Delayed varenicline administration reduces inflammation and improves forelimb use following experimental stroke

Siyi Chen[1, 2], Laura Bennet[3], Ailsa L. McGregor[2, 4], *

[1] School of Pharmacy, and [2] Centre for Brain Research, [3] Department of Physiology
Faculty of Medical and Health Sciences,
University of Auckland,
Private Bag 92019,
Auckland, New Zealand

[4] School of Pharmacy,
Division of Health Sciences
University of Otago,
PO Box 56,
Dunedin, New Zealand

*Corresponding Author:
Dr Ailsa McGregor, School of Pharmacy Division of Health Sciences, University of Otago, Private Bag 56, Dunedin, New Zealand
Ph: +64 3 467 4235
Email: ailsa.mcgregor@otago.ac.nz

Abstract

Pharmacological activation of the cholinergic anti-inflammatory pathway (CAP), specifically by activating α7 nicotinic acetylcholine receptors has been shown to confer short-term improvements in outcome in experimental stroke models. To date, α7 agonists have been administered within 24h of stroke and few studies have investigated drugs approved for use in human patients. We investigated whether delayed administration of varenicline, a high affinity agonist at α7 nicotinic receptors and established therapy for nicotine addiction decreased brain inflammation and improved functional performance in a mouse model of experimental stroke.

CSF-1R EGFP ('MacGreen') mice were subjected to transient middle cerebral artery occlusion and administered varenicline (2.5mg/kg/day for 7 days) or saline (n=10 per group) starting 3 days after stroke. Forelimb asymmetry was assessed in the Cylinder test every 2 days after surgery and structural lesions were quantified at day 10. EGFP and GAP43 immunohistochemistry were used to evaluate the effect of varenicline on inflammation and axonal regeneration respectively.

Varenicline-treated animals showed a significant increase in impaired forelimb use compared to saline-treated animals 10 days after stroke. Varenicline treatment was associated with reduced EGFP expression and increased GAP43 expression in the striatum of MacGreen mice.

Our results show that delayed administration of varenicline promotes recovery of function following experimental stroke. Motor function improvements were accompanied by decreased brain inflammation and increased axonal regeneration in non-penumbral areas. These results suggest that administration of an exogenous nicotinic agonist in the sub-acute phase following stroke may be a viable therapeutic strategy for stroke patients.

Key Words

Cholinergic Anti-inflammatory Pathway, Functional Recovery, Ischaemic Stroke, MacGreen Mice, Varenicline, GAP43

Introduction

Brain injuries, such as stroke, are associated with profound activation of inflammatory pathways. Whilst inflammation is important in resolving injury, prolonged inflammation hinders tissue repair and ultimately, patient prognosis [1-3].

Immune responses and inflammation are regulated in part by neural mechanisms [4]. The parasympathetic nervous system can inhibit cytokine release and prevent tissue injury via an efferent neural signalling pathway termed the cholinergic anti-inflammatory pathway (CAP) [4, 5]. Stimulation of the vagus nerve attenuates production of TNFα and other pro-inflammatory cytokines from macrophages in the spleen through a mechanism dependent on α7 nicotinic acetylcholine receptors (nAChRs) [4, 6-8].

It remains unclear why this homeostatic mechanism fails to limit the extent of brain inflammation that occurs after stroke. Clinically, low heart-rate variability and chronic inflammation correlate with poor neurological outcomes in stroke patients [9-13]. This suggests that parasympathetic nervous system tone is reduced following stroke and that this autonomic imbalance leads to suboptimal activation of the CAP, increased inflammation and inhibition of recovery and repair processes.

Autonomic regulation via stimulation of the vagus nerve has been shown to be a viable strategy for targeting diseases with an inflammatory component such as rheumatoid arthritis and haemorrhagic shock [14, 15]. Vagal nerve stimulation within 30 minutes of injury has also been shown to reduce brain damage in experimental models of stroke [16-18]. More recently direct activation of α7nAChRs has been investigated as a less invasive and more applicable therapeutic strategy to reduce brain inflammation and ultimately promote functional recovery after stroke. Both α7 nAChR agonists and allosteric modulators display neuroprotective and anti-inflammatory effects in rodent models of focal [19, 20] and global ischaemia and traumatic brain injury [21]. To date, these studies have initiated administration within 6h of injury and none have demonstrated functional improvement beyond 7 days post stroke[20]. It is not known whether activation of nAChRs in the sub-acute phase (>24h) after stroke can also modulate brain inflammation and promote recovery of function.

The CSF1R-EGFP ('MacGreen') mouse has been show previously to provide a model system in which to investigate brain inflammation following experimental stroke. The aim of this study was to investigate whether delayed administration of the α7 nAChR agonist varenicline reduced brain inflammation and improved motor function in CSF-1R-EGFP mice.

Materials and Methods

Ethical Statement

All animal work was carried out in accordance with the Animal Welfare act 1999 and was pre-approved by the University of Auckland Animal Ethics committee (Approval number: R842).

Animals

Founder CSF-1R-EGFP (MacGreen) mice were gifted by the Queensland Brain Institute, University of Queensland, Australia. Generation of the strain has been described elsewhere [22, 23]. The MacGreen colony was maintained as homozygote and all offspring were positive for the transgene. The ongoing presence of the transgene within the colony was confirmed every 2 months from tail biopsies using standard PCR methods. Tail tipping was performed under isofluorane anesthesia. DNA extraction from tail biopsies was performed using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, USA) using forward 5'-CTGGTCGAGCTGGACGGCGACG-3' and reverse 5'-CACGAACTCCAGCAGGACCATG-3' primers, producing a 650bp product.

All animals were housed in single sex cage and supplied with standard rodent chow and water available *ad libitum.* Housing was maintained at 20^{o}C with 12 hour day and night cycle. CSF-1R-EGFP 'MacGreen' mice (25-30g, approximately 10-12 weeks) were assigned to treatment groups (varenicline or saline, n=10 per group) using a randomisation table generated by Excel. Body

weight, neurological score and exploratory forelimb use in the cylinder test was recorded at baseline and for 10 days following stroke

Cerebral ischaemia

Monofilament occlusion of the middle cerebral artery (MCA) was performed in adult male MacGreen mice (25–30g, approximately 10–12 weeks) as previously described[24]. Briefly, the right common carotid (CCA), external carotid (ECA) and internal carotid (ICA) arteries and their branches were exposed through a midline cervical incision. A 6-0 silk suture was tied around the CCA proximal to the bifurcation of the ECA and ICA and a second suture tied around the ECA distal to the superior thyroid artery (STA). The STA and occipital artery (OA) were occluded by electrocoagulation. An 8-0 silicone-coated monofilament (diameter 200μm) was introduced into the CCA and advanced 10mm distal to the carotid bifurcation, occluding the origin of the MCA. Mice were subjected to occlusion of the MCA in their dominant hemisphere (based on baseline functional performance) for 45 minutes. Core temperature was maintained at 36.5^{o}C ± 0.5^{o}C throughout the course of the surgery by means of a rectal probe connected to a homoeothermic heating blanket (Harvard Apparatus).

At the end of the occlusion period the monofilament was removed and the surgical site sutured closed. Animals were recovered in a humidified incubator until freely moving and then returned to their home cages.

Drug Delivery

Due to its short half-life in mice (1.4h) [25] varenicline was administered by mini-osmotic pump to maintain a steady plasma concentration. The dose of varenicline used in this study was based on previous studies in mice [26, 27].

Varenicline tartrate (YES Pharma Ltd, Israel) was dissolved in 0.9% saline with a few drops of 0.4M NaOH added to aid dissolution. The quantity of drug required to deliver 2.5 mg/kg/day for 10

days was calculated from the individual body weight of each mouse. Mini-osmotic pumps (Alzet® 1007D, Canada) were filled with saline (n-10) or varenicline (2.5mg/kg/day, n=10) one day prior to insertion surgery and incubated overnight at 37^{o}C. Mice were briefly anaesthetised 3 days after stroke surgery and osmotic pump subcutaneously implanted between the shoulder blades.

Neurological Evaluation

The general welfare of the animals was monitored post-operatively. Animals were weighed and their gross neurological deficit was assessed daily using a 15 point neurological deficit scoring (NDS) system adapted from a modified clinical assessment [28]. This assessment examined general condition (2), mobility (2), spontaneous circling behaviour (1), righting reflex (1), forepaw reach (2), forepaw placement (2), posture re-adjustment on inclined platforms (3), swivel movement of during when rotating by base of tail (2). Normal animals display an NDS of 15 while animals subjected to MCAo typically have neurological scores ranging between 8 and 10.

Cylinder Test

Forelimb asymmetry was assessed in the cylinder test using a modification of the protocol previously described by Sschallert *et al* [29]. Briefly, animals were placed in a Perspex cylinder (10cm x 15cm) and two mirrors were placed behind the cylinder at 90^{o} relative to each other to allow visualisation of all movements. A video camera was used to record all lateral exploratory movements during a 5min trial. One trial was carried out per day on days -1, 2, 4, 6, 8, and 10 relative to stroke surgery. A total of 10 rearing movements were examined on a frame-by-frame basis by an observer blinded to the conditions of the study to identify weight-bearing limbs during lifting, climbing, stepping, and landing movements. Each movement was categorised as predominantly left sided, right sided, or neutral.

Histological Analysis

Ten days after stroke surgery, animals were killed by trans-cardiac perfusion with 4% paraformaldehyde under deep anaesthesia (pentobarbitone, 60 mg/kg i.p.) and the brains removed and processed for quantitative histological analysis. Microtome sections (30 μm) were stained with thionin and examined for damage at nine stereotaxic levels by light microscopy. Image analysis (Neurolucida, MicroBrightField Inc., U.S.A.) was used to determine the volume of damage by an observer blinded to the experimental condition [28].

Incidence maps showing the patterns of ischaemic damage at the level of the striatum (Bregma +0.98) were generated by manually tracing the outline of regions of pallor on thionin stained sections using a Nikon E800 upright microscope with a 4x lens. Outlines were transferred onto an electronic stereotaxic atlas template using Photoshop. The rate of occurrence of histological damage occurring in specific regions were then indicated by colour (regions with histological damage in 25-49% of the animals were coloured light grey; 50-74% of animals, dark grey; 75-100% of animals, black).

Immunohistochemistry

Adjacent free-floating sections were taken for immunohistochemistry as previously described [30, 31]. EGFP transgene expression was enhanced using an antibody to GFP (rabbit anti-GFP, 1:5000 Abcam, UK). Tile scan images of the signal enhanced sections at Bregma +0.74mm were generated using a Nikon Eclipse TE2000E microscope and used to quantify the volume of tissue displaying increased expression of EGFP.

A second series of free floating sections were incubated with an antibody to GAP43 (Rabbit anti-GAP43, Abcam, UK, 1:2000).The secondary antibody used was AlexaFluor 594 (goat anti-rabbit, 1:500, Invitrogen, U.S.A.).

Two representative high power images of EGFP and GAP43 expression were acquired using a Nikon TE2000E Inverted microscope on a 20x objective lens taken with GFP and FITC filters respectively at 500ms exposure time. Images of the ischemic core in the striatum and cortex, and tissue within the immediate vicinity of the ischemic core (peri-infarct) in the striatum and cortex were acquired in the ipsilateral hemisphere and in corresponding areas of the contralateral hemisphere.

Incidence maps showing the general patterns of increased EGFP expression at Bregma +0.98mm were generated using the same principle described for histological damage.

Cell Counts & Signal Intensity

The number of EGFP positive cells within core and peri-infarct regions of the striatum and cortex was assessed semi-quantitatively. Two sections from each animal at Bregma -0.1mm were analyzed at 20x magnification. Three images were taken from predefined areas ($1.2mm^2$) and the number of EGFP positive cell bodies counted manually to determine an estimated cell density/mm^2.

The signal intensity of EGFP immunofluorescence was estimated by converting the acquired images to 8 bit grey scale. A thresholding function was manually defined using ImageJ and the relative area displaying increased EGFP expression, integrated optical density and mean area intensity were measured using ImageJ to acquire fluorescence unit/μm^2.

The signal intensity of GAP43 (fluorescence unit/μm^2) in the ischemic core and peri-infarct region of the striatum and cortex was measured in the ipsilateral and contralateral hemispheres. GAP43 expression in the ipsilateral regions of interest was corrected by subtracting contralateral signal intensity in corresponding regions.

Statistics

Values presented in this study are mean +/- S.E.M. The randomization code was broken after all data was acquired to allow allocation to experimental groups. All data collected were processed using Excel. All statistical analyses were conducted using SPSS (PASW Statistics 18, 2009) and SigmaPlot (version 12.0, Systat Software, Inc.). Two-way repeated measures ANOVA with post-hoc analyses were performed to assess differences in weight, and forelimb asymmetry between saline and varenicline treated animals. Neurological deficit scores were analysed using Friedman one-way repeated measures ANOVA followed by rank sum t-tests (Mann-Whitney). Experimental data for histological and immunohistological analyses were analysed using two-way ANOVA with Bonferonni test for pairwise comparisons. The statistical level of significance was established at $p<0.05$.

Results

Varenicline treatment did not alter the recovery of neurological deficits

This study investigated whether delayed administration of varenicline reduced inflammation and enhanced short-term functional recovery in CSF-1R-EGFP following experimental stroke. All animals showed a decrease (12.2 ± 1.8 %) in body weight immediately following and up to 2 days post-stroke. A gradual recovery of body weight was observed from day 3 to day 10 post-stroke ($F_{11, 155}=19.27$; $p<0.01$, Figure 1A). No significant difference was observed in the pattern of body weight recovery between the two treatment groups ($F_{1, 155}=1.012$; $p=0.336$; Figure 1A).

Daily assessments of gross neurological deficits were performed using a 15 point neurological deficit scoring system (Figure 1B). Non-parametric statistical analysis revealed a significant effect

of time in both varenicline (p<0.001) and saline treated (p<0.001) groups. All animals were neurologically normal at baseline (NDS=15) and showed a reduction in neurological score immediately after stroke (8.7±0.40). Both treatment groups displayed comparable partial recovery at day 4 post stroke (saline 10.7±0.18, varenicline 10.3±0.56, p=0138) and the difference in average neurological score between varenicline (10.5±0.5) and saline-treated animals (11.7±0.18) at day 6 post stroke failed to reach statistical significance (p=0.051). Both groups of animals displayed comparable neurological deficits on day 10 post stroke (saline 11.9±0.14, varenicline 11.8±0.17 p=0.945).

Chronic varenicline administration increased impaired forelimb use

All animals showed comparable baseline performance in the cylinder task prior to stroke surgery (saline: 28.3±6.0%; varenicline: 35.0± 2.2%, t=9.21; p=0.323). Forelimb asymmetry was examined every 2 days post stroke (Figure 1C). Following stroke, but prior to treatment, animals displayed comparable reductions in use of the forelimb contralateral to MCA occlusion (3.3%±2.1%; t=6.457; p<0.01). Saline treated animals showed no change in forepaw asymmetry at any time-point following stroke and use of the impaired forelimb remained around 7.5% ($F_{4,34}$=1.103; p=0.373). In contrast, varenicline-treated animals displayed a statistically significant increase in use of the impaired forelimb at day 10 post stroke (30.0±10.6%; t=2.457; p<0.05).

Delayed varenicline administration did not affect lesion volume

Qualitatively, saline treated and varenicline treated MacGreen mice exhibited comparable patterns of ischaemic damage. All animals subjected to 45 min MCAo displayed striatal and cortical damage (Figure 2A). No damage was observed out with the vasculature territory of the MCA in any of the animals investigated. Quantitative histopathology demonstrated no significant differences in the

area of ischaemic damage across 9 stereotaxic levels through the rostro-caudal extent of the brain in saline and varenicline-treated mice ($F_{1, 116}$=0.00261; p=0.96; Figure 2B).

Varenicline treatment reduced EGFP signal intensity in the ipsilateral hemisphere

MacGreen mice subjected to sham stroke surgery showed a low basal level of EGFP expression (data not shown). MacGreen mice subjected to 45 min MCAo displayed increased EGFP expression in the ipsilateral hemisphere but no change in contralateral expression at 10 days post stroke.

Saline and varenicline-treated mice exhibited qualitatively different patterns of EGFP expression. Saline-treated animals displayed increased EGFP expression in both striatal and cortical regions while EGFP expression was increased in predominantly striatal regions in varenicline-treated mice (Figure 3A). EGFP positive cells appeared densely packed within a central 'core' and displayed a distinct amoeboid morphology with enlarged cell bodies and retracted processes in saline-treated animals. In contrast, EGFP positive cells in varenicline-treated animals displayed a less amoeboid shape and more processes (Figure 3B).

Quantitative analyses showed that the while the volume of tissue showing upregulated EGFP expression was comparable between treatment groups (t=0.611; p=0.547; Figure 4A), we observed a significant reduction in EGFP signal intensity in both the ipsilateral cortex (t=2.987; p<0.01) and striatum (t=2.099; p<0.05) of varenicline-treated animals (Figure 4B). Manual cell counts confirmed that varenicline treatment did not affect the density of EGFP positive cells in these brain regions (F=0.152; p=0.697; Figure 4C).

GAP-43 expression was increased in the striatum of varenicline-treated animals

In order to further investigate the relationship between inflammation and recovery of function, we performed immunohistochemistry with GAP43, a 43 kDa growth associated protein expressed in the plasma membrane of sprouting axons [32, 33] on parallel brain sections.

Qualitative examination of brain sections showed GAP43 was expressed throughout both hemispheres at 10 days post stroke but was down-regulation within and around the infarct area (Figure 5A). In contrast, GAP43 expression appeared further decreased in cortical but increased in striatal regions in varenicline-treated animals compared to saline –treated controls (Figure 5B)

Net changes in GAP43 signal intensity were compared between the ipsilateral and contralateral hemisphere While there was no difference in the intensity of GAP43 expression in boundary (peri-infarct) regions of the striatum or cortex, a significant increases in GAP43 signal intensity was observed in core regions of the striatum in varenicline-treated animals compared to saline control ($t=2.373$; $p<0.05$; Figure 5C). In addition, the density of GAP43 positive cells within this region of varenicline-treated animals was reduced compared to corresponding regions in control-treated animals (Figure 5D; $t=2.337$; $p<0.05$). No difference in GAP43 positive cell density was observed in any other regions investigated (striatum peri-infarct ($t=0.632$; $p=0.528$), cortex ($t=1.808$; $p=0.073$), and cortex peri-infarct ($t=0.281$; $p=0.779$).

Discussion

Brain injuries such as stroke strongly activate inflammatory pathways. While inflammation can help resolve injury, prolonged inflammation hinders tissue repair and ultimately, patient prognosis[1-3]. The parasympathetic branch of the autonomic nervous system inhibits inflammation through a neural signalling pathway termed the *cholinergic anti-inflammatory pathway* (CAP)[4, 5]. Activating the CAP reduces the production of pro-inflammatory cytokines through a mechanism dependent on

α7 nicotinic acetylcholine receptors (nAChRs)[34]. Indeed, acute administration of selective α7 agonists and allosteric modulators has been shown to afford neuroprotection and produce anti-inflammatory effects in models of ischemic stroke [20, 35].

The current study demonstrated that treatment with varenicline, an α7 nAChR agonist and an established therapy for nicotine addiction starting 3 days after stroke significantly reduced brain inflammation in CSF-1R-EGFP (MacGreen) mice. Brain regions demonstrating reduced EGFP expression also displayed up-regulation of GAP43, a marker of neurite outgrowth. This reinforces the observations of previous studies showing that increased GAP43 expression was associated with improved functional performance up to 4 weeks after stroke [32, 36, 37]. GAP43 upregulation is however typically observed in penumbral areas post stroke and few studies report upregulation in core regions [38]. It is possible therefore that the elevated GAP43 expression observed in core regions in the present study may originate from newly generated neurons [39-41]. Varenicline-treated animals also demonstrated an 80% recovery in the use of their impaired paw 10 days after stroke. Quantitative histological analysis confirmed that varenicline did not affect the volume of structural damage and that improved functional performance did not result from a preservation of tissue. Taken together, these results imply that an extended time-window exists in which to modulate brain inflammation and support the hypothesis that reducing inflammation in the subacute phase after stroke can promote recovery.

We have shown previously that EGFP expression is increased in the ipsilateral hemisphere of MacGreen mice up to 35 days post stroke and that the increased EGFP expression post-stroke resulted from changes in both M/M cell density and morphology [28]. Since morphological appearance is an important indicator of functional activation[42], the change in EGFP+ cell appearance from an amoeboid to a more ramified appearance following varenicline treatment suggests that the beneficial effects observed in this study may at least in part be mediated via

activation of a7nAChRs. It cannot be discounted however, that the action of varenicline at other nAChR subtypes may also play a role.

Varenicline is a partial agonist at α4β2, α3β2, α6β2 nAChR receptors[25]. Nicotinic agonists like varenicline increase basal locomotor activity acutely in normal rodents [43, 44]. Dopamine release via activation of striatal α4β2 nAChRs may therefore contribute to the observed improvements in motor function [45].

Agonists at β2 subunit containing nAChRs have also been shown to produce hypothermic effects in mice (de Moura). Therapeutic hypothermia initiated in the acute phase after stroke has been shown to be both neuroprotective and anti-inflammatory [46]. While body temperature was not measured in the days following drug administration, varenicline is a less potent inducer of hypothermia than other nicotinic agonists including epibatidine and nicotine [47]. At the dosing regimen used in the current study, varenicline is likely to have produced minimal effects on body temperature[47]. Further studies are required to determine whether varenicline reduces pro-inflammatory cytokine production and to establish the phenotype of M/M cells.

In addition to its effects at nAChRs, varenicline binds with moderate affinity and acts as a partial agonist at 5HT3 receptors [48, 49]. It is possible therefore that the dual properties of partial efficacy at 5HT3 receptors and a7 blockade synergistically contribute to the potential immunomodulatory properties of varenicline[50].

While the precise mechanisms underlying the beneficial effects in this study remain unresolved, our observations suggest that blockade of inflammatory responses and stimulation of cell survival signalling pathways may underlie the improved functional recovery in varenicline-treated animals.

Conclusion

This study shows that delayed administration of varenicline, an established therapy for nicotine addiction suppressed inflammation, enhanced axonal growth, and improved impaired forepaw use following experimental stroke. This study adds to the current literature that nicotinic agonists have beneficial effects in experimental model of focal ischemia and demonstrates that an extended time-window exists in which inflammation can be modulated pharmacologically. Further studies are warranted to determine whether these beneficial effects are sustained beyond the drug administration period, and whether varenicline can confer longer-term improvements in function in the weeks following stroke.

Designed to be administered in the days after stroke, this therapeutic approach has significant implications for stroke patients who arrive late to hospital and are not suitable for thrombolytic therapy. Identifying an easily administered, well tolerated intervention that is currently available for other treatment indications may simplify the translation from preclinical investigation to clinical practice.

Funding

This study was funded by the University of Auckland Faculty Research Development Fund [grant number FRDF 3700396]

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

The authors would like to thank Jacqui Ross and Hilary Holloway (Biomedical Imaging Research Unit, University of Auckland, New Zealand) for assistance and support with microscopy procedures.

References

1. Candelario-Jalil E. Injury and repair mechanisms in ischemic stroke: Considerations for the development of novel neurotherapeutics. *Current opinion in investigational drugs*. 2009;10:644-654

2. Greifzu F, Schmidt S, Schmidt KF, Kreikemeier K, Witte OW, Lowel S. Global impairment and therapeutic restoration of visual plasticity mechanisms after a localized cortical stroke. *Proc Natl Acad Sci U S A*. 2011;108:15450-15455

3. Jablonka JA, Kossut M, Witte OW, Liguz-Lecznar M. Experience-dependent brain plasticity after stroke: Effect of ibuprofen and poststroke delay. *Eur J Neurosci*. 2012;36:2632-2639

4. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *The Journal of clinical investigation*. 2007;117:289-296

5. Pavlov VA, Tracey KJ. Controlling inflammation: The cholinergic anti-inflammatory pathway. *Biochem Soc Trans*. 2006;34:1037-1040

6. Huston JM, Tracey KJ. The pulse of inflammation: Heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. *Journal of internal medicine*. 2011;269:45-53

7. Cheyuo C, Jacob, A., Wu R., Zhou, M., Coppa GF. Wang, P. The parasympathetic nervous system in the quest for stroke therapeutics. *Journal of Cerebral blood Flow and Metabolism*. 2011

8. Rosas-Ballina M, Tracey KJ. Cholinergic control of inflammation. *Journal of internal medicine*. 2009;265:663-679

9. Chen PL, Kuo TB, Yang CC. Parasympathetic activity correlates with early outcome in patients with large artery atherosclerotic stroke. *J Neurol Sci*. 2012;314:57-61

10. Marthol H, Intravooth T, Bardutzky J, De Fina P, Schwab S, Hilz MJ. Sympathetic cardiovascular hyperactivity precedes brain death. *Clinical autonomic research : official journal of the Clinical Autonomic Research Society*. 2010;20:363-369

11. Norman GJ, Karelina K, Berntson GG, Morris JS, Zhang N, Devries AC. Heart rate variability predicts cell death and inflammatory responses to global cerebral ischemia. *Frontiers in physiology*. 2012;3:131

12. Raimundo RD, de Abreu LC, Adami F, Vanderlei FM, de Carvalho TD, Moreno IL, et al. Heart rate variability in stroke patients submitted to an acute bout of aerobic exercise. *Translational stroke research*. 2013;4:488-499

13. Tang SC, Jen HI, Lin YH, Hung CS, Jou WJ, Huang PW, et al. Complexity of heart rate variability predicts outcome in intensive care unit admitted patients with acute stroke. *Journal of neurology, neurosurgery, and psychiatry*. 2015;86:95-100

14. Li JG, Hu ZF, Du ZH, Zhou Q, Jia BH, Peng ZQ, et al. [protective effect of the cholinergic anti-inflammatory pathway against hemorrhagic shock in rats]. *Zhongguo wei zhong bing ji jiu yi xue = Chinese critical care medicine = Zhongguo weizhongbing jijiuyixue*. 2005;17:24-27

15. van Maanen MA, Vervoordeldonk MJ, Tak PP. The cholinergic anti-inflammatory pathway: Towards innovative treatment of rheumatoid arthritis. *Nature reviews. Rheumatology*. 2009;5:229-232

16. Ay I, Lu J, Ay H, Gregory Sorensen A. Vagus nerve stimulation reduces infarct size in rat focal cerebral ischemia. *Neuroscience letters*. 2009;459:147-151

17. Hiraki T, Baker W, Greenberg JH. Effect of vagus nerve stimulation during transient focal cerebral ischemia on chronic outcome in rats. *Journal of neuroscience research*. 2012;90:887-894

18. Sun Z, Baker W, Hiraki T, Greenberg JH. The effect of right vagus nerve stimulation on focal cerebral ischemia: An experimental study in the rat. *Brain stimulation*. 2012;5:1-10

19. Sun F, Jin K, Uteshev VV. A type-ii positive allosteric modulator of alpha7 nachrs reduces brain injury and improves neurological function after focal cerebral ischemia in rats. *PloS one*. 2013;8:e73581

20. Han Z, Shen F, He Y, Degos V, Camus M, Maze M, et al. Activation of alpha-7 nicotinic acetylcholine receptor reduces ischemic stroke injury through reduction of pro-inflammatory macrophages and oxidative stress. *PloS one*. 2014;9:e105711

21. Gatson JW, Simpkins JW, Uteshev VV. High therapeutic potential of positive allosteric modulation of alpha7 nachrs in a rat model of traumatic brain injury: Proof-of-concept. *Brain research bulletin*. 2015;112:35-41

22. Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, et al. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood*. 2003;101:1155-1163

23. Sasmono RT, Williams E. Generation and characterization of macgreen mice, the cfs1r-egfp transgenic mice. *Methods in molecular biology*. 2012;844:157-176

24. Kerr LE, McGregor AL, Amet LE, Asada T, Spratt C, Allsopp TE, et al. Mice overexpressing human caspase 3 appear phenotypically normal but exhibit increased apoptosis and larger lesion volumes in response to transient focal cerebral ischaemia. *Cell death and differentiation*. 2004;11:1102-1111

25. Obach RS, Reed-Hagen AE, Krueger SS, Obach BJ, O'Connell TN, Zandi KS, et al. Metabolism and disposition of varenicline, a selective alpha4beta2 acetylcholine receptor partial agonist, in vivo and in vitro. *Drug Metab Dispos*. 2006;34:121-130

26. Spiller K, Xi ZX, Li X, Ashby CR, Jr., Callahan PM, Tehim A, et al. Varenicline attenuates nicotine-enhanced brain-stimulation reward by activation of alpha4beta2 nicotinic receptors in rats. *Neuropharmacology*. 2009;57:60-66

27. O'Connor EC, Parker D, Rollema H, Mead AN. The alpha4beta2 nicotinic acetylcholine-receptor partial agonist varenicline inhibits both nicotine self-administration following repeated dosing and reinstatement of nicotine seeking in rats. *Psychopharmacology*. 2010;208:365-376

28. Chen S, Bennet, L, McGregor, A. L. . Macgreen mice: A novel tool to investigate brain inflammation. *Journal of Experimental Stroke and Translational Medicine*. 2015;8:1-10

29. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. Cns plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*. 2000;39:777-787

30. Gordon RJ, Tattersfield AS, Vazey EM, Kells AP, McGregor AL, Hughes SM, et al. Temporal profile of subventricular zone progenitor cell migration following quinolinic acid-induced striatal cell loss. *Neuroscience*. 2007;146:1704-1718

31. Maucksch C, McGregor AL, Yang M, Gordon RJ, Yang M, Connor B. Igf-i redirects doublecortin-positive cell migration in the normal adult rat brain. *Neuroscience*. 2013;241:106-115

32. Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, et al. Overexpression of the neural growth-associated protein gap-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell*. 1995;83:269-278

33. Vitkovic L, Steisslinger HW, Aloyo VJ, Mersel M. The 43-kda neuronal growth-associated protein (gap-43) is present in plasma membranes of rat astrocytes. *Proc Natl Acad Sci U S A*. 1988;85:8296-8300

34. Vida G, Pena G, Deitch EA, Ulloa L. Alpha7-cholinergic receptor mediates vagal induction of splenic norepinephrine. *Journal of immunology*. 2011;186:4340-4346

35. Kalappa BI, Sun F, Johnson SR, Jin K, Uteshev VV. A positive allosteric modulator of alpha7 nachrs augments neuroprotective effects of endogenous nicotinic agonists in cerebral ischaemia. *British journal of pharmacology*. 2013;169:1862-1878

36. Granziera C, D'Arceuil H, Zai L, Magistretti PJ, Sorensen AG, de Crespigny AJ. Long-term monitoring of post-stroke plasticity after transient cerebral ischemia in mice using in vivo and ex vivo diffusion tensor mri. *Open Neuroimag J*. 2007;1:10-17

37. Zhao H, Joo S, Xie W, Ji X. Using hormetic strategies to improve ischemic preconditioning and postconditioning against stroke. *Int J Physiol Pathophysiol Pharmacol*. 2013;5:61-72

38. Xie H, Wu Y, Jia J, Liu G, Zhang F, Zhang Q, et al. Enriched environment preconditioning induced brain ischemic tolerance without reducing infarct volume and edema: The possible role of enrichment-related physical activity increase. *Brain Res*. 2013;1508:63-72

39. Carmichael ST. Cellular and molecular mechanisms of neural repair after stroke: Making waves. *Ann Neurol*. 2006;59:735-742

40. Clarke J, Mala H, Windle V, Chernenko G, Corbett D. The effects of repeated rehabilitation "tune-ups" on functional recovery after focal ischemia in rats. *Neurorehabil Neural Repair*. 2009;23:886-894

41. Jiang Q, Zhang ZG, Ding GL, Silver B, Zhang L, Meng H, et al. Mri detects white matter reorganization after neural progenitor cell treatment of stroke. *Neuroimage*. 2006;32:1080-1089

42. Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. *Nature*.468:253-262

43. Zesiewicz TA, Sullivan KL. Treatment of ataxia and imbalance with varenicline (chantix): Report of 2 patients with spinocerebellar ataxia (types 3 and 14). *Clinical neuropharmacology*. 2008;31:363-365

44. King J, Huang W, Chen W, Heffernan M, Shields J, Rane P, et al. A comparison of brain and behavioral effects of varenicline and nicotine in rats. *Behav Brain Res*. 2011;223:42-47

45. Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, et al. Varenicline: An alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem*. 2005;48:3474-3477

46. Lee JH, Wei ZZ, Cao W, Won S, Gu X, Winter M, et al. Regulation of therapeutic hypothermia on inflammatory cytokines, microglia polarization, migration and functional recovery after ischemic stroke in mice. *Neurobiol Dis*. 2016;96:248-260

47. Rodriguez JS, Cunningham CS, Moura FB, Ondachi P, Carroll FI, McMahon LR. Discriminative stimulus and hypothermic effects of some derivatives of the nachr agonist epibatidine in mice. *Psychopharmacology*. 2014;231:4455-4466

48. Lummis SC, Thompson AJ, Bencherif M, Lester HA. Varenicline is a potent agonist of the human 5-hydroxytryptamine3 receptor. *The Journal of pharmacology and experimental therapeutics*. 2011;339:125-131

49. Price KL, Lillestol RK, Ulens C, Lummis SC. Varenicline interactions at the 5-ht3 receptor ligand binding site are revealed by 5-htbp. *ACS Chem Neurosci*. 2015;6:1151-1157

50. Fakhfouri G, Mousavizadeh K, Mehr SE, Dehpour AR, Zirak MR, Ghia JE, et al. From chemotherapy-induced emesis to neuroprotection: Therapeutic opportunities for 5-ht3 receptor antagonists. *Mol Neurobiol*. 2015;52:1670-1679

Figure legends

Figure 1: Changes in body weight, neurological score and impaired forelimb following stroke in varenicline and saline treated mice. (A) Varenicline treatment did not influence weight loss following stroke (F1, 155=1.012; p=0.336). (B) Recovery of gross neurological deficits were comparable between both treatment groups. (C) Unilateral impaired forelimb use was observed in both groups immediately after stroke (t=6.457; p<0.01). Varenicline significantly increased use of the impaired forelimb 10 days post stroke (t=2.747; p<0.01). Data are presented and mean ± S.E.M, n=10 per group, *p <0.05.

Figure 2: Patterns of ischaemic injury assessed by thionin histology in saline and varenicline-treated MacGreen mice. (A) Incidence maps at the level of the striatum (Bregma +0.98mm) show comparable histological damage in both experimental groups 10 days following a 45 min MCA occlusion. (B) The area of ischemic damage at 9 stereotaxic levels through the rostro-caudal extent of the brain in saline and varenicline-treated mice was comparable in both treatment groups (t=0.0327; p=0.974). Data are presented as mean ± SEM, n=10 per group, *p <0.05.

Figure 3: Patterns of EGFP expression in saline and varenicline-treated MacGreen mice 10 days after MCA occlusion. (A) Incidence maps at the level of the striatum (Bregma +0.98mm) show a lower incidence of EGFP upregulation in cortical regions in varenicline-treated compared to control animals. (B) Tile-scan images showing EGFP expression in the ipsilateral hemisphere of median animals in saline and varenicline treatment groups (scale=500µm). Images were taken at the level of Bregma +0.74mm. Note the decrease in signal intensity in cortical and striatal areas in varenicline-treated animals. Inserts show EGFP positive cell morphology within boxed regions of the respective tile scan images.

Figure 4: Quantification of EGFP expression in saline and varenicline treated MacGreen mice. (A) No statistical difference was observed between lesion volume (t=0.0327; p=0.974) and EGFP expression volume (t=0.611; p=0.547) between treatment groups at 10 days after stroke. EGFP volumes were typically 2.5 fold greater than the volume of infarct outlined thionin histology. (B) EGFP signal intensity was decreased in the ischaemic cortex (t=2.987; p<0.01) and striatum (t=2.099; p<0.05) following varenicline-treatment compared to control animals but was unchanged in peri-infarct areas. (C) There was no significant difference in the number of EGFP expressing cells per unit area in regions of interest between the two treatment groups (F=0.152; p=0.697). Data are presented as mean ± SEM, n=8-9 per group, *p<0.05.

Figure 5: *GAP43 expression in saline and varenicline-treated MacGreen mice 10 days after MCA occlusion treated animals.* Representative photomicrographs showing GAP43 expression in the ipsilateral cortex and striatum of (A) saline and (B) varenicline-treated mice (scale = 500µm). Images were taken at the level of Bregma +0.98mm. Inserts show higher magnification images from boxed regions of the respective photomicrographs (scale = 50µm). (C) GAP43 signal intensity (corrected for contralateral expression) was significantly increased in the ischemic core of the striatum in varenicline-treated animals (t=2.373; p<0.05). (D) The number of GAP43 expressing cells in the ischemic core of the striatum was significantly lower in varenicline-treated animals compared to saline controls (t=2.337; p<0.05). Data are presented as mean ± SEM, n= 8-9 per group, *p<0.05.