Magna Neurologica Vol. 1, No. 2, July 2023

https://journal.uns.ac.id/magna-neurologica
DOI: 10.20961/magnaneurologica.v1i2.647
e-ISSN 2985-3729 p-ISSN 2963-6027

REVIEW ARTICLE **OPEN ACCESS**

ATTENTUATION OF NEURONAL TISSUE DAMAGE IN ISCHEMIC STROKE BY TAT-NR2BCT-CTM AND TAT-NR2B9C

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Article History:
Received: March 12, 2023
Accepted: May 1, 2023
Published: July 1, 2023

Cite this as:
Benedictus, Tan K, Pratama VK P, Sulastyo JD. Attenuation of Neuronal Tissue Damage in Ischemic Stroke by Tat-NR2Bct-CTM and Tat-NR2B9c. Magna Neurologica. 1(2) July 2023: 28-35. 10.20961/magnaneurologica.v1i2.647

ABSTRACT

Background: Worldwide, stroke accounts for 15 million cases annually. Despite improved understanding of its pathophysiology, an effective treatment is yet to be available.
Objective: This review aims to expound ischemic stroke pathophysiology from a bio-molecular perspective, and present two peptides, namely Tat-NR2B9c and Tat-NR2Bct-CTM, which has been proven to attenuate neuronal tissue damage in ischemic stroke.
Methods: Scoping review of several electronic databases: PubMed, Medline, Researchgate, NCBI, SpringerLink, and Google scholar. Search query included: ischemic stroke, NR2B, DAPK1, nNOS, NOX, Tat-NR2Bct-CTM and Tat-NR2B9c.
Results: It is recognizable in a sense, that the pathophysiology pivots on the NR2B-DAPK1 and PSD-95-NR2B interactions. Tat-NR2Bct-CTM and Tat-NR2B9c thwarts NR2B-DAPK1 and PSD-95-NR2B interactions, respectively. These peptides show significant reliability, with the former capable of reducing free DAPK1 by 92,85%, and the latter, the first stroke treatment in two decades to reach Phase 3 clinical trials.
Conclusion: These results strongly support Tat-NR2Bct-CTM and Tat-NR2B9c as effective means to reduce neuronal tissue damage in ischemic stroke.

Keywords: DAPK1, PSD-95, Stroke, Tat-NR2B9c, Tat-NR2Bct-CTM

Introduction

Globally, stroke accounts for 15 million cases annually, with 5 million resulting in permanent disability and another 5 million leading to death. Despite improved comprehension of stroke pathophysiology, an effective treatment remains elusive.[1] A stroke is spawned by a clot, or in 13% of all cases, hemorrhage.[2] Among the multitude of cascading interactions, ischemic stroke pathophysiology repeatedly amplifies intracellular calcium concentration which is preceded by extracellular glutamate flood.[3] Its is also noteworthy that its pathophysiology pivots on the NR2B-DAPK1 and PSD−95-NR2B interactions.

Ischemic stroke pathophysiology therefore can be likened to a domino effect, with the dominos being the cells, and the dominos falling, cells dying. The literatures reviewed presents two peptides, namely Tat-NR2Bct-CTM and Tat-NR2B9c. Both interrupt the falling dominoes at pivotal locations, therefore limiting neuronal cell death and tissue damage by a great quantum. The research on drugs that inhibit glutamate secretion in ischemic stroke. The peptides that we investigate are proven to attenuate neuronal tissue damage in ischemic stroke.

Methods

Study design

This review was reported using the "PRISMA Extension for Scoping Reviews (PRISMA-ScR)" guideline.[39] This comprehensive scoping review was specifically designed and conducted to address the question of whether there is a discernible effect and underlying mechanism associated with the use of Tat-NR2Bct-CTM or Tat-NR2B9c in patients who have experienced an ischemic stroke. This question includes the information of population (patient with ischemic stroke), concept (effect of Tat-NR2Bct-CTM or Tat-NR2B9c on glutamate), and context (brain cell apoptosis and necrosis).

Eligibility criteria

The included studies for this review were following several criteria, including English language articles, using Tat-NR2Bct-CTM or Tat-NR2B9c, and investigating subjects or patients with ischemic stroke. The study was excluded if it did not discuss glutamate and brain apoptosis or necrosis. In addition, duplicate publications and journals with no accessible full text were excluded from the study.

Search strategy

The included studies for this systematic review were collected from three online databases, including PubMed, PubMed Central, SCOPUS, and ScienceDirect, published between 2008 and 2023 in the English language. The keywords used for this systematic review were "ischemic stroke," "NR2B," "DAPK1," "nNOS," "NOX," "Tat-NR2Bct-CTM." and "Tat-NR2B9c." These keywords were combined to search all databases for relevant literature.

Study selection

Data from selected studies were independently extracted by four authors. Disagreements on included journals were resolved through discussion to reach consensus. The collected data were then collated and discussed by all authors to finalize the scoping review.

Results

Identification of studies

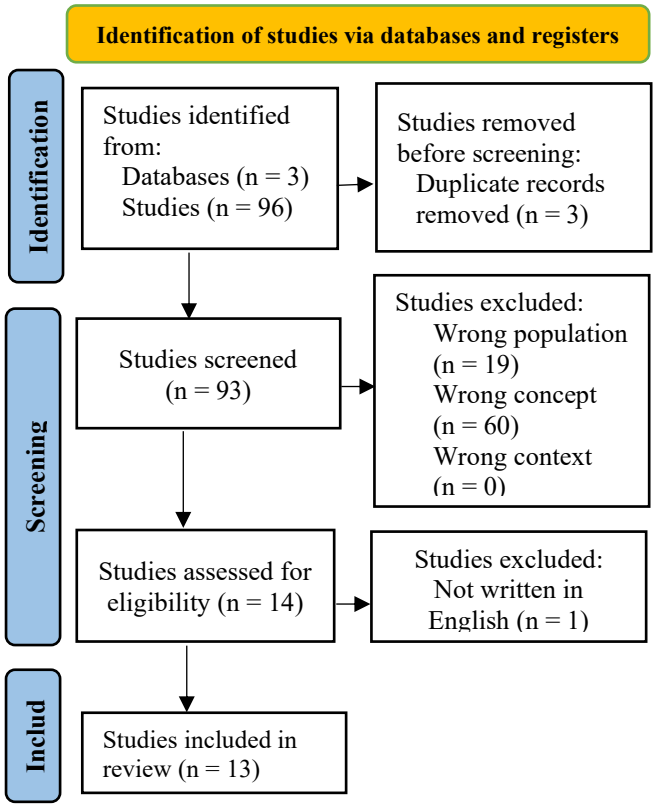

Figure 1. Attentuation of Neuronal Tissue Damage in Ischemic Stroke by Tat-NR2Bct-CTM and Tat-NR2B9c

For our review, we identified 96 studies from three databases and removed 3 duplicates. Of the remaining studies, 19 were excluded for the wrong population and 60 for the wrong concept. For the remaining 14 studies, we assessed their eligibility and removed one study because it was written in a language that is not in English. The remaining 13 studies were included in the qualitative synthesis of this review (Figure 1).

NR2B-DAPK1 complex dependent pro-death signalling in ischemic stroke

When an artery is blocked, the cells normally vascularized undergoes ischemia. Astrocytic and microglial TNFα secretion increases as a response, thereby inhibiting the reuptake of glutamate by excitatory amino acid transporter (EAAT) into the cell (Figure 1).[4,38] Failure of EAAT results in a 150 fold increase in extracellular glutamate concentration, after just thirty minutes of ischemia.[5] The accumulation of glutamate in the extracellular region overstimulates several receptors, namely α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR), and metabotropic glutamate receptor (mGluR).[6]

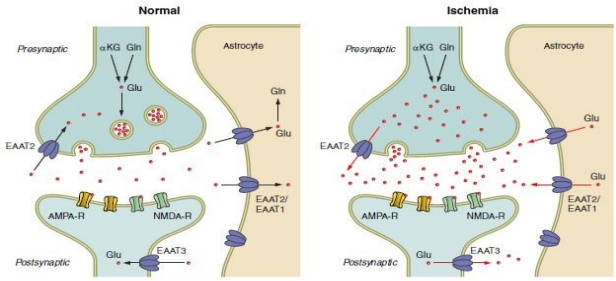

Figure 2. Mechanism of excitatory amino acid transporter (EAAT). In physiological conditions, EAAT reuptakes glutamate in the cell, hence preventing excessive accumulation. In ischemic conditions, TNFα inhibits EAAT function, hence resulting in extracellular glutamate flood[38]

The stimulation of AMPAR is necessary for NMDAR activation. NMDAR requires three factors for its activation: the binding of extracellular glutamate (on the NR2 subunit), co-agonist glycine (on the NR1 subunit) and the removal of the magnesium block by means of intracellular voltage alteration.[6, 7] Stimulation of AMPAR serves the final factor by permitting sodium influx, whereas glycine and glutamate are present outside the cell, the latter in excessive concentration.[6] NMDAR activation allows calcium influx through its subunits, barring NR2B.[8] The NR2B subunit is a dominant benefactor in NMDA-induced calcium influx.[9, 10] Its activation proceeds the elevation of intracellular calcium concentration, attributed to the fast-responding NR2A subunit of NMDAR.[6] Intracellular calcium breaks down the CaM-DAPK1 complex, freeing DAPK1 (this part is to be further discussed) to bind with the NR2B subunit via the

binding site ser-1303.[3, 11] This activates the NR2B channel, allowing further calcium influx.[3] This is instrumental in the bulk of the intracellular calcium spike and downstream pro-death signaling. The increased membrane potential (a consequential effect of the antecedent), activates the voltage-gated calcium channel (VGCC), further cumulating intracellular calcium. Chiefly, it is crucial to understand the concept of source specificity, that a vivid understanding may develop regarding the scale of impact each channel contributes.

NMDAR has a higher attainability of calcium compared to VGCG. Hypothetically, if the same amount of calcium were to pass through both channels, calcium would be more toxic when loaded via the NMDAR, due to the association of its preceding messenger towards pro-death signalling. This clarifies the mentioning of calcium influx through the NR2B subunit of NMDAR as being instrumental in intracellular hypercalcemia and downstream pro-death signalling.[37] Another perpetration of the excess extracellular glutamate is the mGluR, a G protein-coupled receptor (GPCR). Upon activation, it activates the enzyme phospholipase C, which breaks down the cell membrane (Phosphoinositides) into two components, namely diacyl-glycerol (DAG) and inositol triphosphate (IP3).[12,13] Both activate their proceeding messenger, protein kinase C (PKC) and calmodulin, respectively.[12] PKC acts on the L-calcium channel, allowing sustained influx of calcium, whereas the calmodulin-calcium complex activates mGluR7, an integrator of signaling events leading to further calcium influx.[12, 21]

Asides from the aforementioned, ischemia also roots reduced oxygen, glucose, and ATP, subsequently. This is distinctly unfavorable for the ATP-dependent ion channels, namely Na^+/K^+ ATPase and plasma membrane calcium ATPase.[14,15] Failure of the former results in increased membrane potential, attributed to the inability to accommodate sodium influx from AMPAR.[22,23] Whereas the dysfunction of the latter indirectly contributes to increased intracellular calcium, due to its inability to extrude it out of the cell. The upshot of the former is the failure of the sodium-calcium exchanger (NCX), given that it runs on the sodium gradient, nullified by the failure of the Na+/K+ ATPase. As the sodium concentration reemerges in a reversed fashion (higher intracellular sodium concentration), the NCX reoperates (reversed of its normal function), expelling sodium, and admitting calcium, further adding to the intracellular calcium spike.14-16 Eventually, NCX is degraded by calpain, a proteolytic enzyme active in hypercalcemic conditions.[17]

Intracellular hypercalcemia induces both apoptosis and necrosis, through several means. Increased cytosolic calcium concentration triggers the sarco/endoplasmicreticulum calcium ATPase (SERCA) to uptake calcium into the endoplasmic reticulum. Note that SERCA requires ATP to function.[24-27] The net rise in ATP demand results in increased mitochondrial activity leads to necrosis or apoptosis. The former is caused by the byproducts of mitochondrial activity, namely water and oxygen. These by themselves are harmless, however their reaction results in the formation of hydrogen peroxide and superoxide. These substances fully ionize cellular structures, and culminate in cell necrosis by cellular injury (oxidative stress). The latter is due to the activation of the apoptotic pathway (initiated by cytochrome C release), winding the cell into apoptosis.[28-30]

Another pathway leading to necrosis is attributed to cell swelling, and eventually rupture of the cell membrane.[31] Chloride ions diffuse into the cell through chloride channels (passive ionic channel), by electrostatic attraction to intracellular sodium. Polar water molecules then enter the cell through aquaporin (also by electrostatic attraction), exerting considerable force to the cell membrane, eventually bursting the cell.[5] Refer to figure 2 for a schematic summary of ischemic stroke pathophysiology.

Disruption of NR2B-DAPK1 interaction by Tat-NR2Bct-CTM

Tat-NR2Bct-CTM is a targeting peptide composed of the DAPK1 binding domain of the NR2B subunit of NMDAR, and a chaperone-mediated autophagy (CMA) targeting motif (CTM). It is noteworthy that DAPK1 binds to the NR2B subunit in its free form. Tat-NR2Bct-CTM interacts with free DAPK1, disrupting the NR2B-DAPK1 interaction that leads to necrosis and apoptosis, as aforementioned.[32]

Tat-NR2Bct-CTM can enter the cell through the cell membrane permeating domain (CMPD), and binds to the free DAPK1 through its protein binding domain (PBD). This thwarts the NR2B-DAPK1 interaction. The peptide bound DAPK1 is then subjected to lysosomal degradation, via the CTM-CMA machinery interaction, thus decreasing free DAPK1 by 92.85%.[32]

Tat-NR2Bct-CTM presents several virtues that cements its ascendancy over other alternatives. The DAPK1 knockdown can occur two hours upon treatment with the peptide. This is superior in comparison with other DNA or mRNA based methods of protein knockdown. Its reversible and dose-dependent nature also allows for better steering. Lastly, it is superior in its adaptability, in the sense that the synthesis of the peptide can be achieved through several means, without the requirement of high-end molecular biological facilities.[32]

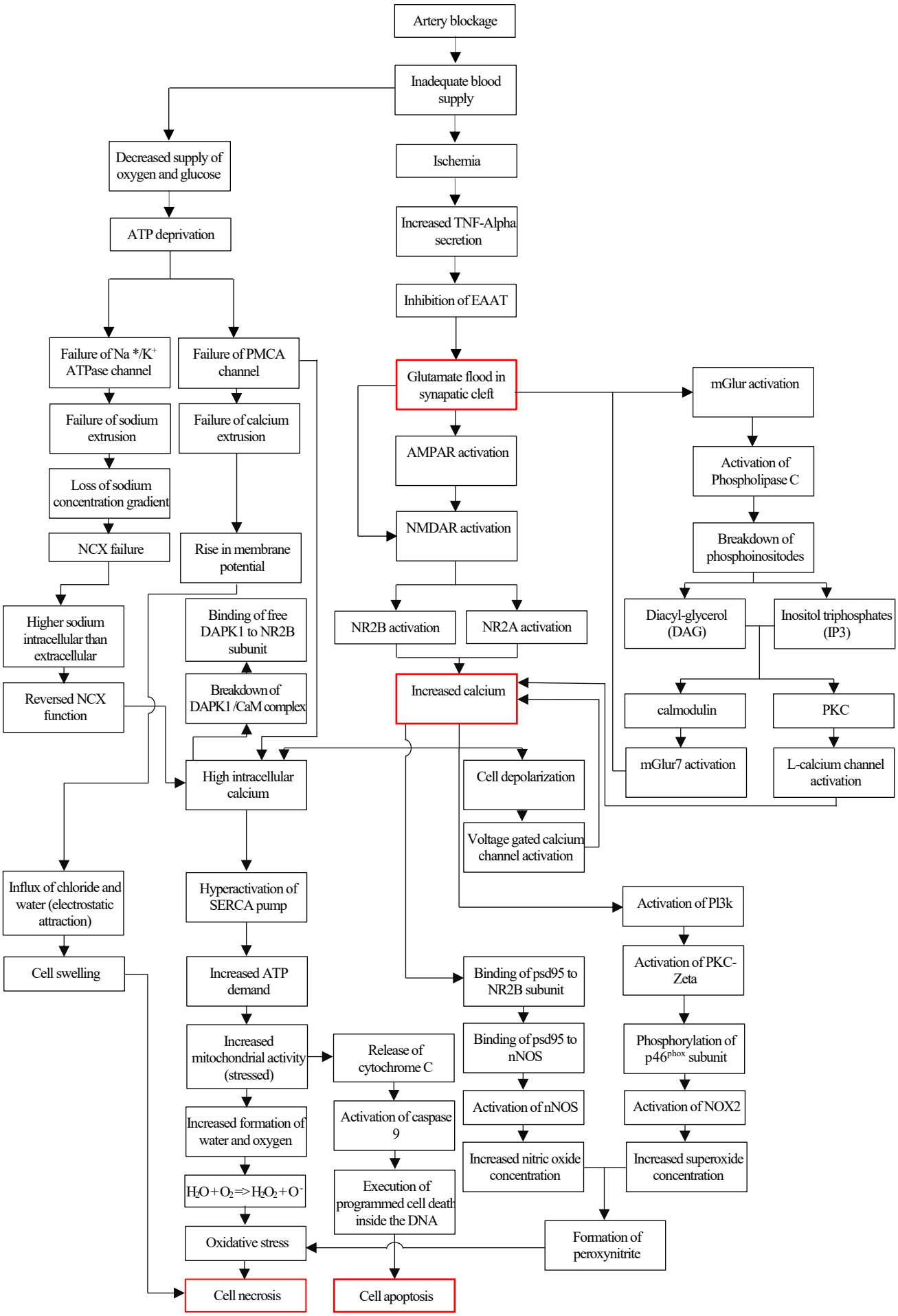

Figure 3. Molecular scheme of ischemic stroke pathophysiology. The ischemic stroke pathophysiology is summarized in this figure (source: personal collection)

Discussion

NR2B-PSD–95-nNOS/NOX2 dependent pro-death signalling in ischemic stroke

Ischemia also leads to the production of peroxynitrite ($ONOO^-$), a highly reactive substance. This is due to the formation of nitric oxide catalyzed by the neuronal nitric oxide synthase (nNOS), and superoxide by NADPH oxidase 2 (NOX2). Note that the two substances in themselves are rather benign, it is the peroxynitrite that is inimical (Figure 3).[18, 19] NR2B-PSD–95-nNOS is a crucial signaling pathway leading to neurotoxicity, following the overstimulation of NMDAR and calcium influx. NR2B binds to nNOS, via PSD–95. PSD–95 is classified into the membrane-associated guanylate-kinase (MAGUK) family, implying its function, that is the regulation of receptor clustering and modulation of receptor functioning. PSD–95 (in its PDZ1-domain of a MAGUK protein) then binds to NR2B subunit via its C-terminal cytoplasmic tail. PSD–95 then binds to nNOS through the PDZ-2 domain, subsequently activating it and initiating the catalyzation of nitric oxide production.[18]

Likewise, the potential hazard of superoxide production is also well reckoned. Calcium influx triggers the activation of phosphoinositide 3-kinase (PI3K), which then binds to the NR2B subunit via PSD–95, forming phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3), accordingly. This forefronts the activation of protein kinase C Zeta (PKCζ), phosphorylating the $p46^{phox}$ subunit of NOX2, thereby activating NOX2. The increased intracellular superoxide reacts with nitric oxide, forming peroxynitrite, leading to cell necrosis by cellular injury (oxidative stress).[20]

Disruption of PSD–95-NR2B interaction by Tat-NR2B9c

The mimetic peptide Tat-NR2B9c shows promising and tangible results in reducing oxidative stress in ischemic stroke. It achieves this by effectively inhibiting the catalyzation of nitric oxide and superoxide production, which are key contributors to the oxidative damage seen in ischemic conditions. This inhibition plays a crucial role in protecting neurons from oxidative injury, potentially offering a therapeutic approach for mitigating the harmful effects of ischemic stroke. Both Tat-NR2B9c and other related compounds operate through the disruption of the PSD-95-NR2B interaction, with the latter specifically working through the prevention of p46phox subunit phosphorylation. Despite the need for further research to clarify its mechanisms of action, the peptide was shown to perturb the PSD-95-NR2B interaction (Figure 4), thereby reducing cellular oxidative stress caused by peroxynitrite, a product of nitric oxide and superoxide.[11, 18-20, 33, 34]

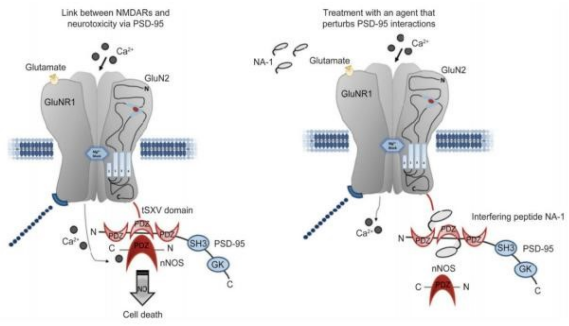

Figure 4. Disruption of PSD–95-NR2B interaction by Tat-NR2B9c. Disruption of PSD–95 and NR2B complex by using Tat-NR2B9c that bind at PDZ-1 and PDZ-2 domain inhibit nitric oxide synthase (nNOS) production of nitric oxide (NO)[18]

Tat-NR2B9c has been tested in cynomolgus macaques, which bears notable structural and behavioral homogeneity to humans. Reassuringly, Tat-NR2B9c has successfully completed ENACT (Evaluating Neuroprotection in Aneurysm Coiling Therapy) Phase 2 clinical trials, making it the first stroke treatment in two decades to proceed to Phase 3 clinical trials.[18,35,36] 182 patients were assessed between 2008 and 2011 (n = 92 TAT-NR2B9c; n = 93 saline). Immidiately over 10 min after the aneurysm repair, patients were given 2.6 mg/kg of TAT-NR2B9c in 0.9% saline. Using primary analysis of all patients, it is found that TAT-NR2B9c treatment significantly reduced the number of all patients with no changes in the lesion volume. Significant effect were found in ruptured aneurysm opposed to unruptured aneurysms in patients treated with TAT-NR2B9c. In patients with ruptured aneurysm, TAT-NR2B9c treatment decreased both lesion number and volume significantly The patient behavioral assessment, significantly shows patients receiving TAT-NR2B9 treatement with ruptured aneurysms receiving a minimal NIHSS score of 0–1 (18/18; 100%), comparable with saline-treated patients with also ruptured aneurysms (13/19; 68%), reflected by the physiological efficacy.[43-45]

Tat-NR2B9c efficacy was evaluated in patients experiencing ischemic stroke undergoing rapid endovascular thrombectomy in a phase III clinical trial. The trial took place during 2017 until 2019 with 549 patients receiving Tat-NR2B9c at the 2.6 mg/kg dose utilized in the ENACT Phase II trial and 556 patients receiving placebo. After randomization TAT-NR2B9c was administered as soon as possible (within 60 min from imaging and randomization). In the subset of patients who were not given rt-PA treatment, there was a positive indication of possible efficacy. Functional independence with a modified Rankin scale of 0-2 (59.3%; 130/219 vs. 49.8%; 113/227) was obtained by a higher percentage of Tat-NR2B9c-treated patients who did not receive rt-PA. Patients who did not receive

rt-PA had an infarct volume that was significantly reduced by Tat-NR2B9c treatment (26.7 mL vs. 39.2 mL), as was mortality (12.8%; 28/219 vs. 20.3%; 46/227).

A recent randomized controlled trial also showed that Tat-NR2B9c significantly reduced mortality and infarction volume and increased functional outcome when administered with alteplase.[40] A Study by Bach et al shows that Tat-NR2B9c at 9 and 30 nmol/g intravenous injection was given 30 min post-ischemia, Tat-NR2B9c at 9 nmol/ g reduced infarct volume by 32% relative to saline-treated.[41] Having said that, studies such as the one by Liu et al demonstrated that NMDAR antagonism (Tat-NR2B9c being one of the peptides studied) failed to exert its neuroprotective effects in the dMCAO (distal middle cerebral artery occlusion) model. This warrants further studies on its most suitable dose, treatment regimen, and stroke type for its neuroprotective effects.[42]

The limitation of the review is that it only addresses the mechanisms of neuronal cell death induced by arterial blockade. As discussed in the introduction, as many as 13% of stroke cases are also caused by bleeding that affects neuron cells and brain parenchyma locally.2 This review has not addressed the domino effect of intraparenchymal hemorrhage on cell death (or its absence), and the role of the two novel therapies Tat-NR2B9c and Tat-NR2B9ct-CTM in hemorrhagic stroke cases. Regarding the mechanism of neuronal death in stroke, as discussed in the flow (Figure 2), these two novel therapies only inhibit a certain aspect of the many multiple aspects of cell death. TatNR2B9c and Tat-NR2B9ct-CTM inhibit the process of cell death by inhibiting one of the pillars, namely by interacting with NR2B-PSD-95 and NR2B-DAPK1.18,32 Even when it's already proven to be effective enough, there are still many pillars that need to be tackled if we desire a "perfect" treatment. Another option to tackle further is to inhibit glutamate accumulation in the synaptic cleft. Although there has been no review yet that discusses the impact between components of the pathomechanism of neuronal death.

Conclusion

Stroke pathophysiology, once an enigmatic riddle, now soundly comprehended. The core mechanism of stroke revolves around the neuronal death process induced by excess intracellular calcium influx and glutamate accumulation in synaptic cleft that led to cellular hyperactivity followed by oxidative stress. However, an effective treatment still evades. Despite revolving around excess extracellular glutamate and intracellular hypercalcemia, its pivotal point lies at the NR2B-DAPK1 and PSD95-NR2B interactions. Thwart these interactions and there will be much reduced intracellular downstream signaling involved in ischemic stroke. The literatures reviewed present two peptides, namely Tat-NR2Bct-CTM and Tat-NR2B9c, that does just that. The former poses multiple advantages, and most remarkably, reduces free DAPK1 by 92,85%.32 The latter is the first stroke treatment in two decades to proceed to Phase 3 clinical trials. These peptides proffer significant scientific evidence regarding its efficacy and functionality, cutting of the falling dominoes at very early and strategic locations, hence significantly reducing neuronal tissue damage in ischemic stroke.

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