Postacute administration of the GABA\(_{\alpha_5}\) antagonist S44819 promotes recovery of peripheral limb fine motor skills after permanent distal middle cerebral artery occlusion in rats

Marta Pace\(^1,2\), Matteo Falappa\(^1\), Patricia Machado\(^3\), Laura Facchin\(^2\), Dirk M Hermann\(^4\), and Claudio L Bassetti\(^2,5\)

Abstract

**Background:** Ischemic stroke causes hypoexcitability in the peri-infarct motor neocortex that stems from increased tonic \(\gamma\)-amino-butyric acid (GABA) activity in neurons. This hypoexcitability, while neuroprotective in the acute phase, may impair neuroplasticity and functional recovery in the subacute phase of stroke. The purpose of this study is to investigate the effect of delayed and prolonged administration of S44819, which is a potent and competitive selective antagonist of GABA\(_{\alpha_5}\) receptors, on the skilled reaching function in a rodent model of stroke. **Methods:** Male Sprague–Dawley rats (\(n = 15\)) were subjected to permanent middle cerebral artery occlusion. Starting 3 days after stroke, a vehicle or S44819 (3 or 10 mg/kg, BID) was delivered orally twice a day for 28 days. All animals were euthanized 2 weeks later after the washout period. A single pellet reaching task (SPR) was performed before (baseline value) and after the ischemic surgery at several time points (3, 10, 17, 24, 31, 38, and 45 days) to assess the motor deficit. Infarct volume and body changes were also evaluated. **Results:** S44819, administered at 10 but not 3 mg/kg, significantly improves SPR results over the 45 days after the ischemic surgery. No effect was observed in the infarct size and in the body weight over time between the groups investigated. **Conclusion:** S44819 at 10 mg/kg significantly enhances motor recovery on a skilled reaching task after sensory-motor cortex lesion. Additionally, our study, in light of the results of the RESTORE BRAIN (Randomized Efficacy and Safety Trial of Oral GABA\(_{\alpha_5}\) antagonist S44819 after Recent ischemic Event) trial, may help clinicians to design clinical studies and stratify variables and patients adequately.

**Keywords**

Stroke, functional outcome, motor function, single pellet reaching task, rats, sensory-motor cortex lesion, permanent middle cerebral artery occlusion, neuroplasticity

Introduction

\(\gamma\)-Amino-butyric acid (GABA), the predominant inhibitory neurotransmitter in the mammalian central nervous system, has a key role in poststroke functional recovery. Ischemic stroke causes hypoexcitability in the peri-infarct motor neocortex that results from increased tonic GABA activity onto neurons.\(^1,2\) Whereas this prolonged elevated inhibition in the peri-infarct region could be useful to protect tissues in the excitotoxic acute phase of stroke, it may impair the neuronal plasticity (remapping of peri-infarct regions, ...)
neurogenesis, angiogenesis, and neurite outgrowth) necessary in a later phase for functional recovery. Therefore, blocking the GABA_A receptor responsible for tonic inhibition should promote neuroplasticity and thus improve functional recovery.3,4

S44819 is a competitive selective antagonist that interacts with GABA_A receptors at the GABA-binding site.5 Ex vivo and in vitro experiments indicate that S44819 shows preferential binding affinity to α5-containing GABA_A receptors.5 S44819 enhances synaptic plasticity in vitro and in vivo, improves memory, and reduces anxiety in a variety of cognitive tests in rodents.5 It has recently been shown that postacute administration per os (p.o.) of S44819 at a dose of 10 mg/kg BID enhances neurological recovery and peri-infarct brain remodeling in the postacute stroke phase in mice exposed to transient proximal middle cerebral artery occlusion (MCAO).6 In this study, a set of behavioral tests evaluating motor coordination and cognitive performance have been studied but skilled forelimb movements were not assessed. The recovery of fine movements is critical for stroke outcome in human patients.

Considering the importance of fine movements for clinical stroke outcome, we now exposed rats to permanent distal MCAO that induces predominantly cortical brain infarcts.7,8 In these rats, we administered S44819 at two doses (3 or 10 mg/kg BID, p.o. starting 3 days post-MCAO) and assessed the effect of S44819 on skilled forelimb movements by the single pellet reaching (SPR) test.

**Material and methods**

**Animals**

All experimental procedures were approved by the Animal Research Committee and Veterinary Office of the Canton of Bern (Switzerland). Male Sprague–Dawley rats (8–11 weeks, 300 ± 50 g) housed under 12-h light/dark cycles (light on 08:00–20:00) at an ambient temperature of 22.0 ± 0.5°C were kept in individual cages. Food and water were provided ad libitum, except for 12-h intervals prior to behavioral tests, in which animals were food deprived. All efforts were made to minimize animal suffering and the number of animals used according to the principles of the 3Rs.9 After a preanalysis of a set of five animals per group, which exhibited statistically significant differences in pellet reaching between groups, further animals were not included in this study.

**Experimental design and drug administration**

Rats were randomly assigned to three experimental groups who was subjected to MCAO and then treated from 3 to 31 days poststroke with vehicle (99.5% aqoat, 0.5% magnesium, suspended in 2% hydroxyethyl cellulose) or S44819 (3 or 10 mg/kg; in 30% aqoat milled extrudate, 69.5% aqoat, 0.5% magnesium, suspended in 2% hydroxyethyl cellulose) at two different dosages (3 or 10 mg/kg body weight; Figure 1(a)). Treatments were administered twice daily at 8 AM and 8 PM by oral gavage. After 2 weeks of washout period (day 45), animals were euthanized. The time window when rats were euthanized was from 9:00 AM to 12:00 AM after the last SPR test. The brains were extracted and immediately frozen using dry ice to assess the infarct volume.

**Induction of focal cerebral ischemia**

Rats were subjected to focal cerebral ischemia induced by the three-vessel occlusion method (3Vo),8,10,11 which predominantly affects the somatosensory cortex. 3Vo combines the permanent occlusion of the distal middle cerebral artery (MCA) by electrocoagulation with the permanent occlusion of the ipsilateral common carotid artery by electrocoagulation and the transient occlusion of the contralateral common carotid artery over 60 min with an aneurysm clip. For stroke induction, rats were anesthetized with 2% isoflurane (in 30% O2). A small piece of skull overlying the MCA was removed and the dura mater was retracted. The MCA and its three main branches were occluded. Stroke was induced in the hemisphere contralateral to the preferred forelimb assessed by the SPR test. Body temperature was maintained between 36.5 ± 0.5°C by a heating pad during the surgery.

**Evaluation of body weight and infarct volume**

Body weight was measured at 3, 10, 17, 24, 31, 38, and 45 days post-MCAO. At day 45 after ischemic surgery, rats were decapitated in deep isoflurane anesthesia. Brains were dissected and frozen immediately on dry ice. Coronal 20 μm sections were cut on a cryostat at six predefined levels (L) with 1 mm interval (L-1: 2.7 mm; L-2: 1.7 mm; L-3: 0.7 mm; L-4: −0.3 mm; L-5: −1.3 mm, and L-6: −2.3 mm from bregma)8,10 (Figure 1(b)). One section from each level was stained with cresyl violet and digitized. To determine infarct area, the healthy tissue of both hemispheres was outlined and tissue areas determined in the lesioned hemisphere subtracted from those in the contralateral hemisphere, as previously described.8,10

**SPR test**

The SPR test was used to assess fine forelimb motor skills. Rats had to use their preferred paw to retrieve a food pellet (45 mg dustless precision pellet; Bio-Serv, Frenchtown, New Jersey, USA) located on a shelf positioned outside the test chamber. The animals were placed in a clear plexiglas box (41 × 27 × 37 cm) with a vertical slit (1 × 15 cm) in the middle of the front wall, 1 cm above the floor. A 2-cm-wide shelf with small wells was mounted at the front of the slit outside the box wall, on which a pellet was placed on the side contralateral to the preferred paw. To establish a
baseline measure of motor performance, rats received daily training sessions for 3–4 weeks on this test. Training sessions comprised the reaching of 50 pellets and the session ended when they made 50 attempts or when 15 min elapsed. Reaches were only considered successful if the rats reached the pellets with the preferred paw, grasped and retrieved the seed, and fed them into their mouth. Performance was defined by: percent success = (number of successful retrievals/number of presented pellets (up to 50)) × 100. The baseline performance was calculated as the average of the 3 days immediately preceding surgery. Animals that failed to reach a baseline level of accuracy of 40% were not exposed to MCAO. Post-MCAO, the test was performed at 3 days prior to S44819 treatment onset and at weekly intervals thereafter (i.e. at 0, 7, 14, 21, 28, 35 and 42 days posttreatment onset). The green arrows indicate days in which single pellets removal test was performed. (b) Representative sets of cresyl violet-stained brain sections from a rat subjected to permanent MCAO. The health tissues delineated by a thin cyan-colored line. (c) Body weight, (d) SPR performance, and (e) infarct area in rats exposed to permanent distal MCAO treated with vehicle or S44819 (3 or 10 mg/kg) from 3 to 31 days post-MCAO. Data are shown as means ± SD. *p < 0.05/ **p < 0.01 compared with vehicle. Dots represent infarct volume of each animal during each time points. GABA: y-amino-butyric acid; SPR: single pellet reaching; MCAO: middle cerebral artery occlusion; SD: standard deviation.

**Statistical analyses**

Data were presented as mean ± standard deviation. Effects of S44819 on infarct size were evaluated by one-way analysis of variance (ANOVA). Effects of S44819 on changes in body weight and motor performance in the SPR task were evaluated by two-way ANOVA with repeated
measures on both factors (groups \times days). Whenever statistical significance was achieved, post hoc Tukey’s tests were performed. GraphPad Prism6 (GraphPad Prism Software Inc., San Diego, California, USA) was used for statistical analysis. \( p \) Values were set at 0.05 to indicate statistical significance.

Results

S44819, administered at 3 or 10 mg/kg, did not influence body weight (Figure 1(d)) but enhanced the recovery of fine motor skills evaluated by the SPR test (effect of treatment group effect: \( F(2,96) = 22.43, p < 0.001 \); Figure 1(e)). Significant improvements of pellet reaching were noted already 1 week after S44819 treatment onset (i.e. at 10 days post-MCAO). Improvements persisted after the discontinuation of S44819 treatment. Representative videos of vehicle-treated and S44819-treated rats are shown in the Supplementary Materials section. Infarct area did not differ between groups (Figure 1(c)).

Discussion

By exposing rats to permanent distal MCAO, we herein show that postacute delivery of the GABA\(_A\) antagonist S44819, which reverses peri-infarct tonic inhibition,\(^5\) promotes the recovery of skilled forelimb movements evaluated by the SPR test, when administered at 10 mg/kg but not 3 mg/kg starting at 3 days post-MCAO for 28 days. Overall, our results suggest that the administration of S44819 positively influences functional outcome, although it should be noted that no neuroplasticity or tonic inhibition, previously described to be induced by S44819, has been tested in this study. Our results extend data from a recent mouse study, in which S44819 was administered at the same dosage during the same time window in mice exposed to transient proximal MCAO.\(^6\) In this study, improvement of motor coordination recovery and cognitive performance was evaluated by Rotarod, tight rope, and Barnes maze tests. Unlike our current study, the recovery of fine movements was not evaluated. In transient proximal MCAO, ischemic injury predominantly affects the striatum and overlying parietal cortex, whereas specifically the motor cortex is spared. As such, the motor deficits are primarily related to subcortical brain injury, which affects the basal ganglia and descending pyramidal tract axons, as well as to injury to sensory and association cortices. Since the recovery of fine movements is critical for clinical outcome in stroke patients, we have now complemented this earlier study\(^6\) with the present study of a permanent distal MCAO model in rats. The combined evidence of both studies suggests that the antagonism of GABAergic tonic inhibition promotes motor recovery both under conditions directly affecting motor cortex and conditions affecting sensory and association cortices.

Following transient proximal MCAO, the promotion of motor coordination recovery by S44819 went along with enhanced structural brain remodeling, that is, reduced secondary neurodegeneration, reduced astrogliosis, and reduced brain atrophy after 45 days survival.\(^6\) By means of infarct planimetry, we have been unable in the present study to detect structural tissue preservation effects that translate into reduced infarct area. Differences in the ischemia model (permanent vs. transient MCAO) may explain the different observations between the previous and present study. Unlike the earlier study, we did not perform in-depth histochemical analyses of brain parenchymal remodeling. The present study may also have been underpowered to detect any modest effects of S44819 on infarct size. Yet, the expected mode of action of S44819, the promotion of peri-infarct neuroplasticity, is independent of structural survival-promoting effects. The main goal of this study was the analysis of skilled forelimb movements, which can be assessed more reliably in rats than mice. With the detection of recovery-promoting effects in permanent distal MCAO in rats and transient proximal MCAO in mice, the two studies fulfill two key requirements of preclinical research, that is, the evaluation of drug effects in at least two species and two-stroke models.\(^13\) Although the results of the current study replicate and strengthen data from the previous mouse study,\(^6\) an important limitation of this study is its small sample size. Highly significant differences between groups were noted in this study supporting the efficacy of S44819 in promoting neurological recovery in the postacute stroke phase.

However, despite the positive results of our preclinical study,\(^14\) the findings of the RESTORE BRAIN study (Randomized Efficacy and Safety Trial of Oral GABA\(_A\) \(\alpha_5\) antagonist S44819 after Recent ischemic Event) have been recently published that did not confirm a positive effect of S44819 on clinical outcomes in patients after ischemic stroke, suggesting that S44819 cannot be recommended for stroke therapy. In light of the findings of this clinical study, the results of our preclinical study assume a relevant role in determining the variables that should be considered to stratify patients and variables in a stroke recovery trial. Indeed, the preclinical study may help clinicians to choose endpoint biomarkers or predictors (serum, genetic, imaging, and neuropsychologic) that may be useful to stratify patients (i.e. age, infarct size and location, and clinical domains outcome), since heterogeneity remains a major challenge for stroke clinical research.

Overall, this preclinical study shows positive results of S44819 in young ischemic rats, in which the stroke lesion is strictly confined in the sensory-motor cortex avoiding subcortical regions and where a single domain points targeting was considered (motor function), and these criteria should be considered for distinguishing stroke subpopulations in relation to poststroke recovery.
Declaration of conflicting interests
The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Dr Machado is an employee of Servier. Drs Bassetti and Hermann received advisory board honoraria and travel expense refunding from Servier.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Institut de Recherches Internationales Servier, Suresnes, France.

ORCID iD
Marta Pace https://orcid.org/0000-0003-1637-4544

References
1. Belelli D, Harrison NL, Maguire J, et al. Extrasynaptic GABAA receptors: form, pharmacology, and function. J Neurosci 2009; 29(41): 12757–12763.
2. Hines RM, Davies PA, Moss SJ, et al. Functional regulation of GABAA receptors in nervous system pathologies. Curr Opin Neurobiol 2012; 22(3): 552–558.
3. Clarkson AN, Huang BS, Macisaac SE, et al. Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. Nature 2010; 468(7321): 305–309.
4. Carmichael ST. Brain excitability in stroke: the yin and yang of stroke progression. Arch Neurol 2012; 69(2): 161–167.
5. Etherington LA, Mihalik B, Palvolgyi A, et al. Selective inhibition of extra-synaptic alpha5-GABAA receptors by S44819, a new therapeutic agent. Neuropharmacology 2017; 125: 353–364.
6. Wang YC, Dzyubenko E, Sanchez-Mendoza EH, et al. Post-acute delivery of GABAA alpha5 antagonist promotes post-ischemic neurological recovery and peri-infarct brain remodeling. Stroke 2018; 49(10): 2495–2503.
7. Hermann DM, Popa-Wagner A, Kleinschnitz C, et al. Animal models of ischemic stroke and their impact on drug discovery. Expert Opin Drug Discov 2019; 14: 315–326.
8. Pace M, Adamantidis A, Facchin L, et al. Role of REM sleep, melanin concentrating hormone and orexin/hypocretin systems in the sleep deprivation pre-ischemia. PloS one 2017; 12(1): e0168430.
9. Tornqvist E, Annas A, Granath B, et al. Strategic focus on 3 R principles reveals major reductions in the use of animals in pharmaceutical toxicity testing. PloS one 2014; 9(7): e101638.
10. Pace M, Camilo MR, Seiler A, et al. Rapid eye movements sleep as a predictor of functional outcome after stroke: a translational study. Sleep 2018; 41(10).
11. Pace M, Baracchi F, Gao B, et al. Identification of sleep-modulated pathways involved in neuroprotection from stroke. Sleep 2015; 38(11): 1707–1718.
12. Chen CC, Gilmore A and Zuo Y. Study motor skill learning by single-pellet reaching tasks in mice. J Vis Exp 2014; (85): 51238.
13. Stroke Therapy Academic Industry R. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999; 30(12): 2752–2758.
14. Chabriat H, Bassetti CL, Marx U, et al. Safety and efficacy of GABAA alpha5 antagonist S44819 in patients with ischaemic stroke: a multicentre, double-blind, randomised, placebo-controlled trial. Lancet Neurol 2020; 19(3): 226–233.