

**Post-acute delivery of NMDA or GABA_A α 5 receptor
antagonists promotes neurological recovery and peri-
infarct brain remodeling after transient focal cerebral
ischemia in mice**

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I. LIST OF ABBREVIATIONS

ACA	Anterior cerebral artery
ABC	Avidin-biotin-peroxidase complex
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Na ⁺ /K ⁺ ATPase	Sodium-potassium adenosine triphosphatase
BA	Basilar artery
BBB	Blood-brain barrier
BCLXL	B-cell lymphoma-extra large
BMSC	Bone marrow-derived stem cell
BDA	Biotinylated dextran amine
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BGT-1	Betaine-GABA transporter 1
CBF	Cerebral blood flow
CD 31+	Cluster of differentiation 31
CNS	Central nervous system
CST	Corticospinal tract
DAB	Diaminobenzidine
DAPI	4'-6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DLL4	Notch ligand delta-like 4
DSC	Dental stem cell
DNA	Deoxyribonucleic acid
DPT	Days post treatment
EAAT	Excitatory amino acid transporter
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

Epo	Erythropoietin
ENSC	Endogenous neuronal stem cell
ESC	Embryonic stem cell
FDA	Food and Drug Administration
FGF	Fibroblast growth factors
FGF2	Fibroblast growth factor 2
GABA	Gamma-amino-butyric acid
GABAR	Gamma-amino-butyric acid receptor
GAT1	GABA transporter protein 1
GAT2	GABA transporter protein 2
GAT3	GABA transporter protein 3
GAT4	GABA transporter protein 4
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
G-CSF	Granulocyte colony-stimulating factor
GLT-1	Glutamate transport 1
GluN1	Glutamate ionotropic receptor NMDA type subunit 1
GluN2A	Glutamate ionotropic receptor NMDA type subunit 2A
GluN2B	Glutamate ionotropic receptor NMDA type subunit 2B
GluN3A	Glutamate ionotropic receptor NMDA type subunit 3A
GluN3B	Glutamate ionotropic receptor NMDA type subunit 3B
G-CSF	Granulocyte-colony stimulating factor
GPI	Glycosylphosphatidylinositol
HMG-CoA	Hydroxymethylglutaryl-CoA reductase
HRP	Horseradish peroxidase
Iba-1	Ionized calcium binding adaptor protein
IGF-1	Insulin growth factor 1
ICA	Internal carotid arteries
ICV	Intracerebroventricular
IGF-1	Insulin growth factor 1

IL-1	Interleukin 1
IQR	Interquartile range
I.P.	Intraperitoneal
IPSC	Inhibitory postsynaptic currents
LDF	Laser Doppler flow
LSD	Least significant differences
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
tMCAO	Transient middle cerebral occlusion
MIT	Mitochondria
MMP	Matrix metalloprotease
MMP9	Matrix metalloprotease 9
MSC	Mesenchymal stem cell
NAD ⁺	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
NeuN	Neuron-specific nuclear protein
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
N ₂ O	Nitrous oxide
NO	Nitric oxide
NOS	Nitric oxide synthase
NP40	Nonylphenoxypolyethoxyethanol 40
NPC	Neural precursor cells
NSC	Neuronal stem cells
O ₂	Oxygen
PCA	Posterior cerebral artery
PARP	Poly-(ADP-ribose) polymerase
ONOO-	Peroxynitrite
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline tween 20
PFA	Paraformaldehyde

PVDF	Polyvinyl difluoride
PSD95	Post synaptic density protein 95
ROI	Region of interest
ROS	Reactive oxygen species
rt-PA	Recombinant tissue-plasminogen activator
S.D.	Standard deviation
SDF-1	Stromal derived factor 1
Stat3	Signal transducer and activator of transcription 3
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SVZ	Subventricular zone
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Triton
TGF- β	Transforming growth factor beta
TrkB	Tropomyosin receptor kinase B
TNF- α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
VA	Vertebral arteries
VEGF	Vascular endothelial growth factor
VGLU	Vesicular glutamate transporter
VSMC	Vascular smooth muscle cell
UCBC	Umbilical cord blood cell

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IV. ABSTRACT

Ischemic stroke is the leading cause of adult disability, but drug therapies that enhance recovery remain lacking. Currently, approximately 15 million people experience a new or recurrent stroke (ischemic or hemorrhagic) worldwide yearly. Untreated ischemic stroke causes death and long-term disability in more than 20% and 70% of patients, respectively; thus, there is a compelling need for novel therapies that promote neurological recovery, brain remodeling and brain plasticity after stroke. The aim of our two studies was to investigate the effects of the delayed delivery of the N-methyl-D-aspartate (NMDA) receptor antagonist memantine and the γ -amino-butyric acid (GABA_A) α 5 receptor antagonist S44819 on functional neurological recovery and potential mechanisms in ischemic stroke. Therefore, we used an experimental paradigm that combines behavioral analyses in mice exposed to intraluminal transient middle cerebral artery occlusion (tMCAO) and histochemical analyses of brain remodeling and plasticity; we have previously utilized this paradigm to evaluate experimental treatments, namely, the delivery of growth factors and cell-based therapeutics. By administering two clinically applicable pharmacological compounds, i.e., memantine and S44819, we aimed to test whether our developed protocols allow for the characterization of the therapeutic effects in a more advanced translational setting. Specifically, we sought to determine whether and how the restorative effects of these two compounds differ from the effects of biological agents.

The first study investigated the NMDA receptor antagonist memantine, which is widely used in human patients for the treatment of Alzheimer's disease and preferentially blocks extrasynaptic NMDA receptors, which are overactivated upon stroke and thought to disturb neuroplasticity. We hypothesized that memantine enhances post-ischemic neurological recovery and brain plasticity. C57BL6/j mice were exposed to tMCAO. Starting 72 hours post-stroke, vehicle or memantine (4 or 20 mg/kg/day) was subcutaneously delivered for 28 days. Neurological recovery, perilesional tissue remodeling and contralesional pyramidal tract plasticity were evaluated for 49 days. Memantine (20 but not 4 mg/kg/day) persistently improved motor coordination and spatial memory. Memantine reduced secondary striatal atrophy. This delayed neuroprotection was associated with reduced astrogliosis and increased capillary formation around the infarct rim. The concentrations of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF) were bilaterally elevated by memantine

in the striatum and cortex. The anterograde tract tracing studies revealed that memantine increased contralesional corticorubral sprouting across the midline in the direction of the ipsilesional red nucleus. In the contralesional motor cortex, the NMDA receptor subunit GluN2B, which is predominantly expressed in extrasynaptic NMDA receptors, was transiently reduced by memantine after 14 days, whereas GluN2A and post synaptic density protein 95 (PSD-95), which preferentially co-localize with synaptic NMDA receptors, were increased after 28 days. Our data support the utility of NMDA antagonists far beyond the acute stroke phase, suggesting that memantine is an attractive candidate for stroke treatment.

The second study investigated whether S44819, which is a GABA_A α 5 receptor antagonist, promotes brain remodeling and neurological recovery during the post-acute stroke phase. GABA, which is the predominant inhibitory neurotransmitter in the adult mammalian central nervous system (CNS), plays a critical role in post-stroke neurological recovery. The tonic inhibition induced by GABA is increased in the peri-infarct cortex, resulting in cerebral hypo-excitability that compromises brain plasticity. In this study, we hypothesized that the blockade of the GABA_A receptor using the selective GABA_A α 5 antagonist S44819 promotes brain remodeling and plasticity during the post-acute stroke phase and enhances neurological recovery. Male C57Bl6/j mice were exposed to intraluminal tMCAO. Starting at 72 hours post-stroke, vehicle or S44819 (3 or 10 mg/kg b.i.d.) was delivered p.o. for 28 days. Neurological recovery and perilesional tissue remodeling were evaluated for 42 days, i.e., up to 14 days after the completion of the S44819 delivery. S44819 (10 but not 3 mg/kg) persistently improved motor coordination and spatial memory. Secondary striatal atrophy was reduced by S44819 (10 mg/kg) 42 days post treatment (dpt), and neuronal long-term survival in the peri-infarct striatum was increased. The delayed neuroprotection was associated with reduced astrogliosis around the infarct rim 14, 28 and 42 dpt and increased brain capillary density 42 dpt. The concentrations of neurotrophic (BDNF and GDNF) and angiogenic (VEGF and fibroblast growth factors; FGF) growth factors were elevated by S44819 (10 mg/kg) in the peri-infarct, but notably not the contralesional, brain tissue 28 and 42 dpt. The GABA_A α 5 abundance was increased by S44819 (10 mg/kg) in the peri-infarct brain tissue 14 dpt. Contralesional pyramidal tract plasticity was not increased by S44819. Our data provide strong evidence supporting that S44819 enhances neurological recovery and perilesional brain remodeling during the post-acute stroke phase.

The present study demonstrates the utility of our experimental paradigms in detecting the restorative effects of clinically applicable drugs. Notably, the GABA_A α 5 antagonist S44819 differed from the NMDA antagonist memantine because S44819, which reversed peri-infarct tonic inhibition, exerted its restorative actions only in the ischemic brain hemisphere, while memantine additionally elevated the growth factor levels and stimulated pyramidal tract sprouting in the contralesional hemisphere. Based on our observations, a controlled randomized phase II study, i.e., RESTORE Brain (<https://clinicaltrials.gov/ct2/show/NCT02877615>), is currently ongoing in human stroke patients on five continents. Similar to this experimental study, the clinical study aims to evaluate the effect of S44819 delivery during the post-acute stroke phase, i.e., starting 3 days post-stroke, on functional neurological recovery as evaluated by the modified Rankin scale.

1. INTRODUCTION

1.1. Pathophysiology of ischemic stroke

The World Health Organization (WHO) defines stroke as “rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin” (WHO, 1988). Currently, approximately 15 million people worldwide suffer from stroke; among these individuals, 6 million people die, and 5 million people are permanently disabled (Mackay and Mensah, 2004). Stroke is the leading cause of adult disability and the second leading cause of death in developed countries (Mozaffarian et al., 2013). However, only tissue plasminogen activator (tPA) is approved by the Food and Drug Administration (FDA) for the treatment of acute ischemic stroke and is used to reduce brain damage and improve the chances of recovery from stroke within 4.5 hours after stroke onset. This therapy is limited by a narrow time window, and only 3% to 8.5% of stroke victims reach the hospital in time to receive tPA treatment (Bambauer et al., 2006; Weintraub, 2006). In late 2014, the results of the Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands (MR CLEAN) trial showed that endovascular mechanical recanalization was effective and safe among patients with ischemic stroke caused by proximal intracranial arterial occlusion, who were treated with endovascular thrombectomy 6 hours after acute ischemic stroke (Berkhemer et al., 2015). The milestone was followed by five more studies which indicated positive results (Goyal., 2015; Campbell BC., 2015; Jovin et al., 2015; Saver et al., 2015; Bracard et al., 2015). Mechanical thrombectomy can be effective in selective patients with occlusion of proximal vessels in the anterior circulation up to 24 h after suspected onset of symptoms (Nogueira et al., 2018; Albers et al., 2018). In order to enhance the neurological recovery after stroke, there is a compelling need to develop novel treatments that promote neurological and functional recovery after ischemic stroke.

1.1.1 Anatomy of mice cerebral arteries

In the CNS, blood is supplied by the internal carotid arteries (ICA) and vertebral arteries (VA); the ICA supplies blood to most of the cerebrum, while the VA supplies blood to the cerebellum,

brainstem, and most of the cerebrum (Alpers et al. 1959; Gillilan, 1972; Lee, 1995) (**Fig. 1**). The left and right VA pass through the skull and then join together to form the basilar artery (BA), which is linked to the ICA in an arterial ring to form the Circle of Willis. The other arteries, including the anterior cerebral artery (ACA), the middle cerebral artery (MCA) and the posterior cerebral artery (PCA), originate from this circle (**Fig. 1**). The ACA supplies blood to most medial portions of the frontal lobes and superior medial parietal lobes. The MCA trifurcates into temporal, frontal, and parietal branches that supply blood to most of the parenchyma of these lobes. The PCA supplies blood to the occipital lobe and the inferior portion of the temporal lobe (Zeman et al., 1963; Lee, 1995) (**Fig. 1**). The communication between the internal carotid and vertebral-basilar systems is an important safety feature in the brain (Alpers et al., 1959; Gillilan, 1972); if one of the major vessels is blocked, it is possible for collateral blood flow to move across the Circle of Willis and prevent brain damage. Since most ischemic strokes (~80%) occur in the territory of the middle cerebral artery, our animal stroke models focus on this MCA territory, including the primary motor and sensory areas of the face, hand and arm (Mohr, 2004).

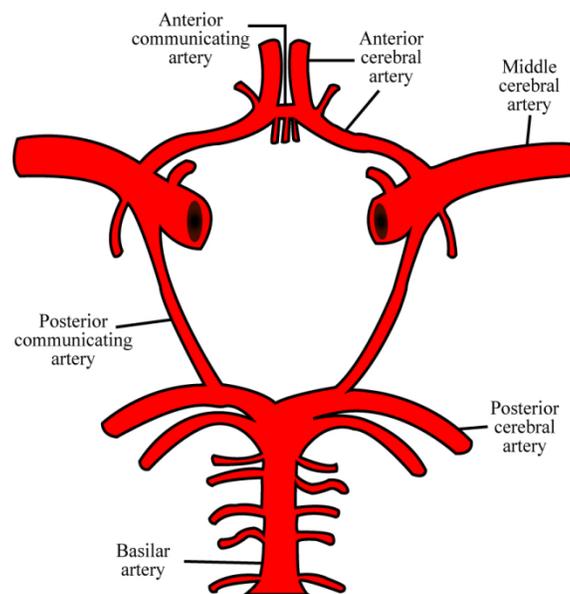


Figure 1. Anatomy of the mice cerebral arteries. Six cerebral arteries and interactions within the Circle of Willis are illustrated.

1.1.2 Concepts of ischemic core and penumbra

At stroke onset, blood flow is blocked by occlusion of a vessel. This blockade limits the delivery of oxygen and glucose, causing adenosine 5-triphosphate (ATP) reduction and energy depletion. The brain regions that suffer the most severe blood flow reduction and experience irreversible damage constitute the ischemic core. In the ischemic core, the neurons die within minutes because of the energy depletion, disruption of ion homeostasis, lipolysis, proteolysis, and cell fragmentation (Martin et al., 1994; Hossmann, 1994) (**Fig. 2**). The ischemic penumbra refers to a region of brain tissue that suffers moderate cerebral blood flow (CBF) reduction and brain function impairment, but the structure remains metabolically active and electrically silent (Ginsberg, 2003), (Astrup et al, 1977; 1981) (**Fig. 2**).

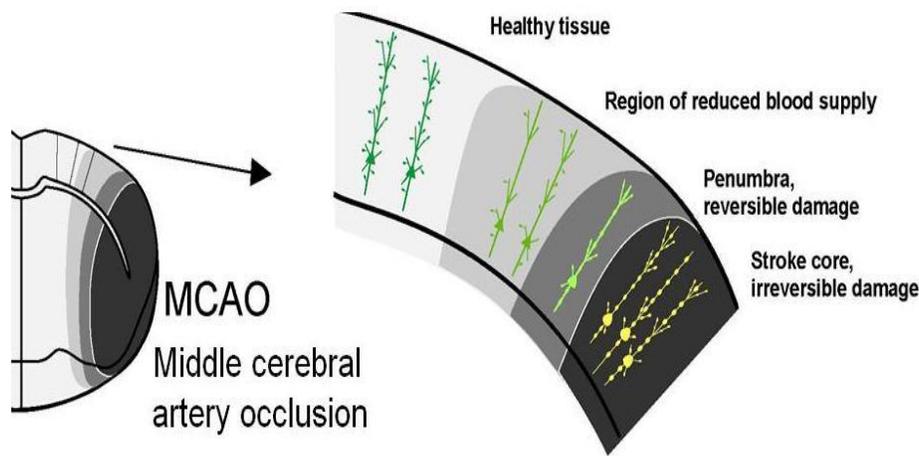


Figure 2. Ischemic core and penumbra (modified from Li P and Murphy TH, 2008).

- ☐ : Damaged structure that can recover during reperfusion
- ☐ : Reduced blood supply but no structural damage during tMCAO or reperfusion
- ☐ : Damaged structure without recovery during reperfusion.

1.1.3 Factors contributing to ischemic injury

Under physiological conditions, the normal average CBF in adult humans is about 50 ml/100g/min, with lower values in the white matter of 20 ml/100 g/min and greater values in the gray matter of 80 ml/100 g/min (Lassen., 1985; Vavilala et al., 2002). However, under ischemic conditions, there is limited or no blood and oxygen supply to the brain, and cerebral auto-regulatory mechanisms have to compensate for the reduction in CBF by local vasodilatation, opening of the collaterals, and extraction of oxygen and glucose from the blood (Astrup et al., 1981; Wise et al., 1983). If cerebral circulation is completely blocked, neurological electrical

activity ceases within seconds, and the energy state and ion homeostasis are disrupted within a few minutes. The balance of Na^+/K^+ transmembrane gradients cannot be maintained by Na^+/K^+ -ATPases, leading to anoxic depolarization and excessive glutamate release (Wise et al., 1983; Heros, 1994). Glutamate, which is immediately uptaken into astrocytes by excitatory amino acid transporters (EAATs) under physiological conditions (Chao et al., 2010), excessively accumulates in the extracellular space. This accumulation, in turn, overactivates amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA glutamate receptors. If this situation is not stopped by blood and oxygen supply, the penumbra surpasses the threshold of irreversible damage and becomes a part of the ischemic core (Weinstein et al., 2004).

During the acute phase of stroke (minutes to hours), reactive oxygen species (ROS) and pro-inflammatory mediators are rapidly released from the injured tissues (Amantea et al., 2009; Kriz, 2006). The ROS further trigger various pro-inflammatory genes and play a crucial role in leukocyte endothelium interactions and secondary post-ischemic brain damage (Pun et al., 2009).

During the subacute (hours to days) phase, leukocytes release ROS and activate matrix metalloproteinase 9 (MMP-9), leading to the extensive activation of infiltration of leukocytes, causing a disruption in the blood-brain barrier (BBB), brain edema and neuronal death (Amantea, et al., 2009; Kriz, 2006). Furthermore, resident microglial cells are swiftly activated after ischemia (Aloisi et al., 2001) and produce interleukin-1 (IL-1) and tumor necrosis factor (TNF) alpha, which exacerbate brain damage (Banati et al., 1993; Barone et al., 1997; Rothwell et al., 1997).

1.2 Role of glutamate and glutamate receptors in ischemic stroke

The excitatory role of glutamate in the mammalian brain and spinal cord has been known since the 1950s (Hayashi, 1952; Curtis and Watkins 1960); furthermore, it has been widely recognized that glutamate is the principal excitatory transmitter within the vertebrate nervous system in the late 1970s (Meldrum, 2000). L-glutamate mediates its effects through two types of glutamate receptors, i.e., ionotropic receptors and metabotropic receptors, which are linked to G proteins found in pre- and post-synaptic neuron membranes in the CNS (Nakanishi, 1992). Ionotropic receptors comprise AMPA and NMDA receptors, and NMDA receptors play a fundamental role in glutamate-mediated excitotoxicity in stroke pathology (Carmichael, 2012; Lai et al., 2011; 2014).

1.2.1 Excitotoxicity induced by glutamate in ischemic stroke

Under physiological conditions, a healthy neuron releases glutamate only as needed to convey a message, and then, glutamate is immediately reabsorbed by EAATs (Chao et al., 2010). Then, glutamate is converted to glutamine and sent back to the presynaptic neuron, where it is reconverted to glutamate before being loaded into synaptic vesicles by Vesicular Glutamate Transporters (VGLUTs) and released as a neurotransmitter. This process is known as the glutamate-glutamine cycle (Shigeri Y et al, 2004; Bak et al., 2006). Under pathological conditions, such as stroke, head trauma, and some other neurological disorders, anoxic depolarization ultimately disrupts the glutamate-glutamine cycle, causing excessive glutamate accumulation in the area of the original damage (Lynch et al., 2002). This accumulation leads to Ca^{2+} influx-mediated excitotoxicity (Arundine et al., 2003; Szydłowska et al., 2010). Ca^{2+} entry via voltage-sensitive channels leads to long-lasting membrane depolarization, synaptic vesicle fusion, the removal of the voltage-dependent magnesium block on the NMDA receptor and, thus, sustained receptor activation (Chizh, 2002). While postsynaptic NMDA-mediated Ca^{2+} entry is a fundamental step in synaptic plasticity (Lau and Zukin, 2007), excessive Ca^{2+} entry can promote further Ca^{2+} release from the endoplasmic reticulum, triggering many neurotoxic cascades, inducing the activation of proteases, lipases, phosphatases and endonucleases (Szydłowska and Tymianski, 2010). Calcium-dependent endonucleases trigger DNA degradation (**Fig. 3**), which activates Poly ADP-ribose polymerase (PARP), leading to the depletion of cellular β -nicotinamide adenine dinucleotide (NAD^+) and initiation of necrosis (Rossi et al., 2000; Schaller et al., 2004). The cytoskeleton is fragmented by calcium-sensitive proteases (e.g., calpain), which leads to the production of arachidonic acids, subsequent free radical, and nitric oxide (NO) (**Fig. 3**). Additionally, the mitochondrial calcium overload induces the release of mitochondrial proapoptotic factors, such as cytochrome C, apoptosis inducing factor (AIF), procaspase 9 and smac/Diablo (Lo et al., 2003; Dirnagl et al., 1999; Lipton et al., 1999; Szydłowska et al., 2010; Nicholls, 2009; Joza ., 2001).

1.2.2 Role of NMDAR receptors in ischemic stroke

Although all members of the glutamate receptor family are thought to be involved in excitotoxicity (Pérez-Otaño and Ehlers, 2005), NMDA receptors play a critical role in cell death (Waxman et al., 2005). NMDA receptors form a heterotetramer that consists of two GluNR1 subunits (glutamate ionotropic receptor NMDA type subunit 1) and two GluNR2 (glutamate ionotropic receptor

NMDA type subunit 2A-D) subunits. According to the conventional view, receptor co-activation requires glutamate and glycine (Monyer et al., 1994; Yamakura and Shimoji, 1999). The NR3 (glutamate ionotropic receptor NMDA type subunit 3) subunit was discovered in 1995 (Ciabarra et al., 1995; Sucher et al., 1995), and new evidence confirms that the functional unit of NMDA receptors consists of NR1 and at least one NR2 or NR1 subunit and both NR2 and NR3 (Henson et al., 2010; Low and Wee, 2010) (**Fig. 4**).

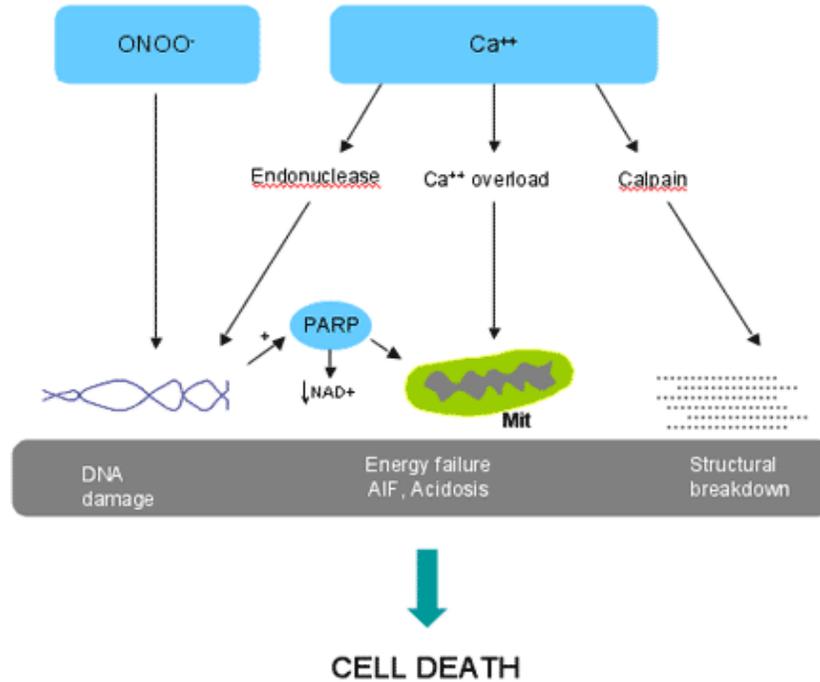


Figure 3. Main effector pathways leading to cell death following the activation of NMDA receptors. Endonucleases are activated by Ca^{2+} and produce DNA damage, which, in turn, activates the DNA repair enzyme PARP. PARP is neurotoxic because it consumes the essential substrate NAD^+ and induces the release of apoptosis inducing factor (AIF) from mitochondria (Mit). Ca^{2+} accumulation in mitochondria results in energy failure and the formation of excess H^+ ions (acidosis). Ca^{2+} -dependent activation of proteases, such as calpain, causes damage to structural proteins (tubulin, spectrin, and focal adhesion kinases), which contribute to the breakdown of the cell (Anrather J and Iadecola C, 2004).

NR1 contains the glutamate binding site, while the NR2 subunit contains the site to which glycine binds (Clements, 1991). NR2A and NR2B play different roles in neuronal survival and neuronal

death after stroke. The NR2A subunit is predominantly located in synaptic glutamate receptors, while extrasynaptic glutamate receptors in the adult brain predominantly contain NR2B (Tu et al., 2010; Martel et al., 2009). Evidence suggests that extrasynaptic glutamate receptors are strongly activated upon stroke, stimulating neuronal death pathways and impeding neuronal plasticity (Bordji et al., 2010; Rush et al., 2014; Wu and Johnson, 2015), while synaptic glutamate receptors have opposite effects in the ischemia brain, including the inhibition of neuronal death pathways and promotion of neuronal plasticity (Lai et al., 2011; 2014). The blockade of NMDARs containing NR2A prevents ischemic tolerance, while the inhibition of NMDARs containing NR2B attenuates neuronal death and induces neuroprotection (Hardingham et al., 2002; Chen et al., 2008) (Fig. 4).

Due to their fundamental role in the initiation of excitotoxic signaling cascades, NMDARs could be excellent targets for therapeutic interventions for stroke. Unfortunately, the clinical translation of NMDA antagonists to human stroke patients has failed (O'Collins et al., 2006; Savitz and Schäbitz, 2008) due to several reasons, including (i) narrow therapeutic time windows of early NMDA antagonists, (ii) cognitive side effects, (iii) underdosing to prevent cognitive side effects and (iv) lack of experimental studies with observation time windows exceeding the acute stroke phase.

Interestingly, in two more recent studies, the delivery of the NMDA receptor competitor inhibitor memantine up to 2 hours post-stroke reduced behavioral deficits without affecting brain injury (Babu and Remanathan, 2009; López-Valdés et al., 2014). Based on these studies, we speculated whether memantine may have restorative effects during the post-acute stroke phase. To address this question, here, we exposed mice to tMCAO, administered different doses of memantine (4 or 20 mg/kg/day) starting 72 hours post-stroke, and evaluated the effects on motor recovery, cognitive performance, peri-infarct tissue remodeling and contralesional pyramidal tract plasticity for 49 days.

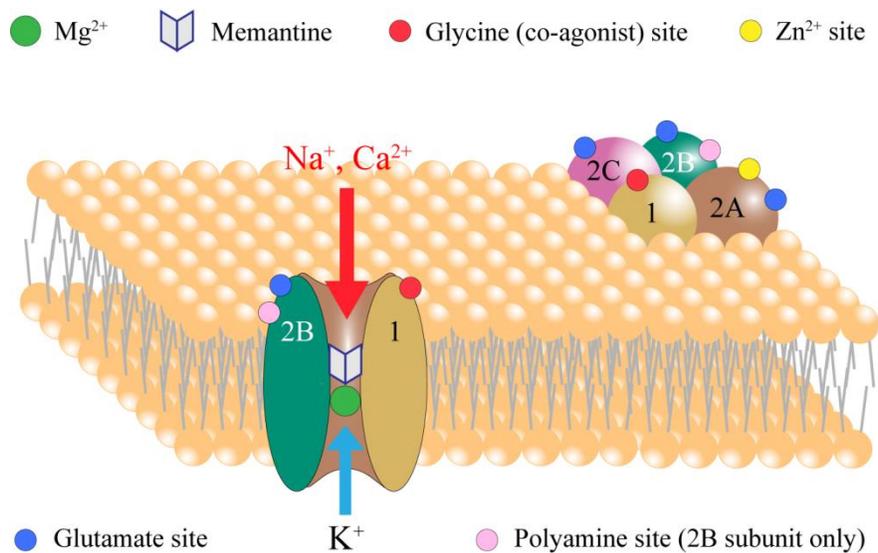


Figure 4. Structure of NMDA receptors. The NMDA receptor forms a heterotetramer among one NR1, one NR2A, one NR2B and one NR2C subunits. Its activation depends on the binding of both glutamate and glycine (modified from Parson CG et al., 1998).

1.3. Role of GABA and GABA_A receptors in ischemic stroke

1.3.1 Tonic inhibition induced by GABA in ischemic stroke

γ -amino-butyric acid (GABA) is the predominant inhibitory neurotransmitter in the adult mammalian CNS (Farrant et al., 2005). GABA mediates its action via distinct receptor systems, including the ionotropic GABA_A and metabotropic GABA_B receptors (Bettler et al., 2004).

Synaptically released GABA activates postsynaptic GABA_A receptors, which allow Cl⁻ influx into the membrane in neurons and trigger fast inhibitory postsynaptic currents (IPSCs), resulting in a reduction in the neural excitability of the brain (Farrant et al., 1994; 2005; Szabadics et al., 2006). GABA_A receptors conventionally mediate most fast synaptic inhibition in the mammalian brain, controlling activity at both the network and cellular levels (Jacob et al., 2008); the tonic inhibition observed in extrasynaptic receptors is also mediated by GABA_A receptors (Clarkson et al., 2010). While GABA_B receptors are indirectly coupled to K⁺ channels, when activated, these receptors can decrease Ca²⁺ conductance and inhibit cAMP production via intracellular mechanisms mediated by G proteins (Luscher et al., 1997). GABA_B receptors can mediate both postsynaptic and presynaptic inhibition. Presynaptic inhibition may occur as a result of GABA_B receptors on

nerve terminals causing a decrease in the influx of Ca^{2+} , thereby reducing the release of neurotransmitters (Padgett & Slesinger, 2010).

GABA receptors are pentameric assemblies that usually consist of three different proteins selected from 19 different genes. There are six α subunits, three β subunits, three γ subunits, three ρ subunits and one ϵ , δ , θ and π subunit (Olsen and Sieghart, 2008; Sigel and Stainmann, 2012; Brickley et al., 2012). The most common subunit combinations are 2α , 2β , and γ , and the γ subunits can be substituted by either ϵ or δ subunits (Bollan et al., 2008; Whiting et al., 2003; Baur et al., 2009; Clarkson, 2012) (**Fig. 6A**). The physiological functions of the receptors and regional and developmental expression patterns are determined by the differences in the subunit gene expression and composition (Hevers and Luddens, 1998; Mody and Pearce, 2004; Brickley and Mody, 2012).

The $\alpha 1-3$, $\beta 1-3$, and $\gamma 1-3$ subunits are located in synaptic GABA_A receptors, while the $\alpha 4-6$, $\beta 1-3$, $\gamma 2$ and δ subunits are located in extrasynaptic GABA_A receptors (Belelli et al., 2009) (**Fig. 6B**). The $\alpha 4\beta\delta$ subunit combination located in the cortex, hippocampus and thalamus and the $\alpha 6\beta\delta$ subunit combination located in the cerebellum play a critical role in GABAergic neurotransmission (Belelli et al., 2009; Clarkson, 2012). The $\alpha 5$ subunit is expressed in the cortex, and $\alpha 5$ -containing GABA_A receptors play a critical role in post-stroke recovery (Clarkson et al., 2010).

The low resting ambient GABA levels present in the extracellular space can activate high-affinity extrasynaptic GABA_A receptors to generate a persistent Cl^- current (and, to a lesser extent, HCO_3^-), which is responsible for generating tonic hyperpolarization and inhibition in cortical neurons (Glykys and Mody, 2006). The GABA levels in the extracellular space are balanced by an interplay between the level of synaptic GABA release and reuptake mediated by membrane GABA transporters, including GAT-2 and BGT-1 in astrocytes, GAT-3 in glia, GAT-1 in axon terminals and vesicular GABA transporter (VGAT), which is involved in its synaptic release. Ultimately, the level of ambient GABA leads to the activation of extrasynaptic GABA_A receptors in the soma/dendrite and even axonal membrane and generates tonic inhibition (Brickley and Mody, 2012) (**Fig. 5**). Tonic inhibition is distinct from the transient activation of synaptic GABA_A receptors, which leads to classical inhibitory postsynaptic currents (phasic inhibition).

Phasic inhibition in postsynaptic neurons is characterized by a fast rising and slow decaying waveform. GABA molecules are released from presynaptic neurons and rapidly diffuse across the synaptic cleft to occupy synaptic GABA_A receptors (Belelli et al., 2009; Brickley and Mody, 2012). Then, the postsynaptic conductance changes, which is reflected by the fast rising and slow decaying waveform, depending on the subunit composition of the synaptic GABA_A receptors and the transmitter profile within the cleft (Brickley and Mody, 2012) (**Fig. 5**).

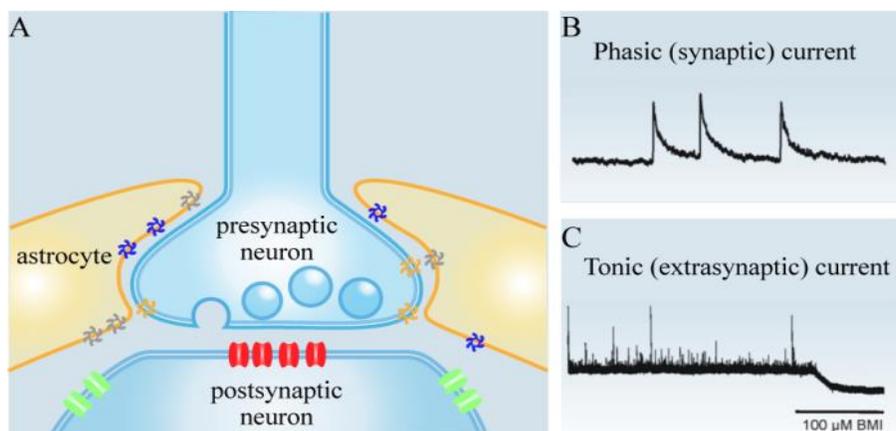


Figure 5. Phasic and tonic GABA_A receptor activation. (A) Synaptic receptors (red) are located on the postsynaptic membrane immediately below the presynaptic release sites, whereas extrasynaptic receptors (light green) are located away from the synaptic junction. The GABA transporter GAT1 (orange pinwheels) is located at presynaptic sites, while the GABA transporters GAT3/4 (blue pinwheels/gray pinwheels) are present on surrounding astrocytes. (B) Phasic (synaptic) inhibitory postsynaptic currents (IPSCs) are rapid events. Three individual IPSCs are shown in a whole-cell voltage-clamp recording obtained from a dentate granule cell. (C) The tonic (extrasynaptic) current in this granule cell is reflected by a baseline shift after the application of the GABA_A receptor antagonist bicuculline methiodide (BMI; 100 μM) (modified from Hunt et al., 2013).

1.3.2. Role of GABA_A receptor in ischemic stroke

GABA accumulates in the extracellular space during transient cerebral ischemia (Phillis et al., 1994; Inglefield et al., 1995). The tonic inhibition mediated by GABA_A receptors, specifically α5-containing GABA_A receptors, plays a critical role in post-stroke recovery (Clarkson et al., 2010).

Although tonic inhibition may protect ischemic tissue from excitotoxic acute brain injury, it impairs neuronal remodeling and plasticity during subsequent stroke recovery (Clarkson et al., 2010, 2012).

In mice exposed to photothrombotic stroke and rats exposed to endothelin microinjection, the deactivation of GABA_A receptors by the inverse GABA_A agonist L655,708 improved functional neurological recovery (Clarkson et al., 2010; Lake et al., 2015). This effect was mediated by the GABA_A α 5 subunit as shown in GABA_A α 5^{-/-} mice, which did not show functional neurological improvements after L655,708 delivery (Clarkson et al., 2010). Increasing evidence indicates that dampening the tonic inhibition induced by the GABA_A receptor can facilitate stroke recovery (Clarkson et al., 2009; 2011; Schmidt et al., 2010; Lake et al., 2015). S44819 is a competitive GABA_A receptor antagonist that preferentially binds the α 5 receptor subunits at their GABA binding site (Darmani et al., 2016). In different rodent models, S44819 enhanced hippocampal long-term potentiation and improved memory when administered at doses between 0.3 and 10 mg/kg body weight (unpublished). In focal cerebral ischemia induced by permanent tMCAO, S44819 reduced the infarct size when administered at doses between 1 and 10 mg/kg body weight (unpublished). S44819 also had no side effects in a phase I dose escalation study involving healthy subjects (unpublished). Here, we exposed mice to transient intraluminal tMCAO, administered S44819 at different doses (3 or 10 mg/kg b.i.d., for 28 days) starting 72 hours post-stroke, and evaluated the effects on motor coordination recovery, cognitive performance and brain remodeling for 42 days.

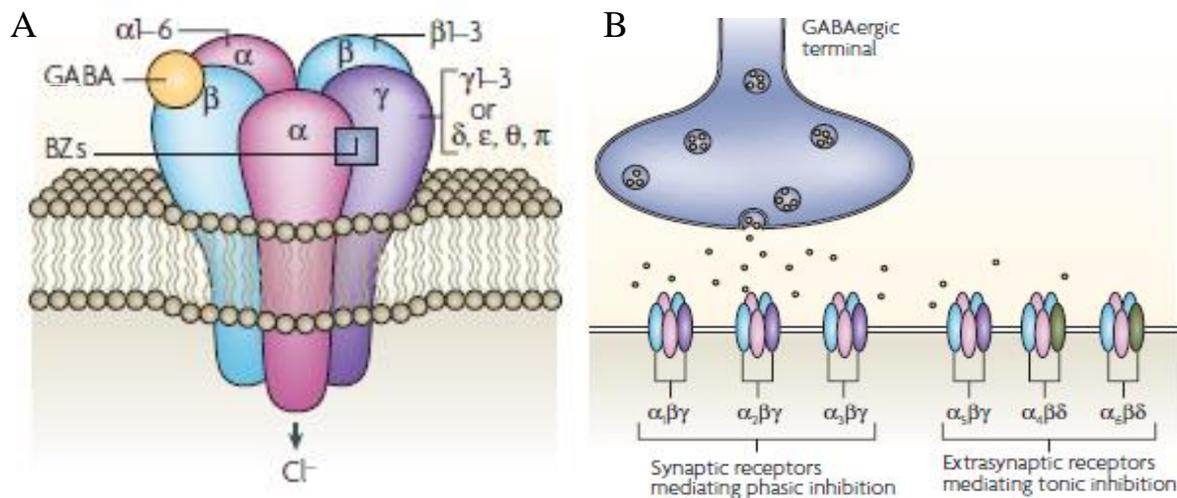


Figure 6. GABA_A receptor structure and neuronal localization. (A) Five subunits from 7 subunit subfamilies (α , β , γ , δ , ϵ , θ , and π) assemble to form a heteropentameric chloride-permeable channel. Despite the extensive heterogeneity of GABA_A receptor subunits, most GABA_A receptors expressed in the brain consist of 2α , 2β , and 1γ subunits, and the γ subunit can be replaced by δ , ϵ or π . The binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, resulting in a rapid influx of chloride ions. BZ binding occurs at the interface between the α (1-3 or 5) and γ subunits and potentiates the GABA-induced chloride flux. (B) GABA_A receptors composed of α (1-3), β and γ subunits are thought to be primarily synaptically localized, whereas $\alpha 5\beta\gamma$ receptors are primarily located at extrasynaptic sites. Receptors composed of the aforementioned subunits are benzodiazepine sensitive. In contrast, receptors composed of $\alpha(4,6)\beta\delta$ are benzodiazepine insensitive and are localized at extrasynaptic sites (modified from Jacob et al., 2008).

1.4 Therapeutic strategies for post-ischemic stroke recovery

1.4.1 Angiogenesis

Angiogenesis is defined as new microvessel formation via the sprouting or division of pre-existing vessels and is mainly responsible for the development of blood vessels after birth. The vascular and nerve systems in the CNS are tightly interwoven and form highly coordinated neurovascular units that consist of endothelial cells (ECs), pericytes, vascular smooth muscle cells (VSMCs), astrocytes, microglia and neurons (Brozzo et al., 2012).

Cerebral angiogenesis is regulated by general angiogenic factors, brain-specific angiogenic factors, mediators and local homeostasis. These factors work together to regulate EC migration, maintain cell identity, and guide cell growth and elongation, and they also align the vessels and nerves (Liu et al., 2014). In the brain and heart, angiogenesis promotion has been correlated with reduced injury in animal models (Banai et al., 1994; Takeshita et al., 1994; Shen et al., 2008; Reitmeir et al., 2012). Angiogenic factors in the brain are expressed by neurons and astrocytes and induce microvascular growth (Forsythe et al. 1996; Marti et al., 2000; Wang et al., 2005; Kilic et al., 2006). Importantly, angiogenesis participates in brain plasticity and functional recovery after stroke (Arai et al., 2009).

Angiogenic (VEGF and FGF) and neurotrophic (BDNF and GDNF) factors play critical roles in stroke recovery. Previous studies in our laboratory have revealed that VEGF and GDNF enhance stroke recovery and brain plasticity (Reitmeir et al., 2011, 2012; Herz et al., 2012; Kilic et al., 2003; 2005). FGF and BDNF are involved in angiogenesis and neurogenesis and are both beneficial for brain plasticity (Chen et al., 2014; Cook et al., 2017).

FGF has been recognized for its ability to stimulate proliferation in endothelial cells (Maciag., et al, 1979). In total, 22 FGF family members have been identified in humans and other vertebrates (Ornitz and Itoh, 2001). FGF2 is expressed ubiquitously in mesodermal and neuroectodermal cells and plays a critical role in proliferation, differentiation and survival of cells in nearly all organ systems (Chen et al., 2004). The downregulation of FGF2 leads to endothelial cell apoptosis, and the deprivation of endogenous FGF2 may lead to dysregulation of activities of other survival- and angiogenesis-related genes (Chen et al., 2004).

FGF2 is significantly upregulated after ischemic stroke in the adult rat brain (Naylor et al., 2005) and patients who died from acute ischemic stroke (Navaratna et al., 2009). Compared with wild type mice, the loss of endogenous FGF2 or the knocking out of FGF2 resulted in a reduction in ischemia-induced progenitor proliferation (Yoshimura et al., 2001). In addition, the overexpression of FGF2 by intracerebroventricular injection or transplantation of FGF2 gene modified MSCs in ischemic rats resulted in enhanced neurogenesis and increased functional recovery (Ikeda et al., 2005). Based on these findings, the endogenous production of FGF2 by endothelial cells may play an important role in post-stroke recovery.

Vascular endothelial growth factor (VEGF) is a critical factor for angiogenesis. VEGF-A is a main component in the VEGF family that stimulates angiogenesis through VEGF receptor-2 (VEGFR-2), which is located at high concentrations at the tip of newly formed capillaries (Nagy et al., 2007, Ferrara, 2009). There are two types of VEGF, i.e., the soluble and matrix bound isoforms of VEGF. Soluble VEGF mainly promotes vessel enlargement, while the matrix bound isoforms stimulate more branching (Carmeliet, 2003). The release of soluble VEGF leads to the upregulation of the expression of the notch ligand delta-like 4 (DLL4), and DLL4 leads to the subsequent activation of NOTCH signaling in adjacent stalk cells and downregulation of VEGFR-2 expression, preventing the uncontrolled sprouting of brain capillaries (Phng et al., 2009) (**Fig. 7**). In human stroke patients, post mortem analyses of brain tissues have revealed that angiogenic activity and angiogenesis seemed more developed in the penumbra (Font et al., 2010). There was a correlation between the numbers of new vessels in the ischemic penumbral regions and prolonged survival, suggesting that activated angiogenesis could be beneficial for the ischemic brain (Liu et al., 2014).

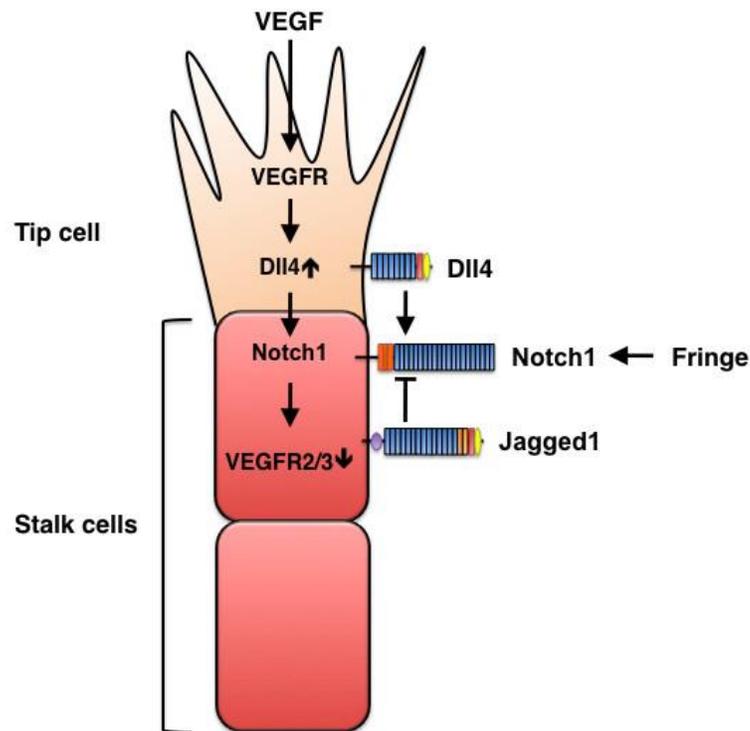


Figure 7. VEGF activates the VEGFR-2 signaling pathway to stimulate angiogenesis. DLL4 binds Notch to negatively regulate sprouting and branching during tumor angiogenesis, resulting

in a functional vascular network. Anti-VEGF treatment inhibits angiogenesis and suppresses tumor growth. In contrast, DLL4 blockade stimulates nonproductive tumor vascularization, resulting in the inhibition of tumor growth. Conversely, Jagged1 antagonizes DLL4-mediated NOTCH activation in stalk cells to increase tip cell numbers and enhance vessel sprouting. The antagonistic effects of the two ligands are controlled by Fringe (from Kume, 2009).

Angiogenesis and tissue remodeling play a critical role in the recovery following stroke, especially in penumbra regions. The partially depolarized tissue adjacent to the core infarct continues to reduce the cerebral blood flow and increase the oxygen extraction rate to fight for survival during a limited time-window. Most recovery after acute ischemic stroke is based on angiogenesis. The stimulation of angiogenesis promotes neurogenesis, neuronal survival, vascular remodeling and final recovery (**Fig. 7**). Indeed, EC proliferation begins as early as 12–24 hours after acute ischemic stroke and continues during the following few weeks (Marti et al., 2000; Hayashi et al., 2003). Meanwhile, microvascular ECs secrete growth factors, which, in turn, support the survival of the newly formed neurons.

VEGF increases the brain capillary density and enhances post-ischemic angiogenesis and neurogenesis (Zechariah et al., 2013; Ma et al., 2012; Crafts et al., 2015; Dzierko et al., 2013; Greenberg et al., 2013). A further understanding of the role of these factors could aid the development of new therapeutics for the treatment of ischemic stroke.

1.4.2 Neurogenesis

Stroke signals stem cell populations in the adult brain to divide and send immature neurons to areas of damage via a process named post-stroke neurogenesis (Carmichael et al., 2008). Mammalian adult neurogenesis occurs in germinal niches in the subventricular zone (SVZ) of the lateral ventricle and the subgranular layer of the dentate gyrus. Neurogenesis is stimulated in response to stroke and is substantially amplified by therapeutic interventions that promote functional recovery (Zhang et al., 2004; 2006; Arvidsson et al., 2002; Chen et al., 2003). Following stroke, the population of neural precursor cells (NPCs) significantly expands in the SVZ. Then, the NPCs are recruited, migrate and differentiate into mature neurons, astrocytes, and oligodendrocytes in the vicinity of the ischemic penumbra (Parent et al., 2002). Growing evidence suggests that cell therapies enhance the proliferation, migration, and differentiation of NPCs and

improve neurological recovery. NPCs enhance neurological recovery and decrease ischemic injury, which could be attributed to effects related to the interaction between neuroblasts and the microvasculature in the vicinity of the ischemic lesion, creating an environment that promotes brain remodeling (Bacigaluppi et al., 2009, 2016; Andres et al., 2011; Thored et al., 2007; Doeppner et al., 2011; Teng et al., 2008).

Ischemic stroke induces coordinated endogenous neurogenesis and angiogenesis, which contribute to brain repair. Among the many neurotrophic factors, BDNF is one of the most widely distributed factors in the CNS and plays a critical role in stroke recovery (Schäbitz., et al. 2007). BDNF acts specifically via a high-affinity cell surface receptor, i.e., tropomyosin receptor kinase B (TRKB), by activating intracellular protein kinase B, mitogen-activated protein kinases, and the extracellular signal-regulated kinases (ERKs). BDNF enhances brain plasticity and repair and influences stroke outcomes in animal models (Schäbitz., et al 2000; Mizuno et al, 2000; Cook et al., 2017). Neurogenesis induced by the intravenous delivery BDNF in vivo has been observed not only in unlesioned healthy animals (Peacea et al, 2001; Zigova et al., 1998; Bernreiss et al., 2001; Scharfman et al, 2005) but also in stroke patients. Intravenous BDNF delivery or hydrogel placement delivered BDNF enhanced neurological recovery post stroke through neurogenesis (Schäbitz., et al. 2007; Cook et al., 2017). The delivery of BDNF promoted SVZ progenitor cell migration to the lesion region and triggered neurogenesis in the SVZ region and hippocampus (Schäbitz., et al. 2007; Cook et al., 2017). The systemic application of BDNF after cortical stroke induces hippocampal neurogenesis and improves functional recovery in several sensorimotor tasks during a 6-week period (Schäbitz., et al. 2007), and another study showed that hydrogel placement delivered BDNF enhanced motor recovery after 3 months of treatment (Cook et al., 2017). These findings confirm that BDNF acts as a modulator of neurogenesis and enhances neurological functional outcomes after cerebral ischemia.

Glial cell line-derived neurotrophic factor (GDNF) was first isolated in dopamine neurons and shown to enhance the survival and morphological differentiation of dopaminergic neurons (Lin et al., 1993). GDNF is activated by the extracellular glycosylphosphatidylinositol (GPI)-linked receptor, GFR α 1, the transmembrane tyrosine kinase and c-Ret (Arvidsson et al., 2001). The GDNF mRNA level and receptor expression were upregulated in penumbral areas, and similar increases in c-Ret and GFR α 1 mRNA have been observed in the striatum after tMCAO (Arvidsson

et al., 2001; Kobayashi et al., 2006). Several findings suggest that the intrastriatal delivery of GDNF enhanced neurogenesis post stroke (Arvidsson et al., 2001; Chen et al., 2005; Kilic et al., 2005, Pahnke et al., 2005). It has been observed that the GFR $\alpha 1$ mRNA levels are upregulated in the ipsilateral SVZ post tMCAO (Arvidsson et al., 2001). The overexpression of GDNF in neural progenitors induces genes involved in migration and differentiation (Pahnke et al., 2005). Intrastriatal GDNF infusions increase cell proliferation in the substantia nigra and hippocampal neurogenesis in the intact brain (Kobayashi et al., 2006; Chen et al., 2005). Finally, our previous work also showed that systemically delivered TAT-GDNF induced neuroprotection in a Bcl-XL-/caspase-3 signaling pathway-dependent manner (Kilic et al., 2005).

In summary, post stroke long term recovery is associated with an increase in angiogenesis and neurogenesis. These processes are inter-independent through the action of pro angio and neurogenic factors released from neurons, glial and endothelial cells. Hence, understanding the mechanisms of release and the effects on their target cells during stroke is critical for an understanding of their role in stroke recovery.

1.4.3 Cortical plasticity

Cortical Plasticity, also known as neuroplasticity, refers to the remarkable ability of the brain to reorganize itself by forming new neural connections based on individual experiences, lifestyle and environment (Livingston RB, 1966; Rakic P, 2002; Kandel ER, 2001; 2004).

Stroke affects both gray and white matter tissue, which invariably results in diverse patterns of brain injury and stroke recovery (Sanchez-Mendoza and Hermann, 2016). Although many patients survive and undergo spontaneous recovery, improving stroke recovery remains challenging because of the limited window for therapeutic interventions following stroke (Murphy and Corbett, 2009).

Parenchymal tissue remodeling plays a crucial role in stroke recovery (Hermann and Chopp, 2012). This remodeling involves (a) the survival of neurons in peri-infarct tissue and whether they re-integrate into the reorganized local neuronal network and the sprouting of neurons distal to the lesion site, (b) the survival of astrocytes surrounding the ischemic lesion and the combined result of their role in the recycling of excitatory neurotransmitters and electrolytes and growth factor

secretion, all of which influence neuronal excitability and enable synaptic plasticity, (c) the outgrowth of axons in the ipsilesional and contralesional hemispheres at various distances from the ischemic lesion and (d) endothelial cell proliferation, which indirectly influences brain homeostasis by promoting proper oxygenation and elimination of brain waste products (Sanchez-Mendoza and Hermann, 2016; Bacigaluppi et al., 2009; Li et al., 2010; 2014; Liu et al., 2010; Reitmeir et al., 2011, 2012; Spudich et al., 2006; Eiali and Hermann, 2011).

The plasticity responses in the post ischemic brain are quite remarkable and seem to be common among species. Nudo and Milliken showed that neurons responsible for distal limb movements before stroke were recruited to aid neuronal networks in proximal limb movements after stroke in primates (Nudo and Milliken, 1996). However, ipsilesional pyramidal tract axons degenerate after stroke (Liu et al., 2013), and the degree of degeneration depends on the severity of the ischemic insult. However, several pharmacological and cell-based therapeutic interventions can promote contralesional pyramidal tract plasticity in rodents (Hermann and Chopp, 2012; Shen et al., 2006, 2008; Cui et al., 2010). Previous work in our laboratory showed that the delivery of the growth factors Epo and VEGF induced the sprouting of midline crossing contralesional pyramidal axon collaterals that accompanied functional neurological recovery but did not promote the outgrowth of ipsilesional pyramidal tract fibers (**Fig. 8**) (Reitmeir et al., 2011, 2012).

The molecular mechanisms regulating axonal and dendritic sprouting largely involve the small RhoGTPase family. Within this family, Rho-A/B, Rac and Cdc42 are the best studied (Sanchez-Mendoza and Hermann, 2016). A very important aspect to consider is that the relationship among these proteins is antagonistic. Thus, neuronal growth is inhibited by RhoA but enhanced by Rac1 and Cdc42 (Tashiro and Yuste, 2004; Ponimaskin et al., 2007; Leemhuis et al., 2010; Sun et al., 2012). Previous work in our laboratory showed that the delivery of Epo promoted contralesional pyramidal tract plasticity post-stroke and enhanced functional neurological recovery in mice (Reitmeir et al., 2011). Epo likely exerted this effect by inhibiting Rho-A and its downstream targets ROCK-1 and ROCK-2 as observed after optical nerve crush, thus promoting axonal growth (Tan et al., 2012). Additionally, Epo promoted axonal and dendritic growth in hippocampal cells by stimulating the PI3K/Akt pathway (Ransome and Turnley, 2008), which promoted microtubule polymerization and, hence, axonal elongation (Yoshimura et al., 2006). Importantly, Rho-GTPase activity was also regulated by glutamate signaling (Ponimaskin et al., 2007). The imbalance in

glutamatergic transmission mediated by VGLUT1 and NMDA receptor overactivation could disrupt neuronal sprouting and lead to excitotoxicity (Sánchez-Mendoza et al., 2010).

Numerous studies suggest that the growth of midline crossing fibers originating from the contralesional hemisphere can be therapeutically promoted to enhance neurological functioning after stroke; for example, the delivery of the growth factors VEGF (Herz et al., 2011; Reitmeir et al., 2012) and Epo (Ehrenreich et al., 2009; Reitmeir et al., 2011), neutralizing anti-Nogo A antibodies (Wiessner et al., 2003; Freund et al., 2006; Kilic et al., 2010; Tsai et al., 2011), neurostimulants (amphetamine) (Papadopoulos et al., 2002) and NMDA receptor antagonists enhanced stroke recovery and promoted the sprouting of midline crossing fibers (Wang et al., 2016). Importantly, neuroplasticity enhanced neurological recovery after stroke not only in young but also in old animals (Markus et al., 2005; Shen et al., 2007; Zhang et al., 2006). Therefore, the therapeutic promotion of neuroplasticity may be a robust tool for stroke recovery in clinical studies.

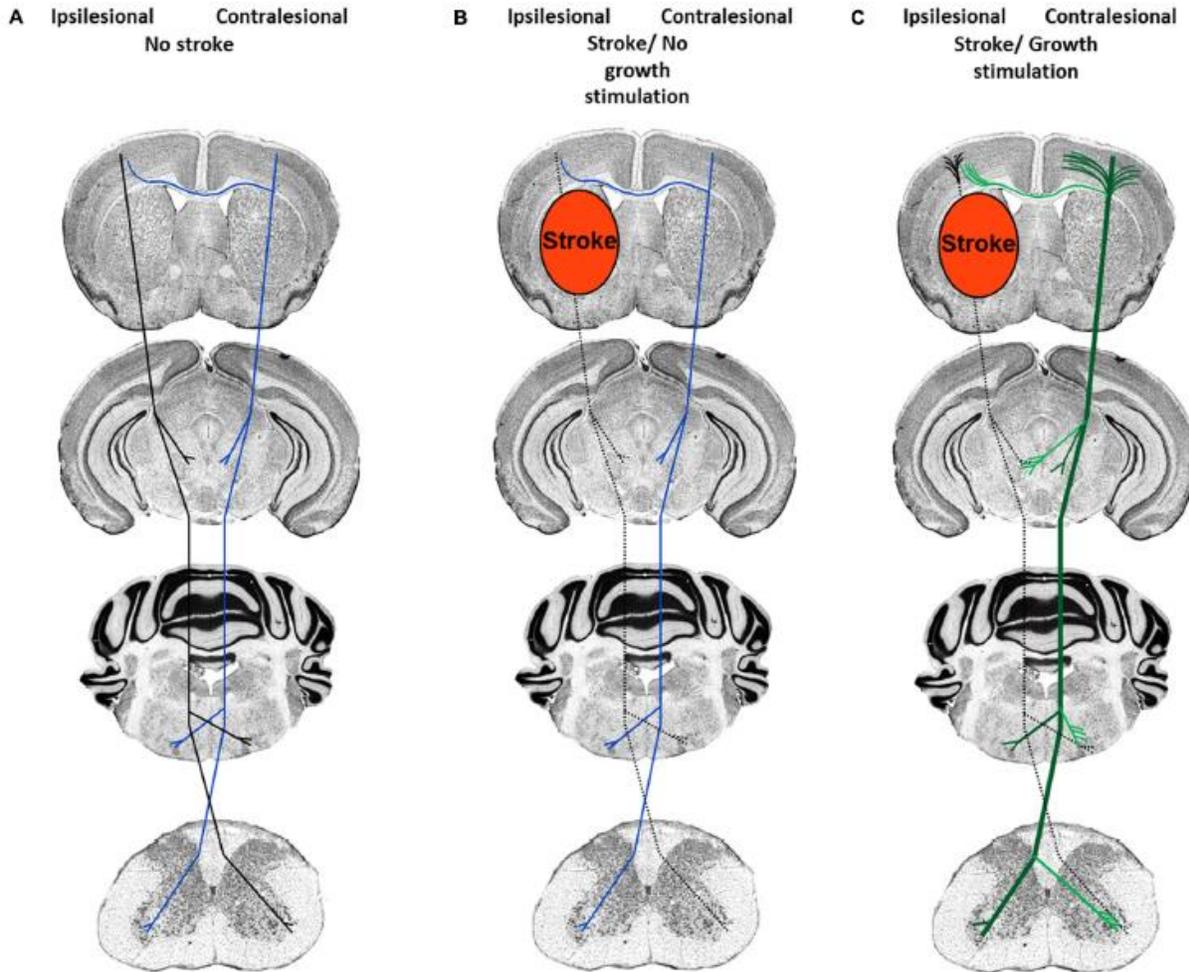


Figure 8. Neuronal plasticity features in cortical neurons in response to stroke and neuronal growth stimulation. (A) Organization of the corticospinal tract previous to stroke. Ipsilesional fibers are depicted in black, and contralesional fibers are depicted in blue. (B) After ischemic stroke induced by middle cerebral artery occlusion, pyramidal tract axons in the ipsilesional hemisphere degenerate (dashed lines), whereas the contralesional pyramidal tract axons and dendrites exhibit scarce sprouting. (C) After growth stimulation, short-distance cortical dendrites exhibit abundant sprouting both ipsilesional (black) and contralesional (green) to the stroke, whereas long-distance axon collaterals grow across the midline (at the level of the red and facial nuclei and spinal cord) in the direction of the denervated target neurons. Post-stroke, the augmentation of contralesional axon collateral sprouting is accompanied by an improvement in motor and coordination deficits (from Sanchez-Mendoza and Hermann, 2016).

1.5 Delayed treatment for stroke recovery evaluated in our laboratory

In contrast to previous beliefs, the brain has a strong potential for endogenous neuroplasticity processes based on the de novo expression of a wide variety of genes related to neuronal growth and synaptic formation (Li et al., 2010). Thus, it has been shown that in addition to the expression of endogenous growth factors, such as BDNF and GDNF, in the perilesional area, genes related to posttranslational modifications of the cytoskeleton (Li et al., 2010), axonal guidance molecules, etc. are also expressed. However, the endogenous capacity of the brain for regrowth must be potentiated to produce optimal recovery results. In our experience, optimal recovery can be achieved by the administration of growth factors, such as Epo, VEGF, and GDNF, or cell-based therapeutics, such as NPCs (Reitmeir et al., 2011, 2012; Herz et al., 2012; Kilic et al., 2005; Bacigaluppi et al., 2016). The timing of the therapeutic intervention is highly important. Indeed, it has been shown that there is a critical time window during which interventions are effective. Thus, interventions that are initiated too early or too late seem to be unsuccessful, while interventions initiated between 7 and 14 days after stroke that are administered on a recurrent basis seem to have the best results (Sanchez-Mendoza and Hermann, 2016; Reitmeir et al., 2011, 2012; Herz et al., 2012).

1.5.1 Post stroke neuroplasticity is potentiated by growth factors

Epo is a protein hormone of approximately 34.4 kD and a member of the class I cytokine family (Ebert and Bunn, 1999). Epo has been shown to increase the transcription of cyclins, inhibit cell cycle and increase the concentration of the anti-apoptotic protein B-cell lymphoma-extra-large (Bcl-XL) (Ebert and Bunn, 1999; Zhande and Karsan, 2007), favoring red cell proliferation and promoting the differentiation of bone-marrow stem cells into circulating mature red cells (Zanjani et al., 1977).

Epo and its receptor are expressed in neurons and astrocytes (Bernaudin et al., 2000); thus, Epo is a candidate due to its neuroprotective role. Recombinant human Epo reduced the infarct volume at a concentration of 5,000 units/kg i.p. after 24 hours of tMCAO in rats (Erbayraktar et al, 2003). Although initial clinical trials classified Epo as both safe and beneficial for acute stroke (Ehrenreich et al, 2002), a subsequent phase II/III German multicenter trial in which Epo was delivered along with rtPA during recanalization produced an unexpected increase in mortality

(Ehrenreich et al., 2009). Our group previously showed that the acute combination of rtPA and Epo increased the permeability of the blood brain barrier and activation of MMP-9 (Zechariah et al. 2010), suggesting the likely mechanism underlying the poor clinical result following Epo administration. However, the subacute delivery of Epo promoted contralesional plasticity and overall positive tissue remodeling, leading to neurological recovery (Reitmeir et al. 2011), which further highlights the critical value of detecting adequate time windows for therapeutic interventions.

The expression of VEGF and VEGF receptors after cerebral ischemia is primarily located in the penumbra (Greenberg and Jin, 2013). VEGF has been reported to reduce the infarct volume and enhance neurological functioning (Kaya et al., 2005; Herz et al., 2012); activated microglia also decreased following the administration of VEGF (Herz et al., 2012; Reitmeir et al., 2012).

Using a series of behavioral tests, our laboratory showed that the delayed delivery of both Epo and VEGF promoted neurological recovery and brain remodeling, enhanced angiogenesis, decreased reactive astrogliosis and prevented scar formation in ischemic tissue (Reitmeir et al., 2011; 2012; Herz et al., 2012). A better recovery of stroke correlates with the recruitment of contralesional brain plasticity in animal studies (Papadopoulos et al., 2002; Wiessner et al., 2003). Combining the anterograde tract tracer BDA and cascade blue (CB) revealed that Epo and VEGF significantly enhanced contralesional corticorubral plasticity and contralesional corticobulbar plasticity but not ipsilesional corticorubral or corticobulbar sprouting (Reitmeir et al., 2011; 2012; Herz et al., 2012). The behavior tests confirmed that both growth factors significantly promoted motor recovery (Reitmeir et al., 2011; 2012). Furthermore, Epo downregulated several pro-inflammatory genes (IL-1b, TNF-a, LIF, TGF-b and IL-6), GFAP and inducible nitric oxide synthase, indicating that anti-inflammation may represent a mode of action (Reitmeir et al., 2011). VEGF reduced brain accumulation of inflammatory leukocytes and decreased microglia activation 14 days after ischemia (Herz et al., 2012).

1.5.2 Post stroke neuroplasticity is potentiated by cell therapy

Regenerative medicine develops new therapeutic strategies for ischemic stroke. NPCs proliferate in germinal niches in the brain after stroke, namely, the subventricular zone in the striatum (Sanchez-Mendoza et al., 2013). Endogenous NPCs migrate to the ischemic lesion where they

enhance brain remodeling (Hermann et al., 2014). However, the proliferation and differentiation of endogenous NPCs are insufficient and unable to promote the full recovery of neurological functioning. Therefore, the transplantation of exogenous NPCs has become a potential tool for stroke treatment. NPCs harvested from adult brains can be used in animal models of stroke. Observations from our laboratory have already shown that the delivery of adult mouse NPCs enhanced neurological recovery, prevented neurodegeneration and decreased glial scar formation. Furthermore, the intravenous (i.v.) delivery of NPCs promoted neurogenesis and stabilized the blood brain barrier (BBB) integrity (Bacigaluppi et al., 2009; Doeppner et al., 2012; Hermann et al., 2014). Grafted NPCs moved to the ischemic lesion and adapted to the new ischemic microenvironment, where they secreted beneficial trophic factors and modulated the innate and adaptive immune responses (Pluchino and Cossetti et al., 2013). However, the interactions between the grafted NPCs and host brain tissue need to be further understood. Whether grafted NPCs survive and integrate into the host brain tissue and how the grafted NPCs affect endogenous NPCs and the microenvironment remain unclear. It is important to identify exogenous NPCs that can be used to promote neurological recovery in a tMCAO model (Hermann et al., 2014). The routes of NPC administration and the time of delivery in stroke therapy are critical for the restorative process in stroke. In comparative studies evaluating intravenous, intraarterial, ipsilateral intrastriatal, contralateral intrastriatal, ipsilateral intraventricular and ipsilateral intracortical transplant of NPCs 6 hours after tMCAO, the intrastriatal delivery resulted in the survival of the highest number of grafted cells, and the intracerebroventricular delivery to the lateral ventricle yielded fewer cells; however, compared with the intravenous NPC delivery route, more cells were found (Doeppner et al., 2015; Hermann et al., 2014). The reasons for these observations may be linked to the differentiation and survival of the grafted NPCs. Intravenously transplanted NPCs stabilize the BBB through a reduction in MMP9 expression and ROS 6 hours post stroke (Doeppner et al., 2012; Bacigaluppi et al., 2009); however, fewer NPCs survived in the ischemic lesion side. Current evidence shows that intravenous delivery can enhance neuronal plasticity even 8 weeks after stroke (Bacigaluppi et al., 2009, 2016; Doeppner et al., 2012), suggesting a strong potential for therapeutic approaches.

The time of the NPC delivery is critical for the grafted NPC survival. There was no difference in NPC proliferation, migration and neuronal differentiation when the NPCs were delivered 48 hours or 6 weeks post stroke; however, the NPC survival significantly reduced following the delayed

delivery (Darsalia et al., 2011). Most intravenously transplanted NPCs remained undifferentiated without the expression of lineage-specific markers, such as MAP2, DCX, GFAP and oligodendroglial transcription factor (Olig2), and even 3 and 10 days post transplantation, a very limited number of exogenous NPCs expressed Olig2 and DCX (Bacigaluppi et al., 2009). Our observations showed that adult mouse NPCs reduced inflammatory responses in the ischemic brain, prevented neuronal degeneration, and enhanced neurological recovery even 3 days post stroke (Bacigaluppi et al., 2009; 2016). More recent data show that delayed intravenous transplantation of NPCs in mice after tMCAO promotes contralesional adaptive plasticity by upregulating glial glutamate transporter-1 (GLT-1) in the peri-ischemic area (Bacigaluppi et al. 2016). The altered expression of VGLUTs and EAATs in the perilesional area has been previously referenced in the literature (Arranz et al, 2010; Sanchez-Mendoza et al, 2010; Sanchez-Mendoza et al, 2013); however, thus far, the importance of the alterations in EAATs, particularly GLT-1, has remained unclear (Chao et al, 2010; Arranz et al, 2010). NPC transplantation increased GLT-1 expression in endogenous astrocytes located within the peri-ischemic area, decreased excitatory neuronal network activity and reduced extracellular glutamate as measured by microdialysis (Bacigaluppi et al., 2016), suggesting that transplanted NPCs may exert restorative effects not only by growth factor release and induction of tissue remodeling (Doeppner et al., 2012; Bacigaluppi et al., 2009) but also by modulating the recycling of excitatory neurotransmitters at their target destination.

Thus, NPCs present unique characteristics applicable to stroke therapy. Transplanted stem cells can promote tissue regeneration by sensing diverse signals in the brain microenvironment, migrating to the lesion, integrating inputs and executing complex responses directed towards remodeling and protecting ischemic tissue (Fischbach et al., 2013; Hermann et al, 2014). Due to the potential side effects, NPC transplantation is currently not suitable for clinical proof-of-concept studies in stroke patients.

2. AIM OF THE STUDY

Experimental data regarding growth factors and cell-based therapies offer promising perspectives for stroke patients. However, much remains unknown about the underlying activated molecular mechanisms, posing considerable difficulties in translating these therapies to clinical practice. Indeed, despite the many positive effects found *in vitro* and *in vivo* following Epo administration on neuronal sprouting and stroke recovery, the acute administration of Epo early after stroke has been reported to enhance mortality when administered along with rtPA (Ehrenreich et al., 2009). This finding highlights the following two important needs in the stroke research field: a) the need to identify suitable molecules to enhance stroke recovery probably among other pharmaceuticals already in use in regular clinical practice and b) the need to emphasize subacute therapies that promote neurological reorganization in light of the vast evidence against the suitability of neuroprotective strategies (Sanchez-Mendoza and Hermann, 2016).

As previously mentioned, during the acute phase, the brain excitability levels are elevated and deleterious. During this phase, blocking glutamate signaling or enhancing GABA signaling promotes neuroprotection, at least in animal models. During the chronic phase, the exact opposite appears to occur. After cell death has transpired, the brain begins to reorganize and repair during the chronic phase of stroke (Carmichael, 2012). Therefore, anti-excitotoxic approaches impeding the overactivation of NMDA receptors or reducing the tonic inhibition induced by GABA could potentially be promising strategies for stroke therapy. Unfortunately, all drugs showing promise as inhibitors of NMDA-mediated excitotoxicity, such as selfotel, aptiganel, and gavestinel, had severe and unacceptable side effects, including drowsiness, hallucinations and even coma. These events lead to the failure of these drugs in clinical trials investigating stroke (Lees et al., 2000; Sacco et al., 2001; Hoyte et al., 2004; Lipton, 1999). Therefore, a new perspective regarding the application of such treatments is necessary.

To be clinically acceptable, the anti-excitotoxic therapy must block the excessive activation of NMDARs while leaving normal functioning relatively intact to avoid side effects (Lipton, 2006). Memantine has been reported to block excitotoxic cell death not only *in vitro* but also in a clinically tolerated manner (Lipton, 2004). Memantine, which is already used in clinical practice for the treatment of dementia, may enhance neurological recovery due to its characteristic low affinity and non-competitive binding to extrasynaptic NMDA receptors; therefore, memantine modulates,

rather than silences, NMDA receptor activity (Lipton, 2006). Many *in vitro* and *in vivo* animal models have shown that memantine is neuroprotective (Parson et al., 1999). Additionally, in a rat model of stroke, memantine delivered up to 2 hours after the ischemic event reduced brain damage by approximately 50% (Chen et al., 1992; 1998). Here, we hypothesized that in addition to its neuroprotective actions, memantine may also have restorative effects during the post-acute stroke phase. To answer this question, here, we exposed mice to tMCAO, administered different doses of memantine (4 or 20 mg/kg/day) starting 72 hours post-stroke, and evaluated the effects on motor recovery, cognitive performance, peri-infarct tissue remodeling and contralesional pyramidal tract plasticity for up to 49 days.

Tonic GABAergic inhibition is mainly mediated by the GABA_A $\alpha 5$ receptor. Many researchers have attempted to design and evaluate GABA_A $\alpha 5$ receptor antagonists for clinical use. The negative allosteric modulator of the $\alpha 5$ GABA_A receptor $\alpha 5$ IA has recently been shown to improve ethanol-induced impaired performance in healthy subjects (Nutt et al., 2007). However, the clinical trial was stopped due to renal toxicity (Atack, 2010). RO4938581 is another negative allosteric modulator of the $\alpha 5$ subunit and is currently under investigation in a phase 1 clinical trial for Down syndrome (the study is sponsored by Roche, and its outcomes have been made publicly available). The inverse GABA_A $\alpha 5$ receptor agonist L655,708 has led to improvement in neurological recovery following delayed subcutaneous or intraperitoneal delivery in stroke models (Lake et al., 2015; Quirk et al., 1995; Clarkson et al., 2010). However, L655,708 was found to be anxiogenic at doses that enhanced cognition (Navarro et al., 2002).

S44819 is a novel competitive selective antagonist of the $\alpha 5$ -GABA_A receptor at the GABA-binding site. In a phase I study, S44819 enhanced cortical excitability in 18 healthy young adults by transcranial magnetic stimulation (TMS) at a single oral dose of 100 mg (Darmani et al., 2016). Here, we hypothesized that S44819 may also have restorative effects during the post-acute stroke phase that improve neurological recovery. To confirm this hypothesis, mice were exposed to tMCAO, and different doses of S44819 (4 or 20 mg/kg/day) were administered starting 72 hours post-stroke. Motor recovery, cognitive performance, peri-infarct tissue remodeling and contralesional pyramidal tract plasticity were evaluated for up to 42 days.

3. MATERIALS AND METHODS

3.1 Legal issues, animal housing, randomization and blinding

All animal experiments were performed with local government approval (Animal Experimentation Committee of Bezirksregierung Düsseldorf) in accordance to E.U. guidelines (Directive 2010/63/EU) for the care and use of laboratory animals in compliance with ARRIVE guidelines. The experiments were strictly randomized. The experimenter performing the animal experiments and histochemical studies was fully blinded at all stages of the study, and another researcher prepared the vehicle and memantine solutions. These solutions received dummy names (solution A, B, and C), which were unblinded after the termination of the study. The animals were kept under a regular 12 h: 12 h light/ dark cycle in groups of 5 animals per cage. The behavioral tests and animal surgeries were always performed in the morning throughout the study.

3.2 Animal groups

3.2.1 Effects of the NMDA receptor antagonist memantine

In this study, memantine was delivered subcutaneously by miniosmotic pumps because miniosmotic pumps can achieve the most stable plasma memantine levels. Memantine is highly stable in miniosmotic pumps at 37°C as previously demonstrated by observations in rats up to three weeks (unpublished data from Merz Pharmaceuticals).

Focal cerebral ischemia was induced in male C57BL6/j mice (8-12 weeks; 23-28 g; Harlan Laboratories, Indianapolis, IN, U.S.A.) by 40 min of left-sided tMCAO. Seventy-two hours post-stroke, one set of mice (animal set 1) received subcutaneous implantations of miniosmotic pumps (Alzet 2004; Alzet, Cupertino, CA, U.S.A.) that were randomly filled with vehicle (normal saline) or memantine (4 or 20 mg/kg/day in normal saline; Merz, Frankfurt, Germany) and left in place for 28 days (n=18 animals/group; **Fig. 9**).

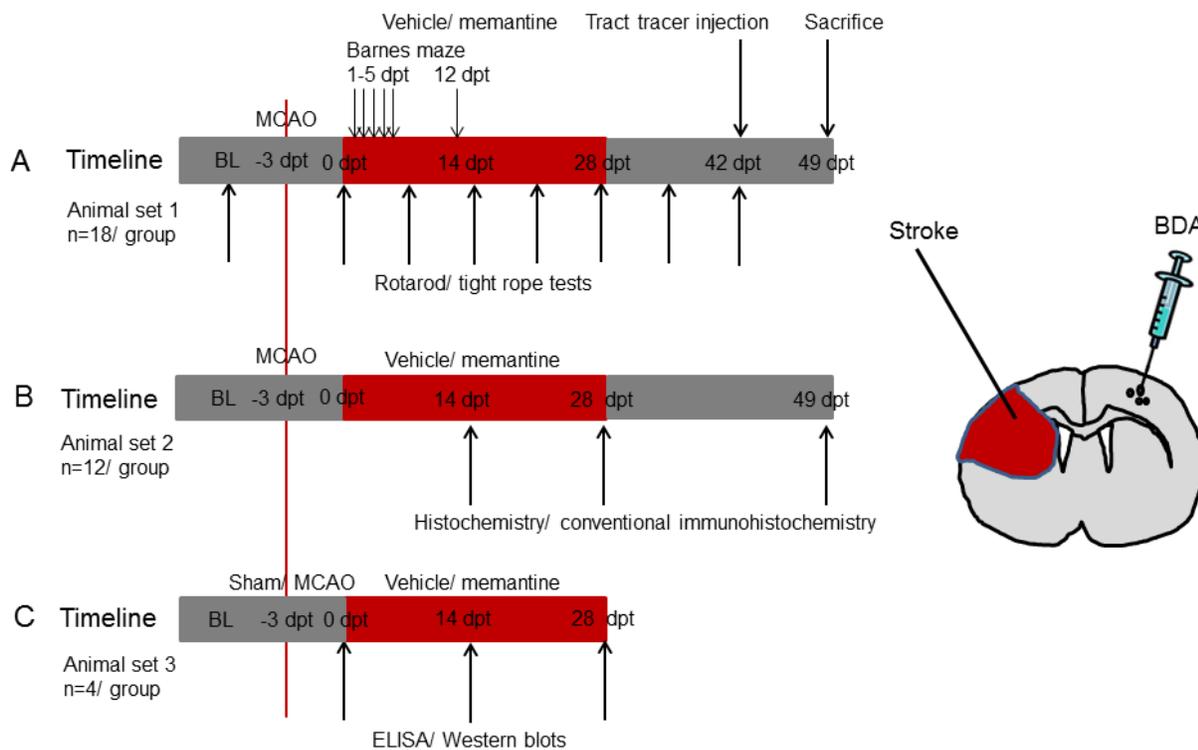


Figure 9. Experimental procedures and animal groups used to evaluate the effect of the NMDA antagonist memantine. Mice subjected to tMCAO were treated with vehicle or memantine (4 or 20 mg/kg/day) starting 72 h after reperfusion for up to 28 dpt and used for (A) behavioral tests (Rotarod, tight rope, and Barnes maze tests) and tract tracing studies, (B) histochemistry and conventional immunohistochemistry, and (C) ELISA and Western blotting. BDA, which is an anterograde tract tracer, was injected into the contralesional cortex to evaluate pyramidal tract plasticity. The numbers of animals used in each group and time-point of the animal sacrifice (days post-treatment onset, dpt) are also shown. BL, baseline.

The animals underwent detailed assessments of the motor coordination and cognitive deficits as described below. To evaluate pyramidal tract plasticity, the anterograde tract tracer BDA

was injected into the contralesional motor cortex 42 days post-treatment onset (dpt). These animals were sacrificed at 49 dpt by transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS).

Another set of mice (animal set 2) was treated as indicated above and sacrificed at different time points to perform detailed histological analyses. The mice were sacrificed at 14 dpt (n=12 animals/group), 28 dpt (n=12 animals/group) or 49 dpt (n=12 animals/group) by transcardiac perfusion with 4% paraformaldehyde in 0.1 M PBS (**Fig. 9**). These animals were used for the histochemical and conventional immunohistochemical studies.

Similarly, a third set of mice (animal set 3) was exposed to 40 min of left-sided tMCAO, followed by implantation of miniosmotic pumps filled with vehicle or memantine (20 mg/kg/day) 72 hours post-stroke. These animals were sacrificed at 14 dpt (n=4 animals/group) or 28 dpt (n=4 animals/group) by transcardiac perfusion with normal saline (**Fig. 9**). Additional animals were subjected to 40 min of left-sided tMCAO or sham surgery (n=4 animals/group). These animals did not receive implantations of miniosmotic pumps. These animals were transcardially perfused with normal saline 72 hours post-stroke or post-sham surgery. These animals were used for the enzyme-linked immunosorbent assays (ELISA) and Western blotting.

The animal flow used in this study is summarized in Table 1. Regarding the exclusion criteria, the animals were removed from the study if they suffered from central respiratory abnormalities (i.e., apneas) or severe motor handicaps with inappropriate nurturing resulting in a weight loss >20%. The excluded animals were replaced with new animals throughout the study.

3.2.2 Effect of the GABA_A α 5 receptor antagonist S44819

Male C57BL6/j mice (8-12 weeks; 25-28 g; Charles River Laboratories, Cologne, Germany) were subjected to 40 min of left-sided tMCAO. From 72 hours to 31 days post-stroke (i.e., from 0 to 28 dpt), one set of mice (animal set 1) received vehicle or S44819 (3 or 10 mg/kg provided by Servier, Suresnes, France) administered b.i.d. by oral gavage (n=18 animals/group; **Fig. 10**). The animals underwent detailed assessments of their motor coordination and cognitive deficits as described below. These animals were sacrificed at 42 dpt by transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS).

Another set of mice (animal set 2) was subjected to 40 min of left-sided tMCAO, followed by treatment with vehicle (as described above) or S44819 (3 or 10 mg/kg b.i.d.; as described above) by oral gavage from 72 hours to 31 days post-stroke (i.e., from 0 to 28 dpt). These animals were sacrificed at 14 dpt (n=6 animals/group) or 28 dpt (n=6 animals/group) by transcardiac perfusion

with 4% paraformaldehyde in 0.1 M PBS (**Fig. 10**). These animals and the animals from set 1 were used for the histochemical studies.

Animal flow chart

Treatment	Animal sets	Animal sacrifice	Before MCAO	After MCAO	After implantation of pumps	After removal of pumps	After tract tracer injection	End of experiments
Vehicle	Set 1	49 dpt	20	19	19	19	18	18
	Set 2	14 dpt	13	12	12	n.a.	n.a.	12
		28 dpt 49 dpt	14 12	12 12	12 12	n.a. 12	n.a. n.a.	12 12
Set 3	14 dpt 28 dpt	4 4	4 4	4 4	n.a. n.a.	n.a. n.a.	4 4	
Memantine 4 mg/kg/day	Set 1	49 dpt	19	18	18	18	18	18
	Set 2	14 dpt	14	12	12	n.a.	n.a.	12
28 dpt 49 dpt		13 12	12 12	12 12	n.a. 12	n.a. n.a.	12 12	
Memantine 20 mg/kg/day	Set 1	49 dpt	20	19	19	19	18	18
	Set 2	14 dpt	14	12	12	n.a.	n.a.	12
		28 dpt 49 dpt	14 13	12 12	12 12	n.a. 12	n.a. n.a.	12 12
Set 3	14 dpt 28 dpt	5 4	4 4	4 4	n.a. n.a.	n.a. n.a.	4 4	
No treatment	Set 3	Sham/ 0 dpt	4	4	n.a.	n.a.	n.a.	4
		MCAO/ 0 dpt	5	4	n.a.	n.a.	n.a.	4
Total			204	188				186

Table 1. Flow chart of animal experiments used to evaluate the effects of memantine. Numbers of animals included at each step of the study are summarized. dpt, day post-treatment onset; n.a., not applicable.

Similarly, a third set of mice (animal set 3) was exposed to 40 min of left-sided tMCAO, followed by treatment with vehicle (as described above) or S44819 (3 or 10 mg/kg b.i.d.; as described above) by oral gavage from 72 hours to 31 days post-stroke. These animals were sacrificed at 14 dpt (n=6 animals/group), 28 dpt (n=6 animals/group) or 42 dpt (n=6 animals/ group) by transcardiac perfusion with normal saline (**Fig. 10**). Additional animals were subjected to 40 min of left-sided tMCAO or sham surgery (n=6 animals/group). These animals did not receive the S44819 treatment. These animals were transcardially perfused with normal saline at 72 hours post-stroke or post-sham surgery. These animals were used for the enzyme-linked immunosorbent assays (ELISA) and Western blotting.

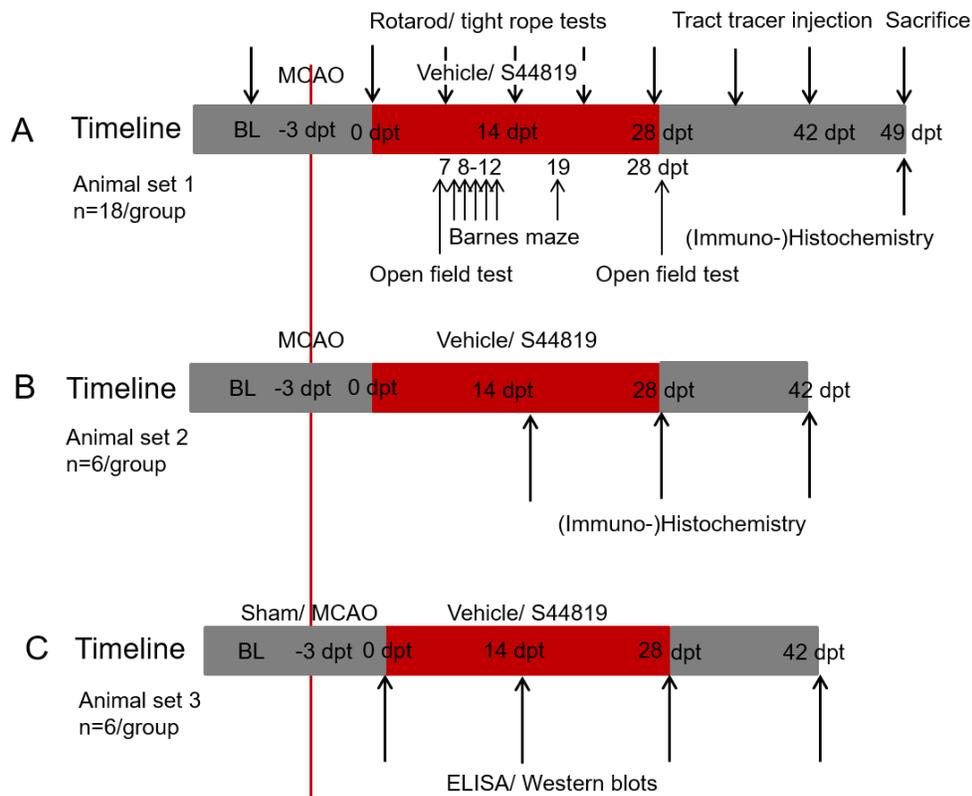


Figure 10. Experimental procedures and animal groups used to evaluate the effect of a $GABA_A \alpha 5$ receptor antagonist. Mice were subjected to tMCAO, treated for 28 days p.o. with vehicle or S44819 (3 or 10 mg/kg b.i.d.) starting 72 hours after reperfusion and used for (A) behavioral tests (Rotarod, tight rope, open field and Barnes maze tests), (B) histochemistry and immunohistochemistry and (C) ELISA and Western blotting. Numbers of animals evaluated in each group and the time-point of animal sacrifice (days post-treatment onset, dpt) are also shown. BL, baseline.

The animal flow used in this study is summarized in Table 2. Regarding the exclusion criteria, animals were removed from the study if they suffered from central respiratory abnormalities (i.e., apneas) or severe motor handicaps with inappropriate nurturing resulting in a weight loss >20%. The excluded animals were replaced with new animals throughout the study to ensure equal animal numbers in all groups.

Animal flow chart

Treatment	Animal sets	Animal sacrifice	Before MCAO	After MCAO	End of treatment	End of experiment
Vehicle	Set 1	42 dpt	19	18	18	18
	Set 2	14 dpt	6	6	6	6
		28 dpt	7	6	6	6
	Set 3	14 dpt	6	6	6	6
		28 dpt	6	6	6	6
S44819 3 mg/kg	Set 1	42 dpt	20	18	18	18
	Set 2	14 dpt	7	6	6	6
		28 dpt	6	6	6	6
	Set 3	14 dpt	6	6	6	6
		28 dpt	6	6	6	6
S44819 10 mg/kg	Set 1	42 dpt	19	18	18	18
	Set 2	14 dpt	6	6	6	6
		28 dpt	6	6	6	6
	Set 3	14 dpt	6	6	6	6
		28 dpt	6	6	6	6
No treatment	Set 3	Sham/ 0 dpt	6	6	6	6
		MCAO/ 0 dpt	7	6	6	6
Total			164	156	156	156

Table 2. Numbers of animals included at each step of the study evaluating the effect of the GABA_A α 5 receptor antagonist S44819 are summarized. dpt, day post-treatment onset; tMCAO, transient middle cerebral artery occlusion.

3.3 Induction of focal cerebral ischemia

In both studies, wounds were carefully instilled with buprenorphine (0.1 mg/kg; Reckitt Benckiser, Slough, U.K.) during all stages of the animal experiments. For the induction of tMCAO, the animals were anaesthetized with 1.0-1.5% isoflurane (30% O₂, remainder N₂O). The rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system (Fluovac, Harvard apparatus). During and after tMCAO, cerebral blood flow was recorded by Laser Doppler flowmetry (LDF) using a flexible probe attached to the skull overlying the core of the MCA territory. The left common and external carotid arteries were isolated and ligated, and the internal carotid artery was temporarily clipped (**Fig. 11**). A silicon-coated nylon monofilament was introduced through a small incision in the common carotid artery and advanced to the carotid

bifurcation for the middle cerebral artery occlusion (**Fig. 11**). Reperfusion was initiated by monofilament removal after 40 minutes. The wounds were carefully sutured, and the animals were returned to their cages. Regarding the sham surgery, a midline neck incision was performed, and the common carotid artery was exposed but left intact, while LDF was recorded. For anti-inflammation, the animals received daily i.p. injections of carprofen (4 mg/kg; Bayer Vital, Leverkusen, Germany) during the first 3 days post-stroke or sham-surgery.

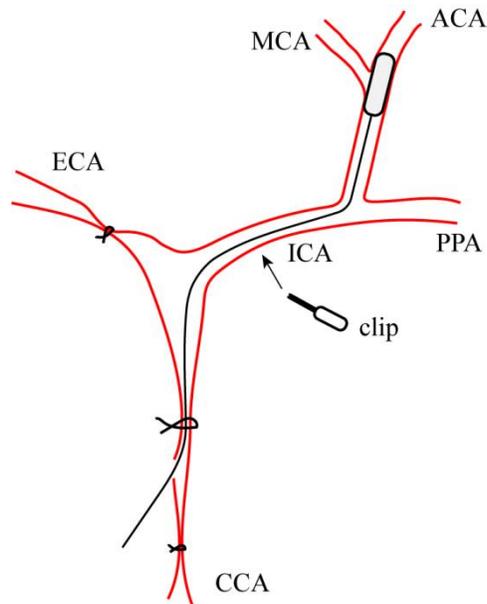


Figure 11. Scheme of an intraluminal suture tMCAO model. tMCAO, transient middle cerebral artery occlusion; ACA, anterior carotid artery; PCOM, posterior communicating artery; PPA, pterygopalatine artery; ECA, external carotid artery; CCA, common carotid artery.

3.4 Drug delivery

3.4.1 Memantine was delivered by miniosmotic pump implantation

Seventy-two hours post-stroke, the animals were re-anesthetized with 1.0-1.5% isoflurane (30% O₂, remainder N₂O). Miniosmotic pumps (Alzet 2004; 0.25 µl/hour) filled with vehicle (normal saline) or memantine (4 or 20 mg/kg/day in normal saline) were subcutaneously implanted (Reitmeir et al., 2011; 2012). The miniosmotic pumps were removed 28 dpt in animals with survival times greater than 49 days.

3.4.2 S44819 delivery by oral gavage

Seventy-two hours post-stroke, the mice received 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; provided by Servier, Suresnes, France) administered b.i.d. by oral gavage (n=18 animals/group).

3.5 Functional neurological tests

In both studies, neurological recovery was analyzed in animal set 1 using RotaRod, tight rope and Barnes maze tests.

Rota rod test: The Rotarod, which consists of a rotating drum with a speed accelerating from 4 to 40 rpm (Ugo Basile, model 47600, Comerio, Italy), was used to evaluate motor coordination skills (Figure 12A) (Kilic E et al., 2010). The maximum speed is reached after 245 seconds, and the time at which the animal falls off the drum is evaluated (maximum testing time: 300 seconds) (Reitmeir et al., 2011; 2012). The measurements were performed three times at each time point, and the mean values were evaluated (Reitmeir et al., 2011; 2012).

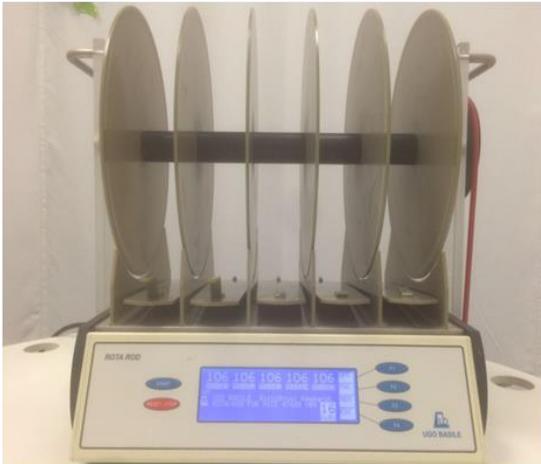
Tight rope test: The animals were placed in the middle of a 60-cm-long rope, which they grasped with both forepaws (Figure 12B). The time until the animals reached the platform, which was composed of wood covered by a white surface without any olfactory cues at the end of the rope, was analyzed. The maximum testing time was 60 seconds (Reitmeir et al., 2011; 2012).

Barnes maze test: The Barnes maze is composed of a circular platform (92 cm diameter) with 20 equally spaced holes (5 cm diameter, 7.5 cm between holes) along a 72 cm perimeter above the ground and is used to evaluate spatial memory. A rectangle box (15.5×9.5 x 6 cm; length, width, and height) was placed under the target hole from which the animals may escape (Figure 12C). Before and after each test, the platform was cleaned with 70% ethanol to remove olfactory cues from the previous mice. The animals were placed into a cylindrical black start chamber in the middle of the maze, which was lifted after 10 seconds with bright light switched on, allowing the animal to explore the maze for 3 minutes. The primary errors in finding the target hole, total errors until escaping into the target hole, primary latency until finding the target hole and total latency until escaping into the target hole were recorded (Barnes, 1979). Whenever an animal escaped into

the target hole, the light was switched off, and the animal was allowed to stay in the hole for 1 min. In an animal did not find the target hole within 3 minutes, the animal was gently placed in front of the hold and stimulated to enter the hole. Visual cues were prepared by rectangle colored paper and placed surrounding the maze. The animals had spatial visual cues endogenous to the room, such as the door, a desk or a computer, and cues intentionally prepared by the experimenter (Berta et al., 2007). These cues were not moved during the whole experiment as these cues served as the animals' reference points for locating the target hole (Herring et al., 2016). Following four days of training (1 to 4 dpt), the target hole was blocked, followed by two testing sessions on 5 and 12 dpt in which the animals were allowed to explore the maze for 90 seconds. During this retention phase, the primary errors, total errors and primary latency were tracked using Video Mot 3D software (version 7.0.1; TSE Systems, Bad Homburg, Germany) (Matsunaga et al., 2015). In the Barnes maze, each animal was examined four times per session. The mean values of these tests were calculated.

Open field test: For the S44819 study, spontaneous motor activity was evaluated by an open field test. The open field arena is a square platform (52 x 52 x 30 cm) subdivided into one center (31.2 x 31.2 cm), four borders (each 10.4 x 31.2 cm) and four corner (each 10.4 x 10.4 cm) fields, and the animals were placed near the wall and observed for 10 minutes (Figure 12D). The number of field entries, duration in each field, speed and distance covered were tracked using Video Mot 3D software (version 7.0.1; TSE Systems, Bad Homburg, Germany) (Matsunaga et al., 2015).

A



B



C



D

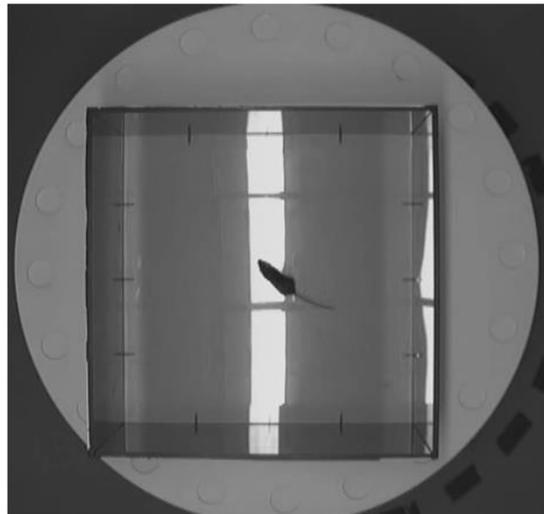


Figure 12. Scheme of devices used in the behavior tests. (A) Rotarod (B) Tight rope (C) Barnes Maze (D) Open field.

3.6 Biotinylated dextran amine (BDA) injection

In both studies, contralesional corticorubral plasticity was examined following injection of the anterograde tract tracer BDA (Reitmeir et al., 2011; 2012). At 42 dpt, the animals in animal set 1 were re-anesthetized with 1.0-1.5% isoflurane (30% O₂, remainder N₂O). A cranial bur hole was drilled 0.5 mm rostral, 2.5 mm lateral and +1.34 mm rostral to bregma (Sanchez-mendoza et al., 2016), and deposits of 10% BDA (MW 10,000; Molecular Probes, Waltham, MA, U.S.A.; diluted in 0.01 M PBS at pH 7.2) were placed into the contralesional motor cortex using stereotactic

microsyringe pressure injections. The syringe (Hamilton, 75N) was held by a stereotactic device at an angle of 45° with respect to the vertical line. The syringe needle was placed in front of the hole under a microscope. The syringe was moved vertically 1.5 mm in 3 sequential steps of 0.5 mm in 30 s intervals until the targeted area was reached. The needle was kept for 1 min in this position before BDA was injected. Three successive 0.2 µl injections of the tracer dilution were performed at intervals of 30 s. After the final injection was completed, the syringe was left in place for 1 min to avoid spillover into the brain. The process was repeated identically at each injection point. The wound was closed, and the mice were returned to a new cage. Ten days after the tracer injection, the animals were transcardially perfused with 4% paraformaldehyde in 0.1 M PBS. Their brains were removed, post-fixed overnight in 4% paraformaldehyde in 0.1 M PBS and cryoprotected by immersion in increasing sucrose concentrations (5%, 10% and 30%) for 3 days. Then, the brains were frozen on dry ice and stored at -80°C.

3.7 Conventional immunohistochemistry

In both studies, 20 µm-thick coronal brain sections obtained from the midstriatal level of the bregma (anterior-posterior +1.0 anterior to Bregma, medial-lateral +/-2.5 lateral to Bregma and ventral 3.5 mm below the skull) from animals sacrificed by transcardiac perfusion with 4% paraformaldehyde (animal set 2) were rinsed three times for 5 minutes with 0.1 M PBS and immersed in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 10% normal donkey serum for 1 hour. The sections were incubated overnight at 4°C with Alexa Fluor 488-conjugated monoclonal rabbit anti-glial fibrillary acidic protein (GFAP) (12389; Cell Signaling, Billerica, MA, U.S.A.), polyclonal rabbit anti-ionized calcium binding adaptor protein (Iba)-1 (Wako Chemicals, Neuss, Germany) or monoclonal rat anti-CD31 (cluster of differentiation 31) (557355; BD Biosciences, Heidelberg, Germany) antibodies that were detected with Alexa Fluor 594 or Alexa Fluor 488 conjugated secondary antibodies in the experiments in which the unconjugated primary antibody was used. The sections were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). The sections were evaluated under a motorized Zeiss AxioObserver Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning. GFAP+ astrogliosis was evaluated by analyzing the optical density of the perilesional (i.e., parietal cortical) scar tissue 3.0 mm lateral to the bregma/ 1.5 mm below the brain surface using Apotome optical sectioning, and the background optical density in homologous tissue contralateral to the stroke was subtracted.

Iba1+ microglia were analyzed in a blinded way by counting the numbers of cells in six defined regions of interests (ROI) in the striatum ipsilateral to the stroke (size: 500 μm x 500 μm ; ROI centered 1.5 mm and 2.5 mm lateral to the midline/ 2.5 mm, 3.25 mm and 4.0 mm below the brain surface) (Reitmeir et al., 2011; 2012). Optical sectioning was used to correct for cell overcounts. CD31+ microvessels were evaluated by counting the numbers of microvessels intersecting grid lines within these ROI (size: 500 μm x 500 μm (10x10) grids; ROI centered 1.5 mm and 2.5 mm lateral to the midline/ 2.5 mm, 3.25 mm and 4.0 mm below the brain surface). For the Iba1+ cell counts and CD31+ microvessel counts, the mean values were calculated for all ROIs by ImageJ software. Finally, the GFAP+ astrogliosis, Iba1+ microglial activation and CD31+ microvessel density were normalized to brain atrophy as described below.

3.8 Analysis of brain atrophy

In both studies, brain atrophy and striatal atrophy were volumetrically evaluated using cresyl violet staining of 20- μm -thick coronal brain sections that had been collected at millimeter intervals throughout the forebrain (from most rostral pole to most caudal end) from animals sacrificed by transcardiac perfusion with 4% paraformaldehyde (animal set 2) (Reitmeir et al., 2011; Bacigaluppi et al., 2009). In the coronal sections obtained from the bregma level, corpus callosum atrophy was further analyzed by determining the tissue area covered by the corpus callosum (Reitmeir et al., 2011; Bacigaluppi et al., 2009).

3.9 Immunohistochemistry for BDA

In both studies, 40- μm -thick coronal brain sections obtained from animals sacrificed by transcardiac perfusion with 4% paraformaldehyde (animal set 1) were rinsed three times for 10 min each with 50 mM Tris-buffered saline (pH 8.0) containing 0.5% Triton X-100 (TBST). The sections were incubated overnight with avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA, U.S.A.). The staining was developed with 3, 3'-diaminobenzidine (DAB) (D4418; Sigma, Deisenhofen, Germany) containing 0.4% ammonium sulfate and 0.004% H_2O_2 for 5 minutes.

3.10 Analysis of corticorubral projections

In the memantine study, the location of the tracer deposits was assessed at the levels of the needle tracks to ensure that the motor cortex had indeed been injected in all animals. To account for variabilities in tracer uptake in different mice, we first evaluated the number of tracer-stained fibers in the pyramidal tract at the level of the red nucleus. Thus, two consecutive sections were analyzed to count the number of fibers crossing the sections in four regions of interest of 2,865 μm^2 each that had been selected in the dorsolateral, ventrolateral, dorsomedial and ventromedial portion of the pyramidal tract. By measuring the total area of the pyramidal tract, we calculated the overall number of labeled pyramidal tract fibers as previously described (Reitmeir et al., 2011; 2012). The corticorubral projections were evaluated at the level of the parvocellular red nucleus (bregma -3.0 to -3.5 mm). A 500 μm long intersection line was superimposed onto the brain midline. Along that line, those fibers crossing into the ipsilesional hemisphere in the direction of the red nucleus were quantified. For each animal, the total number of fibers counted was normalized to the total number of labeled fibers in the pyramidal tract and multiplied by 100, resulting in percent values of fibers crossing the midline. Two consecutive sections were analyzed per animal, and the mean values were determined.

3.11 Enzyme-linked immunosorbent assay (ELISA)

In both studies, tissue samples obtained from the motor cortex or striatum ipsilateral or contralateral to the stroke from animals sacrificed by transcardiac perfusion with normal saline (animal set 3) were homogenized using a lysis buffer consisting of 1% NP40 (nonyl phenoxyethoxyethanol) containing 50 mM Tris (pH 8.0), 150 mM NaCl, protease inhibitor cocktail and phosphatase inhibitor. In these homogenates, the levels of brain-derived neurotrophic factor (BDNF; Promega, Madison, WI, U.S.A.), basic fibroblast growth factor (FGF; R&D Systems, Minneapolis, MN, U.S.A.), glial cell line-derived neurotrophic factor

(GDNF; Promega) and vascular endothelial growth factor (VEGF; R&D Systems) were determined using commercial mouse ELISA kits according to the manufacturers' instructions (Doepfner et al., 2012).

3.12 Western blot analyses

In both studies, tissue samples obtained from the motor cortex ipsilateral or contralateral to the stroke from animals sacrificed by transcardiac perfusion with normal saline (animal set 3) were homogenized using lysis buffer as previously described. The protein concentration was measured using the Bradford method (BioRad, Hercules, CA, U.S.A.). Equal amounts of 20 µg protein were used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by protein transfer onto Protran BA83 nitrocellulose blotting membranes (GE Healthcare Life Sciences, Freiburg, Germany). The membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 0.1% (TBS-T) for 1 hour at room temperature, washed three times with TBS-T and incubated overnight with a polyclonal rabbit anti GluN2A antibody (612-401-D89; Rockland, Limerick, PA, U.S.A.), polyclonal rabbit anti-GluN2B antibody (AGC-003; Alomone Labs, Jerusalem, Israel), monoclonal mouse anti-PSD95 antibody (124011; Synaptic Systems, Göttingen, Germany), monoclonal mouse anti-tau5 antibody (ab80579; Abcam, Cambridge, U.K.), polyclonal rabbit anti-β-actin antibody (#4967L; Cell Signaling Technology, Danvers, MA, U.S.A) or monoclonal rabbit anti-GADPH antibody (#2118S; Cell Signaling Technology, Danvers, MA, U.S.A). The membranes were washed three times, incubated with blocking solution containing secondary antibody conjugated with peroxidase, washed three times and developed using a chemiluminescence kit (RPN2235; GE Healthcare Life Sciences) according to the manufacturer's instructions. The optical density of each protein was measured using the ImageJ program in 4 independent blots, which were corrected for protein loading using β-actin or GAPDH blots. The mean values of these blots were calculated and used for further data analysis.

3.13 Statistical analysis

The statistical planning was performed using a sample size calculator (<https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/samplesizecalculators.aspx>). For the behavioral tests, specifically the Rotarod and tight rope tests (subsequently evaluated in animal set 1), we predicted an effect size of 25% of the mean value and an expected standard deviation of 30% of the mean value, which required a sample size of 18 animals/ group with an alpha error level of 5% and beta error (1 – statistical power) of 20%. For the histochemical studies (subsequently performed in animal set 2), we expected an effect size of 30% of the mean value and a standard deviation of 30% of the mean value, which demanded a sample size of 12 animals/

group (alpha error of 5%, beta error of 20%). For the ELISA and Western blot analyses (performed in animal set 3), we expected an effect size of 50% of the mean value and a standard deviation of 30% of the mean value, which required a sample size of 4 animals/ group (alpha error of 5%, beta error of 20%).

The behavioral tests were analyzed using two-way repeated measurement analysis of variance (ANOVA) to evaluate the main effects of treatment and time and interaction effects of treatment x time starting on day 3 post-stroke (i.e., the time-point of minipump implantation). For all tests, significant main effects of treatment or interaction effects of treatment x time at the $p < 0.05$ level were noted. Two-tailed t-tests were then performed for each time point. The tract tracing studies were evaluated by one-way ANOVA, followed by least significant difference (LSD) tests. All other histochemical data were analyzed by two-way ANOVA to evaluate the main effects of treatment and time and interaction effects of treatment x time starting at 14 dpt. If a main effect of treatment or interaction effect of treatment x time was significant at the $p < 0.05$ level, two-tailed t-tests were performed for each time point. The data are presented in the graphs as the mean \pm standard deviation (SD) (longitudinal comparisons within the same animals) or box blots as median/ mean \pm interquartile range (IQR) with minimum and maximum data (all other comparisons between animals or tissue samples). Throughout the study, p-values < 0.05 were considered significant.

4. RESULTS

4.1 NMDA receptor antagonist Memantine promotes neurological recovery, peri-infarct tissue remodeling, and contralesional plasticity after stroke

4.1.1 Post-acute Memantine delivery improves post-ischemic neurological recovery

Body weight and LDF above the core of the tMCAO lesion did not reveal any difference between animals treated with vehicle or Memantine (4 or 20 mg/kg/day) (**Fig. 13 A, B**). In all groups, LDF decreased to approximately 15-20% of baseline during tMCAO, followed by rapid recuperation after reperfusion. The mortality rate of mice was 8.8% during the entire experiment (Table 1). Rotarod and tight rope tests revealed a clearly defined motor-coordination deficit after tMCAO that was attenuated by Memantine delivered at 20 mg/kg/day but not 4 mg/kg/day (**Fig. 13 C, D**). Interestingly, motor-coordination improved rapidly, achieving significance between groups by 7 days post-treatment (dpt).

4.1.2 Post-acute Memantine delivery enhances post-ischemic spatial memory

In line with motor-coordination improvement, spatial memory in the learning and retention phase of the Barnes maze test improved significantly in mice receiving 20 mg/kg/day of Memantine but not in mice receiving vehicle or 4 mg/kg/day (**Fig. 14**). Better performance was similarly noted in primary errors in finding the target hole, total escape errors, primary latency until finding the target hole, and total escape latency.

4.1.3 Post-acute Memantine delivery promotes peri-infarct tissue remodelling

To assess perilesional remodeling in response to Memantine, histochemical studies were performed. Cresyl violet stains revealed localized infarcts restricted to the striatum and lateral parietal cortex. Notably, the secondary atrophy of the striatum, which represents the core of the MCA territory, was significantly reduced by 20 mg/kg/day of Memantine at 49 dpt (**Fig. 15A**), while atrophy of whole brain (**Fig. 16A**) and the corpus callosum (**Fig. 16B**) were not significantly altered. Peri-infarct astrogliosis, evaluated by GFAP immunohistochemistry (**Fig. 15B**), was also significantly decreased by the high dose of Memantine at 49 dpt, while capillary density, evaluated by CD31 immunohistochemistry, was significantly increased (**Fig. 15C**).

However, Microglial activation, evaluated by Iba1 immunohistochemistry, was not altered by Memantine (**Fig. 16C**).

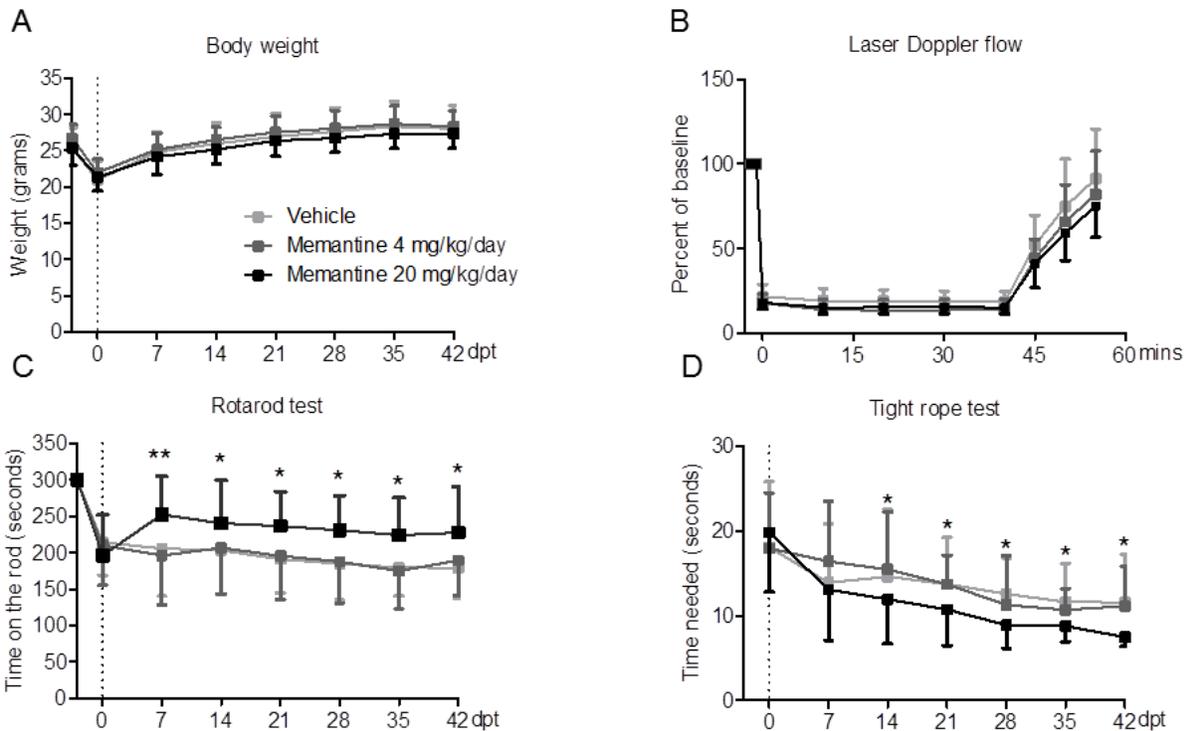


Figure 13. Delayed delivery of memantine at 20 mg/kg/day, but not 4 mg/kg/day promotes post-ischemic recovery of motor-coordination deficits. (A) Body weight, (B) LDF recordings above the core of the MCA lesion and (C, D) coordination skills evaluated by RotaRod and tight rope tests in animals exposed to tMCAO. Vehicle or Memantine (4 or 20 mg/kg/day) were subcutaneously administered over 28 days starting 72 hours post-reperfusion onset. Note the rapid improvement of coordination skills in animals receiving 20 mg/kg/day Memantine. LDF recordings (A) and body weight (B) do not differ between groups. Results are means \pm SD values (n=18 animals/ group). Data were analyzed by two-way repeated measurement ANOVA, followed by two-tailed unpaired t-tests for individual time-points. *p<0.05/ **p<0.01 compared with ischemic vehicle.

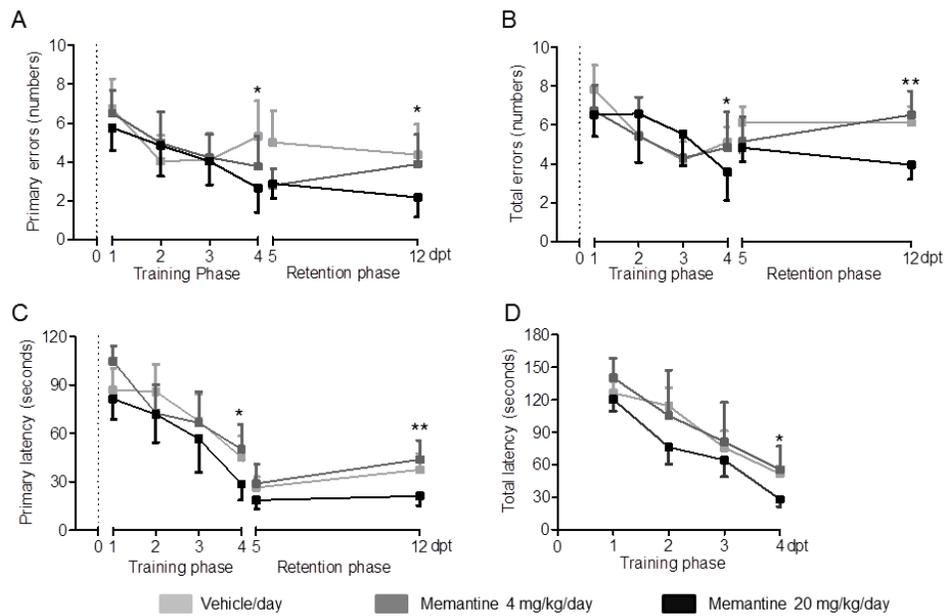


Figure 14. Delayed delivery of memantine at 20 mg/kg/day enhances post-ischemic spatial memory. (A) Primary errors in finding the target hole, (B) total escape errors, (C) primary latency until finding the target hole and (D) total escape latency in the Barnes maze test for animals exposed to tMCAO. Vehicle or Memantine (4 or 20 mg/kg/day) was subcutaneously administered over 28 days starting 72 hours after reperfusion onset. A memory training phase was followed by a memory retention phase, in which the target hole was blocked. Results are means \pm SD values (n=18 animals/ group). Data were analyzed by repeated measurement ANOVA, followed by two-tailed unpaired t-tests for individual time-points. *p<0.05/ **p<0.01 compared with ischemic vehicle.

4.1.4 Post-acute Memantine delivery increases growth factor levels in ipsilesional and contralesional brain hemisphere

To further characterize restorative responses to post-acute Memantine at the dose of 20 mg/kg/day, concentrations of selected growth factors were evaluated in the ipsilesional and contralesional cortex and striatum prior to treatment onset and at 14 and 28 dpt. Interestingly, concentrations of BDNF, GDNF and VEGF, but not FGF, were significantly increased at the dose of 20 mg/kg/day group in the ipsilesional striatum at 14 and 28 dpt and in the ipsilesional cortex, contralesional cortex and contralesional striatum at 28 dpt (Fig. 17 A-D).

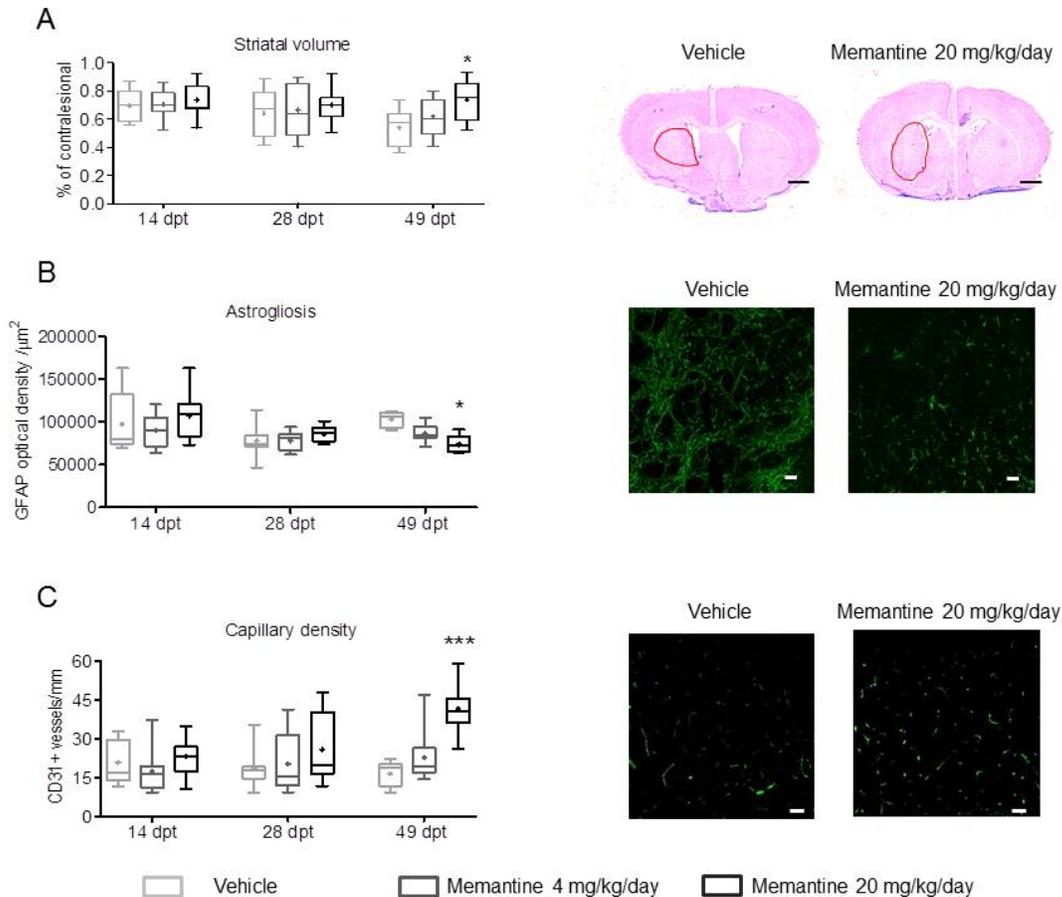


Figure 15. Delayed delivery of memantine at 20 mg/kg/day promotes peri-infarct brain remodeling. (A) Striatal volume, measured on cresyl violet-stained brains sections, (B) diffuse astroglial activation, evaluated by GFAP immunohistochemistry, and (C) capillary density, evaluated by CD31 immunohistochemistry, in animals exposed to tMCAO that were subcutaneously treated with vehicle or Memantine (4 or 20 mg/kg/day) starting 72 hours after reperfusion onset. Photomicrographs at 49 dpt are also shown. Optical density in (B) was evaluated in the peri-infarct (parietal) cortex, whereas capillary density in (C) was assessed in a total of six ROIs within the striatum, as described in the Materials and Methods section. Representative microphotographs from these brain regions are shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=12 animals/ group). Data were analyzed by two-way ANOVA followed by two-tailed unpaired t-tests for individual time-points. *p<0.05/ ***p<0.001 compared with ischemic vehicle. Bars, 1000 μm (A)/ 25 μm (B, C).

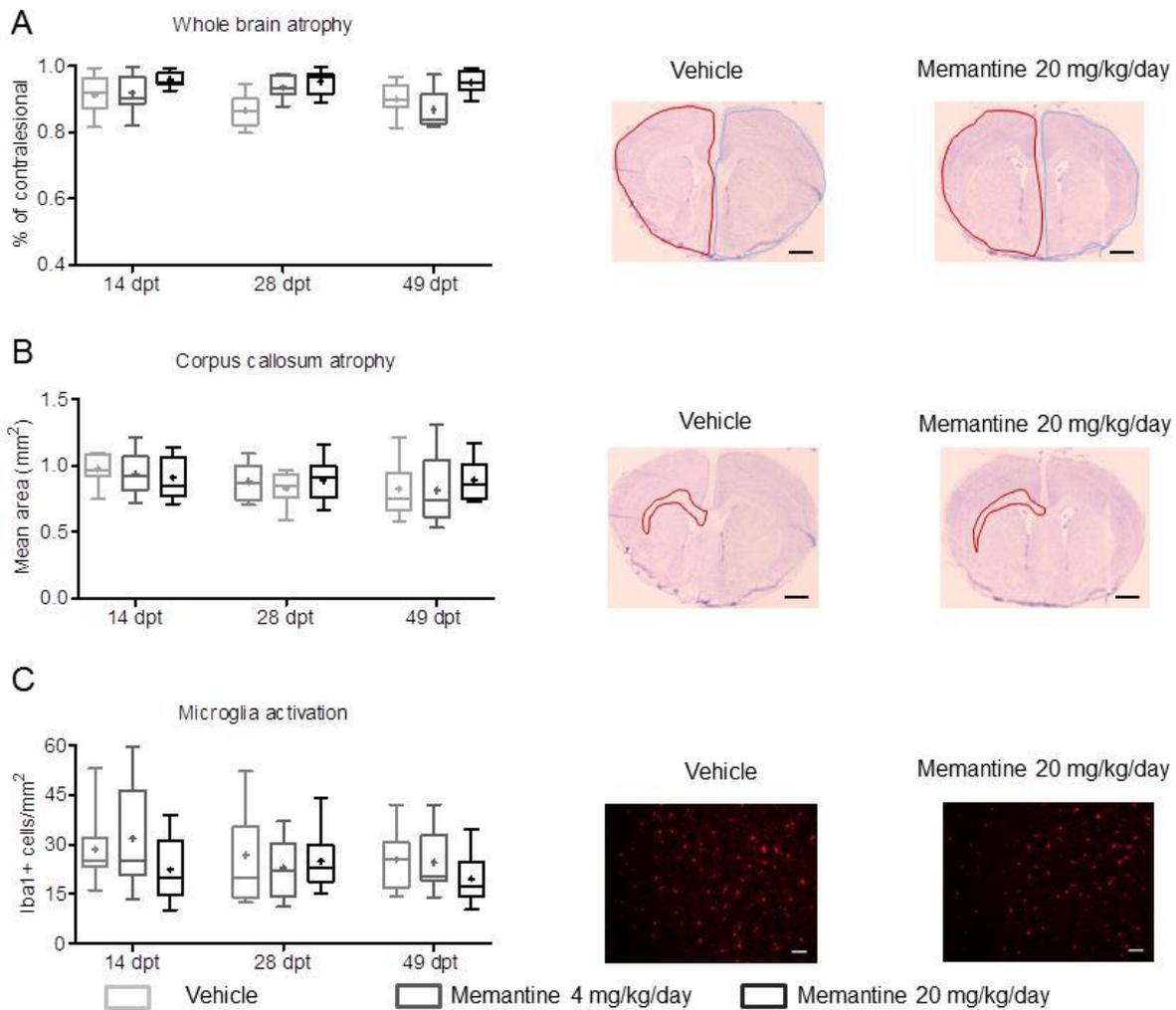


Figure 16. Memantine does not influence whole brain atrophy, corpus callosum atrophy, or microglial activation. (A) Whole brain atrophy and (B) corpus callosum atrophy, evaluated on cresyl violet-stained brains sections, as well as (C) microglial activation, evaluated by Iba1 immunohistochemistry, in animals exposed to tMCAO that were subcutaneously treated with vehicle or Memantine (4 or 20 mg/kg/day) starting 72 hours after reperfusion onset over 28 days. In (C), the density of Iba1+ activated microglia was assessed in a total of six regions of interest (ROI) in the striatum, as described in the Materials and Methods section. Representative microphotographs from these brain regions are shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm interquartile ranges (IQR; boxes) and minimum/ maximum data (elongation lines) (n=12 animals/ group). Data were analyzed by two-way analysis of variance (ANOVA) tests followed by two-tailed unpaired t-tests for individual time-points. No significant differences were observed. Bars, 1000 μ m (A, B)/ 25 μ m (C).

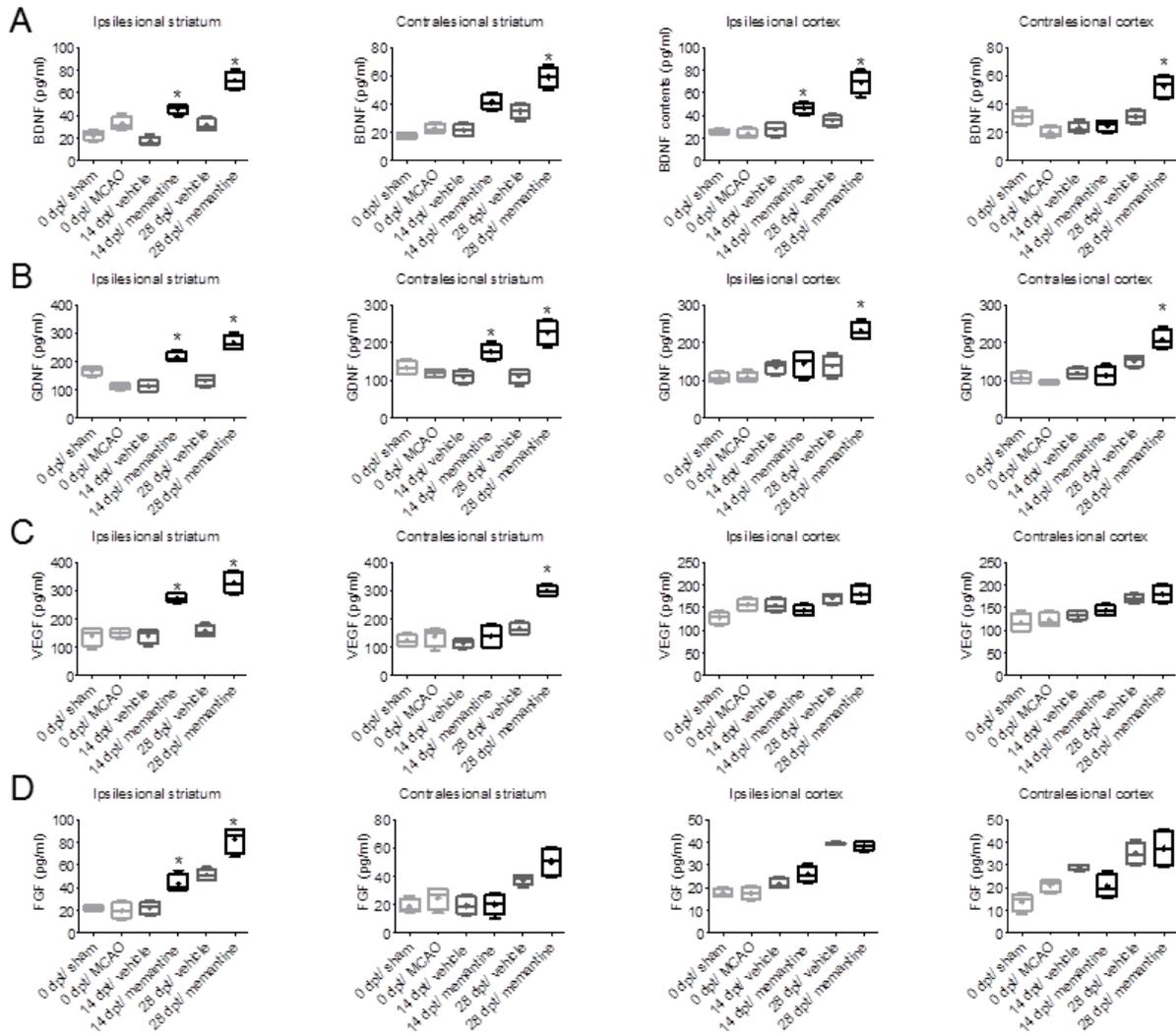


Figure 17. Delayed delivery of memantine at 20 mg/kg/day elevates growth factor concentrations both ipsilateral and contralateral to the stroke. Concentrations of (A) BDNF, (B) GDNF, (C) VEGF and (D) FGF in the ipsilesional striatum, contralesional striatum, ipsilesional motor cortex and contralesional motor cortex, evaluated by ELISA in animals exposed to tMCAO or sham-surgery that were subcutaneously treated with vehicle or Memantine (20 mg/kg/day) starting 72 hours after reperfusion onset. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=4 animals/ group). Data were analyzed by two-way ANOVA followed by two-tailed unpaired t-tests for individual time-points. *p<0.05 compared with ischemic vehicle.

4.1.5 Memantine promotes contralesional corticorubral plasticity

In response to growth factors, namely erythropoietin and VEGF, increased ipsilesional sprouting of contralesional pyramidal tract axons across the midline and towards brainstem targets has previously been reported (Reitmeir et al., 2011; 2012). Because we found elevated growth factors in the contralesional motor cortex, we asked whether Memantine influenced contralesional pyramidal tract plasticity as evaluated by the anterograde tract tracer BDA, which we injected into the contralesional motor cortex at 42 dpt (**Fig. 18A**). BDA-stained fibers originating from the contralesional motor cortex crossed the pyramidal tract to branch off dorsomedially at mesencephalic levels, terminating as previously described in the contralesional parvocellular red nucleus (Reitmeir et al., 2011). We quantified the number of midline-crossing terminal fibers emanating from this fiber bundle ipsilesional to the parvocellular red nucleus and normalized them to the total number of fibers in the contralesional pyramidal tract at the same rostrocaudal level. Compared with animals receiving vehicle, animals receiving 20 but not 4 mg/kg/day Memantine had significantly more midline crossing fibers (**Fig. 18B, C**). The pyramidal tract contralesional to the stroke was not altered by Memantine (**Fig. 18D**), indicating that Memantine did not affect pyramidal tract integrity.

4.1.6 Post-acute Memantine delivery prevents accumulation of tau protein

Tau5 accumulated in the peri-infarct area after the tMCAO (Fujii et al., 2016). Delivery of Memantine at 20 mg/kg significantly reduced tau5 during the treatment which may enhance cognition (**Fig. 19**).

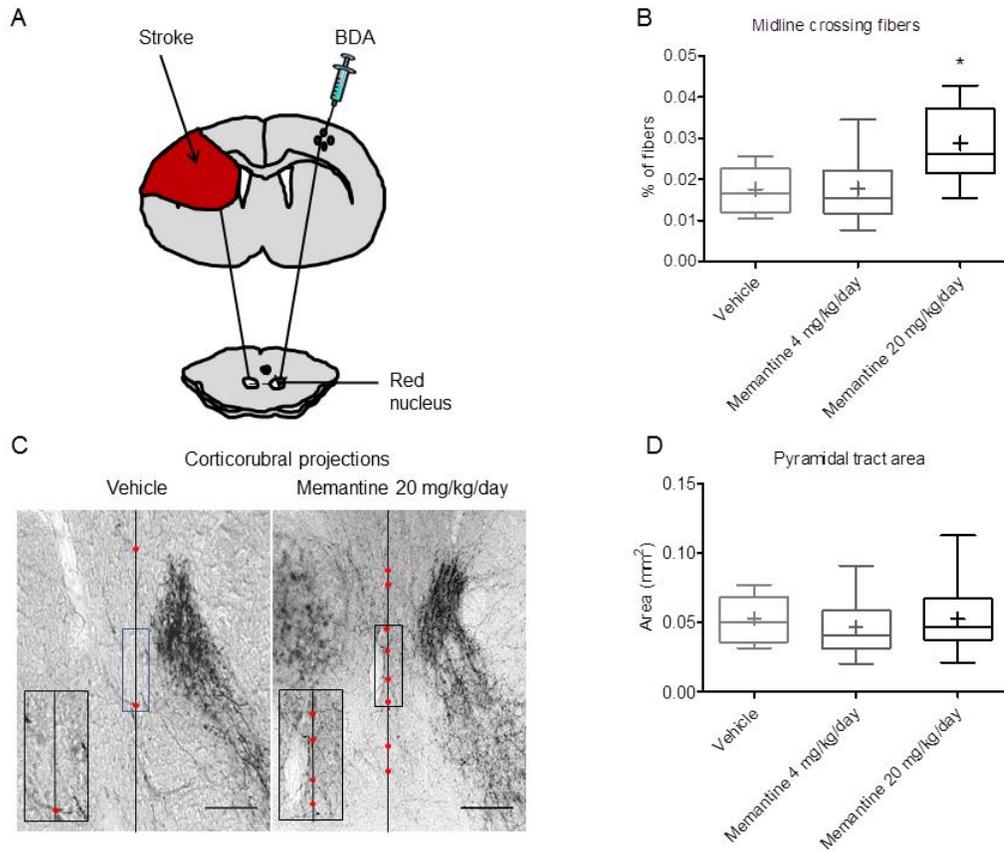


Figure 18. Delayed delivery of memantine at 20 mg/kg/day promotes contralesional corticorubral plasticity. (A) Midline-crossing fibers originating from the contralesional motor cortex ipsilesional to the parvocellular red nucleus were evaluated by injection of the anterograde tract tracer BDA into the contralesional motor cortex at 42 dpt. (B) Percentage of BDA-labeled midline-crossing fibers after subcutaneous delivery of vehicle or Memantine (4 or 20 mg/kg/day). Note the significant increase of midline-crossing fibers in response to 20 mg/kg/day, but not 4 mg/kg/day Memantine (representative microphotographs with magnified inlets are shown in (C)). (D) Total area of the contralesional pyramidal tract at the level of the red nucleus, which does not change in response to Memantine, indicating the absence of contralesional corticospinal tract degeneration. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=18 animals/ group). Data were analyzed by one-way ANOVA followed by LSD tests. *p<0.05 compared with ischemic vehicle. Bar, 100 μ m (B). In the ipsilesional motor cortex, Memantine markedly reduced the accumulation of tau protein at 28 dpt. In the contralesional motor cortex, tau protein was undetectable at all time-points.

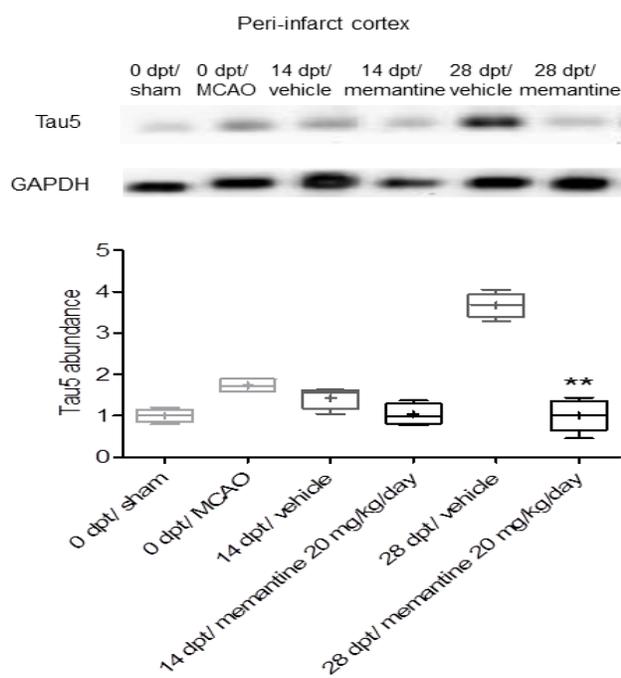


Figure 19. Delayed delivery of Memantine at 20 mg/kg/day reduces peri-infarct accumulation of tau protein. Western blot for tau protein in the peri-infarct (motor) cortex of animals exposed to tMCAO or sham-surgery that were subcutaneously treated with vehicle or Memantine (20 mg/kg/day) starting 72 hours after reperfusion onset. Note that the tau accumulation in the brains of vehicle-treated mice is markedly reduced by Memantine at 28 dpt. Representative Western blots are shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=4 independent blots). Data were analyzed by two-way ANOVA followed by two-tailed paired t-tests for individual time-points. **p<0.01 compared with corresponding ischemic vehicle.

4.1.7 Memantine regulates contralesional motor cortical levels of GluN2B, GluN2A, and PSD95

Because Memantine induced lesion-remote brain plasticity, we next examined how Memantine at a dose of 20 mg/kg/day influenced the abundance of the NMDA receptor subunits GluN2B, which predominantly localizes to extrasynaptic NMDA receptors targeted by Memantine, and GluN2A, which is preferentially found in synaptic NMDA receptors (Rush and Buisson, 2014). Additionally, we measured post-synaptic density protein-95 (PSD-95), which consolidates

synaptic plasticity upon NMDA receptor binding (Lai et al., 2011). In the contralesional motor cortex, the overall abundance of GluN2B, GluN2A and PSD-95 proteins was generally lower at 3days post-stroke than after sham-surgery (**Fig. 20A-C**). By 14 and 28 dpt, protein abundance was partially restored in vehicle-treated animals. Interestingly, Memantine reduced GluN2B, but not GluN2A or PSD-95 abundance in the contralesional motor cortex at 14 dpt (**Fig. 20A-C**). Conversely, Memantine increased GluN2A and PSD-95, but not GluN2B in the contralesional motor cortex at 28 dpt (**Fig. 20A-C**).

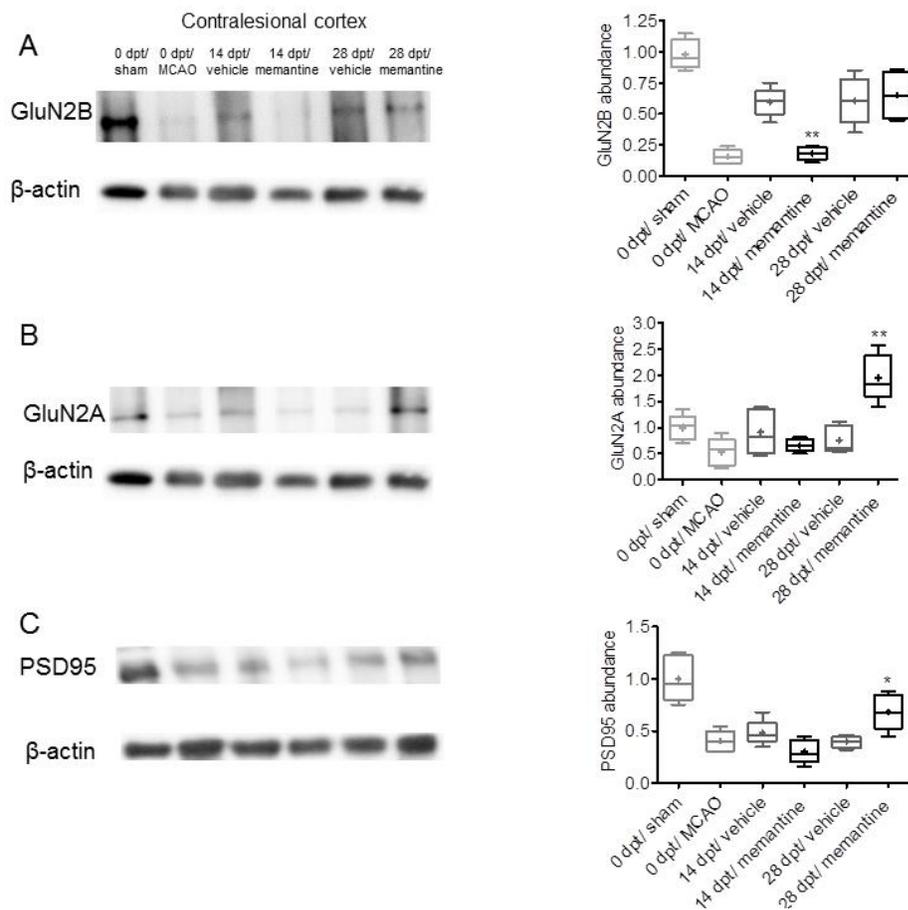


Figure 20. Delayed delivery of memantine at 20 mg/kg/day regulates contralesional motor cortical NMDA receptor components. Western blots for (A) the NMDA receptor subunit GluN2B, which predominantly localizes in extrasynaptic NMDA receptors targeted by Memantine, (B) GluN2A, which is preferentially found in synaptic NMDA receptors, and (C) PSD-95, which consolidates synaptic plasticity upon binding to NMDA receptors, in the contralesional motor cortex of animals exposed to tMCAO or sham-surgery. Note that in response

to stroke, all three proteins are reduced at 3 days after reperfusion. Protein levels are partly restored in vehicle-treated animals at 14 and 28 dpt. Interestingly, Memantine reduces GluN2B abundance at 14 dpt and increases GluN2A and PSD-95 abundance at 28 dpt. Representative blots are also shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=4 independent blots). Data were analyzed by two-way ANOVA followed by two-tailed paired t-tests for individual time-points. *p<0.05/ **p<0.01 compared with ischemic vehicle.

α 5 subunit receptor promotes the neurological recovery, peri-infarct tissue remodeling, and contralesional plasticity

4.2.1 Post-acute delivery of S44819 improves post-ischemic recovery

Body weight and LDF above the core of the middle cerebral artery did not reveal any differences between animals treated with vehicle or S44819 (3 or 10 mg/kg) (**Fig. 21 A, B**). In all groups, LDF decreased to ~15% of baseline during tMCAO, followed by a rapid blood flow recovery after reperfusion. Rotarod and tight rope tests revealed a clearly defined motor-coordination deficit after tMCAO that was attenuated by S44819 delivered at a dose of 10 mg/kg but not 3 mg/kg (**Fig. 21 C, D**).

4.2.2 Post-acute delivery of S44819 prevents anxiety

The improvement of motor-coordination skills was rapid, reaching significance by 14 dpt. Additionally, spontaneous motor activity, measured by speed and total distance covered, was mildly increased in the open field test at 7 dpt but not 28 dpt by 10 mg/kg, but not 3 mg/kg S44819 (**Fig. 22A, B**). Anxiety, measured time spent in the center of the open field arena, was significantly reduced by 10 mg/kg S44819 (**Fig. 22 D**). The time spent in the border and corner of the open field arena was unchanged in response to S44819 treatment (**Fig. 22 E, F**). However, this effect was not significant after Bonferroni correction for the number of comparisons made.

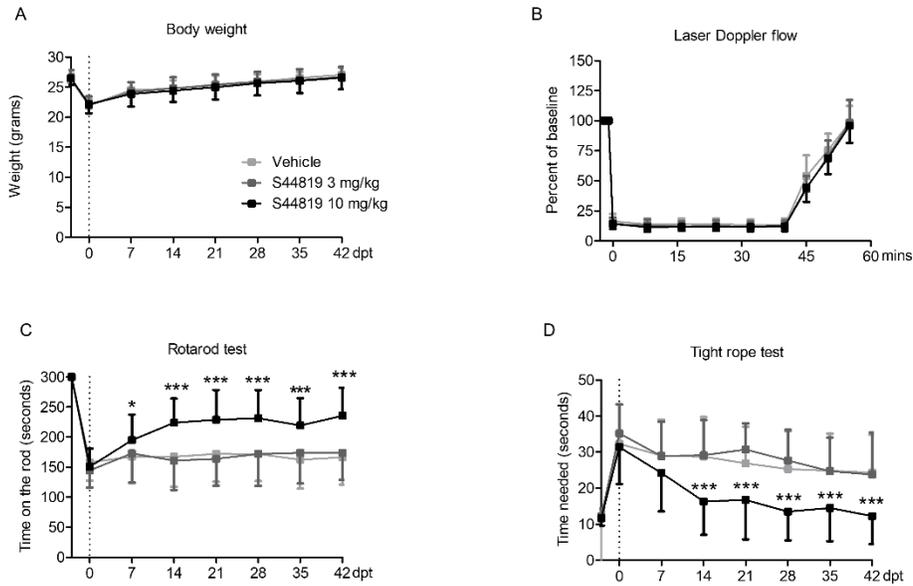


Figure 21. Delayed delivery of S44819 at 10 mg/kg, but not 3 mg/kg promotes post-ischemic recovery of motor-coordination deficits. (A) Body weight, (B) LDF recordings above the core of the middle cerebral artery and (C, D) coordination skills evaluated by RotaRod and tight rope tests in mice exposed to tMCAO. Vehicle or S44819 (3 or 10 mg/kg b.i.d.) were administered over 28 days starting 72 hours after reperfusion. Note the rapid improvement of coordination skills in mice receiving 10 mg/kg S44819. LDF recordings (A) and body weight (B) do not differ between groups. Results are means \pm SD values (n=18 animals/ group). Data were analyzed by two-way repeated measurement ANOVA, followed by Bonferroni-corrected two-tailed t-tests at individual time-points. * $p < 0.05$ / ** $p < 0.01$ compared with ischemic vehicle.

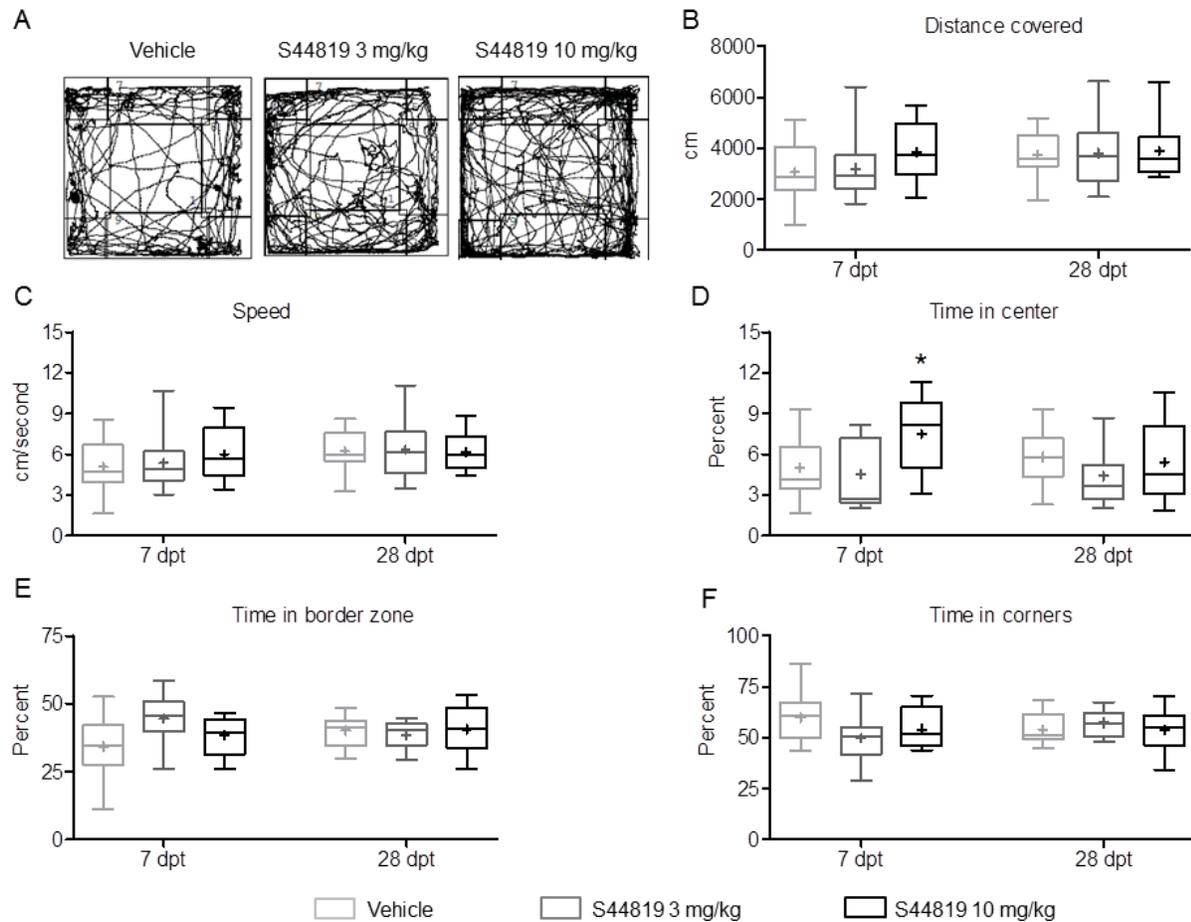


Figure 22. Delayed delivery of S44819 at 10 mg/kg, but not 3 mg/kg enhances post-ischemic exploration behavior. (A) Distance covered, (B) speed, (C) time in center, (D) time in border zone and (E) time in corner in open field tests of mice exposed to tMCAO. Vehicle or S44819 (3 or 10 mg/kg b.i.d.) was administered over 28 days starting 72 hours after reperfusion. Note the increased percentage of time spent in the center of the open field arena in mice receiving 10 mg/kg S44819, indicative of reduced anxiety. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=18 animals/group). Data were analyzed by one-way ANOVA for both time-points, followed by Bonferroni-corrected two-tailed t-tests. *p<0.05 compared with ischemic vehicle.

4.2.3 Post-acute delivery of S44819 enhances cognitive improvement

Spatial memory, evaluated as the number of errors in the retention phase of the Barnes maze test, was improved by 10 mg/kg, but not 3 mg/kg S44819 (**Fig. 23 A**). Latency until finding the target hole, on the other hand, did not change after S44819 delivery (**Fig. 23 B**).

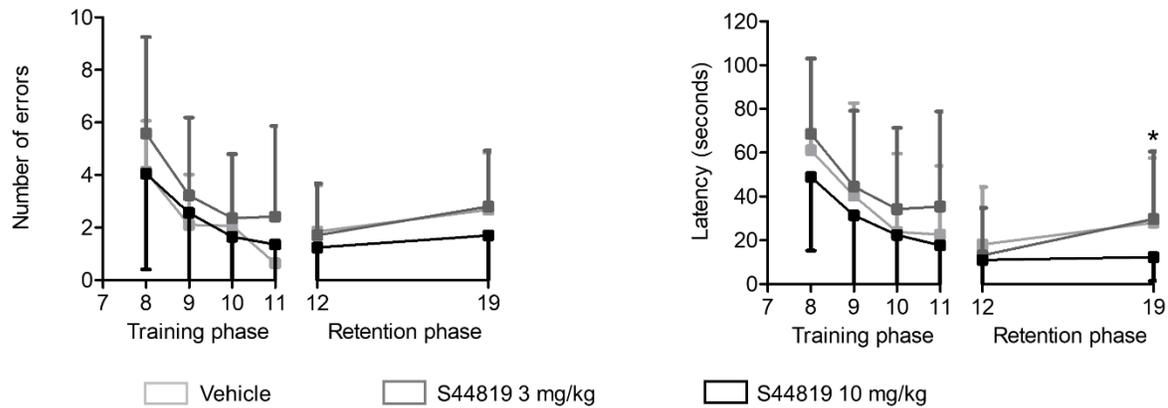


Figure 23. Delayed delivery of S44819 at 10 mg/kg, but not 3 mg/kg enhances post-ischemic spatial memory. (A) Number of errors and (B) latency until finding the target hole of the Barnes maze test in animals exposed to tMCAO. Vehicle or S44819 (3 or 10 mg/kg b.i.d.) were administered over 28 days starting 72 hours after reperfusion. A memory training phase was followed by a retention phase, in which the target hole was blocked, and memory was evaluated. Results are means \pm SD values (n=18 animals/ group). Data were analyzed by repeated measurement ANOVA, followed by Bonferroni-corrected two-tailed t-tests at individual time-points. *p<0.05 compared with ischemic vehicle.

4.2.4 Post-acute delivery of S44819 promotes peri-infarct tissue remodeling

We next performed histochemical studies to assess perilesional remodeling in response to S44819. Cresyl violet stains revealed localized infarcts that were restricted to the striatum and lateral parietal cortex. Notably, secondary atrophy of the striatum, which represents the core of the middle cerebral artery territory, was significantly reduced by 10 mg/kg, but not 3 mg/kg S44819 at 42 dpt (**Fig. 24A**), while whole brain atrophy was not significantly altered.

Neuron survival in the striatum was significantly increased by 10 mg/kg, but not 3 mg/kg S44819 at 42 dpt, as shown by immunohistochemistry for the neuronal marker NeuN (**Fig. 24B**). Post-acute S44819 delivery reduces peri-infarct astrogliosis and increases brain capillary density peri-infarct astrogliosis, evaluated by GFAP immunohistochemistry in brain sections. These results were significant at 10 mg/kg, but not 3 mg/kg S44819 at 14, 28 and 42 dpt (**Fig. 24C**). While brain capillary density, evaluated by CD31 immunohistochemistry, was significantly increased at 42 dpt (**Fig. 24D**).

4.2.5 Post-acute delivery of S44819 increases growth factor levels in the ipsilesional striatum but does not affect the contralesional striatum

To further characterize restorative responses to post-acute S44819, we next evaluated the concentrations of growth factors in peri-infarct brain tissue prior to treatment onset, at 14, 28, and 42 dpt. Concentrations of both the neurotrophic growth factors BDNF and GDNF and the preferentially angiogenic growth factors VEGF and FGF were significantly elevated in peri-infarct brain tissue by 10 mg/kg, but not 3 mg/kg S44819 at 28 dpt (GDNF, FGF) and 42 dpt (all four growth factors) (**Fig. 25A-D**). However, S44819 did not affect growth factor levels in the contralesional striatum (**Fig. 26E-H**).

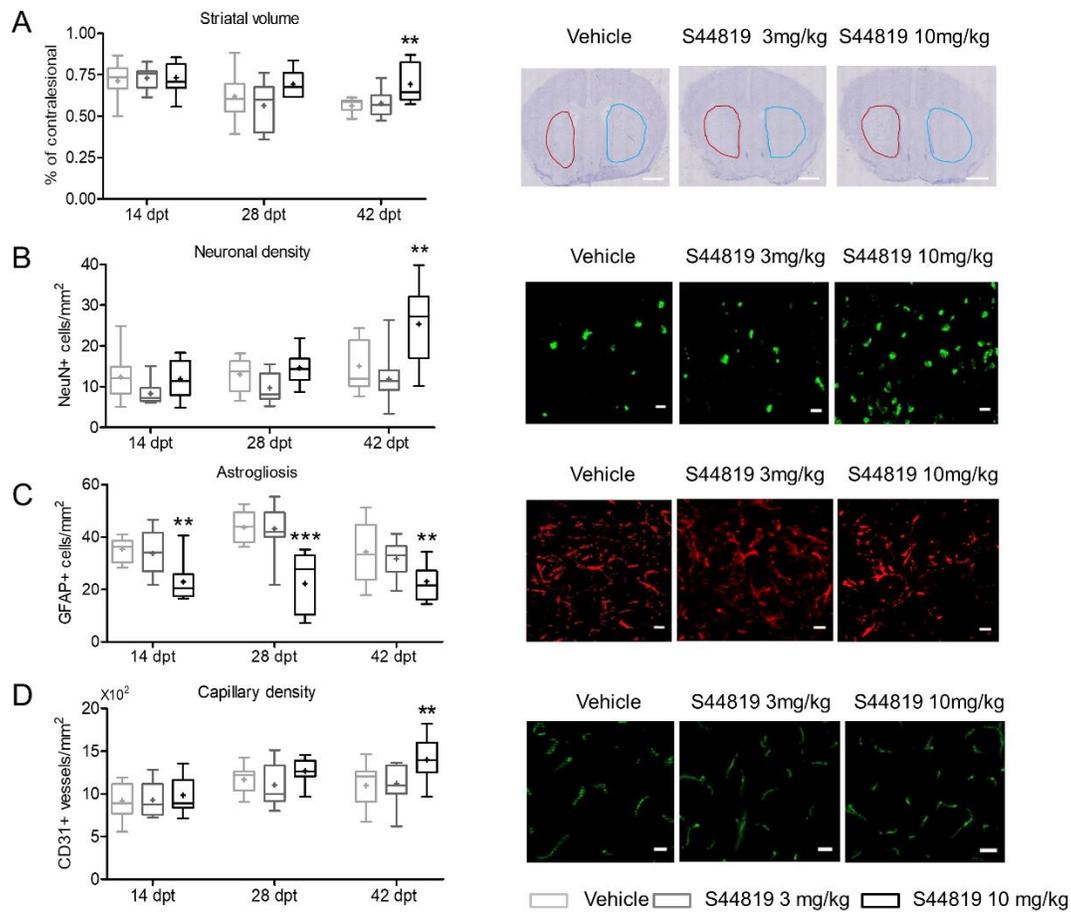


Figure 24. S44819 prevents delayed neurodegeneration and promotes peri-infarct brain remodeling. (A) Striatal volume, measured on cresyl violet-stained brain sections, (B) neuron survival, assessed by NeuN immunohistochemistry, (C) astrogliosis, examined by GFAP immunohistochemistry, and (D) capillary density, evaluated by CD31 immunohistochemistry, in mice exposed to transient tMCAO that were treated with vehicle or S44819 (3 or 10 mg/kg b.i.d.) 72 hours after reperfusion. Photomicrographs at 42 dpt are also shown. Cell and capillary densities were assessed in six ROIs within the striatum, as described in the Materials and Methods section. Representative microphotographs are shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) ± IQR (boxes) and minimum/ maximum data (elongation lines) (n=6 [14 and 28 dpt] or n=18 [42 dpt] animals/group). Data were analyzed by two-way ANOVA followed by Bonferroni-corrected two-tailed t-tests at individual time-points. *p<0.05/**p<0.01/**p<0.001 compared with ischemic vehicle. Bars, 200 μm (A, B)/ 50 μm (C, D).

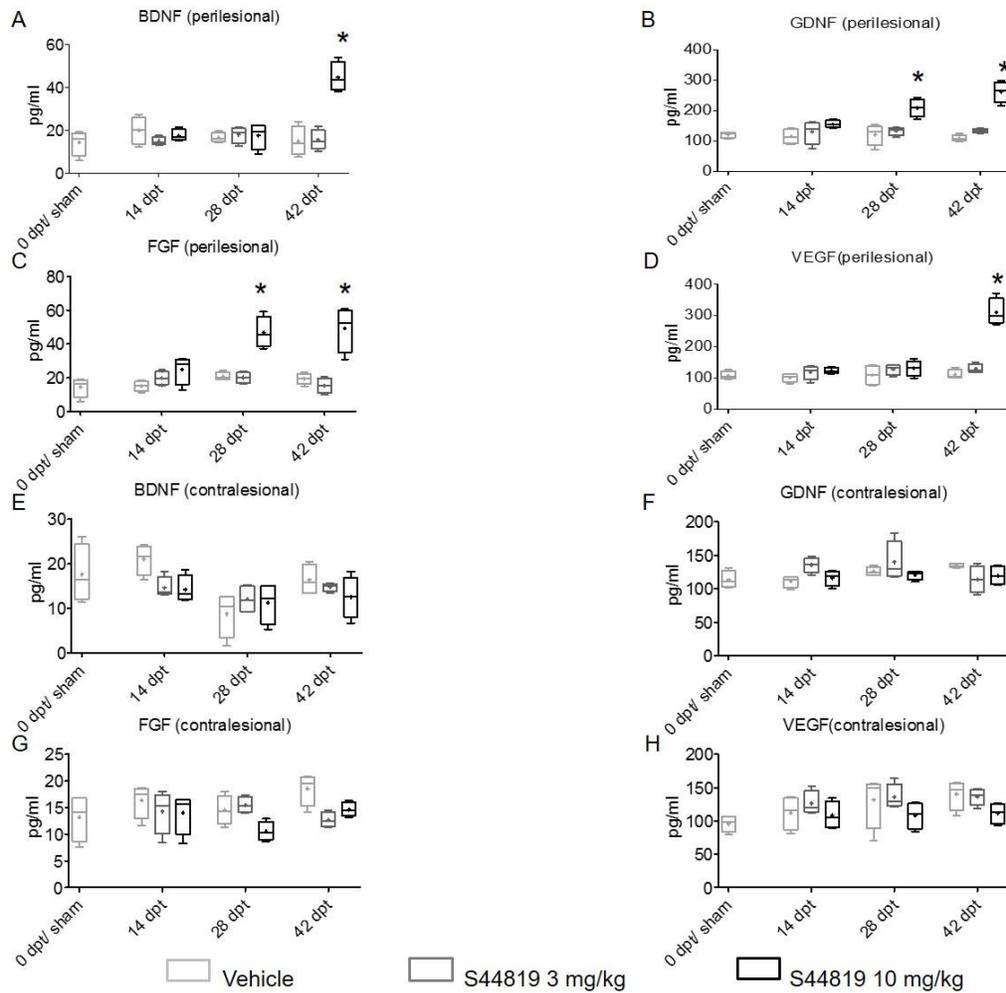


Figure 25. Delayed delivery of S44819 at 10 mg/kg, but not 3 mg/kg elevates neurotrophic and angiogenic growth factors in peri-infarct brain tissue, but not in contralesional striatum. Concentrations of (A) BDNF, (B) GDNF, (C) VEGF and (D) FGF in peri-infarct brain tissue and (E) BDNF, (F) GDNF, (G) FGF and (H) VEGF evaluated by ELISA in mice exposed to tMCAO or sham-surgery that were treated with vehicle or S44819 (3 or 10 mg/kg b.i.d.) 72 hours after reperfusion. Note the increase of neurotrophic (BDNF, GDNF) and angiogenic (VEGF) growth factors in response to S44819 delivery. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=4 animals/group). Data were analyzed by two-way ANOVA followed by Bonferroni-corrected two-tailed t-tests at individual time-points. *p<0.05 compared with ischemic vehicle.

4.2.6 Post-acute delivery of S44819 does not affect contralesional corticorubral plasticity

Because growth factors were elevated in the contralesional motor cortex (Reitmeir et al., 2011; 2012), we next asked whether S44819 influenced contralesional pyramidal tract plasticity, which was assessed by the anterograde tract tracer BDA, through injection of BDA into the contralesional motor cortex at 42 dpt (Fig. 26A). We quantified the number of midline-crossing terminal fibers emanating from the level of the red nucleus that were ipsilesional to the parvocellular red nucleus and normalized them to the total number of fibers in the contralesional pyramidal tract at the same rostrocaudal level at 49 dpt. There were no significant differences in the number of midline crossing fibers for animals given vehicle or S44819 (3 or 10mg/kg/day) (Fig. 26B, C). In addition, the pyramidal tract area contralesional to the stroke was not altered by S44819 (Fig. 26D).

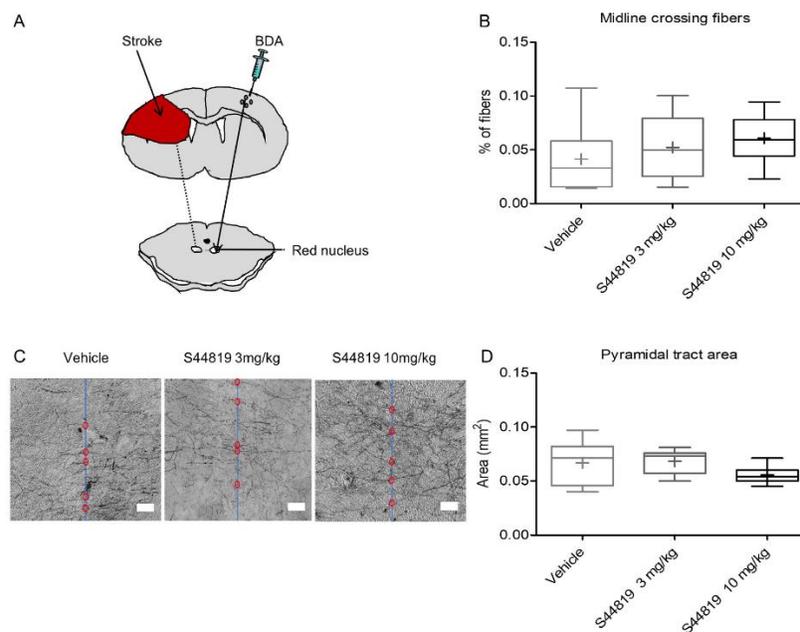


Figure 26. Delayed delivery of S44819 at 10 mg/kg/day does not affect contralesional corticorubral plasticity. (A) Midline-crossing fibers originating from the contralesional motor cortex ipsilesional to the parvocellular red nucleus were evaluated by injection of the anterograde tract tracer BDA into the contralesional motor cortex at 42 dpt. (B) Percentage of BDA-labeled midline-crossing fibers after delivery of vehicle or Memantine (3 or 10 mg/kg/day). Note the midline-crossing fibers in response to treatment (representative microphotographs with magnified

inlets are shown in (C)). (D) Total area of the contralesional pyramidal tract at the level of the red nucleus. The total area did not change in response to S44819, indicating the absence of contralesional corticospinal tract degeneration. Results are medians (lines inside boxes)/ means (crosses inside boxes) \pm IQR (boxes) and minimum/ maximum data (elongation lines) (n=18 animals/ group). Data were analyzed by one-way ANOVA followed by LSD tests. *p<0.05 compared with ischemic vehicle. Bar, 100 μ m (B). In the ipsilesional motor cortex, Memantine markedly reduced the accumulation of tau protein at 28 dpt. In the contralesional motor cortex, tau protein was undetectable at all time-points.

4.2.7 Post-acute delivery of S44819 promotes GABA_A subunit expression

S44819 increases GABA_A α 5 and Signal transducer and activator of transcription 3 (Stat3) abundance in peri-infarct brain tissue. Because S44819 induced functional neurological recovery, we next examined effects of S44819 on the abundance of the GABA_A α 5 receptor subunit, which is the molecular target of S44819, and of Stat-3, which controls GABA_A receptor transcription in a BDNF-dependent fashion, using Western blots. At a dose of 10 mg/kg, S44819 increased the abundance both of GABA_A α 5 subunit and Stat-3 in peri-infarct brain tissue at 14 dpt (**Fig. 26**). Our data indicate an endogenous compensatory reaction through the reduction of GABA_A receptor activity in response to S44819 treatment.

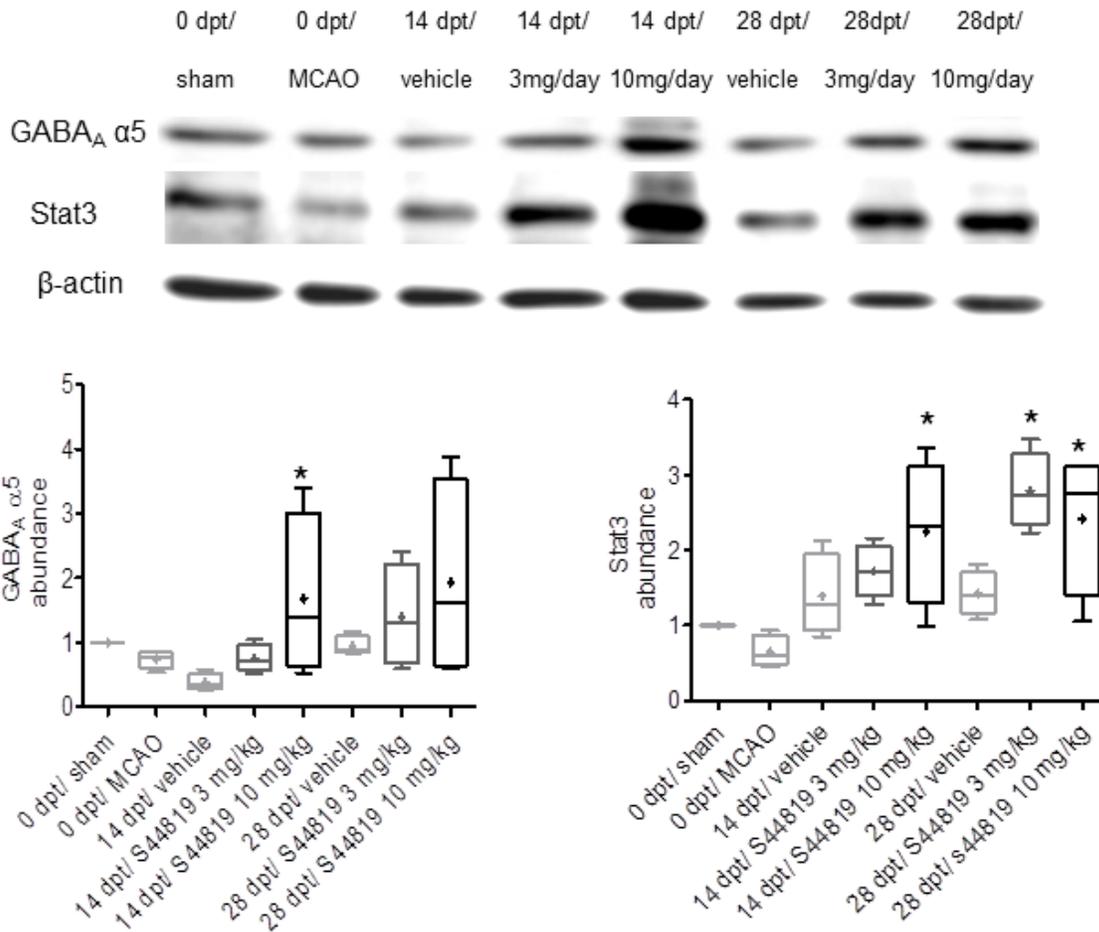


Figure 27. Delayed delivery of S44819 at 10 mg/kg, but not 3 mg/kg increases GABA_A α5 and Stat-3 abundance in peri-infarct brain tissue. Western blots for (A) the α5 subunit of the GABA_A receptor and (B) Stat-3, which controls GABA_A receptor transcription, in the peri-infarct brain of mice exposed to tMCAO or sham-surgery treated with vehicle or S44819 (3 or 10 mg/kg b.i.d.) 72 hours after reperfusion. Note that 10 mg/kg S44819 increases GABA_A α5 and Stat-3 abundance in peri-infarct brain tissue at 14 dpt. Representative blots are also shown. Results are medians (lines inside boxes)/ means (crosses inside boxes) ± IQR (boxes) and minimum/maximum data (elongation lines) (n=4 separately processed blots). Data were analyzed by two-way ANOVA followed by Bonferroni-corrected two-tailed t-tests at individual time-points. *p<0.05 compared with ischemic vehicle.

5. DISCUSSION

5.1 General comments on post-acute therapy

Thrombolysis with intravenous tPA is still the only FDA approved treatment for acute ischemic stroke (Murata et al., 2008). However, its widespread application is constrained by narrow treatment time windows 4.5h after stroke onset and the related risks of cerebral hemorrhage (Bambauer et al., 2006; Weintraub, 2006). In late 2014, results from the MR CLEAN trial indicated that endovascular mechanical recanalization was effective and safe among patients with ischemic stroke caused by proximal intracranial arterial occlusion, who were treated with endovascular thrombectomy 6 hours after acute ischemic stroke (Berkhemer et al., 2015). The following five more studies also confirmed its positive results (Goyal., 2015; Campbell BC., 2015; Jovin et al., 2015; Saver et al., 2015; Bracard et al., 2015). Furthermore, mechanical thrombectomy can be effective in selective patients with occlusion of proximal vessels in the anterior circulation up to 24 h after suspected onset of symptoms (Nogueira et al., 2018; Albers et al., 2018). Previous work in our lab demonstrated that delivery of EPO and VEGF reduced the astrogliosis, inflammation, and promoted contralesional plasticity and neurological recovery (Reitmeir et al., 2011; 2012; Herz et al., 2012). However, a phase II/III German multicenter trial in which EPO was delivered together with rtPA during recanalization produced an unexpected increase in mortality (Ehrenreich et al., 2009). We confirmed this in a model of experimental stroke by demonstrating that EPO administered together with rtPA increases brain edema, blood-brain barrier permeability, and MMP-9 activity, providing insight into the unexpected fatal response found in patients (Zechariah et al., 2010). Despite finding that suggest VEGF promotes angiogenesis, enhances neurological function, and increases contralesional plasticity, there are currently no clinical trials testing for the therapeutic benefit of VEGF in stroke (Reitmeir et al., 2012; Herz et al., 2012). The current state of the field highlights the need to identify therapeutic time windows for stroke therapy and for the clear characterization of molecular mechanisms that are modulated by potential therapeutic agents (Sanchez-Mendoza and Hermann, 2016).

Memantine and S44819 both compounds with safe profiles and thus have high translational potential in the clinical stroke practice. Memantine, which is already used in the treatment of dementia, has no reported side effects (Lipton, 2006). S44819 passed a phase I clinical trial,

demonstrating that it increased cortical excitability in healthy human subjects without side effects (Darmanni et al., 2016). Currently, Memantine is undergoing a phase I clinical stroke trial in the United States (<https://clinicaltrials.gov/ct2/show/NCT02144584>), while S44819 is in a phase II study spanning 86 centers in 14 countries (<https://clinicaltrials.gov/ct2/show/NCT02877615>) based the data presented here. Our data demonstrate that delivery of both Memantine and S44819 starting at 72 hours post-stroke enhance motor coordination and spatial memory, prevent degeneration, reduce peri-infarct astrogliosis, and increase brain capillary density, thus confirming that both Memantine and S44819 could be promising therapeutic agents for stroke patients.

5.2 Post-acute delivery of Memantine promotes post-ischemic neurological recovery, peri-infarct tissue remodeling, and contralesional brain plasticity

Using a series of behavioral tests to evaluate the effect of the NMDA receptor antagonist Memantine for stroke recovery, we found that delivery of Memantine at 72 hours post stroke enhanced motor coordination as early as 7 dpt, and spatial memory at 12dpt (**Fig. 13, 14**). Accordingly, Memantine produced tissue remodeling, preventing neuronal degeneration, reducing peri-infarct astrogliosis, and increasing brain capillary density (**Fig. 15**). Interestingly, Memantine increased the concentrations of the growth factors BDNF, GDNF and VEGF in both hemispheres (**Fig. 17**). Additionally, anterograde tract tracer analysis showed that Memantine enhanced contralesional corticorubral plasticity (**Fig. 18**). Molecular studies revealed that delivery of Memantine reduced the expression of NR2B at 14 dpt and increased the expression of NR2A and PSD95 at 28 dpt, while tau5 accumulation at 28 dpt was reduced (**Fig. 19, 20**).

In contrast to previous work in our lab, we were surprised that Memantine enhanced motor-coordination almost immediately within 7-14 dpt (**Fig. 13**). In this respect, Memantine differed from studies using growth factors EPO and VEGF (Hermann et al., 2001; Reitmeir et al., 2011; 2012), NPCs (Hata et al., 2000), and hydroxymethylglutaryl-CoA reductase (HMG-CoA) inhibitor delivery (Kilic et al., 2014), where we previously observed slow evolving motor-coordination improvements that became detectable only after several weeks of treatment using the same tMCAO model. The most likely explanation for such an early effect is that Memantine has an immediate moderate inhibitory effect on extrasynaptic NMDA receptors, which likely focused glutamatergic signaling on NR2A subunits. Such subunits have been previously linked to neuroprotection and enhanced neuronal plasticity (Lipton, 2004). We did not find growth factors

significantly increased levels until after 14dpt (**Fig. 17**). Previous work from our lab demonstrated that intraventricular delivery of VEGF at a dose of 20 ng per day enhanced motor coordination at 28 dpt (Reitmeir et al., 2012; Herz et al., 2012). In the current study, Memantine increased the concentration of GDNF, VEGF and BDNF to concentrations ranging between 0.06 to 0.3 ng/ml starting at 14 dpt (**Fig. 17**), one week after motor coordination had already improved, suggesting that growth factor secretion might support but not initiate neurological recovery.

Memantine also enhanced spatial memory. Alzheimer's disease is a devastating neurodegenerative disorder that is triggered by the accumulation of the amyloid- β peptide and hyperphosphorylation of Tau (Chabrier, 2014, Snaphaan et al., 2009). Hyperphosphorylated Tau has also been found in ischemic stroke (**Fujii et al., 2016**) where Tau phosphorylation causes Tau to remove the tubulin cytoskeleton, giving access to katanin to cut down axons. Thus, hyperphosphorylation of Tau is indirectly related to memory. Our Western blot results demonstrated that the delivery of Memantine reduced Tau5 accumulation at 28 dpt (**Fig. 19**). Therefore, Memantine might have enhanced spatial memory by reducing phosphorylated Tau accumulation. Lowering hyperphosphorylated tau may be protective by preventing axonal breakdown by Katanin and Spastin. Because most patients have cognitive degeneration after stroke, Memantine could prevent the progression of dementia in patients who survive stroke by delaying Tau hyperphosphorylation. Structural neuroprotective effects have been found after acute Memantine delivery after focal cerebral ischemic in rats and mice (Lipton, 2004; Wu and Johnson, 2015; Chen et al., 1998). Interestingly, in two more recent studies, Memantine reduced behavioral deficits without affecting structural brain injury, when delivered up to 2 hours post-stroke (Babu and Ramanathan, 2009; López-Valdés et al., 2014), suggesting that Memantine may have restorative actions in the acute phase of stroke in addition to its neuron survival promoting effects. In one study using photothrombotic stroke in mice (López-Valdés et al., 2014), Memantine decreased peri-infarct astrogliosis and increased capillary density. Considering the early Memantine delivery, underlying neuroprotective effects may also be considered as part of the overall response of the brain in our study. Prevention of secondary brain atrophy, reactive astrogliosis, microglial activation, and promotion of angiogenesis have repeatedly been described as surrogates of brain remodeling post-stroke (Hata et al., 2000; Hermann et al., 2001; Reitmeir et al., 2011; 2012).

The promotion of contralesional corticorubral plasticity, which outlasted the period of Memantine delivery, suggests the effects of Memantine are long-lasting. Importantly, our group has previously

shown that EPO and VEGF promoted contralesional pyramidal tract plasticity after stroke (Reitmeir et al., 2012; Herz et al., 2012). Following acute Memantine delivery, increased abundance of BDNF and its receptor Trk-B (tyrosine receptor kinase B) were associated with ipsilesional sensory forepaw map remodeling (López-Valdés et al., 2014). Indeed, enhanced sprouting of BDA-labeled corticorubral fibers originating from the contralesional motor cortex ipsilesional to the red nucleus was noted in Memantine-treated mice (**Fig. 18**). Thus, by demonstrating changes in growth factors and pyramidal tract plasticity contralateral to the stroke, we provide evidence that Memantine induces profound neuronal rewiring that is not restricted to the lesion rim.

Mechanistically, Memantine may have promoted neuron survival and neuronal plasticity by inhibiting the activation of cell death pathways and by favoring the interaction of NR2A with PSD95. NR2A and NR2B subunits containing NMDA receptors have been proposed to play a key role in both neuronal cell function and dysfunction (Rush et al., 2014). The synaptically located NR2A subunit inhibits neuronal death pathways and promotes neuronal plasticity by activation of downstream signals which involve RhoGTPases (Ponimaskin et al., 2007). In contrast, the extrasynaptic NR2B glutamate receptors are strongly activated upon stroke and stimulate neuronal death pathways and impede neuronal plasticity (Tu et al., 2010; Martel et al., 2009; Bordji et al., 2010; Rush et al., 2014; Wu et al., 2015). As a non-competitive antagonist of NR2B, Memantine preferentially blocks neurotoxicity mediated by the extrasynaptic NMDARs without disrupting physiological synaptic activity (Hardingham et al., 2002; Chen et al., 2008). Our data demonstrate that delivery of Memantine reduced the NR2B expression at 14 dpt and increased the NR2A expression at 28 dpt (**Fig. 20**), coinciding with a significant increase of VEGF, GDNF and BDNF (**Fig. 17**). Thus, Memantine blocked neuronal death pathways and promoted the survival pathways. Indeed, Memantine has been found to suppress the activation of the calpain-caspase-3 pathway and cell apoptosis in tMCAO model (Chen et al., 2017). These data provide a molecular basis for the role of Memantine in reducing neuronal apoptosis and enhancing neuron survival, suggesting that Memantine may be a promising therapy for stroke patients (Chen et al., 2017).

Additionally, synaptic NMDA receptors form large and dynamic signaling complexes, mainly through the interaction of their intracellular NR2 C-terminus with PSD-95, which as a scaffolding protein attached to the NR2 C-terminus in its postsynaptic membrane (Gascón et al., 2008; Cousins

and Stephenson, 2012). Previous reports showed downregulation of the NR1 and NR2B receptor subunits and PSD95 after the ischemia (Gascón et al., 2008), which were in line with our data. PSD-95 uncoupled from NR2B can prevent neuronal death without blocking normal NMDA receptor function (Aarts et al., 2002). Furthermore, NR2A subunits interact directly with PSD-95 membrane-associated guanylate kinase via the C-terminal conservative amino acids motif, while NR2B does not (Cousins and Stephenson, 2012). Our data demonstrated that Memantine also enhanced the PSD95 and NR2A abundance in parallel at 28 dpt, while reducing NR2B abundance at 14 dpt (**Fig. 20**). Therefore, one additional mechanism through which Memantine may have promoted neuron survival and neuronal plasticity could be through the enhancement of the interaction between PSD95 and NR2A, which then leads to the activation of secondary cell survival pathways.

The clear strength of this study is the efficacy of an NMDA antagonist that is already clinically approved for patients suffering from Alzheimer's disease. By demonstrating that Memantine promotes post-ischemic neurological recovery, brain remodeling, and plasticity, we provide a rationale for the use of Memantine in stroke patients in the post-acute stroke phase. The dose of Memantine we used was higher than what is given to human patients (20 mg per patient per day). Yet, in view of the far longer plasma half-life of Memantine in humans (60-100 hours) compared to rodents (2-5 hours), Memantine plasma levels are similar (~0.5-1.0 μM), with brain levels being ~30-40% lower (25). The main limitation of this study was the use relatively simple motor-coordination tests (compared to what might be used in humans), in which we were able to document an improvement of stroke recovery with a statistical power (1 – beta error) of 80%, given that the alpha error was 5%. Our data open fascinating perspectives for clinical studies, in which NMDA receptor antagonists might be re-evaluated in the post-acute stroke setting.

5.3 Post-acute delivery of GABA_A $\alpha 5$ antagonist S44819 promotes neurological recovery and peri-infarct brain remodeling after transient focal cerebral ischemic in mice

Behavioral testing was used to confirm the hypothesis that delivery of S44819 at 72 hours post stroke should enhance motor coordination (**Fig. 21**). We also observed that mice treated with S44819 explored more in the open field test (**Fig. 22**) and had improved spatial memory in the

Barnes maze test. (**Fig. 23**). Functional neurological recovery with treatment of S44819 was associated with tissue remodeling, preventing neuronal degeneration, reducing peri-infarct astrogliosis, and increasing brain capillary density (**Fig. 24**). Interestingly, delivery of S44819 at a dose of 10 mg/kg also increased the concentrations of VEGF, FGF, BDNF and GDNF in the peri-infarct tissue, but not in contralesional tissue, indicating that responses were specific to the damaged hemisphere (**Fig. 25**). In line with the lack of growth factor elevations in the contralesional hemisphere, S44819 did not promote contralesional corticorubral sprouting (**Fig. 26**). Tonic inhibition is a perilesional event. Hence, its reversal by S44819 suggests brain remodeling mainly in the surrounding of the ischemic brain infarct. Peri-lesional tissue responses were associated with peri-lesional molecular responses to S44819 delivery. Hence, our data revealed that S44819 increased the GABA_A receptors alpha5 and Stat3 abundance in the perilesional brain tissue (**Fig. 27**). Our data indicate that modulation of tonic GABAergic inhibition or deactivation the GABA_A α 5 could be a promising strategy for stroke recovery (Clarkson et al., 2010).

In models of permanent tMCAO, i.e., photothrombotic stroke in mice and cortical endothelin microinjection in rats, improvement of neurological recovery has been previously reported following delayed subcutaneous or intraperitoneal delivery of the inverse GABA_A agonist L655,708 (Lake et al., 2015; Quirk et al., 1995), which was similar to S44819, attenuated tonic GABAergic inhibition. The recovery-promoting effect of L655,708 was absent in ischemic GABA_A α 5^{-/-} mice, indicating that the deactivation of the GABA_A α 5 subunit was critical for neurological recovery (Clarkson et al., 2010). In contrast to L655,708, S44819 is a competitive selective antagonist of the α 5 GABA_A at the GABA binding site which can be safely used in humans (Darmani et al., 2016). S44819 can be orally administered (unlike L655,708), which makes it attractive for clinical use.

Besides improving motor and coordination recovery post-stroke, S44819 reduced anxiety and increased explorative behaviors (**Fig. 21, 22**). Notably, our data showed delivery of S44819 enhanced spatial memory (**Fig. 23**). α 5 GABA_A receptors are located largely at extrasynaptic sites and play a specific role in memory and learning (Chambers et al., 2003; Collinson et al., 2016). It seemed alpha5-containing GABA_A receptors play a key role in cognitive processes by controlling a component of synaptic transmission in the CA1 region of the hippocampus (Collinson et al.,

2002). Interestingly, our results showed that delivery of S44819 increased $\alpha 5$ expression in striatum at 14 dpt compared to control animals, which showed decreased levels of the receptor (**Fig. 27**). The $\alpha 5$ overexpression induced by the S44819 treatment probably reflects a compensatory mechanism to GABAergic signalling inhibition. Indeed, $\alpha 5$ containing GABA_A receptor abundance was reduced post stroke which could be due to the disruption of synaptic transmission following the stroke (**Fig. 27**).

Neurological recovery induced by S44819 was associated with profound structural brain remodeling, i.e., reduced secondary brain atrophy, increased long-term neuron survival, reduced astrogliosis, and increased brain capillary density (**Fig. 24**). We were surprised to see that brain capillary density was increased by GABA_A $\alpha 5$ deactivation (**Fig. 24**). This finding may result from increased brain concentrations of angiogenic growth factors VEGF and FGF in the peri-infarct brain tissue that promotes the remodeling not only of neural but also of microvascular tissue.

Similar to the GABA_A $\alpha 5$ antagonist S44819, the NMDA antagonist Memantine promoted brain remodeling, attenuated secondary brain atrophy, reduced peri-infarct astrogliosis, and increased brain capillary density over a time-window of 7 weeks (Wang et al., 2016). Interestingly, the inhibition of astrogliosis after S44819 delivery was faster than that after Memantine delivery. Brain astrocytes express GABA_A receptors (Höft et al., 2014), and they may attenuate tonic inhibition under pathophysiological conditions via GABA uptake (Pandit et al., 2015). These observations raise the possibility that the effects of S44819 on neurological recovery might at least be partly mediated by astrocytes.

Mechanistically, our data revealed that delivery of S44819 increased the concentration of BDNF which induced Stat3 expression (**Fig. 27**). Besides that, delivery of S44819 also increased $\alpha 5$ abundance (**Fig. 27**). The clear strength of this study is the use of a stringent study protocol that involves well-defined batteries of functional neurological tests and sets of histochemistry analyses, which our group previously used in the evaluation of other experimental therapies. This study was adequately powered to detect improvements of neurological recovery with a statistical power (1 – beta error) of 80% and an alpha error of 5%. This study complied with state-of-the-art principles of animal randomization and data blinding. Hence, our data provide strong evidence that S44819 promotes post-stroke neurological recovery and brain remodeling in mice, raising the question whether this GABA_A $\alpha 5$ antagonist may also be effective in human stroke patients.

6. SUMMARY

The reasons for the failure in translation of therapeutic strategies into clinic have been intensely discussed (Lipton, 1999; Hermann and Chopp, 2012). This argues in favor of a paradigm shift in the approach taken to stroke treatment and recovery to models. The brain has a remarkable but limited capacity for post stroke reorganization that is largely limited by the balance of several signals. Neurotransmitter release, both excitatory and inhibitory, is severely altered after stroke and plays a critical role in neurological recovery. Therefore, the identification of appropriate time windows to promote brain remodeling and plasticity, by using modulators of neurotransmission, for example, is a more promising approach for the treatment of stroke compared to acute neuroprotective interventions (Hermann and Chopp, 2012).

We used the specific NMDA receptor antagonist Memantine and the specific GABA_A $\alpha 5$ antagonist S44819 to assess neurological recovery, tissue remodeling, and brain plasticity in a tMCAO model beyond the acute stroke phase. Importantly, subacute delivery of both antagonists sustained post-stroke neurological recovery, prevented brain atrophy, promoted brain remodeling and (in case of Memantine, but not S44819) enhanced contralesional pyramidal tract plasticity. Memantine, which is clinically approved for the treatment of Alzheimer's disease, could easily be repurposed for stroke patients to treat post-stroke dementia and possibly promote improvements in motor coordination due to the activation of underlying plasticity mechanisms. S44819 had similar effects in the promotion of motor coordination, reduction of anxiety, promotion of exploratory behaviors, and promotion of tissue remodeling. Based on the data presented within this thesis, S44819, which already passed preclinical phase I studies without side-effects, is currently undergoing a controlled randomized international multicenter phase II trial in human stroke patients. Thus, this thesis has truly contributed to clinical translation. This thesis makes a strong argument for therapies that modulate neurotransmission, since such strategies, as shown here, may potentially promote the restorative processes in the ischemic brain.

ZUAMMENFASSUNG

Gründe für das Scheitern der Übertragung von Ergebnissen der Grundlagenforschung in die klinische Anwendung wurden intensiv diskutiert. Ein Paradigmenwechsel bei der Modellierung des ischämischen Schlaganfalls und dessen Erholung ist erforderlich. Das Gehirn hat eine bemerkenswerte, jedoch begrenzte Kapazität zur Regeneration nach einem Schlaganfall, welche zu großen Teilen durch das Gleichgewicht verschiedener Prozesse beeinflusst wird. Die Neurotransmitterfreisetzung, sowohl exzitatorisch als auch inhibierend, ist nach einem Schlaganfall deutlich verändert und spielt eine entscheidende Rolle bei der Reorganisation des Gehirns. Daher könnte die Identifizierung geeigneter Zeitfenster für Therapien zur Förderung der Regeneration des Gehirns nach einem Schlaganfall, beispielsweise durch den Einsatz von Neurotransmitter-Modulatoren, einen besser geeigneter Behandlungsansatz darstellen als akute neuroprotektive Therapie (Hermann und Chopp, 2012).

Wir verwendeten den spezifischen NMDA-Rezeptor-Antagonisten Memantine und den spezifischen GABAA- α 5-Antagonisten S44819, um die funktionelle und strukturelle Reorganisation und Plastizität des Gehirns in einem transienten Okklusionsmodell der A. cerebri media (tMCAO) jenseits der akuten Schlaganfallphase zu untersuchen. Die subakute Verabreichung beider Antagonisten beginnend 72 Stunden nach Schlaganfall förderte die langfristige funktionelle neurologische Funktionserholung und strukturelle Hirngewebe-Reorganisation nach dem Schlaganfall, was eine Hirnatrophie verhinderte, die Reorganisation des Hirnparenchyms förderte und im Fall von Memantine, nicht aber S44819, die Plastizität des kontralateralen Pyramidenbahnsystems steigerte. Memantine, welches klinisch zur Behandlung der Alzheimer-Krankheit zugelassen ist, könnte auch bei Schlaganfallpatienten zur Behandlung der Schlaganfallerholung eingesetzt werden. Basierend auf den in dieser Arbeit präsentierten Daten wird S44819, welches bereits erfolgreich präklinischen Phase-I-Studien unterzogen wurde, derzeit in vierzehn Ländern weltweit in einer kontrollierten randomisierten internationalen multizentrischen Phase-II-Studie an Schlaganfallpatienten untersucht (RESTORE Brain, <https://clinicaltrials.gov/ct2/show/NCT02877615>), in welcher die Substanz analog zur vorgelegten Studie in der Schlaganfall-Postakutphase verabreicht wird. Somit hat diese Dissertation erfolgreich zur Übertragung von Ergebnissen aus dem Forschungslabor in die klinische Anwendung beigetragen. Diese Dissertation liefert ein starkes Argument für Therapien, welche

die Neurotransmission modulieren, da diese Therapien die Reorganisation des Gehirns nach einem ischämischen Schlaganfalls durchgreifend beeinflussen.

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8. PERMISSION AND IMAGES SOURCE

Parts of materials and methods, results and discussion are based on the accepted version of publications, one of which is already published and the one which has recently been in press.

Citation: Post-acute delivery of Memantine promotes post-ischemic neurological recovery, peri-infarct tissue remodeling, and contralesional brain plasticity. Wang YC, Sanchez-Mendoza EH, Doeppner TR, Hermann DM. *J Cereb Blood Flow Metab.* 2016 May 11. pii: 0271678X16648971.

The cortical plasticity cited from (Sanchez-Mendoza and Hermann, 2016) with permission.

Figure 2. The ischemic core and penumbra (with permission).

Figure 3. Main effector pathways leading to cell death following activation of NMDA receptors (with permission).

Figure 6. GABA_A receptor structure and neuronal localization (with permission).

9. Lebenslauf

„Aus datenschutzrechtlichen Gründen ist der Lebenslauf in der elektronischen Version nicht enthalten“

10. PRESENTATIONS

Oral presentations:

NMDA antagonist Memantine induces functional neurological recovery in the post-acute ischemic brain. Wang YC, Sanchez-Mendoza EH, Doeppner TR, Hermann DM. The Joint Congress of European Neurology, Istanbul, Turkey (2014)

NMDA antagonist Memantine induces post-ischemic neurological recovery, peri-ischemic tissue remodeling and contralesional pyramidal tract plasticity. Wang YC, Sanchez-Mendoza EH, Doeppner TR, Hermann DM. The 9th International Congress on Vascular Dementia. Ljubljana, Slovenia (2015)

Post-acute delivery of GABA_A receptor alpha5-antagonist S44819 promotes motor-coordination recovery, prevents secondary brain atrophy and reduces astrocytic scar formation after transient middle cerebral artery occlusion in mice. Wang YC, Sanchez-Mendoza EH, de Carvalho TS, Kaltwasser B, Machado P, Bassetti CL, Hermann DM. American Academy of Neurology, Boston (2017)

Post-acute delivery of GABA_A receptor alpha5-antagonist S44819 promotes motor-coordination recovery, prevents secondary brain atrophy and reduces astrocytic scar formation after transient middle cerebral artery occlusion in mice. Wang YC, Sanchez-Mendoza EH, de Carvalho TS, Kaltwasser B, Machado P, Bassetti CL, Hermann DM. European Academy of Neurology, Amsterdam (2017)

Posters:

NMDA antagonist Memantine induces functional neurological recovery in the post-acute ischemic brain. Wang YC, Sanchez-Mendoza EH, Doeppner TR, Hermann DM. The Joint Congress of European Neurology, Istanbul, Turkey (2014)

NMDA antagonist Memantine induces post-ischemic neurological recovery, peri-ischemic tissue remodeling and contralesional pyramidal tract plasticity. Wang YC, Sanchez-Mendoza EH,

Doepfner TR, Hermann DM. Deutsche Gesellschaft für neurologie conference, Düsseldorf, Germany (2015)

NMDA antagonist Memantine induces post-ischemic neurological recovery, peri-ischemic tissue remodeling and contralesional pyramidal tract plasticity. Wang YC, Sanchez-Mendoza EH, Doepfner TR, Hermann DM. The 9th International Congress on Vascular Dementia. Ljubljana, Slovenia (2015)

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11. PUBLICATIONS

Higher levels of kallikrein-8 in female brain may increase the risk for Alzheimer's disease-related pathology. Keyvani K, Mrain may increase the risk forl "aff-1" secondary brain atrophy and rehamed Karout M, Hammesfahr P, **Wang YC**, Hermann DM, Teuber-Hanselmann S, Herring A. Brain Pathol. 2018. doi: 10.1111/bpa.12599.

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12 ERKLÄRUNG:

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Post-acute delivery of NMDA or GABA_A α 5 receptor antagonists promotes neurological recovery and peri-infarct brain remodeling after transient focal cerebral ischemia in mice“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Yachao Wang des Doktoranden) befürworte.

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