets of miR-204-5p (Supplementary Fig. S1A–D) and analysed the intersection of the predicted target genes.

GO enrichment analysis showed enrichment of biological processes related to cell death, including apoptosis (GO:0043525, $P = 4.38 \times 10{-}4$), whereas the enrichment for autophagy did not reach conventional statistical significance (GO:0010506, P = 0.058) (Supplementary Fig. S1E – G). (Supplementary Fig. S1E–G). Using a specific probe for miR-204-5p, we co-labelled miR-204-5p with NeuN, GFAP and Iba-1 and found that miR-204-5p was predominantly detected in neurons and was less abundant in astrocytes and microglia (Fig. 1J). miR-204-5p signal was also observed in the cytoplasm and mitochondrial compartment of neurons. We next examined apoptosis-associated and mitophagy-related changes after miR-204-5p supplementation. ICH increased LC3-II, Parkin, PINK1, cleaved PARP and Bak, together with increased TUNEL and LC3-II signals (Fig. 2A–D). miR-204-5p agomir reduced these changes. Transmission electron microscopy showed more autophagosome-like structures and mitochondrial structural abnormalities after ICH, whereas miR-204-5p treatment was associated with preservation of mitochondrial morphology (Fig. 2E). miR-204-5p agomir also improved mNSS and corner-test performance and reduced brain water content and Evans blue leakage (Fig. 2F–I). Collectively, these data indicate that miR-204-5p overexpression is associated with improved neurological outcomes and reduced apoptosis-associated and mitophagy-related changes. Because autophagic flux was not directly measured, these findings should not be interpreted as definitive evidence of increased or decreased mitophagic flux.



270

271 Fig.2

272 **3.3 miR-204-5p directly targets CAMK1 and TAOK1**

273 To identify candidate functional targets of miR-204-5p, we combined target prediction

274 with pathway analysis and expression screening. Among the predicted candidates, 11

275 genes were present in the intersection of the selected target lists (Supplementary Fig.

S1I). PIP4K2B and TAOK1 were associated with apoptosis-related pathways, whereas RPS6KA3 and CAMK1 were associated with mitochondrial quality-control pathways. All four proteins were increased after ICH, while TAOK1 and CAMK1 mRNA levels were comparable between ICH and sham groups (Fig. 3A–B), suggesting post-transcriptional regulation. Conserved miR-204-5p binding sites were identified in the 3′UTRs of TAOK1 and CAMK1 (Fig. 3E,G). In HEK293T cells, miR-204-5p mimic reduced luciferase activity from WT, but not MUT, TAOK1 and CAMK1 3′UTR reporters (Fig. 3F,H). miR-204-5p agomir also reduced TAOK1 and CAMK1 protein levels in perihaematoma tissue (Fig. 3I–J). These results support direct post-transcriptional regulation of TAOK1 and CAMK1 by miR-204-5p. However, rescue experiments were not performed; therefore, TAOK1 and CAMK1 should be regarded as direct targets rather than definitively established mediators of all miR-204-5p effects.

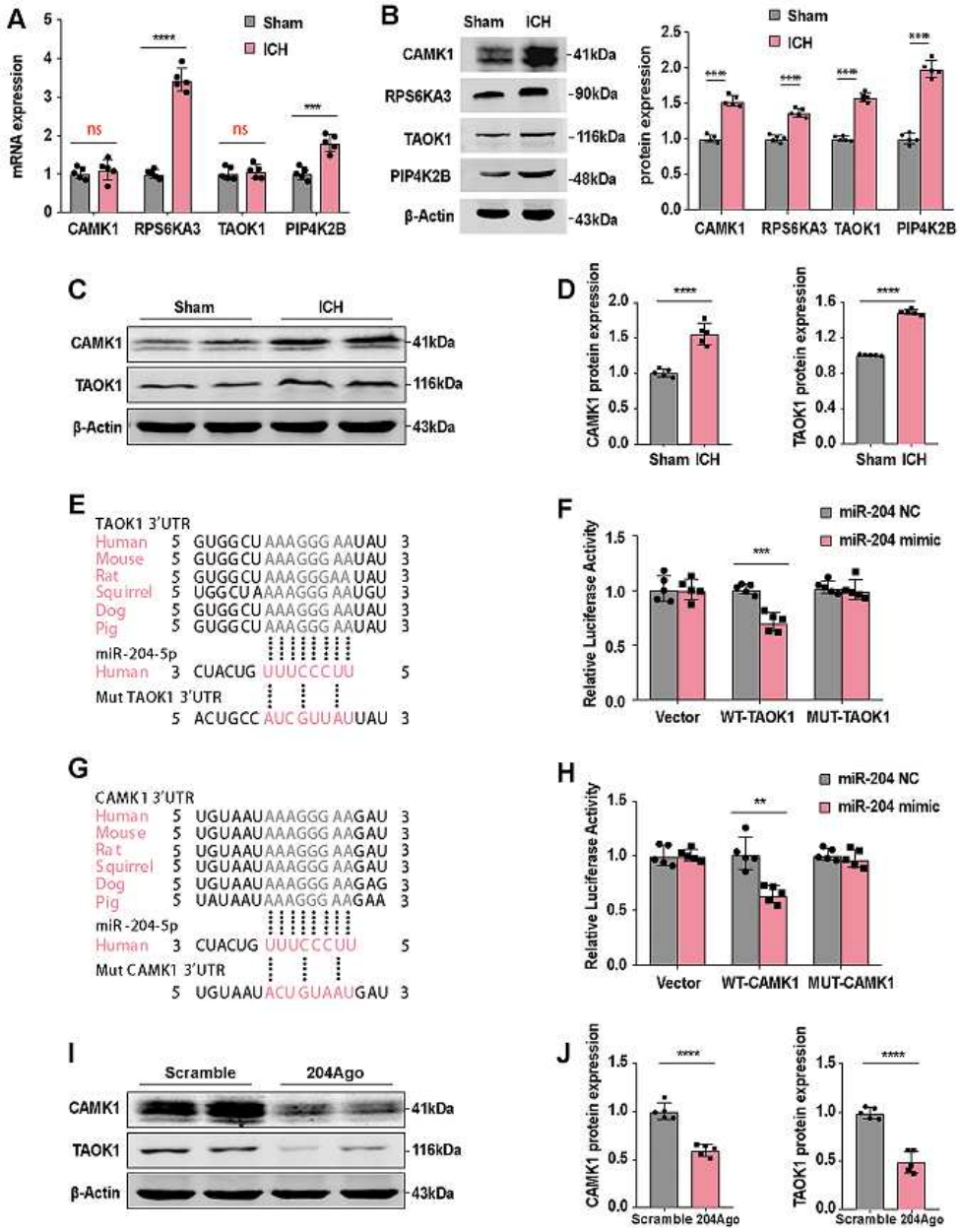
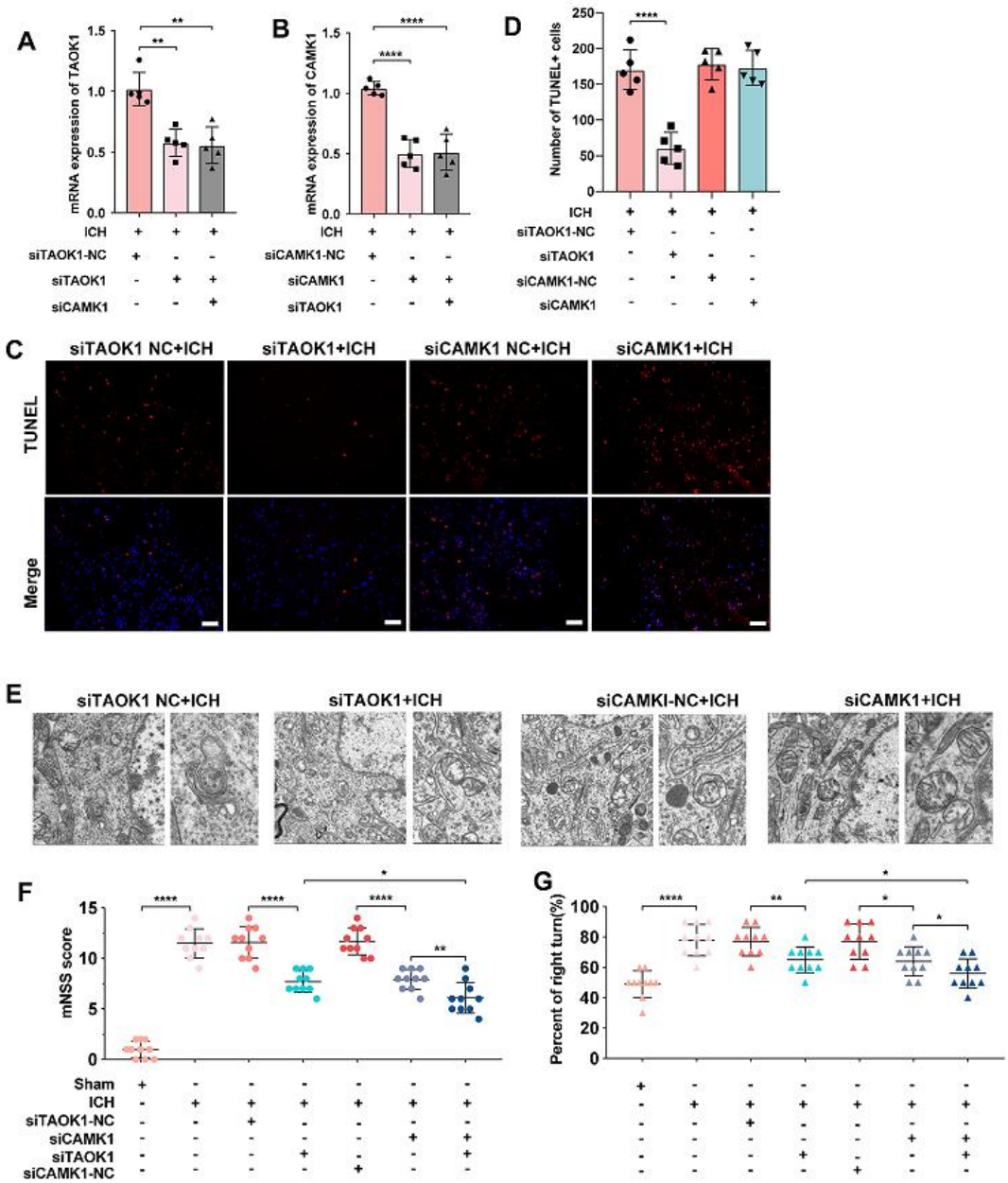


Fig.3

3.4 TAOK1 and CAMK1 knockdown phenocopies selected effects of miR-204-5p after ICH

To examine the functional contribution of TAOK1 and CAMK1, we injected AAV vectors carrying shRNAs targeting TAOK1 or CAMK1, or corresponding control vectors, into the basal ganglia before ICH induction. TAOK1 knockdown reduced

TUNEL-positive signals, whereas CAMK1 knockdown was associated with preservation of mitochondrial ultrastructure and fewer abnormal autophagosome-like structures (Fig. 4C–E). Neurological deficits were improved by TAOK1 or CAMK1 knockdown and were further reduced when both targets were inhibited (Fig. 4F–G). These results show that TAOK1 and CAMK1 knockdown can phenocopy selected protective effects associated with miR-204-5p overexpression. However, because target re-expression experiments were not performed, the data do not establish epistatic mediation or exclude contributions from additional miR-204-5p targets.



303

304 Fig.4

305 **3.5 GATA3-associated regulation of miR-204-5p and exploratory effects of**

306 **donepezil**

307 To explore upstream regulation of miR-204-5p, we cloned 1-kb, 2-kb and 3-kb

308 upstream regions into a pGL3 reporter (Supplementary Fig. S1J). The 2-kb construct

309 showed the strongest promoter activity (Supplementary Fig. S1K). RegRNA and

LASAGNA prediction identified potential binding motifs for GATA1, GATA3, AP-1, Cart-1, NF-1 and PU.1; among the candidates tested, GATA3, but not the other tested factors, increased miR-204-5p promoter reporter activity (Supplementary Fig. S1L and Fig. 5A). Mutation of the tested GATA3-associated region reduced miR-204-5p promoter activity relative to the corresponding construct (Fig. 5B). GATA3 mRNA and protein levels were reduced after ICH (Fig. 5C–E). These findings support a regulatory association between GATA3 and miR-204-5p transcription, but direct chromatin binding was not assessed by ChIP. We next screened four compounds reported to influence GATA3-related signalling and found that only donepezil increased GATA3 expression in the tested cell system (Fig. 5F). Donepezil was administered intraperitoneally for 14 days before ICH induction. Pretreatment partially improved neurological scores, restored GATA3 mRNA expression and reduced apoptosis-associated and mitophagy-related molecular changes (Fig. 5G–L). Because donepezil was administered before ICH and no miR-204-5p loss-of-function experiment was performed, these data provide pharmacological support but do not establish that donepezil neuroprotection requires the GATA3–miR-204-5p pathway.

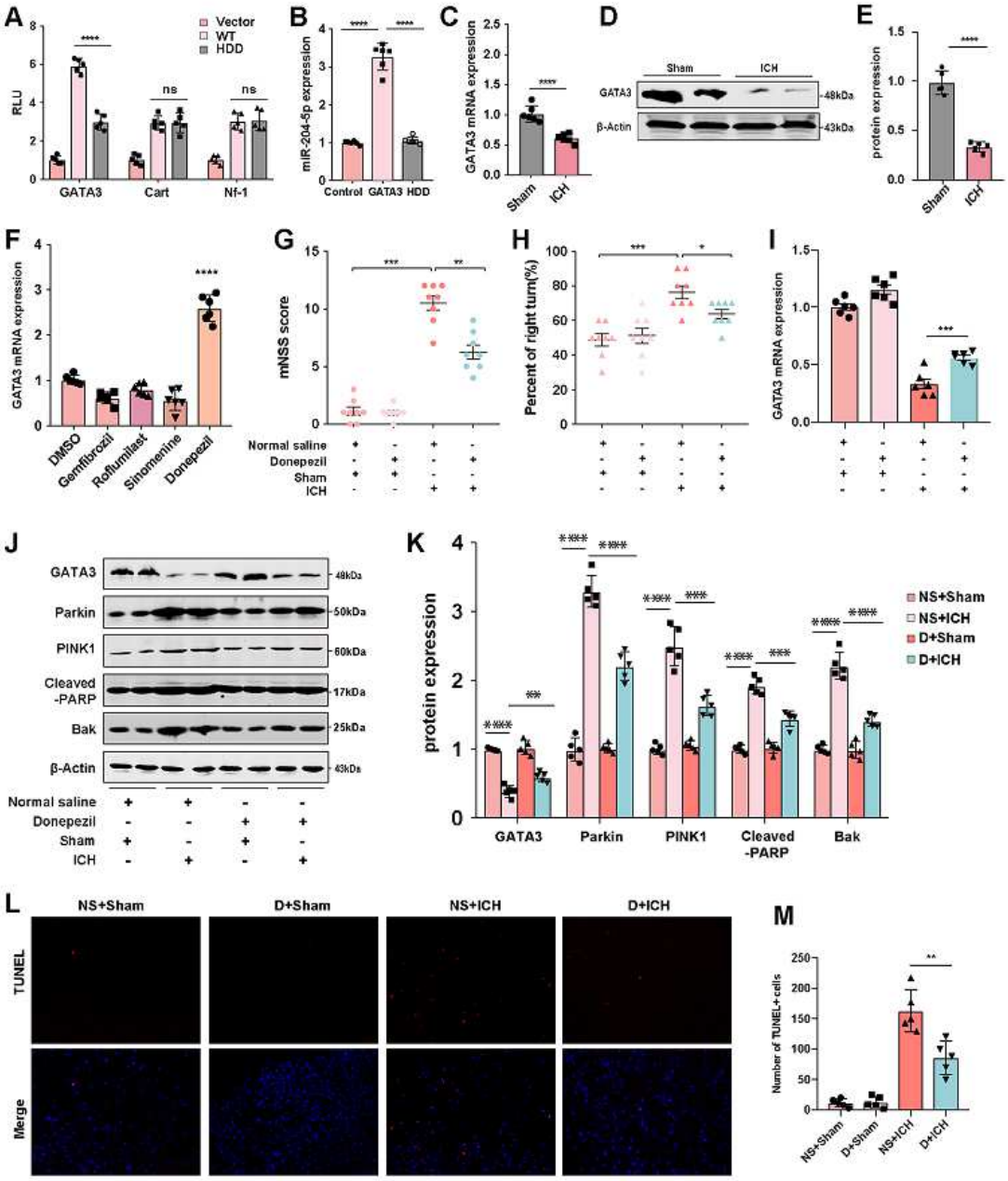


Fig.5

4Discussion

Our findings support a regulatory model in which miR-204-5p is reduced after ICH and its supplementation is associated with improved neurological outcomes and reduced apoptosis-associated and mitophagy-related changes. We identified TAOK1 and CAMK1 as direct post-transcriptional targets and found that their knockdown

phenocopied selected protective effects. We further identified GATA3 as a candidate upstream regulator of miR-204-5p promoter activity and found that donepezil pretreatment increased GATA3 expression. Importantly, the present study should be interpreted as a mechanistic framework rather than a fully established linear pathway because mitophagic flux, target-rescue/epistasis and pharmacological dependency were not directly tested.

4.1 Reduced miR-204-5p after ICH is associated with neurological impairment

Our group has previously reported miR-181c, miR-21-5p and miR-23a-3p as regulators of inflammatory or blood-brain barrier-related responses after ICH[15-17]. The present study addresses a different biological question by focusing on miR-204-5p and its relationship with apoptosis-associated and mitochondrial quality-control phenotypes. miR-204-5p was reduced in perihaematoma brain tissue and in serum from patients with ICH, and lower serum levels were associated with higher NIHSS and mRS scores in the available samples. The neuronal localization of miR-204-5p and the effects of miR-204-5p supplementation provide a rationale for further investigation. Nevertheless, serum miR-204-5p cannot be assumed to reflect neuronal miR-204-5p directly because the present study did not examine cerebrospinal fluid or neuron-derived extracellular vesicles.

4.2 miR-204-5p is associated with apoptosis and mitochondrial quality-control changes after ICH

Excessive neuronal apoptosis contributes to secondary injury after ICH, while mitochondrial damage caused by oxidative stress, inflammation and excitotoxicity can further compromise neuronal survival[24,27]. Mitophagy is an important component of mitochondrial quality control, but its biological effect depends on the balance between mitochondrial clearance and degradation capacity. Our increases in LC3-II, Parkin and PINK1, together with electron-microscopic changes, indicate altered autophagy/mitochondrial quality-control signalling after ICH. However, these measurements alone cannot distinguish increased mitophagy from impaired autophagic flux. The reduction of these markers and improvement in mitochondrial morphology after miR-204-5p supplementation therefore support an association with improved mitochondrial homeostasis, but not a definitive conclusion about the direction of mitophagic flux. Future studies using flux reporters, lysosomal inhibition, mitochondrial-lysosomal colocalization and functional mitochondrial measurements will be required.

4.3 TAOK1 and CAMK1 are direct post-transcriptional targets of miR-204-5p

By combining target prediction, pathway analysis and expression screening, we identified TAOK1 and CAMK1 as candidate targets of miR-204-5p. Conserved 3′UTR binding sites, WT/MUT reporter assays and reduced endogenous protein levels after miR-204-5p agomir support direct post-transcriptional regulation. Knockdown experiments further showed that TAOK1 and CAMK1 contribute to distinct phenotypic components: TAOK1 knockdown was associated with reduced TUNEL signals,

whereas CAMK1 knockdown was associated with improved mitochondrial ultrastructure. These findings are consistent with previous reports linking TAOK1 to apoptosis and CAMK-related proteins to mitochondrial quality control[30,31]. Nevertheless, target re-expression using 3′UTR-less constructs was not performed. Thus, the present data support target regulation and phenotypic contribution but do not prove that TAOK1 and CAMK1 are the exclusive or obligatory mediators of miR-204-5p-mediated protection.

4.4 GATA3 and donepezil: a hypothesis-generating upstream regulatory component

GATA3 is a transcription factor involved in cell differentiation, apoptosis and immune regulation[32-34]. Our promoter-reporter experiments suggest that GATA3 can increase miR-204-5p promoter activity, and GATA3 expression was reduced after ICH. These findings support GATA3 as a candidate upstream regulator, but direct promoter occupancy was not demonstrated by ChIP and the effects of GATA3 loss- or gain-of-function on endogenous pri-miR-204 were not examined. We therefore regard the GATA3–miR-204-5p relationship as supportive but not definitive. Donepezil pretreatment increased GATA3 expression and was associated with improved neurological outcomes in our adult rat model. However, the treatment was initiated before ICH, the therapeutic window after ICH was not assessed, and the dependence of the effect on miR-204-5p was not tested. Molecular docking suggested an interaction with GATA3, but this interaction was not validated by mutational or biochemical

experiments. Accordingly, donepezil should be considered a hypothesis-generating pharmacological observation rather than evidence for a miR-204-5p-dependent therapeutic mechanism.

4.5 Study limitations

Several limitations should be considered. First, the present study used 18-week-old adult rats and therefore does not specifically represent aged ICH. Second, although changes in LC3-II, PINK1, Parkin and mitochondrial ultrastructure were observed, mitophagic flux was not directly assessed. Therefore, these findings should be interpreted as mitophagy-related changes rather than definitive evidence of altered mitophagy flux. Third, although TAOK1 and CAMK1 were identified as direct targets of miR-204-5p and their knockdown reproduced selected effects of miR-204-5p overexpression, rescue or epistasis experiments were not performed to establish a definitive causal relationship. Similarly, the regulatory relationship between GATA3 and miR-204-5p was not further validated by ChIP or endogenous transcriptional assays. Finally, the effects of donepezil were examined only in a pretreatment setting, and whether its effects depend on miR-204-5p remains to be determined. Further studies using aged animals, direct mitophagy-flux assays, rescue experiments and post-ICH treatment paradigms are needed to validate these findings.

Abbreviations

ICH:intracerebral hemorrhage,BBB:blood brain barrier,KEGG:Kyoto Encyclopedia of Genes and Genomes,CAMK1:calcium/calmodulin-dependent protein kinase 1,CAMKs:calcium/calmodulin-dependent protein kinases,TAOK1:Thousand and One Kinase 1

Figure Legends

Figure 1:**Expression and subcellular location of miR-204-5p,and its relationship with neurological deficits**.(**A**)Heat map showed the 10 downregulated candidate miRNAs after ICH with | log2 fold change | > 2 and $p < 0.05$ as the filter condition from the expression profile of miRNAs in perifocal brain tissue of rats between the ICH and sham group.(**B**)RT-qPCR was performed to validate the expression of the 10 miRNAs around the perifocal tissue,suggested that 4 miRNAs - miR-501-3p,miR-409-3p,miR-204-5p,miR-150-5p were found significantly decreased.Using the $2^{-\Delta\Delta CT}$ method with U6 expression levels as an internal reference.All results are presented as mean ± SEM of five independent experiments.(**C**)We established ICH rat models at different time points on day 1,day 3 and day 7,evaluated neurological damage like motor nerves,sensory function,reflex ability,abnormal activity with mNSS scores.(**D**)Assessed the degree of hemiplegia with right turn behavior experiment.(**E**)Detection of miR-204- 5p expression level in each group.(**F**)miR-204- 5p expression in the serum of patients at different time points after the onset

of ICH.(**G-H**)Correlation between the expression of miR-204-5p and ICH patient's NIHSS and mRS score.(**I**)Use TargetScan(http://www.targetscan.org/),miRDB(http://mirdb.org/)and miRWalk(http://mirwalk.umm.uni-heidelberg.de/)database to predicted the target genes of these four miRNAs,took target genes intersection and enriched them with GO function ,suggesting that only miR-204-5p target genes are mainly involved in the regulation of cell death biological processes including neuron apoptosis(GO:0043525,p=4.38E-04)and autophagy(GO:0010506,p=0.05800458).(**J**)The subcellular distribution of miR-204-5p was visualized by RNA fluorescence in situ hybridization(FISH).astrocytes,microglia,neurons and mitochondria were identified by GFAP,Iba-1 and NeuN,Tom20 antibody,respectively.miR-204-5p is primarily localized in the nucleus and mitochondria of neurons.Scale bar=20um.***p < 0.001.****p<0.0001.

Figure 2:**Neuron apoptosis and mitophagy disorders increased 24h post-ICH,miR-204-5p overexpression rescued these change and improved neurological deficits.**(**A**)Western blotting detection of LC3II,Parkin,PINK1,Cleaved-PARP and Bak expression in sham-operated ,ICH group,Scramble+ICH group and miR-204-5p agomir(204Ago)+ICH group.(**B**)Quantitative analysis of western blotting results.**(C)**Apoptosis was visualized by the TUNEL Assay,and the TUNEL-

positive(red)cells were quantitatively analyzed.Scale bar=20um.(**D**)Immunofluorescence detection and quantification of LC3II levels in brain sections.Scale bar=20um.(**E**)Ultrastructure of neuronal mitochondria in perifocal brain tissue was observed by transmission electron microscope(TEM).Mitochondrial autophagosomes increased,cristae structures of some mitochondria swollen and disappeared,mitochondrial structure was seriously damaged in ICH or Scramble+ICH group,while that of 204Ago+ICH group was restored.Scale bar=500nm .**p<0.01,****p<0.0001.The neurological damage of rats in each group was evaluated by mNSS score(**F**)and corner behavioral test(**G**),n=10.(**H**)Dry-wet weight method was used to detect the brain water content of the ipsilateral and contralateral lesions of the rats in each group,n=5.(**I**)Evans blue assay to detect the permeability of the perifocal blood-brain barrier,n=5.

Figure 3:**miR-204-5p directly targets TAOK1 and CAMK1.**(**A**)mRNA and(**B**)protein expression levels of apoptosis-related genes(PIP4K2B,TAOK1)and mitophagy-related genes(RPS6KA3,CAMK1)were detected.The mRNA expression levels of TAOK1 and CAMK1 showed no significant change but protein expression increased when ICH versus sham group,implying post-transcriptional regulation.(**C**)Western blotting measure and(**D**)statistical analysis of TAOK1 and CAMK1 protein expression.(**E**)Predictive binding sites in TAOK1 3′UTR on mammalian miR-204-5p by bioinformatics analysis.(**F**)Constructed luciferase reporter

plasmid,psi-CHEK2 containing wild-type(WT-TAOK1)or mutant(MUT-TAOK1)3'UTR of TAOK1,transfected them to HEK293T cells with the mimic or negative control(NC)of miR-204-5p respectively for 48 hours,then performed luciferase reporter assay.Bar graph shows relative luciferase activities.(**G**)Putative binding sites in CAMK1 3′UTR on miR-204-5p among mammals.(**H**)Similar procedure to verify the direct bind relationship between CAMK1 and miR-204-5p.Relative luciferase activities were measured.(**I**)Protein expression of TAOK1 and CAMK1 around hematoma of ICH rats after injecting miR-204-5p agomir or negative scramble.(**J**)Quantification analysis of TAOK1 and CAMK1 protein levels.**p<0.01,***p<0.001,****p<0.0001.

Figure 4 **:Knockdown of TAOK1 inhibits neuronal apoptosis,and knockdown of CAMK1 improves mitophagy disorder,protecting neurological function.**(**A**)TAOK1 mRNA expression in rats 24h post-ICH after TAOK1 knockdown virus,corresponding control virus or TAOK1 knockdown virus + CAMK1 knockdown virus injected 14 days later.(**B**)CAMK1 mRNA expression 24h post-ICH with injection of CAMK1 knockdown virus,corresponding control virus or TAOK1 knockdown virus + CAMK1 knockdown virus for 14 days.(C)TUNEL staining apoptotic cells around the focus of rats in the TAOK1 knockdown virus + ICH group and control virus + ICH group,Scale bar=50um.(D)Counts the number of TUNEL positive(red)cells.(**E**)Ultrastructure of neuron mitochondria in the perihametoma of

ICH-induced rats with injection of CAMK1 knockdown virus or control virus.Scale bar=500nm.(E)mNSS score and(**F**)Corner test to evaluate the neurological function damage of rats in each group.*p<0.05,**p<0.01,****p<0.0001.

Figure5 **Donepezil promoted GATA-3 expression and drove miR-204-5p to work.(A)**Predicted the transcription factors that might bind to the promoter region of miR-204-5p,constructed plasmids containing binding domain or its homeodomain deletion mutation(HDD)of the transcription factor,and transfected it to 293T cells with the plasmid containing miR-204-5p promoter for 48 hours，then use luciferase assy to detect fluorescence intensity.Only the group containing GATA-3 binding domain in relative light unit(RLU)pronouncedly enhanced compared with its mutant group.(**B**)Transfected the plasmid containing GATA-3 binding domain or its HDD into 293T cells,RT-qPCR detected miR-204-5p expression levels.(**C**)GATA-3 mRNA and(**D**)protein levels in sham group and ICH group.(**E**)Quantitative analysis of western blotting results.(**F**)GATA-3 mRNA expression after 4 drugs(Gemfibrozil,Roflumilast,Sinomenine and Donepezil)to 293t cells separately.Injected Donepezil or normal saline into rats once a day intraperitoneall for 14 days,then established ICH models,mNSS score(**G**)and Corner test(**H**)to evaluate the neurological deficits of rats in each group.(**I**)Detection expression of GATA-3 mRNA.(**J**)Western blotting detection of GATA3,Parkin,PINK1,Cleaved-PARP and Bak expression and(**K**)Quantitative analysis.**(L)**Apoptosis was visualized by the

518 TUNEL Assay,and the TUNEL-positive(red)cells were quantitatively analyzed.Scale

519 bar=50um.**p<0.01,***p<0.001,****p<0.0001

Declarations

Ethics approval and informed consent

This study was approved by the Ethics Committee of the Affiliated Hospital of Jiujiang University.Written informed consent was obtained from all the participants.

Consent for publication

All authors and patients have given their consent for publication.

Data availability

Please contact the corresponding author for data requests.

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Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' contributions

Xiao-ping Yin and Zhiying Chen conceived and designed the work.Dongling Li,Yetong Ouyang,Qinghua Luo and Min Yin conducted the experiments.Dongling Li,Xiaoqing Wen,Manqing Zhang and Yuqin Zhong analysed and interpreted the data.Bing Bao,Xianming Cao,Zhengfang Qin and Moxin Wu advised on the experimental design and contributed materials and animals.Xiaoping Yin and Zhiying Chen prepared the manuscript, while Song Wenqi handled the submission.All authors revised the manuscript and approved the final version.

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