GAT3 selective substrate l-isoserine upregulates GAT3 expression and increases functional recovery after a focal ischemic stroke in mice

Lie, Maria Ek; Gowing, Emma K; Johansen, Nina B; Dalby, Nils Ole; Thiesen, Louise; Wellendorph, Petrine; Clarkson, Andrew N

Published in:
Journal of Cerebral Blood Flow and Metabolism

DOI:
10.1177/0271678X17744123

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

Original Article

JCBFM

Journal of Cerebral Blood Flow & Metabolism
2019, Vol. 39(1) 74–88

DOI: 10.1177/0271678X17744123
journals.sagepub.com/home/jcbfm
SAGE

GAT3 selective substrate L-isoserine upregulates GAT3 expression and increases functional recovery after a focal ischemic stroke in mice

Maria EK Lie[1,2], Emma K Gowing[2], Nina B Johansen[1], Nils Ole Dalby[1], Louise Thiesen[1], Petrine Wellendorph[1] and Andrew N Clarkson[2,3]

Abstract

Ischemic stroke triggers an elevation in tonic GABA inhibition that impairs the ability of the brain to form new structural and functional cortical circuits required for recovery. This stroke-induced increase in tonic inhibition is caused by impaired GABA uptake via the glial GABA transporter GAT3, highlighting GAT3 as a novel target in stroke recovery. Using a photothrombotic stroke mouse model, we show that GAT3 protein levels are decreased in peri-infarct tissue from 6 h to 42 days post-stroke. Prior studies have shown that GAT substrates can increase GAT surface expression. Therefore, we aimed to assess whether the GAT3 substrate, L-isoserine, could increase post-stroke functional recovery. L-Isoserine (38 μM or 380 μM) administered directly into the infarct from day 5 to 32 post-stroke, significantly increased motor performance in the grid-walking and cylinder tasks in a concentration-dependent manner, without affecting infarct volumes. Additionally, L-isoserine induced a lasting increase in GAT3 expression in peri-infarct regions accompanied by a small decrease in GFAP expression. This study is the first to show that a GAT3 substrate can increase GAT3 expression and functional recovery after focal ischemic stroke following a delayed long-term treatment. We propose that enhancing GAT3-mediated uptake dampens tonic inhibition and promotes functional recovery after stroke.

Keywords

Photothrombotic stroke, GAT3 substrate inhibitor, L-isoserine, tonic inhibition, functional recovery

Received 31 May 2017; Revised 30 October 2017; Accepted 31 October 2017

Introduction

Stroke is a leading cause of disability and death with patients exhibiting varied levels of functional recovery.[1] Despite this, no pharmacological treatment options exist to promote functional recovery, beyond the use of remedial therapies. The tissue region surrounding the infarct, the peri-infarct, is a structurally intact but functionally impaired region critical for recovery. Targeting this region can facilitate plasticity and cortical remapping resulting in the formation of new structural and functional circuits between related cortical regions.[2–4] The process of plasticity is highly influenced by $GABA_A$ receptor signaling and tonic GABA-mediated inhibition, which maintains and shapes the level of neuronal excitability and facilitates the remapping of brain regions.[5–8] Accordingly, alterations in GABAergic signaling and tonic inhibition are triggered by an ischemic stroke in both animals and humans.[6,8–10] Of particular importance, dampening extrasynaptic α_5-containing $GABA_A$ receptors from three to seven days post-stroke and onwards, but not acutely within hours, increases motor functional

[1]Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark
[2]Department of Anatomy, Brain Health Research Centre and Brain Research New Zealand, University of Otago, Dunedin, New Zealand
[3]Faculty of Pharmacy, The University of Sydney, Sydney, New South Wales, Australia

Corresponding authors:
Petrine Wellendorph, Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen 2100, Denmark.
Email: pw@sund.ku.dk
Andrew N Clarkson, Department of Anatomy, University of Otago, PO Box 913, Dunedin 9054, New Zealand.
Email: andrew.clarkson@otago.ac.nz

recovery in animal stroke models.[6,11] Further, these studies parallel observations in human patients where functional recovery correlates with decreased GABAergic signaling.[10,12]

Although GABA agonists have failed to translate into positive clinical outcomes promoting neuroprotection in humans,[13] GABA modulation, and in particular tonic inhibition, has attracted new attention as a putative approach for promoting recovery during the sub-acute phase (3–7 days) after ischemia.[6,8,11,14,15] Interventions targeting the sub-acute phase offers a much broader therapeutic window than the thrombolytic treatment by tissue plasminogen activator (t-PA),[16] and most if not all patients will have been assessed and available to receive the treatment.[17] Recent clinical advances have confirmed that decreasing tonic inhibition by inhibiting α_5-containing $GABA_A$ receptors results in an increase in cortical excitability.[15] We now await outcomes from phase 2 trials to confirm if, indeed, such treatments promote neural repair post stroke.[18,19]

Tonic inhibition is in most cases regulated at the $GABA_A$ receptor level and little attention has been given to GABA transporters (GATs),[14] which can regulate ambient GABA levels and hence affect tonic inhibition.[6,20–22] The glial GABA transporter, GAT3, has previously been suggested to contribute to an increase in tonic inhibition, as after stroke there is decreased expression and impairment of GAT3 function. By contrast, GAT1, the most abundant GAT in the brain, displays unaffected expression and function.[6,8,23] Therefore, GAT3 could be a target for regulating tonic inhibition in ischemic stroke. An alternative approach to using GAT inhibitors is to use substrate inhibitors for GATs, which have been reported to increase GABA uptake in neuronal and glial cultures via an increase in the surface expression of GAT1 and GAT3, respectively.[24] We report here that a delayed chronic administration of the GAT3 substrate, L-isoserine,[25] from 5 to 32 days post-stroke, increases functional recovery in mice in a concentration-dependent manner. Furthermore, we demonstrate that L-isoserine induces long-lasting effects by increasing the expression of GAT3 in peri-infarct regions, which we hypothesize contributes to a dampening of tonic inhibition and promotes remapping of cortical networks.

Material and methods

In vitro studies

Materials. L-Isoserine, (*S*)-SNAP-5114, GABA, tiagabine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NO-711 and picrotoxin (PTX) were purchased from Tocris Bioscience (Bristol, UK). [2,3-^{3}H(*N*)]GABA (35.0 Ci/mmol), [2,2-^{3}H(*N*)]taurine (19.1 Ci/mmol), [2-^{3}H]glycine (47.0 Ci/mmol) and [methylene-^{3}H(*N*)]muscimol (30.2 Ci/mmol) were purchased from PerkinElmer (Boston, MA, USA). [4-^{14}C]creatine hydrate (57.0 mCi/mmol) was purchased from Biotrend (Köln, Germany).

DNA encoding the human taurine transporter (TauT) was kindly provided by Dr. Nanna MacAulay (University of Copenhagen, Denmark). Dr. Robert J Vandenberg (University of Sydney, Australia) generously provided DNA constructs encoding the human glycine transporters, GlyT1 and GlyT2. Human creatine transporter (CreaT) DNA was purchased from Origene (Rockville, MD, USA). All were subcloned into the pUNIV vector (Addgene, Cambridge, MA, USA) as described earlier.[26]

Cell cultures and transfection. Mouse and human GATs were stably expressed in HEK-293 and CHO cells, respectively, and tsA201 cells were cultured as described previously.[27] tsA201 cells were transfected with plasmid DNA (8 µg per 10 cm dish) encoding human GAT3-pcDNA5/FRT,[28] human GAT3 epitope-tagged extracellularly with the hemagglutinin (HA) (GAT3-HA)[29] and human TauT,[26] using 40 µl PolyFect transfection reagent according to the manufacturer's protocol (Qiagen, West Sussex, UK).

Cortical astrocytes were isolated from one- to two-day-old NMRI mice (Janvier Labs, Le Genest-Saint-Isle, France) using the neuronal tissue dissociation Kit (P) and anti-ACSA-2 Microbead Kit according to manufacturer's protocols (MACS Miltenyi Biotec, Lund, Sweden) and cultured as described previously.[26]

In vitro competition uptake of L-isoserine at recombinant SLC6A transporters. The [^{3}H]GABA competition uptake assay at mouse and human GATs and in cortical astrocyte cultures was performed as previously described.[26–28] Cortical astrocyte cultures were pre-incubated prior to the [^{3}H]GABA assay with compounds diluted in Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 1 mM CaCl and 1 mM MgCl, 0.1% DMSO (pH 7.4) for 60 min at 37°C in a humidified incubator with 5% CO_2.

Further, *Xenopus laevis* oocytes recombinantly expressing related SLC6A members, including GlyT1, GlyT2, TauT and CreaT, were also used to assess the specificity for the GATs.[26] [^{3}H]taurine competition uptake assay was performed at human TauT transiently expressed in tsA201 cells following the protocol for the [^{3}H]GABA competition uptake assay described previously[27,28] with the exception of exchanging [^{3}H]GABA with 22 nM [^{3}H]taurine for 5 min.

The FLIPR® membrane potential assay. The FMP assay was performed as previously described.[27]

[^{3}H]Muscimol binding. The assay of [^{3}H]muscimol binding to the $GABA_A$ receptors was performed as previously described.[30]

GAT3 surface expression in vitro. Surface expression of GAT3-HA upon pre-incubation with GAT3 compounds was determined using an ELISA against the HA-epitope. tsA201 cells recombinantly expressing GAT3 was used to ensure that there was a significant difference in the signal between GAT3-HA and GAT3 on the day of assay. Cells were washed with HBSS supplemented with 20 mM HEPES, 1 mM CaCl and 1 mM MgCl (pH 7.4) (HBSS-HEPES) and incubated with compounds diluted in HBSS-HEPES to study the effect of 5 to 120 min pre-incubation and in growth media to study the effect of the compounds following 24 and 48 h pre-incubation, at 37°C in a humidified incubator with 5% CO_2. The cells were fixed with 4% PFA, and 0.05% Triton X-100 was added when the total pool of GAT3-HA (intracellular pool and surface fraction) was determined. The cells were blocked for 60 min in 3% skim milk, incubated 45 min at room temperature (RT) with anti-HA (anti-HA.11, 1:1000, BioLegend, San Diego, CA, USA), washed and incubated with a HRP-conjugated antibody (anti-mouse, 1:1500, P0447, Dako, Glostrup, Denmark) for 45 min at RT. SuperSignal™ ELISA Femto substrate (ThermoFisher Scientific, Waltham, MA USA) was added with PBS, and chemiluminescence was measured on an EnSpire2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA). Chemiluminescence is depicted as a percentage of the control response.

MTT assay. The viability of the compound-treated tsA201 cells expressing GAT3-HA was tested using MTT. Compound-treated cells were incubated with 0.5 mg/ml MTT for 2 h at 37°C in a humidified incubator with 5% CO_2 and solubilized with isopropanol before reading the absorbance at 550 nm on an EnSpire2300 Multilabel Reader (PerkinElmer).

Slice preparation. Slices were prepared from C57BL/6 J male mice (Janvier Labs, Le Genest-Saint-Isle, France) aged 5 ± 1 weeks and dissected as described earlier[31] with minor modifications in the carbonated bicarbonate buffer, with the composition (mM): NMDG (100), NaCl (26), KCl (2.5), NaH_2PO_4 (1.25), Glucose (10), $CaCl_2$ (1), $MgCl_2$ (3), $NaHCO_3$ (26), kynurenate (1), ascorbate (0.3) and pyruvate (0.1). Slices were stored in regular carbonated artificial cerebrospinal fluid (ACSF) at 28–29°C (composition as above, with NMDG replaced by NaCl and without kynurenate) and used for recordings in the period of 1–5 h after cutting.

Whole-cell patch-clamp electrophysiology. In the recording chamber, slices were perfused at a speed of 2.8–3 mL/min with a modified carbonated ACSF at 33–34°C and osmolarity adjusted to 310 ± 3 mOsm. Recording ACSF contained in addition to storage ACSF: kynurenate (1 mM), CGP54626 (1 μM), atropine (1 μM), TTX (0.5 μM), strychnine (1 μM), A804598 (1 μM), GABA (1 μM). Pyramidal neurons in layer 2/3 of the cingulate cortex were visually identified under IR video-microscopy and recorded from using borosilicate pipettes (1.5/1.1 OD/ID mm) with a CsCl-based intracellular solution of the composition (mM): CsCl (135), NaCl (4), $MgCl_2$ (2), EGTA (1), HEPES (10), ATP (2), GTP (0.5), TEA (5), QX-314 (5), adjusted to pH 7.2 and 292 ± 3 mOsm. Recording electrodes had a resistance of 5–8 MOhm in this solution and recording of baseline activity began 6–7 min after establishing the whole cell configuration. Recordings were made using a Multiclamp 700A amplifier (Axon, Molecular Devices, Sunnyvale, CA, USA), digitized at 10 kHz on a 1322 digidata (Molecular Devices) and filtered at 3 kHz (8-pole Bessell). After stable baseline was obtained, recordings consisted of 3 min baseline, 6 min of NO-711, 6 min of L-isoserine and 5 min of PTX. Neuron capacitance and series resistance (SR) were noted every 3–4 min and compensated 70% for changes in SR. Neurons for which the SR changed >30% during the recording were omitted.

Recordings were analyzed in pClamp/Clampfit and Origin 9 (Molecular Devices). Holding current was assessed in a 60 s interval at the end of each baseline and drug period as the center of a single Gaussian of points sampled at a 100 Hz frequency. Differences in tonic current upon drug perfusion are depicted as difference in holding current relative to baseline and normalized to neuron capacitance. Data are based on recordings from 8 cells/8 slices from four animals.

In vivo and ex vivo studies

Animals and surgical procedures. All *in vivo* procedures described in this study were carried out in accordance with guidelines on the care and use of laboratory animals set out by the University of Otago, Animal Research Committee and the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996). All *in vivo* studies were approved by the University of Otago Animal Ethics Committee and are reported according to the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines.

Focal stroke was induced by photothrombosis in young (2–4 months old) male C57BL/6J mice

(Hercus Taieri Research Unit, Dunedin, New Zealand) weighing approximately 25–29 g as previously described.[6] Under isoflurane anesthesia (2–2.5% in O_2), mice were placed in a stereotactic apparatus, the skull exposed through a midline incision, cleared of connective tissue and dried. A cold light source (KL1500 LCD, Zeiss) attached to a 40× objective giving a 2 mm diameter illumination was positioned 1.5 mm lateral from Bregma and 0.2 ml Rose Bengal solution (10 g/L in normal saline, i.p.: Sigma-Aldrich) was administered. After 5 min, the brain was illuminated through the intact skull for 15 min, while keeping body temperature at 37.0 ± 0.3°C by a heating pad (Harvard apparatus, Holliston, MA, USA).

Mice were housed under a 12 h light/dark cycle with ad libitum access to food and water. All animals (36 in total) were randomly assigned to a treatment group five days post-stroke (sham + vehicle (n = 6) and sham + L-isoserine high (n = 6); stroke + vehicle (n = 8), stroke + L-isoserine low (n = 8) and stroke + L-isoserine high (n = 6)). All assessments were carried out by observers blinded to the treatment group.

Compound administration and processing of tissue. A micro-osmotic pump (model 1004, administration for 28 days at 0.11 µl per hour, Alzet, Cupertino, CA, USA) was connected to a cannula, placed directly into the infarct, and was implanted on the back of the mice on day 5 post-stroke. L-isoserine was dissolved in sterile DPBS with 0.3% DMSO and administered at a concentration of 38 µM and 380 µM corresponding to the IC_{50} and $10 \times IC_{50}$ values at recombinant mouse GAT3 (Table S1, Supplementary material). Control mice received the vehicle containing DPBS with 0.3% DMSO. The mice were individually housed after the implantation of the micro-osmotic pump to avoid littermates tampering with the pumps. On day 42 post-stroke, all mice were sacrificed by transcardial perfusion with 4% PFA, brains extracted and processed for cresyl violet staining and immunofluorescent labeling.

Behavioral assessment. Recovery of forelimb motor function was determined by the cylinder and grid-walking tasks to assess their exploratory behavior and walking, respectively, as previously reported.[6] The mice were tested approximately seven days prior to stroke to establish a baseline performance level and then after 7, 14, 28 and 42 days post-stroke at approximately the same time each day. Observers blinded to the treatment group scored behaviors as previously described.[6]

Immunofluorescent labeling of GAT3 and GFAP and infarct volume. Immunofluorescent labeling of GAT3 and GFAP was performed 42 days post stroke. Brain sections, every sixth section through the stroke with a thickness of 30 µm, were rinsed in tris buffered saline (TBS) and transferred to 1% sodium tetraborate in TBS for 20 min at RT. The sections were blocked for 60 min in TBS containing 5% goat and donkey serum with 0.3% Triton X-100 and incubated in TBS with 2% goat and donkey serum and 0.3% triton X-100 containing primary polyclonal antibodies (rabbit anti-mouse GAT3, 1:100, AB1574 and chicken anti-mouse GFAP, 1:3000, AB5541, Millipore, Darmstadt, Germany) for 24–48 h at 4°C. The rinsed sections were incubated for 2 h at RT in the dark in TBS with 2% normal serum and 0.3% triton X-100 containing appropriate fluorescent secondary antibodies (anti-rabbit 1:1000, 711-485-152, Abacus ALS, Auckland, New Zealand; anti-chicken 1:1000, SA5-10071, ThermoFisher Scientific) and nuclear stained in Hoechst (1:1000, Sigma-Aldrich) in TBS for 5 min at RT. Images were taken with an Olympus BX61 microscope, and two sections from each animal were included in the analysis. Changes in GAT3 intensity/cell and GFAP staining were investigated at day 42 post-stroke in layers 2/3 and 5 in the peri-infarct and 400 µm and 800 µm from the peri-infarct in 6 squares of 90×180 µm. Using the software FIJI Image J (National Institutes of Health, USA), the integrated density value (IDV) was measured in all six regions of interest (ROIs) on duplicate slides across the three channels. Using the blue channel, a selection of at least 10 representative nuclei in the ROI were outlined and measured for integrated density. Dividing the blue channel IDV of each ROI by the mean nucleus IDV gives the average number of cells present in each ROI. The fluorescent intensity of the GAT3 is expressed as a per cell measure by dividing the IDV of each ROI by its respective average cell number. GFAP staining is expressed as IDV only. Infarct volumes were determined 42 days post-stroke using cresyl violet staining and Image J analysis by an observer blinded to the treatment groups.[6] The analysis is based on obtaining measurements from every sixth section through the entire infarct (area in mm^2), and infarct volume was quantified as follows: $\text{infarct volume } mm^3 = \sqrt{area\ mm^2 \times section\ thickness \times section\ interval}$. All analyses were performed by an observer blinded to the treatment groups.

GAT3 Western blotting. Another group of C57BL/6J male mice (2–4 months old) were subjected to a photothrombotic stroke as described to investigate GAT3 expression in naïve animals and animals subjected to stroke. The animals were sacrificed by a pentobarbital overdose, followed by rapid extraction of the brain after

1, 3 and 6 h and 1, 3, 7, 14 and 28 days post-stroke, to determine the temporal change in GAT3 levels compared to sham operated mice. The brains were cut on a cryostat (CM1860, Leica) at −20°C from anterior to posterior, and the top quadrant of the left hemisphere containing the peri-infarct and surrounding tissue were collected. Protein was purified using RIPA-buffer supplemented with cOmplete™ and homogenized using a bullet blender.

Western blotting was performed using the NuPAGE® system from Invitrogen (Carlsbad, CA, USA). Protein samples mixed with 2× Laemmli with 0.1 M DTT were sonicated for 5–10 s and left at RT for 10 min before loading. Ten micrograms of protein was loaded in 4–12% Bis-Tris gels and separated by SDS PAGE for 60 min at 170–180 V. The gel was blotted on a PVDF membrane (ThermoFisher Scientific) at 25–30 V for 60 min. The rinsed membranes were blocked 60 min in TBS containing 5% skimmed milk. The top part of the membranes was incubated with blocking buffer containing the polyclonal GAT3 antibody (rabbit anti-mouse GAT3, 1:1000, AB1574, Millipore) and the bottom part of the membranes with the monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (mouse anti-mouse GAPDH, 1:12000, NB600-502SS, Biologicals, Littleton, CO, USA) overnight at 4°C. The rinsed membranes were incubated with appropriate HRP-conjugated secondary antibodies (anti-rabbit, 1:2000, A16096, ThermoFisher Scientific; anti-mouse, 1:2000, P0447, Dako) for 60 min at RT, rinsed and incubated with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Biosciences, Pittsburg, PA, USA) for 4 min prior to the detection of chemiluminescence using FlourChem® HD2 (Alpha Innotech, San Leandro, CA, USA). Image J was used to quantify optical density using GAPDH as a reference, which was unaffected by the stroke and stably expressed at all time points. Each group contained 5–6 animals with 1–2 technical replicates.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 7.0c, San Diego, CA, USA). One-way and two-way ANOVA followed by Dunnett's multiple comparison test and unpaired t-test were used when as appropriate and specified accordingly. $P < 0.05$ was considered statistically significant.

Group sizes for the behavioral analysis were determined by power analysis calculations using the G Power Software (version 3.1.5) based on data obtained previously in the grid-walking and cylinder tasks.[6,32,33] The following parameters: $\alpha = 0.05$, effect size = 1.5, two-sided for behavioral experiments were used. To ensure a power >80%, we included six animals in each of the two sham groups and 6-8 animals in each of the stroke groups.

Results

GAT3 protein expression levels are decreased in the peri-infarct and surrounding region

Previous studies have shown that GAT3 protein levels are decreased in the peri-infarct cortex after a photothrombotic stroke to the motor cortex coinciding with an increase in tonic inhibition.[6,8] To extend the regional and temporal pattern of GAT3 expression after stroke, we examined GAT3 protein expression in the peri-infarct cortex of mice after a photothrombotic stroke to the forelimb motor cortex. Using Western blotting, we found that the GAT3 protein expression decreased in the peri-infarct cortex and surrounding tissue from 1 h onwards, reaching a significant reduction by 6 h, with a maximal decrease in expression (46% decrease) observed seven days post-stroke (Figure 1(a) and (b)). A small non-significant decrease in expression was observed at 14 and 28 days post-stroke. For the Western blotting, we isolated the top quadrant of the brain containing the peri-infarct and surrounding tissue (Figure 1(b)). As a result, we were not able to assess region-specific changes in GAT3 expression, which could also be masked given the way the tissue was collected. Thus, to more accurately assess the GAT3 expression specifically in the peri-infarct cortex and subsequent regions, we investigated immunofluorescent staining of GAT3 42 days post-stroke in layers 2/3 and 5 in the peri-infarct and 400 μm and 800 μm away from the peri-infarct region. We show that the photothrombotic stroke induced long-lasting effects after 42 days primarily in the peri-infarct cortex, where the GAT3 expression was significantly decreased by 48% and 63% in layers 2/3 and 5, respectively (GAT3 intensity/cell: sham + vehicle vs. stroke + vehicle, layer 2/3: 2436 vs. 1253 and layer 5: 3916 vs. 1445), as well as in the subsequent regions in layer 5 by 28–41% (GAT3 intensity/cell: sham + vehicle vs. stroke + vehicle, 400 μm: 3733 vs. 2211 and 800 μm: 3439 vs. 2463) (Figure 1(c)). Although GAT3 protein expression levels determined by Western blotting were not different from sham levels after 14 days, it is clear from the immunofluorescent staining of GAT3 that a stroke to the motor cortex did indeed induce long-lasting decreases in GAT3 expression. These long-lasting changes in GAT3 were mainly in the peri-infarct region up to 800 μm away from the infarct and may very well affect the tonic currents in these given regions accordingly as long as 42 days post-stroke.

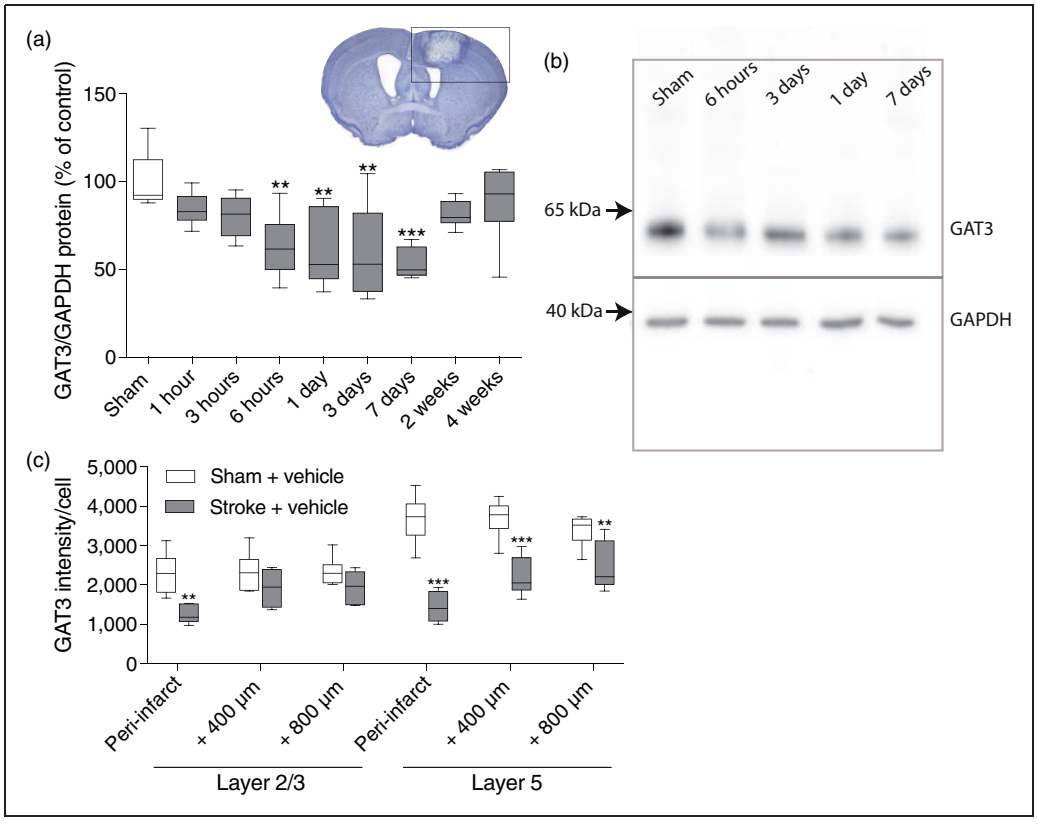

Figure 1. Temporal changes in GAT3 expression in the peri-infarct region and surrounding tissue after a photothrombotic stroke to the motor cortex in male mice. (a) Western blotting data showing that GAT3 expression decreased significantly from 6 h to 7 days post-stroke including the depiction of the outlined region isolated for Western blotting containing the peri-infarct and surrounding tissue (black box). (b) Representative Western blots of GAT3 and GAPDH. One-way ANOVA followed by Dunnett's multiple comparison test comparing sham to the different time points post-stroke. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ ($n = 6$ for sham and 1, 3 and 6 h and 1, 14 and 28 days, $n = 5$ for 3 and 7 days). (c) GAT3 intensity/cell 42 days post-stroke in the peri-infarct region and +400 µm and +800 µm from the peri-infarct in layers 2/3 and 5 (see Figure 4(a)). GAT3 intensity/cell was significantly decreased in the peri-infarct in layers 2/3 and 5 and outside the peri-infarct in layer 5, but not in layer 2/3. Two-way ANOVA with stroke and region as independent factors and region as repeated measures followed by Dunnett's multiple comparison test comparing sham + vehicle to stroke + vehicle in the specific regions. **$P < 0.01$, ***$P < 0.001$ ($n = 6$). Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).
GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

In vitro characterization of the GAT3 substrate L-isoserine

Supported by our findings in Figure 1, impaired GAT3-mediated uptake leads to a chronic increase in tonic inhibition in the peri-infarct cortex hindering functional recovery.[6] Previous studies have shown that GAT substrates can enhance GABA uptake in neuronal and glial cultures via an increase in the surface expression of GAT1 and presumably also GAT3.[24,34,35] The only GAT3 substrates currently available are L-isoserine and 3-guanidinopropionic acid (3-GPA),[36] but the *in vivo* usability of 3-GPA is limited as it induces weight loss in animals[37] and inhibits both the TauT and the CreaT (data not shown). Thus, we chose to use L-isoserine to investigate if a GAT3 substrate could enhance the expression of GAT3 and increase functional recovery after a motor cortex stroke in mice. To determine the dosing of L-isoserine, we first determined the selectivity profile of L-isoserine at recombinant GATs expressed in cell lines using a standard [^{3}H]GABA uptake assay. We show that L-isoserine, by competing with [^{3}H]GABA, has an IC_{50} value of 38.2 µM at mouse GAT3 displaying >26-fold and >19-fold selectivity over GAT1 and BGT1, respectively. However, L-isoserine has a similar potency at GAT2 as GAT3, making it a GAT2/3 preferring substrate inhibitor (Table S1, Supplementary material), similar to 3-GPA, although L-isoserine does not inhibit CreaT (Table S2, Supplementary material).[36] For comparison, we also determined the profile of L-isoserine on human GATs and found the selectivity profile of L-

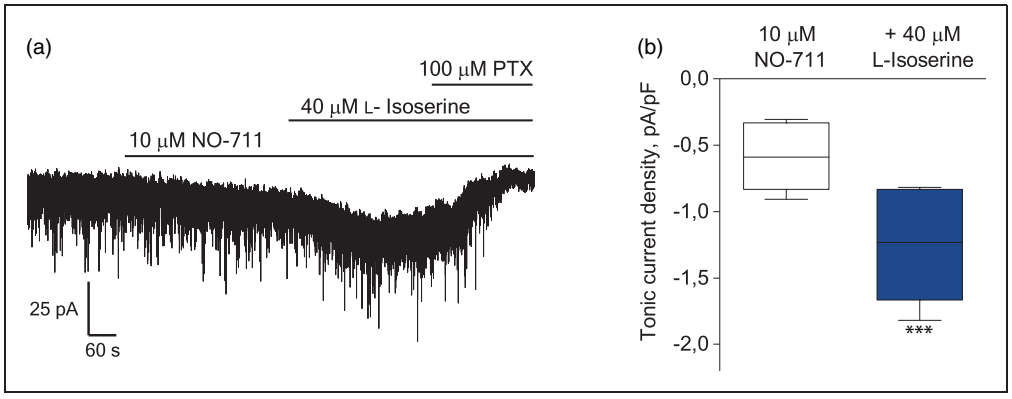

Figure 2. Tonic currents in pyramidal neurons in layer 2/3 and the effect of L-isoserine. Whole-cell patch-clamp recordings were made on slices from naïve mice. (a) Representative traces showing the tonic inhibitory currents and the sequential inhibition of GAT1 by NO-711 and GAT3 by L-isoserine followed by blockade of the $GABA_A$ receptors by picrotoxin (PTX) as indicated by the horizontal bars. (b) The GAT1 inhibitor NO-711 and L-isoserine both enhanced the tonic currents via $GABA_A$ receptors as the change in tonic currents were abolished by PTX. Unpaired t test comparing the change in tonic currents in response to NO-711 and L-isoserine. ***$P < 0.001$. Recordings are from 8 cells/8 slices from 4 mice. Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).

isoserine to be retained at the human orthologues (Table S1, Supplementary material). Using the FMP assay, we verified that L-isoserine is indeed a substrate for GAT3 with a potency similar to GABA itself (EC_{50} (pEC50 ± S.E.M.): 10.7 μM (4.97 ± 0.04), Figure S1(a), Supplementary material) and will be transported by GAT3 in competition with endogenous GABA making it a substrate inhibitor. Despite L-isoserine's structural resemblance to GABA, L-isoserine has very weak affinity for the $GABA_A$ receptors ($IC_{50} > 1000$ μM in [^{3}H]muscimol binding, Figure S1(b), Supplementary material).

The GAT2/3 inhibitor (*S*)-SNAP-5114 has been shown to increase tonic inhibition,[6,21,22,38] while the effect of a GAT3 substrate inhibitor on tonic currents is unknown. To confirm that GAT3 interaction leads to modulation of tonic inhibition in cortex, we tested the effect of L-isoserine on tonic currents in layer 2/3 pyramidal neurons. Indeed, by means of whole-cell patch-clamp recordings on slices from naïve mice, we show that L-isoserine is able to modulate tonic currents via $GABA_A$ receptors as the change in tonic currents was abolished in the presence of PTX (Figure 2). Therefore, L-isoserine exposure, being a substrate inhibitor of GAT3 with negligible binding to $GABA_A$ receptors at 1000 μM, plausibly modulates tonic currents by competing with GABA present during the recordings for GABA uptake via GAT3, hence increasing ambient GABA levels.

Due to the structural resemblance of L-isoserine to GABA, which has a zwitterionic nature at physiological pH, brain penetration is unlikely.[36] Thus, in the subsequent experiments, we administered L-isoserine directly into the infarct, which at the same time eliminates any GAT2-mediated inhibition, since GAT2-mediated GABA uptake is absent from brain parenchyma.[39]

The GAT3 substrate L-isoserine increases post-stroke recovery

Having verified that GAT3 expression is subjected to long-lasting changes following an ischemic stroke, we wanted to investigate the modulatory role of GAT3 in stroke recovery using L-isoserine. Therefore, we set out to investigate the effect of a delayed chronic administration of L-isoserine on functional recovery after a photothrombotic focal stroke to the motor cortex. L-isoserine was administered from five days post-stroke, the beginning of the critical period for neuroplasticity in rodents post-stroke,[40] with administration continuing for 28 consecutive days. Significant forelimb motor deficits contralateral to the hemisphere with stroke were observed throughout the 42 days in the grid-walking and cylinder tasks in vehicle-treated animals with minor spontaneous recovery (Figure 3). Thus, chronic impairment of the affected forelimb was observed in response to a photothrombotic stroke mimicking what others have reported in humans.[17] Chronic delayed treatment with the high concentration of L-isoserine increased motor performance of the affected forelimb from day 7 in the grid-walking task and from day 14 in the cylinder task. The low concentration of L-isoserine increased motor performance from day 14 in both behavioral tasks (Figure 3) illustrating a concentration-dependent improvement in motor function in response to L-isoserine treatment. The total increase after 42 days for the low and high concentration compared to the vehicle-treated mice,

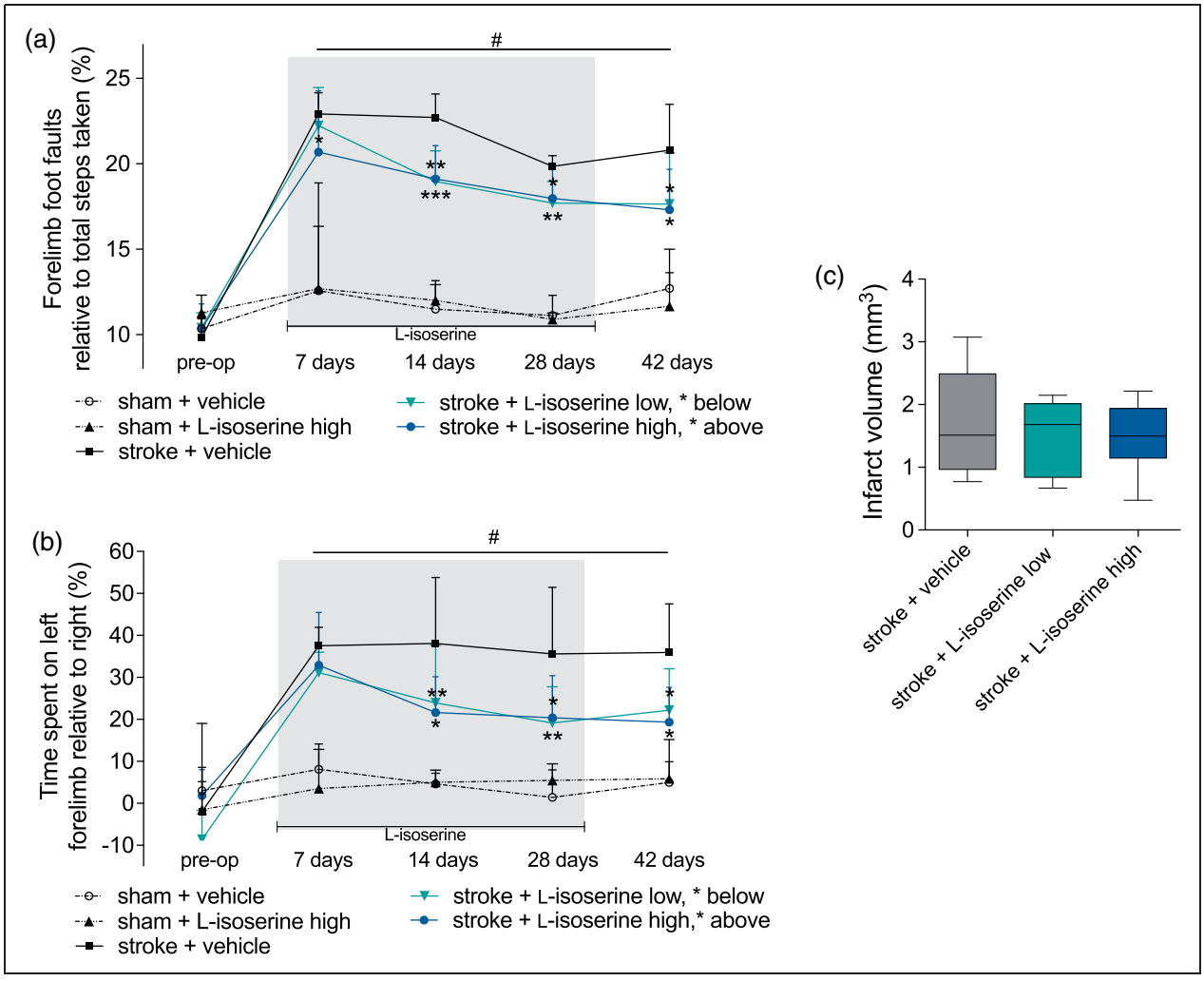

Figure 3. Motor function pre-op (before) and 7, 14, 28 and 42 days after a photothrombotic stroke and infarct volume. Motor function was assessed by analyses of (a) foot faults and (b) forelimb asymmetry in the grid-walking and cylinder tasks, respectively. Significant forelimb deficits ($P < 0.001$), contralateral to the hemisphere with stroke, were observed for at least 42 days post-stroke on both behavioral tasks. Chronic treatment with the high concentration of L-isoserine from day 5 and for 28 consecutive days (gray box) improved motor function from day 7 and onwards in the grid-walking task and from day 14 in the cylinder task. The low concentration of L-isoserine improved motor performance in both tasks from day 14. Two-way ANOVA followed by Dunnett's multiple comparison test with time and treatment as independent factors and time as repeated measures. # $P < 0.001$ stroke + vehicle compared to sham + vehicle, *$P < 0.05$, **$P < 0.01$, $P < 0.001$, stroke + vehicle compared to stroke + L-isoserine low or high (sham + vehicle ($n = 6$) and sham + L-isoserine high ($n = 6$); stroke + vehicle ($n = 8$), stroke + L-isoserine low ($n = 8$) and stroke + L-isoserine high ($n = 6$)). Data are expressed as mean ± SD. (c) Infarct volume 42 days post-stroke was unaffected by L-isoserine treatment. One-way ANOVA followed by Dunnett's multiple comparison test comparing stroke + vehicle to stroke + L-isoserine low or high (stroke + vehicle ($n = 8$), stroke + L-isoserine low ($n = 8$) and stroke + L-isoserine high ($n = 6$)). Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).

respectively, was 44% and 53% in the grid-walking task (forelimb foot faults relative to total steps taken (%): stroke + vehicle vs. stroke + L-isoserine, low: 43% vs. 27% and high 43% vs. 26%, Figure 3(a)) and 36% and 39% in the cylinder task (time spent on left forelimb relative to right (%): stroke + vehicle vs. stroke + L-isoserine, low: 88% vs. 49% and high: 88% vs. 41%, Figure 3(b)). No signs of seizures or seizure-like behavior were observed in any of the mice throughout the 42 days and no changes in infarct volumes upon L-isoserine treatment were observed (Figure 3(c)).

L-Isoserine increases the GAT3 intensity/cell outside the peri-infarct region

To investigate whether L-isoserine increased GAT3 expression in the stroke animals, we assessed the

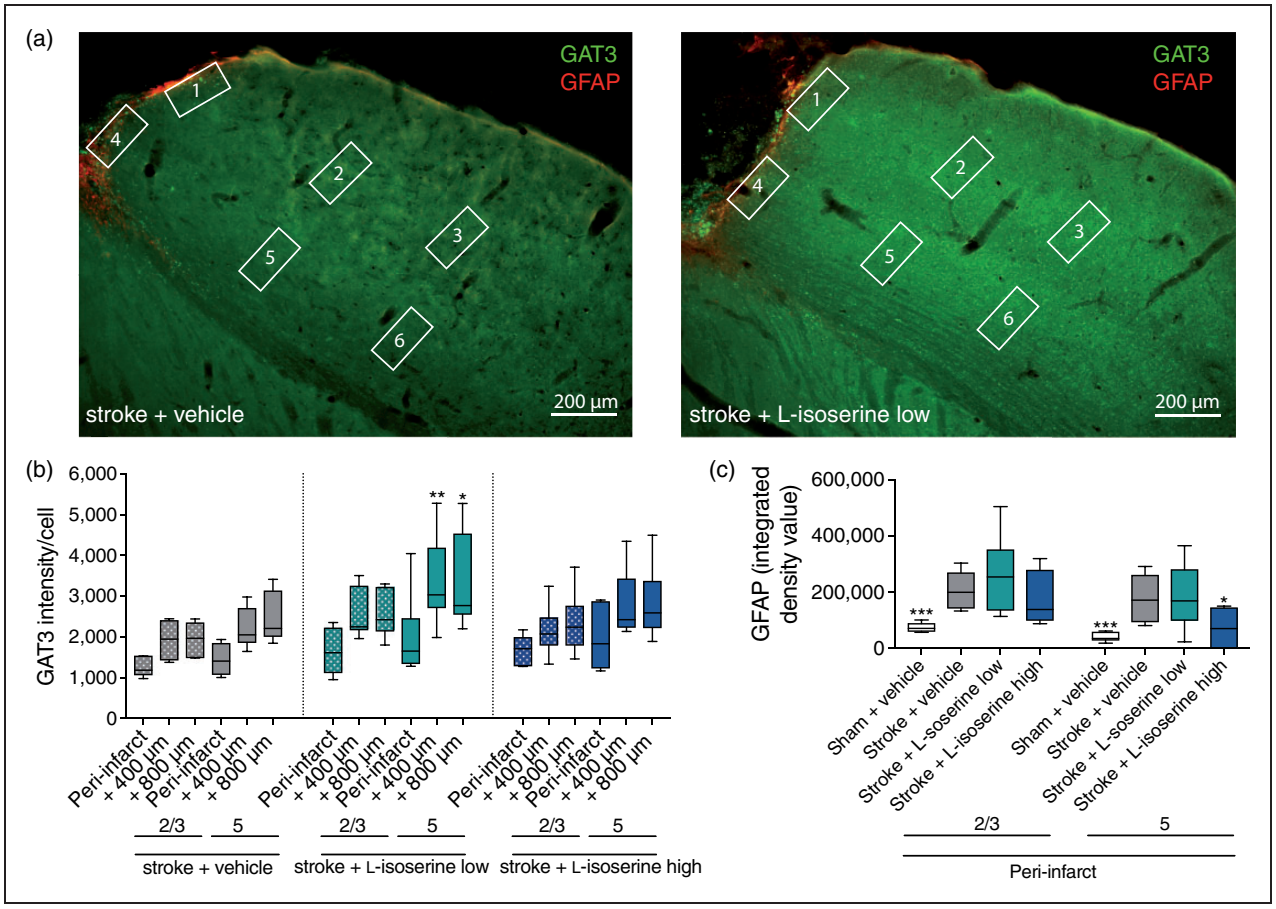

Figure 4. GAT3 intensity/cell and GFAP expression 42 days post-stroke. (a) Representative staining of GAT3 and GFAP and the six squared regions, where the GAT3 intensity/cell and GFAP intensity were determined; peri-infarct (1 and 4) and 400 µm (2 and 5) and 800 µm (3 and 6) from the peri-infarct in layers 2/3 and 5, respectively. (b) L-Isoserine increased the GAT3 intensity/cell compared to stroke + vehicle in layer 5 outside the peri-infarct, while the GAT3 intensity/cell was unaffected in layer 2/3. (c) GFAP is significantly increased post-stroke in the peri-infarct compared to sham + vehicle at day 42. This increase is reduced by the high concentration of L-isoserine in layer 5, but not in layer 2/3, compared to stroke + vehicle layer 5, while the low concentration had no effect. Two-way ANOVA with treatment and region as independent factors and region as repeated measures. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (sham + vehicle ($n = 6$) and sham + L-isoserine high ($n = 6$); stroke + vehicle ($n = 6$), stroke + L-isoserine low ($n = 8$) and stroke + L-isoserine high ($n = 6$)). Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).
GFAP: glial fibrillary acidic protein;

GAT3 expression using immunofluorescent labeling 42 days post-stroke, after the animals had completed the last behavioral tasks, corresponding to 10 days after discontinuing the L-isoserine treatment. We found that L-isoserine treatment generally increased GAT3 expression by 33–53% at the low concentration and 12–37% at the high concentration in different regions (Figure 4). At the low concentration of L-isoserine, this increase was significant in layer 5, 400 µm and 800 µm from the peri-infarct where GAT3 expression was increased by 53% and 37%, respectively (GAT3 intensity/cell: stroke + vehicle vs. stroke + L-isoserine, 400 µm: 2211 vs. 3381 and 800 µm: 2463 vs. 3379).

With immunofluorescent GAT3 staining, we studied the effect of L-isoserine at a fixed time point 10 days after the treatment was discontinued and in relation to the total pool of GAT3. We next wanted to investigate if L-isoserine had an effect on the total GABA uptake or GAT3 surface expression *in vitro*. Using cerebral astrocyte cultures, we found no effect on the total [^{3}H]GABA uptake after 60 min pre-incubation with L-isoserine (10–200 µM) (Figure 5). These data indicate that short-term exposure to L-isoserine, although being a substrate inhibitor of GAT3 as demonstrated in the FMP assay, does not induce lasting changes in GAT3 activity or in GAT3 surface expression in astrocyte cultures. This is in contrast to previous findings with the GAT1/3 substrate nipecotic acid, which has been found to slow down the internalization of GAT1 by a direct transporter-dependent mechanism requiring direct

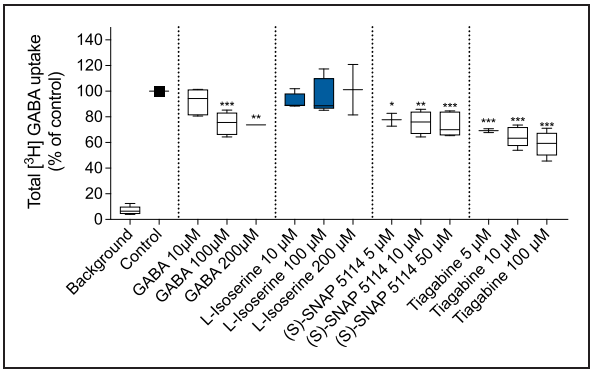

Figure 5. Total [^{3}H]GABA uptake in cortical astrocyte cultures. Pre-incubation (60 min) with L-isoserine had no effect at the concentrations tested, while GABA (100 and 200 μM), (*S*)-SNAP-5114 (5,10 and 50 μM) and tiagabine (5, 10 and 100 μM) decreased the total [^{3}H]GABA uptake significantly. Experiments were performed in triplicates in five independent experiments except for (*S*)-SNAP-5114 and tiagabine at 5 μM and L-isoserine and GABA at 200 μM that were tested in two independent experiments. One-way ANOVA followed by Dunnett's multiple comparison test comparing all compounds to the control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).

tyrosine phosphorylation of GAT1.[24] Interestingly, more chronic exposure to L-isoserine did result in an increased GAT3 expression as indicated by immunohistochemistry on tissue collected after completion of the behavioral testing. This indicates that substrate inhibitors of GAT3 can alter the expression of GAT3 *in vivo*. However, we did find that pre-incubation with the GAT3 inhibitor (*S*)-SNAP-5114 decreased the total [^{3}H]GABA uptake (Figure 5), in line with previous findings,[24] suggesting that GAT3 is subjected to the same kind of regulation as GAT1, as revealed by the GAT1 inhibitor tiagabine, when it comes to inhibitors but not substrate inhibitors. This is supported by a decreased total [^{3}H]GABA uptake in response to high GABA concentrations (Figure 5).

To investigate if the surface expression of GAT3, which, in large, dictates ambient GABA levels *in vivo*, is modulated by L-isoserine, we turned to recombinant settings using ELISA and tsA201 cells transiently expressing an extracellularly epitope-tagged transporter, GAT3-HA. We found that L-isoserine had no effect on the surface expression of GAT3 *in vitro* within a period of 5–120 min, 24 or 48 h exposure (Figure 6), although an intracellular pool of GAT3-HA was available (data not shown). In addition, GABA and (*S*)-SNAP-5114 had no effect on GAT3 surface expression, although we were unable to study the long-term effects of (*S*)-SNAP-5114 due to toxic effects (observed as a decreased viability of the cells after 24 h) (Figure 6).

L-Isoserine modulates the glial scar

Last, we wanted to investigate if L-isoserine has an effect on the glial scar. The glial scar has many roles after a stroke has occurred, both as a regulator of the inflammatory response and in relation to axonal sprouting, which is critical for cortical remapping.[41–43] We observed a clear up-regulation of the GFAP expression 42 days post-stroke compared to sham-operated mice in the peri-infarct in layer 2/3 and 5 (Figure 4(c)). Chronic treatment with the high concentration of L-isoserine decreased the GFAP expression in the peri-infarct in layer 5, while the low concentration had no effect (Figure 4(c)). The GFAP expression outside the peri-infarct was not increased in the stroke animals compared to shams and was not affected by L-isoserine treatment (data not shown).

Discussion

Recent studies have shown that tissue adjacent to the stroke, the peri-infarct cortex, is a region of heightened plasticity and contributes to the limited capacity of the brain to repair following a stroke.[17,43–45] The peri-infarct cortex is a region where patterns of evoked neuronal activation shift, correlating to improved functional recovery.[33,43] This process involves changes in brain excitability, which underlies changes in the way the brain represents motor and sensory function. In particular, a reduction in cortical inhibition promotes recovery of function in peri-infarct cortex by facilitating the formation of new functional and structural circuits to enhance motor remapping.[46] Consistent with this, we have recently shown that a delayed reduction in GABA-mediated tonic inhibition, by inhibiting extrasynaptic $GABA_A$ receptors, can increase the recovery of motor function,[6,47] and that the changes in GABA signaling in the peri-infarct region and resultant increase in tonic inhibition after an ischemic stroke is in part due to changes in GAT3 expression and function.[6]

GAT3 expression is decreased post-stroke

In the present study, we show that GAT3 is subjected to long-lasting changes in response to ischemia, primarily affecting the cells found in close proximity to the ischemic core. In this area, GAT3 expression is already down-regulated after 6 h and remains suppressed 42 days post-stroke, indicating that it is most likely the palisading astrocytes, which form the glial scar, that display a reduced GAT3 expression. These temporal changes in GAT3 levels match the reported change in tonic inhibition,[6] further supporting the hypothesis that impaired GAT3-mediated uptake is contributing to the

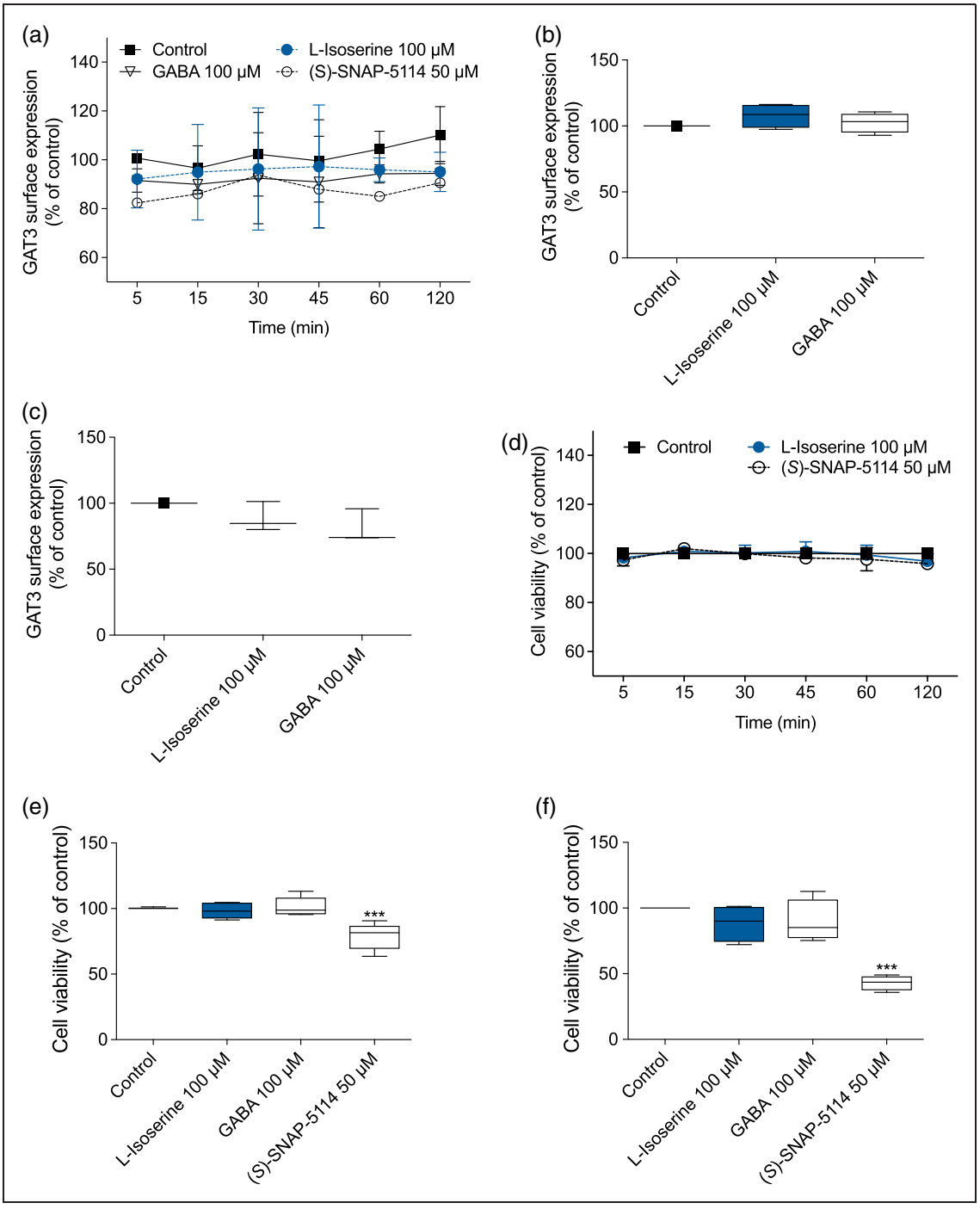

Figure 6. GAT3 surface expression in tsA201 cells recombinant expressing GAT3-HA and their corresponding cell viability. (a to c) The surface expression of GAT3-HA was assessed by ELISA, (d to f), while cell viability after pre-incubation at different time points with the compounds was assessed by MTT. (a to c) 5 min to 48 h pre-incubation with L-isoserine and GABA had no effect on the surface level of GAT3 compared to control. (a) (*S*)-SNAP-5114 also had no effect on GAT3 surface expression after 5–120 min pre-incubation, (e and f) while we could not see any long-term effect of (*S*)-SNAP-5114 (50 µM) due to toxic effects after 24 h. Experiments were performed in triplicate in at least three independent experiments. Two-way ANOVA with treatment and time as independent factors and no repeated measures followed by Dunnett's multiple comparison test comparing all compounds to the control (5–120 min) and one-way ANOVA (24 and 48 h) followed by Dunnett's multiple comparison test comparing the effect of the compound to the control. *$P < 0.05$, ***$P < 0.001$. Box plot (boxes, 25–75%; whiskers, minimum and maximum; lines, median).

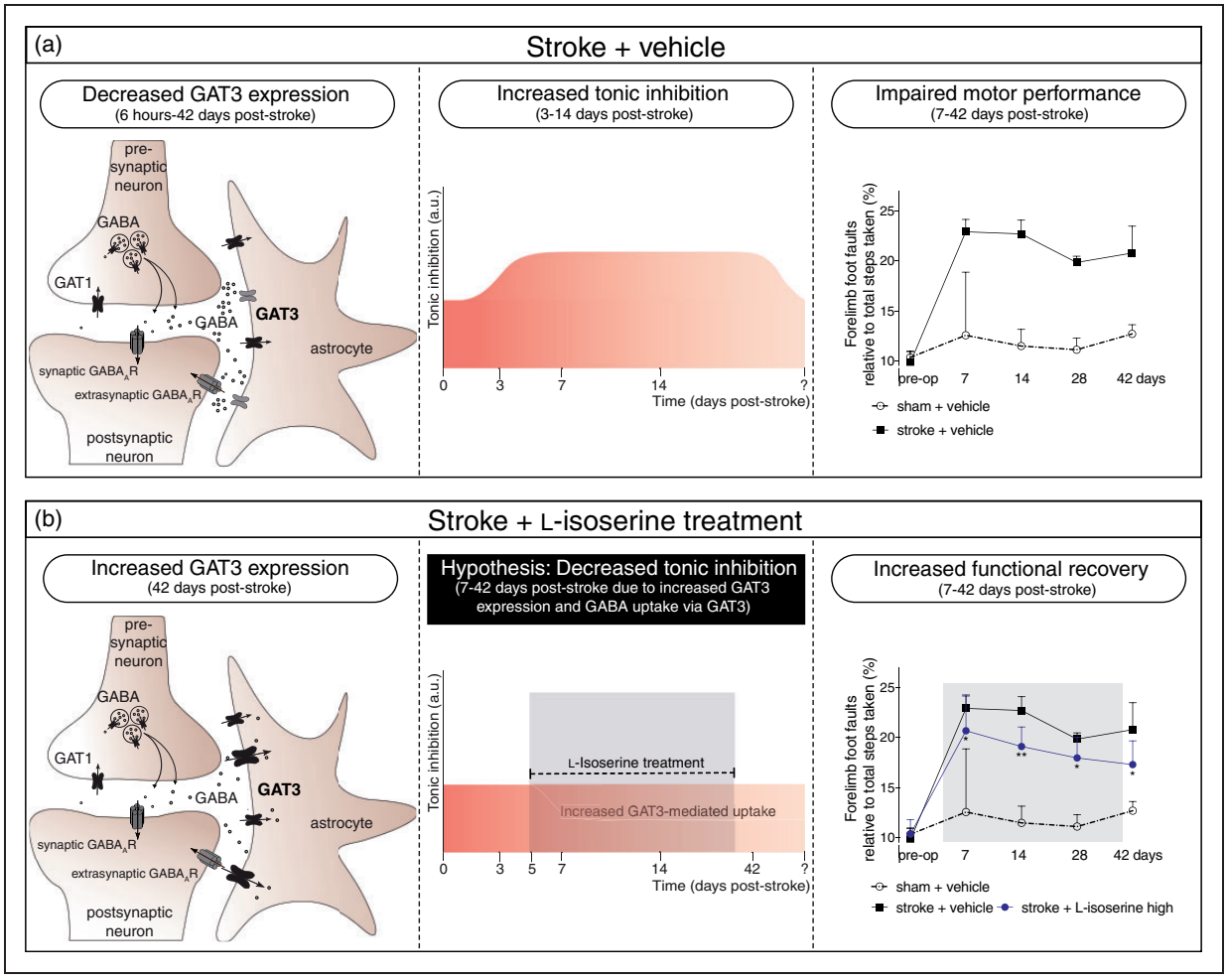

Figure 7. Schematic summary of key results and the proposed hypothesis stating how L-isoserine increases functional recovery after a photothrombotic stroke. (a) A focal stroke to the motor cortex decreases GAT3 expression in the peri-infarct and surrounding regions from 6 h to 42 days post-stroke (indicated by a lighter shading in a subset of GAT3) and impairs motor function from 7 to 42 days post-stroke (stroke + vehicle compared to sham + vehicle). Also, we have previously shown that tonic inhibition is increased in the peri-infarct region from 3 to 14 days after a focal stroke due to impaired GAT3-mediated GABA uptake.[6] (b) Chronic L-isoserine treatment increases GAT3 expression at day 42 in stroke animals (indicated by a subset of GAT3 being larger), 10 days after the treatment was discontinued, and alongside increases functional recovery from day 7 and onwards (stroke + vehicle compared to stroke + L-isoserine high). We hypothesize that the increase in functional recovery in response to chronic L-isoserine treatment (indicated by the gray boxes) from day 7 and onwards is mediated by an increase in GAT3 surface expression already from day 7, which ultimately leads to a greater removal of ambient GABA by GAT3-mediated uptake leading to a decrease in tonic inhibition.

chronic elevation in tonic inhibition after ischemia. We also show that changes in the total pool of GAT3 correlates with equal changes in tonic inhibition and that the down-regulation of GAT3 precedes the changes in tonic inhibition reported by Clarkson et al.[6] and extends for as long as 42 days post-stroke, much longer than previous thought.[6] Yet, we cannot exclude that other sources may contribute to the increase in ambient GABA levels including reactive astrocytes, which have been reported to be able to produce and subsequently release GABA via the anion channel bestrophin1 (Best1)[48] and GAT3 efflux.[38,49]

Delayed treatment with a GAT3 substrate, L-isoserine, increases GAT3 expression and post stroke recovery

With GAT3 being a direct regulator of ambient GABA levels and tonic inhibition[14] and being involved in the pathology of an ischemic stroke,[6,8] we wanted to investigate the therapeutic potential of a GAT3 substrate inhibitor. We are the first to show that the GAT3 substrate inhibitor, L-isoserine, increases functional recovery of the affected forelimb in a concentration-dependent manner from day 7 without any rebounds

in motor function when discontinued after at day 32 post-stroke, indicating that L-isoserine induces lasting plastic changes in the brain. The L-isoserine treatment was initiated at a delay (5 days) post-stroke at the time of the critical period for neuroplasticity in rodents[40] and within the time window where positive effects of lowered tonic inhibition via the α_5-containing $GABA_A$ receptors using L-655,708 have been reported in respect to post-stroke recovery.[6,11] Thus, this study supports that increased recovery of function following stroke can be achieved not only by dampening tonic inhibition directly by targeting extrasynaptic $GABA_A$ receptors[6,11] but also through a delayed modulation of GAT3, which could be a putative target for the treatment of stroke.

Ambient GABA levels and tonic inhibition are controlled by the reuptake of GABA into surrounding astrocytes and neurons via GATs.[14,20] We show that L-isoserine generally increased the level of GAT3 expression when assessed 42 days post-stroke, corresponding to 10 days after discontinuing the treatment. These data indicate lasting changes in GAT3 expression following chronic treatment with L-isoserine that preferentially affects the cells furthest away from the ischemic core suggesting that palisading astrocytes are less susceptible to substrate-induced changes in GAT3 expression. While L-isoserine is a substrate for GAT3, it still allows the transport of GABA, and although L-isoserine modulates tonic GABA currents in slices from naïve mice, the effect in animals subjected to a stroke still remains unknown. However, based on the data at hand, we hypothesize that L-isoserine increases the surface expression of GAT3 from day 7, which leads to an overall increase in GABA uptake via GAT3, which in turn dampens tonic inhibition and improves functional recovery (Figure 7). Our previous findings, showing that a reduction in tonic inhibition via α_5-containing $GABA_A$ receptors increases functional recovery,[6] supports this hypothesis, but awaits further clarification. These studies will indeed be complicated further, since GAT3 may be functionally impaired in some regions[6] or reversed in reactive astrocytes,[38,49] although the latter is doubtful as the resting membrane potential and GABA reversal potential have been shown to be unaffected in the peri-infarct region.[6]

In contrast to the effects of L-isoserine *in vivo*, we found no effect of short-term L-isoserine exposure on GAT3 surface expression *in vitro* and GABA uptake in astrocyte cultures. These findings suggest that the increase in GAT3 expression in the stroke animals in response to L-isoserine treatment depends both on chronic exposure as well as factors present *in vivo*. However, it remains unclear if the increase in GAT3 expression following *in vivo* treatment with L-isoserine was mediated by a transporter-dependent mechanism, e.g. affecting the degradation of GAT3 or was indirectly linked to enhanced GAT3 activity.

Tonic inhibition is not the only parameter controlling stroke recovery and there is a plethora of factors that influence the outcome besides tonic inhibition per se. The reactive astrocyte in particular has many roles in ischemia and is highly involved in the regulation of the inflammatory response,[42] where chronic neuroinflammation impede post stroke recovery.[50] A recent study has also shown that reactive astrocytes are needed for axonal sprouting,[41] indicating that some astrogliosis is necessary for recovery. For this reason, we also looked at the modulation of the glial scar in the stroke mice at day 42 and investigated the effect of L-isoserine. We show that the high concentration of L-isoserine decreased the GFAP level in the peri-infarct in layer 5, but not in layer 2/3. Nevertheless, given that the low concentration of L-isoserine had no effect on the GFAP level, and the two concentrations of L-isoserine had similar effects on motor performance post-stroke questions whether L-isoserine promoted recovery by enhancing the resolution of the glial scar. Also, the changes in GFAP and GAT3 expression in response to L-isoserine treatment do not seem to be linked, since the change in GFAP occurred in the peri-infarct region, where the smallest increase in GAT3 expression was observed.

Conclusion

We show that a photothrombotic stroke to the motor cortex produces long-lasting effects by reducing the GAT3 protein level thus impairing the GAT3 uptake, persisting for as long as 42 days after the ischemic insult. We are the first to demonstrate that a GAT3 substrate inhibitor, L-isoserine, administered at a delay post-stroke, increases GAT3 expression and facilitates an improvement in recovery of the affected forelimb, presumably through enhanced GABA uptake via GAT3 potentially dampening tonic inhibition and hence enabling cortical remapping.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Lundbeck Foundation (grant R118-A11564), the Carlsberg Foundation (PW), the Drug Research Academy, the New Zealand Neurological Foundation and the Royal Society of New Zealand Project Grant (ANC).

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

MEKL, ANC and PW designed the studies. MEKL, EKG and ANC performed and analyzed the *in vivo* studies. MEKL, EKG, NBJ, NOD and LT performed and analyzed the *in vitro* studies. MEKL drafted the manuscript. PW and ANC critically revised the manuscript. All authors reviewed, edited and approved the final version of this manuscript.

Supplementary material

Supplementary material for this paper can be found at the journal website: http://journals.sagepub.com/home/jcb

References

1. WHO. The top 10 causes of death, www.who.int/media-centre/factsheets/fs310/en/ (2015, accessed 1 March 2017).
2. Murphy TH and Corbett D. Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci* 2009; 10: 861–872.
3. Lo EH. A new penumbra: transitioning from injury into repair after stroke. *Nat Med* 2008; 14: 497–500.
4. Lim DH, LeDue JM, Mohajerani MH, et al. Optogenetic mapping after stroke reveals network-wide scaling of functional connections and heterogeneous recovery of the peri-infarct. *J Neurosci* 2014; 34: 16455–16466.
5. Hensch TK. Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* 2005; 6: 877–888.
6. Clarkson AN, Huang BS, Macisaac SE, et al. Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature* 2010; 468: 305–309.
7. Alia C, Spalletti C, Lai S, et al. Reducing $GABA_A$-mediated inhibition improves forelimb motor function after focal cortical stroke in mice. *Sci Rep* 2016; 6: 37823.
8. Jaenisch N, Liebmann L, Guenther M, et al. Reduced tonic inhibition after stroke promotes motor performance and epileptic seizures. *Sci Rep* 2016; 6: 26173.
9. Kett-White R, O'Connell MT, Hutchinson PJA, et al. Extracellular amino acid changes in patients during reversible cerebral ischaemia. *Acta Neurochir Suppl* 2005; 95: 83–88.
10. Blicher JU, Near J, Næss-Schmidt E, et al. GABA levels are decreased after stroke and GABA changes during rehabilitation correlate with motor improvement. *Neurorehabil Neural Repair* 2015; 29: 278–286.
11. Lake EMR, Chaudhuri J, Thomason L, et al. The effects of delayed reduction of tonic inhibition on ischemic lesion and sensorimotor function. *J Cereb blood flow Metab* 2015; 35: 1601–1609.
12. Kim YK, Yang EJ, Cho K, et al. Functional recovery after ischemic stroke is associated with reduced GABAergic inhibition in the cerebral cortex: a GABA PET study. *Neurorehabil Neural Repair* 2014; 28: 576–583.
13. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. *Neuropharmacology* 2008; 55: 363–389.
14. Brickley SG and Mody I. Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* 2012; 73: 23–34.
15. Darmani G, Zipser CM, Böhmer GM, et al. Effects of the selective α_5-$GABA_AR$ antagonist S44819 on excitability in the human brain: a TMS–EMG and TMS–EEG phase I study. *J Neurosci* 2016; 36: 12312–12320.
16. Emberson J, lees K, Lyden P, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet* 2014; 385: 1929–1935.
17. Cramer SC. Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. *Ann Neurol* 2008; 63: 272–287.
18. Roche. A study to evaluate the safety and efficacy of basmisanil in adults with severe motor impairment following an ischemic stroke, https://clinicaltrials.gov/ct2/show/record/NCT02928393 (2016, accessed 14 May 2017).
19. Servier. Efficacy and safety trial with S 44819 after recent ischemic cerebral event, https://clinicaltrials.gov/ct2/show/NCT02877615 (2016, accessed 17 May 2017).
20. Lie M, Al-Khawaja A, Damgaard M, et al. Glial GABA transporters as modulators of inhibitory signalling in epilepsy and stroke. *Adv Neurobiol* 2017; 16: 137–167.
21. Kersanté F, Rowley SCS, Pavlov I, et al. A functional role for both γ-aminobutyric acid (GABA) transporter-1 and GABA transporter-3 in the modulation of extracellular GABA and GABAergic tonic conductances in the rat hippocampus. *J Physiol* 2013; 591: 2429–2441.
22. Song I, Volynski K, Brenner T, et al. Different transporter systems regulate extracellular GABA from vesicular and non-vesicular sources. *Front Cell Neurosci* 2013; 7: 23.
23. Frahm C, Haupt C, Weinandy F, et al. Regulation of GABA transporter mRNA and protein after photothrombotic infarct in rat brain. *J Comp Neurol* 2004; 478: 176–188.
24. Whitworth TL and Quick MW. Substrate-induced regulation of γ-aminobutyric acid transporter trafficking requires tyrosine phosphorylation. *J Biol Chem* 2001; 276: 42932–42937.
25. Kragler A, Höfner G and Wanner KT. Novel parent structures for inhibitors of the murine GABA transporters mGAT3 and mGAT4. *Eur J Pharmacol* 2005; 519: 43–47.
26. Al-Khawaja A, Haugaard A, Marek A, et al. Pharmacological characterization of [^{3}H]ATPCA as a substrate for studying the functional role of the betaine/GABA transporter 1 and the creatine transporter. *ACS Chem Neurosci* (Accepted).
27. Al-Khawaja A, Petersen JG, Damgaard M, et al. Pharmacological identification of a guanidine-containing β-alanine analogue with low micromolar potency and selectivity for the betaine/GABA transporter 1 (BGT1). *Neurochem Res* 2014; 39: 1988–1996.
28. Kvist T, Christiansen B, Jensen AA, et al. The four human γ-aminobutyric acid (GABA) transporters: pharmacological characterization and validation of a

highly efficient screening assay. *Comb Chem High Throughput Screen* 2009; 12: 241–249.
29. Jørgensen L, Al-Khawaja A, Kickinger S, et al. Structure-activity relationship, pharmacological characterization, and molecular modelling of non-competitive inhibitors of the betaine/γ-aminobutyric acid transporter 1 (BGT1). *J Med Chem* 2017; 60: 8834–8846.
30. Wellendorph P, Høg S, Greenwood JR, et al. Novel cyclic γ-hydroxybutyrate (GHB) analogs with high affinity and stereoselectivity of binding to GHB sites in rat brain. *J Pharmacol Exp Ther* 2005; 315: 346–351.
31. Dalby NO, Volbracht C, Helboe L, et al. Altered function of hippocampal CA1 pyramidal neurons in the rTg4510 mouse model of tauopathy. *J Alzheimers Dis* 2014; 40: 429–442.
32. López-Valdés HE, Clarkson AN, Ao Y, et al. Memantine enhances recovery from stroke. *Stroke* 2014; 45: 2093–2100.
33. Clarkson AN, López-Valdés HE, Overman JJ, et al. Multimodal examination of structural and functional remapping in the mouse photothrombotic stroke model. *J Cereb Blood Flow Metab* 2013; 33: 716–723.
34. Bernstein EM and Quick MW. Regulation of γ-aminobutyric acid (GABA) transporters by extracellular GABA. *J Biol Chem* 1999; 274: 889–895.
35. Law RM, Stafford A and Quick MW. Functional regulation of γ-aminobutyric acid transporters by direct tyrosine phosphorylation. *J Biol Chem* 2000; 275: 23986–23991.
36. Damgaard M, Haugaard A, Kickinger S, et al. Development of non-GAT1 selective inhibitors; challenges and achievements. *Adv Neurobiol* 2017; 16: 315–332.
37. Meglasson MD, Wilson JM, Yu JH, et al. Antihyperglycemic action of guanidinoalkanoic acids: 3-guanidinopropionic acid ameliorates hyperglycemia in diabetic KKAy and C57BL6Job/ob mice and increases glucose disappearance in rhesus monkeys. *J Pharmacol Exp Ther* 1993; 266: 1454–1462.
38. Wu Z, Guo Z, Gearing M, et al. Tonic inhibition in dentate gyrus impairs long-term potentiation and memory in an Alzhiemer's disease model. *Nat Commun* 2014; 5: 4159.
39. Conti F, Zuccarello LV, Barbaresi P, et al. Neuronal, glial, and epithelial localization of γ-aminobutyric acid transporter 2, a high-affinity γ-aminobutyric acid plasma membrane transporter, in the cerebral cortex and neighboring structures. *J Comp Neurol* 1999; 409: 482–494.
40. Biernaskie J, Chernenko G and Corbett D. Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. *J Neurosci* 2004; 24: 1245–1254.
41. Anderson M, Burda J, Ren Y, et al. Astrocyte scar formation aids central nervous system axon regeneration. *Nature* 2016; 532: 195–200.
42. Sofroniew MV. Astrocyte barriers to neurotoxic inflammation. *Nat Rev Neurosci* 2015; 16: 249–263.
43. Brown CE, Aminoltejari K, Erb H, et al. *In vivo* voltage-sensitive dye imaging in adult mice reveals that somatosensory maps lost to stroke are replaced over weeks by new structural and functional circuits with prolonged modes of activation within both the peri-infarct zone and distant sites. *J Neurosci* 2009; 29: 1719–1734.
44. Dijkhuizen RM, Singhal AB, Mandeville JB, et al. Correlation between brain reorganization, ischemic damage, and neurologic status after transient focal cerebral ischemia in rats: a functional magnetic resonance imaging study. *J Neurosci* 2003; 23: 510–517.
45. Nudo RJ. Mechanisms for recovery of motor function following cortical damage. *Curr Opin Neurobiol* 2006; 16: 638–644.
46. Cicinelli P, Pasqualetti P, Zaccagnini M, et al. Interhemispheric asymmetries of motor cortex excitability in the postacute stroke stage: a paired-pulse transcranial magnetic stimulation study. *Stroke* 2003; 34: 2653–2658.
47. Clarkson AN. Perisynaptic GABA receptors: the overzealous protector. *Adv Pharmacol Sci* 2012; 2012: 708428.
48. Jo S, Yarishkin O, Hwang YJ, et al. GABA from reactive astrocytes impairs memory in mouse models of Alzheimer's disease. *Nat Med* 2014; 20: 886–896.
49. Hernandez-Rabaza V, Cabrera-Pastor A, Taoro-Gonzalez L, et al. Neuroinflammation increases GABAergic tone and impairs cognitive and motor function in hyperammonemia by increasing GAT-3 membrane expression. Reversal by sulforaphane by promoting M2 polarization of microglia. *J Neuroinflammation* 2016; 13: 83.
50. Neumann S, Shields N, Balle T, et al. Innate immunity and inflammation post-stroke: an α_7-nicotinic agonist perspective. *Int J Mol Sci* 2015; 16: 29029–29046.