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Excitatory and inhibitory amino acid neurotransmitters in stroke: from neurotoxicity to ischemic tolerance

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The search for neuroprotection in acute ischemic stroke has been dramatically disappointing, with virtually all clinical trials failed for excessive toxicity or lack of efficacy of the tested drug; whereby, current treatments are exclusively based on reperfusion. Given the crucial role of amino acid neurotransmission in ischemic pathobiology, numerous failed strategies were aimed at blocking ionotropic glutamate receptor-mediated excitotoxicity or potentiating GABA-mediated inhibition. Recent work has revived the interest of pharmacologists toward glutamate and GABA receptors, due to a better understanding of subtype-specific toxicity and their involvement in ischemic tolerance. Thus, blocking receptor stimulation through glutamate grabbing, inhibiting downstream transduction pathways or selectively antagonizing detrimental NMDA receptor subpopulations represent promising strategies to rescue ischemic brain injury with limited side effects.

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Introduction

Ischemic stroke is a major cause of mortality for both men and women, and represents the first largest cause of long-term disability worldwide. Due to the paucity of effective therapies, medical and social care consume considerable healthcare resources for patients' management. In fact, currently accepted and utilized treatments for acute ischemic stroke are exclusively based on reperfusion therapies and include thrombolysis with recombinant tissue plasminogen activator (rt-PA) or other proteins with similar activity [1,2], and endovascular procedures (thrombectomy or embolectomy) alone or in combination with thrombolysis [3–8].

The thrombolytic rt-PA, first approved in 1996, is effective up to 4.5 h after a stroke [1,2]. However, it is underutilized in almost all communities worldwide as it is estimated that less than 10% of stroke patients are being treated with rt-PA [9]. Despite the patients' improvements assessed by the National Institute of Health Stroke Scale (NIHSS), rt-PA administration has some limitations, including a significant risk of haemorrhagic events [10], neurotoxicity under certain circumstances [11,12•,13,14] and lack or minimal neuroprotective effects [15–17]. This is a crucial point because in addition to endovascular approaches aimed at removing the cause of ischemia, the progression of brain damage needs to be prevented through strategies aimed at rescuing and protecting neurons. Accordingly, the concept of neuroprotection refers to any pharmacological or interventional approach that mitigates or blocks the injurious molecular and cellular events leading to irreversible cerebral ischemia [18].

Over the past two decades, crucial findings from animal models have improved our understanding of the pathobiological mechanisms contributing to ischemic brain damage following stroke [19]. Cerebral ischemia is typically triggered by an arterial occlusion causing reduced cerebral blood flow, oxygen deprivation and activation of a cascade of events that ultimately lead to tissue damage. The interruption of cerebral circulation leads within seconds to cessation of neuronal electrical activity and within few minutes to deterioration of the energy state and ion homeostasis. Mitochondrial dysfunction and bioenergetic collapse are associated with decreased adenosine triphosphate production and failure of the sodium-potassium pump. This leads to deterioration of membrane ion gradients, excessive calcium influx and extracellular release of amino acids, including toxic concentrations of the excitatory neurotransmitters glutamate and aspartate [20–22]. Thus, excitotoxicity, peri-infarct depolarizations, oxidative stress and inflammation actively participate to the spatiotemporal progression of ischemic brain damage and have been exploited as potential targets for the development of stroke therapies [18,23].

Despite these significant advances in the knowledge of the mechanisms underlying ischemic brain damage, the search for effective neuroprotection or adjuvant neuroprotection has been dramatically disappointing, since virtually all clinical trials performed to date have failed due to toxicity or lack of efficacy of the tested drug [18,24,25]. Trials were focussed on assessing safety

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and/or efficacy of drugs with demonstrated efficacy in stroke models, on the basis of their ability to block crucial mechanisms implicated in ischemic brain damage, including excitotoxicity, calcium overload, oxidative stress, inflammation, apoptosis, vascular dysfunction [24,26]. Therefore, novel approaches need to be identified and, despite the apparent failure of some molecular targets, such as those involving excitatory and inhibitory neurotransmitters, there has been a recent re-evaluation of their therapeutic potential in stroke.

Targeting ionotropic glutamate receptors for neuroprotection

The excessive release of glutamate, coupled to the drastic disruption of glutamate transporters, that occurs early after the ischemic insult is toxic to neurons, mainly through the activation of ionotropic receptors and intracellular calcium overload that trigger detrimental cascades causing 'excitotoxic' neuronal death [27,28]. At millimolar concentration range (excitotoxic levels), glutamate induces an over-activation of N-methyl-D-aspartate (NMDA) receptors and AMPA/KA receptors not only on neuronal cells, but also on other cellular components of the neurovascular unit [29•]. The relevance of ionotropic glutamate receptor blockade in ischemic stroke treatment was first demonstrated in the late 80s by the evidence that the high-affinity uncompetitive NMDA antagonist dizocilpine (MK-801) significantly reduced histological lesion volume caused by focal cerebral ischemia in rodents [30]. Since then, agents that bind to several binding sites of the NMDA receptor complex have been tested in stroke clinical trials, including antagonists to the glutamate and glycine binding sites, high-affinity and low-affinity uncompetitive antagonists to the ion channel site, magnesium ions, subunit-specific antagonists (usually to the NR2B subunit), and antagonists to the polyamine modulatory site (Table 1).

Diverse pharmacological agents acting as NMDA receptor antagonists provide neuroprotection in animal models of cerebral ischemia when administered before or up to 2 h after the insult [31]. Nevertheless, systematic re-evaluation of these preclinical studies has highlighted a number of methodological flaws, mainly regarding lack of rigorous randomization and blinding, incorrect statistical assessment and power analysis, as well as biases affecting selection, performance, measurement, attrition and outcome reporting [24,26,31,32]. As a result, clinical trials conducted with NMDA receptor antagonists did not demonstrate any advantage of the tested drug versus placebo in terms of reduced proportion of patients dead or dependent after stroke [33]. Moreover, these studies confirmed the occurrence of typical psychotomimetic adverse effects including nausea, vomiting, agitation, hallucinations and hypertension. Safety concerns led to premature termination of some studies. In particular, those involving selfotel (competitive NMDA antagonist) and aptiganel (non-competitive NMDA antagonist) demonstrated a trend toward higher mortality for both drugs and a worse functional outcome for aptiganel [34,35].

The responses evoked by NMDA receptor blockage are better understood following a detailed analysis of receptor expression changes occurring after ischemia. Unilateral carotid artery occlusion reduces the [3H]-MK801 binding in the gerbil hippocampus at late stages (i.e., 6 and 22 days) after the insult, suggesting that NMDA receptors located on pyramidal cells are vulnerable to ischemia [36]. With their pioneer work, Bowery *et al.* anticipated the evidence that NMDA receptor availability remains abnormally low for weeks after the insult [37–39], thus providing a likely explanation for the long-lasting cognitive and neurological deficits often observed in stroke victims. This evidence also gives an explanation to the fact that NMDA receptor antagonists act in a restricted therapeutic window, since access to these receptors *in vivo* may be sufficiently impaired within 2 hours of ischemic insult, whereas at later time-points a persistent post-stroke decline in NMDA receptor density occurs [40]. Accordingly, given its pivotal role in plasticity and memory formation, NMDA receptor stimulation, rather than inhibition, proved to be beneficial in the subacute period after stroke [41,42]. However, despite the blockade of mechanisms of post-ischemic repair, the major reason for failure of clinical trials with NMDA antagonists was related to immediate toxicity rather than delayed recovery [31].

Despite the initial disappointments, the exploration of strategies for blocking excitotoxicity has continued. Given its role as an endogenous calcium antagonist, including its ability to block NMDA receptors, it was postulated that magnesium could theoretically provide neuroprotection in stroke. However, the Field Administration of Stroke Therapy – Magnesium (FAST-MAG) trial showed that pre-hospital administration of intravenous magnesium sulphate was safe although there was no improvement in mortality or functional outcome at 90 days [43]. A crucial concept that will aid the development of NMDA-focussed strategies consists of the evidence that receptor subunit composition differentially influences downstream targets. At the synaptic level, activation of the GluN2A subunit-containing NMDA receptor leads to activation of the pro-survival signalling proteins Akt, ERK, and CREB. Whereas, the ischemia-induced abrupt elevation of extracellular glutamate concentration prompts stimulation of the extrasynaptic GluN2B-containing NMDA receptor that triggers excitotoxic neuronal death [28]. In this context, a promising target downstream NMDA receptor activation is the post-synaptic density protein PSD-95, a scaffolding protein implicated in the functional interaction between the GluN2B subunit and neuronal nitric oxide synthase (nNOS). Following the demonstration of its neuroprotective efficacy in rodents and non-human primates exposed

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Table 1

Clinical trials for neuroprotection with drugs acting on excitatory and inhibitory amino acids-mediated neurotransmission

Drug/mechanism	Date	Trial	Results/status	Reference
Drugs acting on the glutamatergic system				
ACEA 1021 (Licostinel)/NMDA glycine site antagonist	1999	–	Confirmation of safety; similar improvement of NIHSS scores versus placebo	[96]
AR-R15896AR/noncompetitive NMDA receptor antagonist (channel blocker)	2001, 2002	–	No significant difference in neurological recovery; more side effects than placebo	[97,98]
CBG000592 (riboflavin/vitamin B2)/ Glutamate grabber	2016	Administration of CBG000592 (riboflavin/vitamin B2) in patients with acute ischemic stroke	Completed	–
CGS 19755 (Selfotel)/Competitive NMDA antagonist	2000	Acute Stroke Trials Involving Selfotel Treatment (ASSIST)	No significant difference versus placebo in neurological recovery; might have neurotoxic effect	[34]
CNS-1102 (Aptiganel, Cerestat)/Non-competitive NMDA antagonist	2001	Aptiganel Acute Stroke Trial	No significant difference in neurological recovery, trend toward higher mortality and a worse functional outcome versus placebo	[34,35]
CP101, 606 (Traxiprodil)/NR2B-selective N-methyl D-aspartate receptor antagonist	2004	A Study to Evaluate the Efficacy and Safety of CP-101,606 in Subjects With an Acute Stroke	Terminated, unreported results	[99]
Dextrorphan/non-competitive NMDA antagonist	1995	–	Dose ranging study; no difference in neurological outcome versus placebo	[100]
Dextromethorphan/low-affinity, non-competitive NMDA receptor antagonist	2011	Evaluation of the neuroprotective effect of dextromethorphan in the acute phase of ischemic stroke	Lack of neuroprotection; neurological outcome was not worsened; reduction in seizures, and increase of myocardial infarction and renal failure versus placebo	[101]
Eliprodil (SL 82.0715)/NMDA polyamine site blocker	1996	Eliprodil trial	No significant difference in neurological recovery versus placebo	–
Gavestinel (GV150526A)/NMDA glycine site antagonist	2000	Glycine Antagonist in Neuroprotection (GAIN)	No improvement of outcome as compared to placebo	[55]
Magnesium/NMDA ion channel blocker, Calcium antagonist	2015	Field Administration of Stroke Therapy – Magnesium (FAST-MAG) trial showed that pre-hospital administration of intravenous magnesium sulphate	Safe, no improvement in mortality or functional outcome at 90 days	[43*]
Memantine/low-affinity uncompetitive use-dependent NMDA antagonist	2017	Memantine for Enhanced Stroke Recovery	Recruiting	–
	2017	Evaluation of Memantine Versus Placebo on Ischemic Stroke Outcome (EMISO)	Not yet recruiting	–
NA-1 (Tat-NR2B9c)/PSD-95 inhibitor	2012	ENACT	Fewer ischemic infarcts versus placebo	[44]
	2016	FRONTIER	Recruiting	–
NPS 1506/NMDA ion channel blocker	1999	Phase Ib Trial of NPS 1506	Safety confirmation: no serious adverse effects	[102]
Piracetam/AMPA allosteric modulator, nootropic agent	1997	Piracetam in Acute Stroke Study (PASS)	Early treatment group (within 7 hours of onset) showed behavioural improvements versus placebo	[103]
	2016	PASS II	Active, not recruiting	
ZK200775 (MPQX)/AMPA receptor antagonist	2002	Phase II Safety Study of ZK200775	Transient worsening of neurological conditions	[104]
Zonampanel (YM872)/AMPA receptor antagonist	2006	AMPA Receptor Antagonist Treatment in Ischemic Stroke (ARTIST MRI and ARTIST+ in association with rtPA)	Abandoned after failing an interim futility analysis	–

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Table 1 (*Continued*)

Drug/mechanism	Date	Trial	Results/status	Reference
Drugs acting on the gabaergic system				
Baclofen/GABA-B agonist	2001	–	Decreases spastic hypertonia as compared to placebo	[77]
	2016	Spasticity In Stroke Study — Randomized Study (SISTERS)	Completed	–
	2016	Study of the Effects on Motor Recovery of Early Poststroke Spasticity Treatment (BacloTox)	Recruiting	–
Clomethiazole/GABA-A agonist	2002	Clomethiazole Acute Stroke Study in ischemic stroke (CLASS-I)	No evidence of efficacy on neurological outcome compared with placebo.	[67]
Diazepam/Positive allosteric modulator of GABA-A receptor	2006	Early GABA-ergic Activation Study in Stroke (EGASIS)	Beneficial in cardioembolic infarct patients, safe in acute ischemic stroke, but may better be avoided in intracerebral haemorrhage	[68]

to brain ischemia, and after the preliminary observation of its safety and efficacy in human, the PSD-95 inhibitor NA-1 (Tat-NR2B9c) is currently under evaluation in a phase IIb/III clinical trial (Field Randomization of NA-1 Therapy in Early Responders, FRONTIER) [44].

With the aim of selectively targeting the detrimental cascades triggered by extrasynaptic NMDA receptors, the well-tolerated drug memantine, a low-affinity uncompetitive use-dependent NMDA antagonist was tested in rodent models of cerebral ischemia, where it significantly enhanced post-stroke recovery [45,46•]. More recently, the NitroMemantines were shown to be both well tolerated and effective against ischemic stroke in rodent models via a dual allosteric mechanism of an open-channel block and nitric oxide/redox modulation of the NMDA receptor. Targeted S-nitrosylation of the NMDA receptor by NitroMemantine is potentiated by hypoxia and thereby selectively directed at ischemic neurons [47•]. If NA-1 and memantine will prove clinically effective, they will cause a paradigm shift in stroke therapy toward safe and effective targeting of excitotoxicity.

Targeting the strychnine-insensitive glycine binding site

Likewise glutamate, cerebral and serum levels of glycine increase under ischemic conditions both in animals and man, being however smaller but more persistent than glutamate [48,49]. The presence of a strychnine-insensitive [3H]-glycine binding site in supraspinal regions of the rat brain was initially discovered by Bowery *et al.* [50] and it was later demonstrated to overlap with NMDA receptor distribution, thus corresponding to a modulatory site on this receptor [51]. Activation of the glycine site causes a shift in the NMDA receptor from an 'antagonist-' to an 'agonist-preferring' state or conformation both in rodent and human brain [52]. Thus, the 'excitotoxic index,' namely the ratio of (glutamate x glycine) to γ-aminobutyric acid (GABA), has been suggested to be a better predictor of neuronal damage than either glutamate or glycine alone [53]. More importantly, being glycine a co-agonist for glutamate on the NMDA receptor, blocking its binding would result in a wider therapeutic window and a more consistent efficacy. The selective antagonist of the strychnine-insensitive glycine binding site of the NMDA receptor, GV150526 (Gavestinel) was reported to reduce the ischemic damage produced by middle cerebral artery occlusion (MCAo) in rodents when administered up to 6 h after the insult [54]. Nevertheless, Gavestinel did not affect ischemic infarction and did not improve outcome when tested in acute stroke patients [55,56].

Grabbing glutamate

Given the numerous clinical failures, studies in the last two decades have expanded beyond the NMDA receptor, seeking ways to control the upstream glutamate concentration as well as downstream protein signals [57].

Blood glutamate grabbing is considered a novel and attractive protective strategy to reduce the excitotoxic effect of excess extracellular glutamate that accumulates in the brain following an ischemic stroke [29•]. This hypothesis stems from the original evidence that glutamate plasma concentrations above 200 μM act as an important predictor of neurological deterioration in ischemic stroke patients [49], followed by a number of evidence documenting the existence of a dynamic interplay between brain and blood glutamate metabolism. Excess glutamate is efficiently removed from the extracellular space by astrocytes and endothelial cells that are endowed with uptake and metabolizing functions. When endothelial glutamate concentration becomes higher than in the blood, glutamate is transported into the blood through facilitated diffusion that, in turn, facilitates its removal from the brain [29•]. Therefore, grabbers (or

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scavengers) that decrease blood glutamate levels elevate the blood/brain gradient, thus facilitating clearance of extracellular glutamate from the brain. The neuroprotective potential of this strategy has been demonstrated in preclinical stroke studies that have shown the efficacy of compounds that grab glutamate by increasing the activity of the blood-resident enzymes that metabolize glutamate, namely glutamate-oxaloacetate and transaminase glutamate-pyruvate transaminase [29•]. To demonstrate the proof of concept, a clinical study with the glutamate-grabbing drug riboflavin is currently ongoing in patients with acute ischemic stroke (EudraCT number: 2014-003123-22).

Potentiating inhibitory GABA-mediated neurotransmission

Like most neurotransmitters, a rapid and transient elevation of GABA occurs in the extracellular space during cerebral ischemia [48,58]. Conversely, expression of both GABA-A and GABA-B receptors after ischemia is decreased to various extent in distinct brain regions of rodents subjected to transient MCAo [59]. The disruption of GABA-mediated neurotransmission early during reperfusion might contribute to ongoing neuronal excitability and possibly to neuronal death [60,61]. Accordingly, neuroprotection is achieved in the preclinical setting by GABAergic drugs acting through various mechanisms, that is, GABA receptors agonists or positive modulators, GABA transaminase inhibitors or GABA transporter blockers [60].

Hypothermia produced by systemic administration of the benzodiazepine drug diazepam was suggested to contribute to neuroprotection in the short term [62,63], whereas its long-term beneficial effects appear to be independent of body temperature modifications [64]. The GABA-A agonist clomethiazole, administered at doses that do not produce hypothermia, was shown to ameliorate the level of functional disability and to reduce the size of infarct caused by focal cerebral ischemia both in rodents and in non-human primates [65,66]. Nevertheless, in the CLASS-I trial, patients with large ischemic strokes receiving clomethiazole within a 12-h time-window did not display any outcome amelioration as compared to placebo [67]. Similar disappointing findings were obtained with diazepam administered to stroke patients within 12 h of onset [68]. More well-designed randomized controlled trials with large samples of participants with total anterior circulation syndrome are required to determine if GABA-A agonists or positive modulators are beneficial for this subgroup [69].

Interestingly, when endogenous levels of GABA are pharmacologically elevated, activation of both GABA-A and GABA-B receptors contributes to neuroprotection [70]. Whether in global cerebral ischemia *in vivo*, or in oxygen or in oxygen glucose deprivation (OGD) *in vitro*, coapplication of muscimol with baclofen protects neurons through down-regulating NMDA receptor function via attenuating tyrosine phosphorylation of the NR2A subunit [71]. In addition to its ability to inhibit excitatory transmission, GABA may exert distinct protective effects both in the acute and in the chronic phase after stroke. In fact, by promoting non-rapid eye movement sleep, delayed repeated treatment with baclofen after stroke has been shown to promote both neuroplasticity and functional outcome in rats [72]. Nevertheless, studies on GABA-B agonists in stroke have been contradictory [73] and, although baclofen may be neuroprotective, its utility is complicated by a number of side effects, including post-ischemic hypertension and cerebral haemorrhage [74].

GABA plays an important function in modulating brain repair; in fact, inhibiting tonic (extrasynaptic) GABA signalling during the repair phase enhances functional recovery, whereas potentiation of phasic GABA (synaptic) inhibition improves behavioural recovery in mice [75,76•]. This may have important implications during rehabilitation. In addition, continuous intrathecal delivery of baclofen was shown to effectively decrease spastic hypertonia as compared to placebo in stroke patients [77]. The abnormal and excessive muscle tone, together with motor weakness that often occurs after recovery from a stroke, significantly impairs mobility and function. In this context, baclofen may represent an effective alternative to pharmacological agents typically used for the management of spastic hypertonia [78], such as drugs that reduce the cholinergic tone at the neuromuscular junction (botulinum toxin), inhibit the release of calcium from the sarcoplasmic reticulum (dantrolene) or act centrally to elevate the inhibitory tone [79].

The role of glutamate and GABA in ischemic tolerance

Triggering mechanisms implicated in endogenous ischemic tolerance represents a very promising strategy to protect neurons against an ischemic insult [80]. In this context, preconditioning, or the more clinically feasible postconditioning, with a brief period of ischemia or with a chemical challenge is an effective approach to decrease neuronal death caused by a more severe ischemic episode [81,82]. The same concept was applied to excitotoxicity, whereby a mild glutamate-induced stress acts as a preconditioning stimulus to prompt a tolerant state that mitigates injury triggered by a subsequent, more severe glutamate exposure. Previous *in vitro* studies have demonstrated that activation of NMDA receptors underlies preconditioning induced by exposure to glutamate or to sublethal oxygen-glucose deprivation (OGD) in cultured neurons [83–85]. A constitutive moderate excess of synaptic release of endogenous glutamate also has a relevance in ischemic preconditioning *in vivo*, conferring resistance to an ischemic episode in adult and aged mice,

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through increased proteasome activity and microtubule-associated protein 2A synthesis and transport [86•]. This latter evidence underlines the concept that complete blockade of glutamate, that is, NMDA, receptors is ineffective in the clinical setting, whereas mild activation of these pathways may represent a more effective strategy to protect the brain. Intriguingly, action potential-driven co-activation of primarily synaptic NMDA receptors and L-type voltage-gated Ca^{2+} channels was suggested to trigger a potent, prolonged but reversible, OGD-tolerant phenotype in cultured cortical neurons [87]. Conversely, other authors have demonstrated that OGD-induced preconditioning in organotypic rat hippocampal slices is strongly dependent on metabotropic mGlu1 receptor activation, while mGlu5 or NMDA receptor does not appear to be involved [88]. In this context, it is important to highlight that activation of both mGlu1 and mGlu5 receptor subtypes underlies post-conditioning induced by exposure to 3,5-dihydroxyphenylglycine in rat organotypic hippocampal slices challenged with 30-min OGD [89].

Thus, the receptor pathways involved in ischemic tolerance appear to be strongly dependent on the type of conditioning stimuli. In fact, *in vivo*, delayed post-conditioning (3 min ischemia) administered 2 days after global cerebral ischemia in adult male rats, induces neuroprotection and cognitive enhancement through activation of NR2A-type NMDA receptors and downstream prosurvival pathways [90•]. One mechanism underlying NMDA receptor-mediated preconditioning is the rapid adaptation of voltage-dependent calcium flux. Since neurodegeneration induced by hypoxia/ischemia is triggered by intracellular calcium overload, mild NMDA receptor activation promotes rapid calcium adaptation in preconditioning that may alleviate cell damage prompted by calcium overload [80]. Glutamate-independent Ca^{2+} and Na^{+} cellular overload have also been associated with neuronal ischemic cell death, whereas distinct isoforms of the sodium-calcium exchangers (NCX) play a role in both pre-conditioning and post-conditioning [91].

In addition to its effects on glutamate neurotransmission, ischemic preconditioning was found to enhance GABA synthesis and release during lethal cerebral ischemia [80,92]. Ischemic tolerance is associated with a transient (between 30 min and 48 h of recirculation) increase of ligand binding to inhibitory GABA-A receptors in the preconditioned gerbil hippocampus [93]. Accordingly, weak antagonism of the GABA-A receptor with bicuculline abolished neuroprotection conferred by ischemic preconditioning in rat hippocampal slices [94]. Conversely, activation of GABA-B receptors has been suggested to play a role in neuroprotection of organotypic hippocampal slices [92]. Thus, ischemic tolerance depends on functional modifications of GABA synapses, involving both the pre-synaptic and post-synaptic elements that ultimately contribute to a shift in the glutamate/GABA balance toward inhibition in the preconditioned brain [95].

Conclusions

Insights gained from experimental and clinical studies of neuroprotective agents have provided crucial guidance for future exploitation of amino acid neurotransmitters' modulators in ischemic stroke. Drugs that specifically target ischemia-activated pathways, such those triggered by extrasynaptic NMDA receptors, will allow to selectively block detrimental mechanisms, allowing to reduce the risk of toxicity. Alternatively, controlling the upstream glutamate concentration, as well as its downstream protein signals, or modulating amino acid-mediated neurotransmission through pre-conditioning or post-conditioning may represent promising strategies in the clinical setting. Thus, despite virtually all the clinical trials performed to date have failed to demonstrate the validity of neuroprotection in stroke patients, there has been a recent re-evaluation of the therapeutic potential of targeting excitatory and inhibitory neurotransmission to rescue ischemic brain damage.

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Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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