

Lentivirus- or AAV-mediated gene therapy interventions in ischemic stroke: A systematic review of preclinical in vivo studies

Skukan, Laura; Brezak, Matea; Ister, Rok; Klimaschewski, Lars; Vojta, Aleksandar; Zoldoš, Vlatka; Gajović, Srećko

Source / Izvornik: **Journal of Cerebral Blood Flow & Metabolism, 2022, 42, 219 - 236**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

<https://doi.org/10.1177/0271678X211039997>

Permanent link / Trajna poveznica: <https://um.nsk.hr/um:nbn:hr:105:642820>

Rights / Prava: [Attribution-NonCommercial 4.0 International/Imenovanje-Nekomercijalno 4.0 međunarodna](#)

Download date / Datum preuzimanja: **2024-07-17**



Repository / Repozitorij:

[Dr Med - University of Zagreb School of Medicine Digital Repository](#)





Lentivirus- or AAV-mediated gene therapy interventions in ischemic stroke: A systematic review of preclinical *in vivo* studies

Journal of Cerebral Blood Flow & Metabolism
2022, Vol. 42(2) 219–236
© The Author(s) 2021



Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/0271678X21103997
journals.sagepub.com/home/jcbfm



Laura Skukan^{1,*}, Matea Brezak^{1,*} , Rok Ister¹,
Lars Klimaschewski², Aleksandar Vojta³, Vlatka Zoldoš³ and
Srećko Gajović¹ 

Abstract

Due to the limited therapeutic options after ischemic stroke, gene therapy has emerged as a promising choice, especially with recent advances in viral vector delivery systems. Therefore, we aimed to provide the current state of the art of lentivirus (LV) and adeno-associated virus (AAV) mediated gene interventions in preclinical ischemic stroke models. A systematic analysis including qualitative and quantitative syntheses of studies published until December 2020 was performed. Most of the 87 selected publications used adult male rodents and the preferred stroke model was transient middle cerebral artery occlusion. LV and AAV vectors were equally used for transgene delivery, however loads of AAVs were higher than LVs. Serotypes having broad cell tropism, the use of constitutive promoters, and virus delivery before the stroke induction via stereotaxic injection in the cortex and striatum were preferred in the analyzed studies. The meta-analysis based on infarct volume as the primary outcome confirmed the efficacy of the preclinical interventions. The quality assessment exposed publication bias and setbacks in regard to risks of bias and study relevance. The translational potential could increase by using specific cell targeting, post-stroke interventions, non-invasive systematic delivery, and use of large animals.

Keywords

Adeno-associated virus, gene therapy, infarct, ischemic stroke, lentivirus

Received 18 February 2021; Revised 14 July 2021; Accepted 20 July 2021

Introduction

Ischemic stroke occurs due to an occlusion in the brain vasculature leading to decreased oxygen and glucose supply of brain tissue. The extent of the resulting infarction is correlated to the site of occlusion, blood pressure, vascular structure, and amplitude of collateral circulation.¹ The most commonly affected blood vessel in the brain is the middle cerebral artery, which in humans provides blood supply to a portion of the frontal, temporal, and parietal lobes as well as the caudate nucleus, internal capsule, and thalamus. If the circulatory obstruction is prolonged, it will lead to depletion of cellular energy, resulting in ionic disruption and metabolic failure, ultimately causing neuronal loss and brain damage.² Currently, tissue plasminogen

activator (tPA) mediated thrombolysis is the only approved drug treatment, which can only be administered in a hospital setting within 4.5 hours of the onset

¹Croatian Institute for Brain Research, University of Zagreb School of Medicine, Zagreb, Croatia

²Institute of Neuroanatomy, Medical University of Innsbruck, Innsbruck, Austria

³Department for Molecular Biology, University of Zagreb Faculty of Science, Zagreb, Croatia

*These authors contributed equally to this work.

Corresponding author:

Srećko Gajović, Croatian Institute for Brain Research, University of Zagreb School of Medicine, Šalata 12, 10000 Zagreb, Croatia.
Email: srecko.gajovic@hiim.hr

of symptoms.³ Nevertheless, it has several side effects, such as the opening of the blood-brain barrier (BBB), neuroinflammation, ROS generation, and hemorrhagic transformation.⁴ As an alternative, mechanical removal of the obstruction can be achieved by thrombectomy, while the dedicated supportive care in a stroke unit provides important benefits to patients.⁵

Due to the short therapeutic window and limited applicability of both thrombolysis and thrombectomy, there is an ongoing need for additional therapeutic approaches. As a potential breakthrough, gene therapy has become a promising emerging technology applicable for a variety of diseases.⁶ The principal mechanism of gene therapy is a modification of endogenous gene activity or an introduction of a therapeutic transgene into patient cells using viral or non-viral vectors. The viral vectors allow for high transfection efficiency and stable long-term expression; therefore, they currently represent the dominant delivery system in preclinical and clinical research.⁷

The viruses used in preclinical research differ by their cell type affinity, immunogenicity, transduction efficacy, capacity for gene delivery, and biosafety. Due to their features, the usage of adeno-associated viruses (AAVs)⁸ and lentiviruses (LVs)⁹ in preclinical studies of brain diseases is currently preferred. Both AAVs and LVs are efficient delivery systems for gene integration into the host cell. New generations of these vectors have broad tropism, the capability to infect dividing and nondividing cells, minimal immune response, and increased biosafety compared to the preceding generations.^{10,11} The improvements in viral vector biosafety are achieved by the development of new viral generations with the genes crucial for replication removed, reduced cellular toxicity and immunogenicity as well as the use of modified viral envelopes or capsids.¹² LV vectors can be pseudotyped with a variety of different cell affinity glycoprotein envelopes, while peptides inserted in AAVs capsid can determine cell tropism.^{13,14} An important advantage of AAVs and LVs lies in their capability of stable gene expression as they enter the nucleus of infected cells through a nuclear pore complex in both dividing and nondividing cells, which is especially important when targeting neurons.¹⁵ After integration, the gene expression starts within days and lasts for many weeks.⁹ Alternatively, these vectors can be altered to not integrate into the host genome, therefore, having a reduced risk of insertional mutagenesis with the downside of transient and relatively short-term gene expression.¹⁶ AAVs can be generated in high-grade titers and have higher transduction efficiency; however, they have a limited cloning capacity (i.e. 4.5 kb). In contrast, LVs have higher cloning capacity (i.e. 8-11 kb), but cannot be produced in as high titers as AAVs.^{17,18}

Therapeutic applications of LVs and AAVs have been already approved for a variety of genetic disorders and cancers. An example of an approved virus-mediated gene therapy is a treatment for spinal muscular atrophy. Noninvasive systematic administration of AAV serotype 9 (AAV9) vector, which has the capability of crossing the BBB, was used to insert *SMN1* gene without bi-allelic mutation.¹⁹ Similarly, LV was used for the transduction of autologous T cells with the CAR gene in treating patients with acute lymphoblastic leukemia.²⁰ Considering the extent of AAV and LV usage for gene integration in preclinical studies of the stroke and their translational potential, we aimed to provide a systematic overview of the current state of the art in this rapidly advancing field. A systematic approach was applied to analyze original research articles published until December 2020 and to present the current methodologies and rationales applied in virus-mediated preclinical ischemic stroke interventions.

Methods

Search strategy

The SYRCLE²¹ and PRISMA²² guidelines were taken into account during the realization of the systematic review. Here, we sought out preclinical *in vivo* studies that employed lentivirus (LV) or adeno-associated virus (AAV) mediated gene therapy approaches for the treatment of cerebral ischemic stroke. A systematic search of online databases PubMed, Scopus, and Web of Science (All Databases) was performed on April 10, 2020, and updated on January 7, 2021, to cover all publications published until the end of 2020. Titles, abstracts, and keywords were searched by the following syntax: (genes OR gene) AND (therapy OR therapeutics) AND (AAV OR lentivirus) AND (brain OR neurons OR neuron OR astrocytes OR neuroglia OR glia OR microglia) AND (stroke OR ischemia OR ischemic). No language or publication date restrictions were applied. Two investigators performed the search independently (LS and MB).

Inclusion criteria

The following inclusion criteria were used to screen the retrieved titles and the abstracts: (1) the study produced new, original results (primary research article), (2) it was a preclinical ischemic stroke study, i.e., it used *in vivo* ischemic stroke animal model, (3) the viral gene transfer was used as a therapeutic intervention, (4) types of viral vectors used were LV or AAV, and (5) the virus was applied directly into the animal. Excluded were perinatal studies or on pups before

reaching sexual maturity, and all in vitro application of viruses, even to the cells subsequently transplanted to the animals. In case of doubt whether the publication meets the inclusion or exclusion criteria, the full-text screening was performed. All discrepancies were resolved through discussion with the third investigator (SG). Finally, reference lists from publications fulfilling the inclusion criteria were used to identify additional relevant studies.

Data extraction

The content of publications identified by the search were analyzed in detail. We extracted the information about the viral vector type, vector envelope, virus titer, and gene promoter type used for gene therapy. The applied intervention was reconstructed including a virus delivery route, location, and time point of administration as well as total volume and number of viral particles injected. Additionally, we coded data on animal species, strain, age, sex, stroke model, and ischemia duration. Lastly, we recorded details about transduction efficiency assessment (methods, time point, and outcome) and outcome measures regarding infarct volume, neurobehavioral outcomes as well as any other relevant results. The data extracted from selected publications by one investigator were additionally revised by another investigator, and all disagreements were resolved through discussions with the third investigator (LS, MB, and SG).

Meta-analysis

From the studies selected for the qualitative synthesis based on the above-described methods, a subset of the studies were selected for the meta-analysis. The study inclusion criteria for meta-analysis was the presence of information on infarct volume and group size in the main article or the supplementary materials. All methods of infarct volume assessment, such as histological staining by cresyl violet, 2,3,5-triphenyl tetrazolium chloride (TTC), and magnetic resonance imaging (MRI) were included. Mean infarct volume and reported standard deviations or errors (mm^3 or percentage) at the last reported time point were used for the analysis. If the numerical data regarding infarct volume was not provided in the text, the online graphical tool WebPlotDigitizer²³ was used for data extraction from published figures. Moreover, the sample size of each treatment and control group was noted, and if the size was reported as a range, the lower value was used.

The pooled data were analyzed using R under RStudio graphical environment.²⁴ In all studies that reported it, SEM was converted to SD using the

given formula: $SD = SEM \times \sqrt{N}$. For all model fitting and plotting, “metafor” package within R environment was used as freely available under CRAN repository.²⁵ Outcome measurements from included studies were considered continuous and compared using standardized mean differences (SMD) and fitted using the random-effects model. Heterogeneity was assessed using the I^2 statistic and confirmed using the Q test. Subgroup analysis was done in the same manner to further explore probable sources of heterogeneity.

Quality assessment

Assessment of the quality of the selected studies included the evaluation of risk of bias, and study relevance for all selected publications, and publication bias for the subset of studies included in the meta-analysis.

The risk of bias was evaluated using the modified version of the SYRCLE risk of bias tool²¹ adapted for the specificity of this systematic review. The modifications of the risk of bias tool and the creation of categories used for study relevance assessment was based on Ripley et al. (2021).²⁶ The questions of the SYRCLE risk of bias tool were rephrased in simple quotes specific for the preclinical stroke research: (1) subject randomization method, (2) groups balanced before stroke or virus injection, (3) personnel blinded to the group allocation, (4) animals randomly distributed in housing room, (5) all procedures performed blind to the stroke status, (6) animals chosen randomly for the infarct assessment, (7) personnel blinded for the infarct assessment, (8) group allocation of the excluded animals, (9) reporting of the all outcomes including the infarct volume and mortality, and (10) statement regarding potential conflict of interest, funding sources and animal welfare regulations requirements. Moreover, as an indicator of the translational potential, study relevance was evaluated according to the following items: (1) the use of adult animals (3 months and older mice, or 6 months and older rats), (2) information regarding virus volume or titer reported, (3) virus administration after stroke induction, (4) monitoring of the physiological parameters during the stroke, (5) short-term outcome assessment (up to 14 days after stroke induction), (6) long-term outcome assessment (after 14 days after stroke induction), (7) the infarct and neurobehavioral assessment. The risk of bias and study relevance assessments were used to generate specific scores by rating the items from each publication with 1 (yes), 0 (no or NA, meaning that there was no information to perform the assessment).

Publication bias was evaluated using funnel plots and confirmed using Egger’s regression test. Trim and fill analyses were applied in the next step, wherever significant asymmetry was detected. To ascertain

possible correlations of effect sizes with viral load, study relevance, risk of bias, and study quality score, meta-regression was done by including those variables as moderators in random-effect models.

All statistical tests were reported as significant if $P < 0.05$.

Results

Study selection process

The literature search of PubMed, Scopus, and Web of Science databases resulted in a total of 440 scientific publications (Figure 1). After removal of duplicates and screening of titles and abstracts, 205 publications were excluded resulting in 135 publications that were subsequently screened at the full-text level. This process identified 72 publications that matched the inclusion criteria. Lastly, reference lists of these 72 publications

were screened for additional relevant studies, resulting in a total of 87 publications that met the prespecified inclusion criteria (listed in Supplementary Table 1). The selected studies were used for the qualitative synthesis, and a subset of 55 studies were used for the meta-analysis. The oldest publication was from the year 2000, and more than 4 publications per year were published since 2011 (Supplementary Figure 1).

Animal characteristics

The qualitative synthesis was performed on the 87 selected studies dealing with virus-mediated preclinical interventions in ischemic stroke. Animal species were reported in all included studies: 50.6% (44/87) of the studies used mice and 48.3% (42/87) used rats, whereas only one study used Mongolian gerbils and another Rhesus macaques as an animal model. Animal strains were not reported in 3 studies (3.4%), age was not

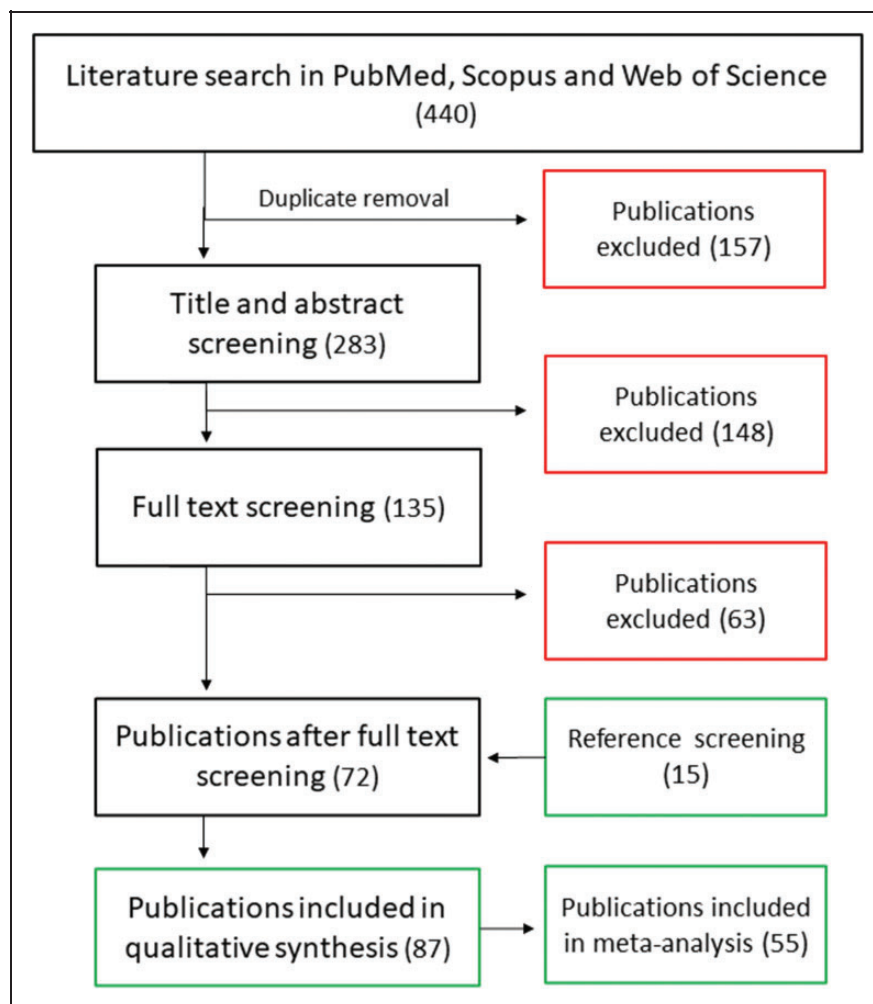


Figure 1. Flow chart of selection process of the analyzed articles. A systematic search in online databases yielded 440 publications. After duplicates removal and application of inclusion criteria total of 87 publication was used for qualitative and 55 for quantitative synthesis.

declared in 17 (19.5%), and sex in 9 (10.3%) publications. The most commonly used mouse strains were C57BL/6 (22.6%; 19/84), CD-1 (13.1%; 11/84), and ICR (11.9%; 10/84), while the mostly used rat strains were Sprague Dawley (36.9%; 31/84) and Wistar (8.3%; 7/84). Males were the predominantly used sex (97.4%; 76/78), with only two studies (2.6%; 2/78) using female animals. Regarding age, the use of young adults (2–3 months for mice, 2–6 months for rats) and adults (3–16 months for mice, 6–20 months for rats) prevailed, representing 38.5% (27/70) and 60.0% (42/70) of the selected publications, respectively. Only two studies (2.9%; 2/70) used aged animals. According to this analysis, the preferred practice was the use of adult male animals, C57BL/6 mice, or Sprague Dawley rats.

Stroke characteristics

All studies reported on the type of surgical approach used for induction of ischemic stroke, but the duration of ischemia was not reported in 5 studies (5.7%). The middle cerebral artery occlusion (MCAO) was the most frequently used model of cerebral ischemic stroke used in 88.5% (77/87) of the studies, while 5 studies (5.7%) used a three-vessel occlusion (3VO; bilateral common carotid arteries and middle cerebral artery) and 2 studies (2.3%) used a four-vessel occlusion (4VO; two vertebral arteries and two common carotid arteries) stroke model. Of the 77 studies that used the MCAO model, 88.3% (68/77) used a proximal occlusion while others used the distal occlusion method. Furthermore, permanent occlusion was used in 16.1% (13/81) of the studies and transient was used in 83.9% (68/81). The duration of transient occlusion ranged from 30 to 120 minutes, with 60 minutes for mice (38.6%; 17/44) and 120 for rats (43.9%; 18/41) being most frequently used. The preferred experimental model of stroke in the analyzed studies was transient proximal MCAO used in 65.5% (57/87) of the selected publications.

Viral vector characteristics

Regarding viral vector information, all studies reported a type of viral vector, however, 37.9% (33/87) of the selected publications did not report on the promoter that drove gene expression and 72.4% (63/87) of the selected publications did not declare viral vector envelop or capsid type. The two types of viruses, AAV (46.1%; 41/89) and LV (53.9%, 48/89) were evenly used for a vector delivery (Figure 2(a)).

All of the studies that reported the type of LVs envelopes used LV pseudotyped with vesicular stomatitis virus-G protein (VSV-G). The VSV-G envelope has a broad cell tropism allowing LVs to transduce all central

nervous system (CNS) cell types. Among AAV vectors, capsid serotypes 1, 2, 5, 7, 8, 9, and rh10 were used. The majority of the studies used serotypes 1 and 2 (53.8%; 7/13), which are characterized by high transduction efficiency and a wide range of tropism. In the 56 studies that reported promoters, 89.3% (50/56) used constitutive promoters (CVM, RSV, CAG, etc.), while 10.7% (6/56) used cell-specific promoters for expression in neurons, astrocytes, and microglia (Table 1).

Regarding the viral vectors, the preferred practice in the analyzed studies was the use of serotypes having broad cell tropism and the use of constitutive promoters, however no preferences toward AAVs or LVs were present.

Vector delivery to the brain

Only one study (1.1%) did not provide information about the way of viral delivery to the animals, while 3 studies (3.4%) did not contain information on the time point nor on viral administration location. Additionally, 16.1% (14/87) of the studies did not mention details about viral titer, and 10.3% (9/87) of the studies about the volume of the injected viral load. The preferred method for viral vector delivery was stereotaxic injection (96.5% 83/86), while only 2 studies used an intravenous injection (2.3%; 2/86) and one study used intracerebral micro-infusion (1.16%; 1/86, Figure 2(b)). Virus injections into the brain were performed at various time points before or after stroke induction; however, the majority of applications occurred before the stroke (78.6%; 66/84, Figure 2(c)). The location of viral injections were reported as either stereotaxic coordinates or by stating the name of the brain region to which an injection was delivered (Figure 2(d)). Regardless of reporting type, the cortex and striatum were the dominant regions for brain injections (64.3%; 54/84).

The volume of the injected virus did not depend on the animal species or the type of the viral vector (Figure 3(a)). The most frequently used volumes for parenchymal and ventricular injections were between 2 and 4 μ L (51.3%; 40/78, Figure 3(b)). Regarding the concentration of viral particles injected per animal, a titer ranged from 10^3 to 10^{11} transduction units per mL (TU/mL) and this amount was injected in the volumes ranging from 1 to 10 μ L (Figure 3(c)). The most frequently used dose of LV particles injected into the mouse and rat brain was 10^6 TU (57.9%; 22/38). Regarding AAV particles, the most frequent dose was 10^9 TU (84.2%; 6/19) in the mouse model and 10^{10} TU (47.1%; 8/17) in the rat model.

Taken together, the preferred practice for virus application was by the stereotaxic injection in the cortex and striatum of 10^6 TU of LVs and 10^9 TU or 10^{10} TU of AAVs in mice and rats respectively.

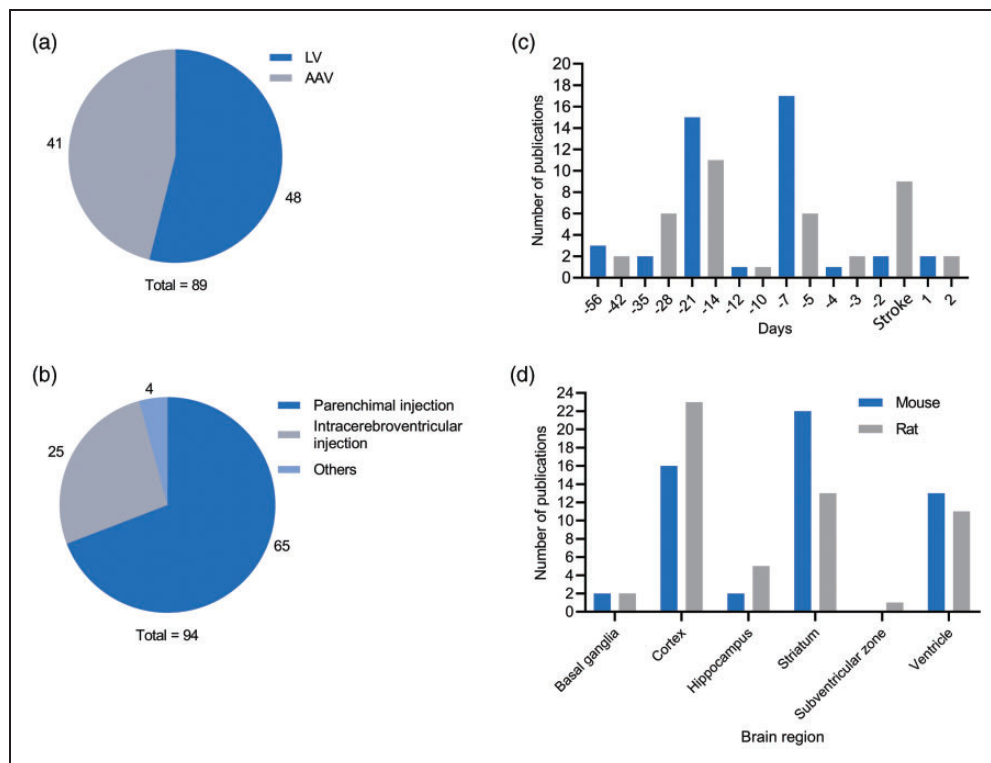


Figure 2. Data related to the viral vector construction and administration. (a) Virus type used in selected studies; total number was 89 as some studies used more than one virus type, (b) virus delivery route; total number was 94 as some studies used several routes of administration, (c) timepoint of virus administration, (d) brain location to which virus was stereotactically injected. LV: lentivirus; AAV: adeno-associated virus.

Table 1. List of promoters driving gene expression reported in 87 analysed studies.

Promoter	Abbreviation	Primary used for	Expression	Number of publications
Cytomegalovirus	CMV	General expression	Constitutive	28
Chemokine (C-X3-C motif) receptor 1	CX3CR1	Microglia specific expression	Microglia	1
Elongation factor 1- α	EF-1 α	General expression	Constitutive	2
Glial fibrillary acidic protein	GFAP	Astrocyte specific expression	Astrocytes	4
HI	HI	Small RNA expression	Constitutive	2
Hybrid CMV/ β -actin	CAG	General expression	Constitutive	6
Hypoxia-inducible factor 1-alpha	HIF-1 α	General expression	Constitutive (upregulated by hypoxia)	1
Neuron-specific enolase	NSE	Neuron specific expression	Neurons	1
Phosphoglycerate kinase	PGK	General expression	Constitutive	2
Rous Sarcoma Virus	RSV	General expression	Constitutive	1
Simian vacuolating virus 40	SV-40	General expression	Constitutive	3
U6	U6	Small RNA expression	Constitutive	5

Transduction efficacy and therapy outcome validation

All publications reported a method for evaluating the therapeutic interventions applied, however, 12.6%

(11/87) of the publications did not report any method for evaluating transduction efficacy. Viral cell transduction was confirmed, and its efficacy was determined mainly by immunohistochemical staining (75.0%; 57/76), Western blot (63.2%; 48/76), qRT-PCR (26.3%;

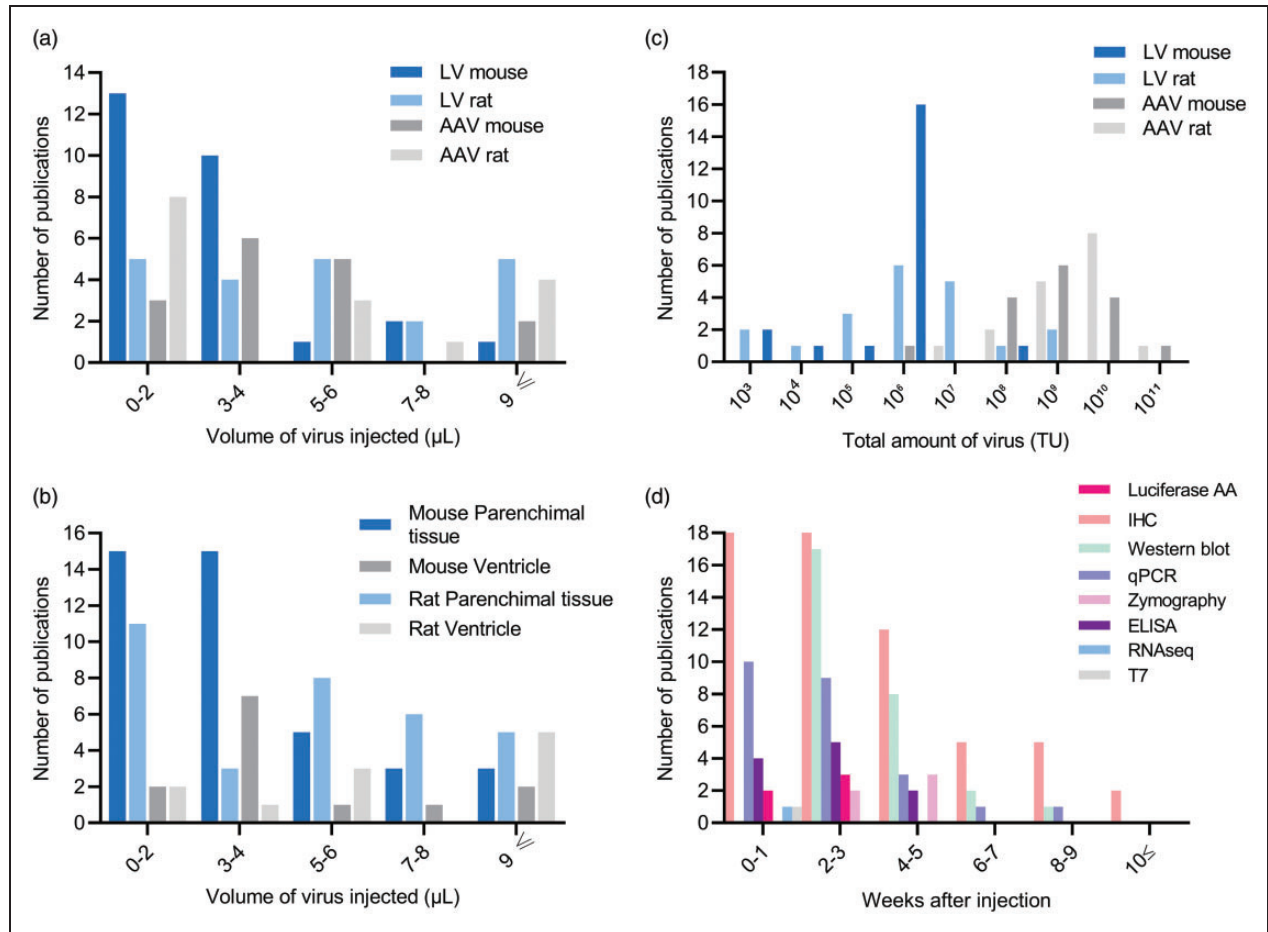


Figure 3. Data related to the viral vector construction and administration. (a) Total amount of viral vector injected per animal, (b) total volume of viral vector injected in brain parenchymal tissue or ventricular system, (c) volume of AAV or LV injected per animal, (d) timepoint and methods used for evaluation of virus transduction efficacy.

LV: Lentivirus; AAV: Adeno-associated virus; Luciferase AA: luciferase activity assay; IHC: Immunohistochemical staining; WB: Western blot; qPCR: Quantitative polymerase chain reaction; ELISA: The enzyme-linked immunosorbent assay; RNAseq: RNA sequencing; T7: T7E1 mutation detection assay.

20/76), ELISA (6.6%; 5/76), and less frequently by gelatin zymography (5.3%; 4/76), T7E1 mutation detection assay (1.3%; 1/76), RNA sequencing (1.3%; 1/76) and luciferase activity assay (1.3%; 1/76). The above-mentioned methods were mostly performed within the first two weeks of viral injection (60.0%; 46/78), however, two studies (2.6%; 2/78) evaluated and confirmed gene activity even after 11 and 12 weeks following viral injection (Figure 3(d)).

The candidate therapeutic genes in experimental stroke included neurotrophic factors, antiapoptotic, antioxidative, anti-inflammatory, and proangiogenic genes (e.g. *Bdnf*, *Gdnf*, *Bcl2l2*, *Bcl2*, *G6pdx*, *Nox1*, *Irf4*, *Il1rn*, *Vegfa* and *Angpt1*; Supplementary Table 2). A total of 64 different genes were analyzed in the included studies, out of which 15 were investigated in multiple studies. The effects of the *Igf1* gene on stroke outcome were investigated in two species of model

animals, both females and males. The effects of gene delivery before and after stroke induction, and in transient as well as a permanent model of ischemic stroke, were evaluated.^{27–29} We noticed a similar approach in the group of studies investigating possible therapeutic effects of *Ntn1*,^{30–34} *Angpt1*^{35–37} and *Vegfa*.^{36–39}

Another candidate therapeutic gene is NeuroD1, a transcription factor claimed to enable transdifferentiation of reactive astrocytes to fully functioning neurons. This was the only case in which an animal model other than a rodent was used. Viral vector-mediated NeuroD1 delivery and subsequent transdifferentiation were studied in rodent⁴⁰ and non-human primate models.⁴¹ The use of Rhesus macaques (*Macaca mulatta*) was particularly challenging as there was no clear-cut methodological approach to induce and evaluate the ischemic stroke: the ischemic lesion was achieved by endothelin-1 induced arterial occlusion,

Table 2. Therapy outcome assessment. Most frequently assessed outcomes after virus mediated gene therapy correlated with preferential time points of assessment for rodents.

Outcomes assessed	Timing mode, <range>	Number of publications
Oxidative stress	3 h, <3 h–3 days>	3
Blood-brain barrier permeability	1 day, <1–21 day>	18
Inflammation	1 day, <1–35 days>	16
Cell death	1 day, <1–56 days>	38
Infarct volume	1 day, <1–60 days>	62
Neurobehavioral outcome	1 day, <1–60 days>	63
Angiogenesis	14 days, <1–56 days>	19
Neurogenesis	14 days, <1–90 days>	25
Astrogliosis	14, 35 days, <1–35 days>	4

the viral vector was administrated by stereotaxic injection into the motor cortex more than a week after injury, and long-term effects were monitored.

In the majority of the selected studies, the therapeutic outcome was estimated by measuring the infarct volume (71.3%; 62/87) and neurobehavioral deficits (71.3%; 62/87). Depending on the gene function, other outcome measures, such as the extent of apoptosis, cell proliferation, oxidative stress, inflammation, or BBB permeability were determined to address the mechanism of the therapeutic effect (Table 2).

Effect size analysis

The 55 studies included in the meta-analysis provided information on the lesion size and group size allowing to address the effects of the applied interventions. However, the interventions were highly heterogeneous in the sense of both applied virus-mediated gene intervention and applied preclinical model of the ischemic stroke. Subsequently, the I^2 statistic showed increased interstudy heterogeneity ($I^2 = 84.2\%$). Due to this heterogeneity, the performed meta-analysis provides only orientational values on the efficacy of the rather diverse group of interventions. The meta-analysis showed a reduction of infarct volume in treated animals compared to the control ($-1.82 [-2.25, 1.39]$, Figure 4).

Publication bias, risk of bias, and study relevance assessment

To address the quality of the analyzed studies, the 87 selected publications were evaluated for risk of bias and study relevance, while publication bias was assessed for the 55 publications included in the meta-analysis.

The risk of bias assessment was done according to the questions defined by the SYRCLE Risk of Bias Tool.²¹ These questions were used as a basis for the specific items applied in this study (similar to Ripley et al. (2021)²⁶). The items used include: (1) subject randomization method, (2) groups balanced before stroke or

virus injection, (3) personnel blinded to the group allocation, (4) animals randomly distributed in housing room, (5) all procedures performed blind to the stroke status, (6) animals chosen randomly for the infarct assessment, (7) personnel blinded for the infarct assessment, (8) group allocation of the excluded animals, (9) reporting of the all outcomes including the infarct volume and mortality, and (10) statement regarding potential conflict of interest, funding sources and animal welfare regulations requirements (Figure 5).

According to the risk of bias assessment, 36.8% (32/87) of the studies reported whether random group allocation was applied and only 3 stated if the animals were chosen randomly for infarct assessment (3.4%). Also, 34.5% of the studies (30/87) reported if the experimental groups were similar for baseline characteristics. Only one study (1.1%) reported the random distribution to animal cages in housing rooms. Furthermore, blinding of the group allocation was reported by 16.1% (14/87) of the studies, whereas blinding for stroke status in 28.7% (25/87) and blinding for infarct volume assessment in 26.4% (23/87) of the publications. Group allocation of excluded animals was reported in 13.8% (12/87) and all outcomes, including infarct volume and mortality, were specified in 41.4% (36/87) of the publication. Almost all included studies 87.4% (76/87) reported information regarding animal welfare regulations, conflicts of interest, and funding sources. The median risk of bias score for all included studies was 3 (IQR = 2.5), ranging from 1 to 8.

As a specific measure for the analyzed studies expected to contribute to the translation of the intervention from the preclinical to the clinical settings, we have addressed the study relevance. The study relevance was assessed by 7 items: (1) the use of adult animals, (2) information regarding virus volume or titer reported, (3) virus administration after stroke induction, (4) monitoring of the physiological parameters during the stroke, (5) short-term outcome assessment (up to 14 days after stroke induction), (6)

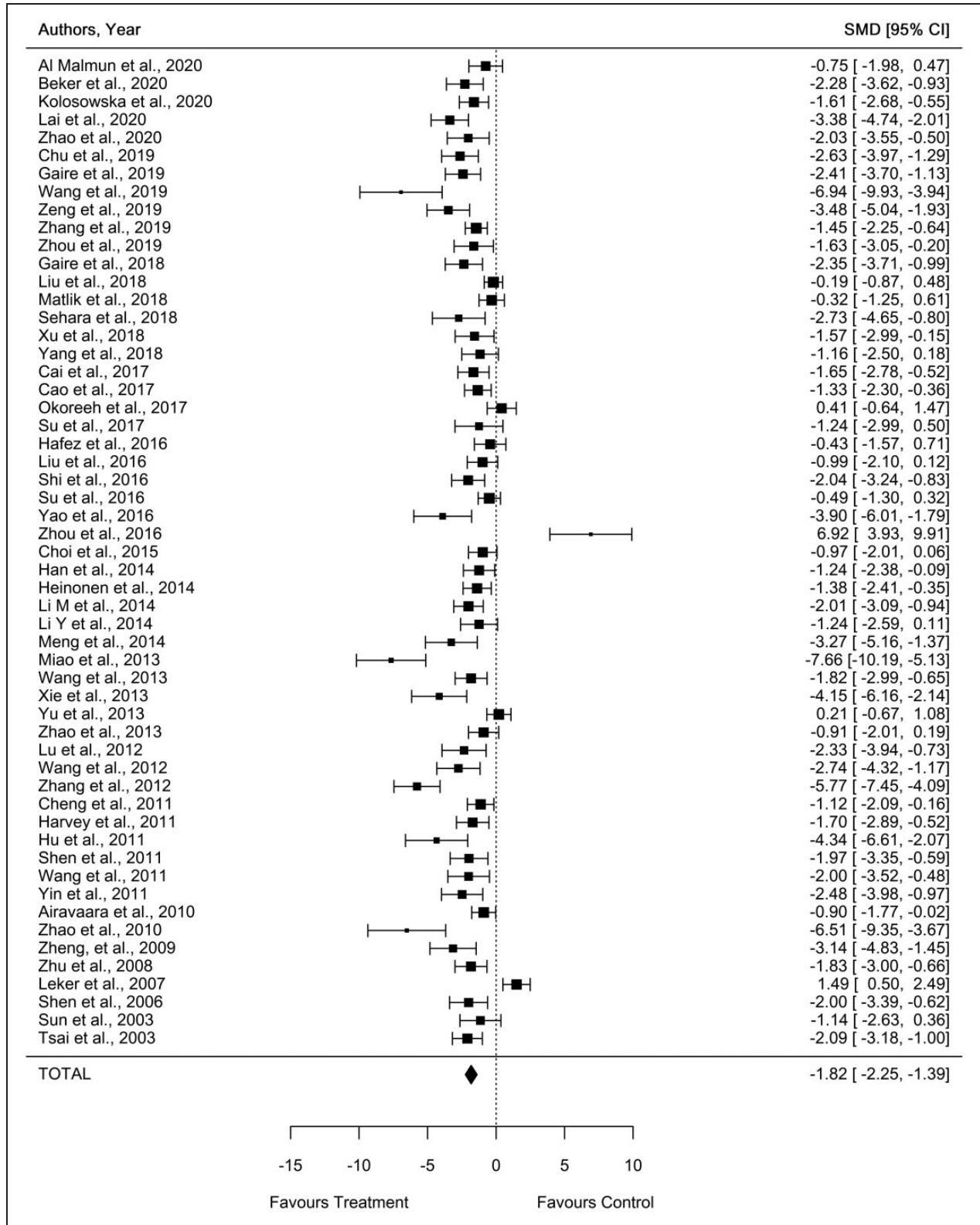


Figure 4. Overall effect of viral vector mediated gene therapy on infarct volume. Forest plot of standardized mean differences (SMD) with 95% confidence intervals (CI).

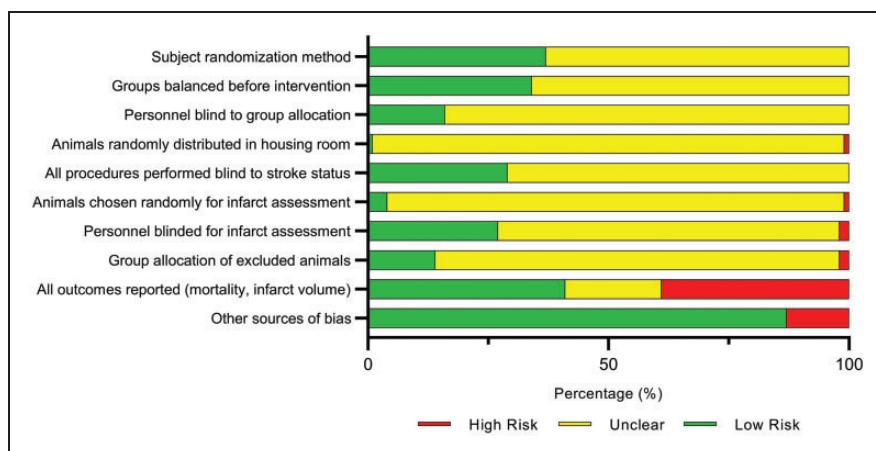


Figure 5. Risk of bias assessed by modified SYRCLC criteria. The category “Other sources of bias” includes conflict of interest, funding sources and compliance to animal welfare regulations.

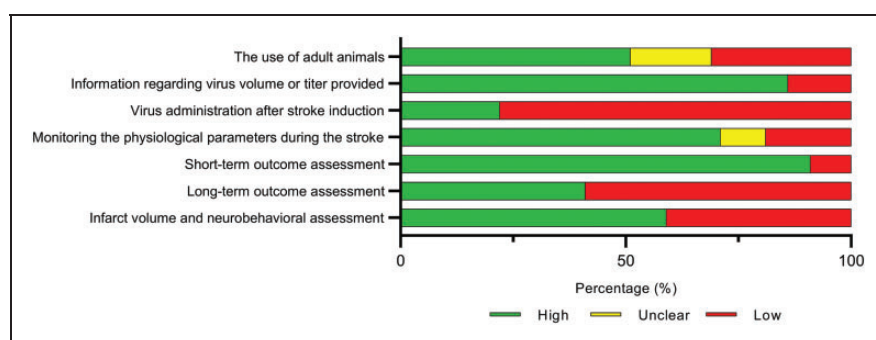


Figure 6. Study relevance assessment.

long-term outcome assessment (14 days or more after stroke induction), (7) the infarct and neurobehavioral assessment (Figure 6). The majority of studies fulfilled the given criteria, i.e. performing short-term outcome evaluation (90.8%; 79/87), providing the information about the volume of viral vector injected or viral vector titer (86.2%; 75/87), monitoring the physiological parameters during the stroke induction (71.3%; 62/87) assessing both infarct volume and neurobehavioral outcomes (58.6%; 51/87), and using the (not young-) adult animals (50.6%; 44/87). A minority of studies provided long-term assessment (41.4%; 36/87) or administered virus vector particles after the stroke induction (21.8%; 19/87). The median study relevance score for all included studies was 4 (IQR = 1.5), ranging from 1 to 7.

To address the association of the effect size with the assessed parameters, several analyses were performed. Again, due to the high heterogeneity of the analyzed studies, these analyses serve only for orientational purposes.

In the first step, the effect size of several subgroups were compared: virus type (LV vs. AAV), the time

point of virus administration (before vs. after stroke induction), animal species (rats vs. mice), and measurement unit of stroke (absolute vs. relative), however, the effect sizes of the infarct volume were not significantly different than the overall estimation (Figure 7). A slight reduction of effect size was observable when the virus was administered after stroke induction (-1.44 [$-2.05, -0.83$]) and the measurement unit of infarct volume was in mm^3 (-1.47 [$-1.90, -1.03$]). In the second step, metaregression analysis was performed to analyze the correlation between stroke effect size and dose for both virus vectors, LV (Supplementary Figure 2) and AAV (Supplementary Figure 3), analyzed separately due to the different doses used. Again, a statistically significant correlation was not noticed ($P(\text{LV}) = 0.4322$, $P(\text{AAV}) = 0.1903$).

In the final step of the analysis the sources of heterogeneity of the effect sizes, and the possible association of the quality of publications with the effect size, was addressed. The presence of the publication bias in reporting of infarct volume-outcome was confirmed by the analysis of the corresponding funnel plot (Figure 8). The publication bias related heterogeneity

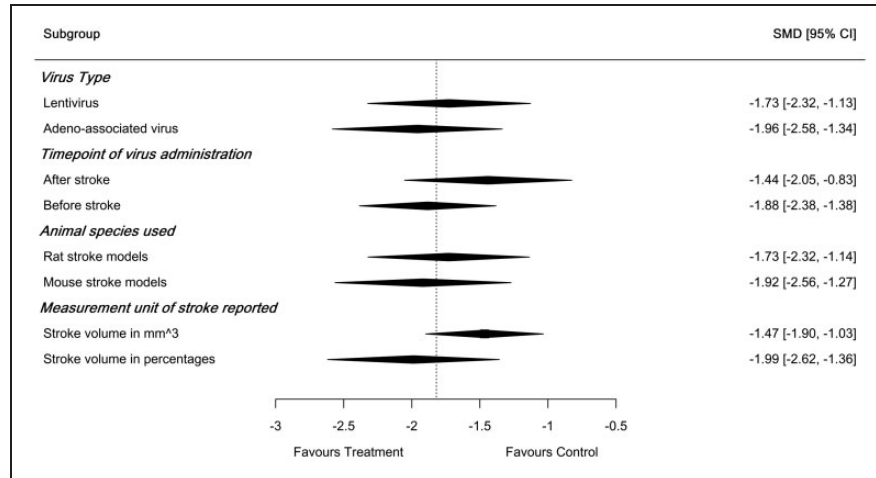


Figure 7. Subgroup comparisons on the effect of the viral vector mediated gene therapy on infarct volume. Forest plot of standardized mean differences (SMD) with 95% confidence intervals (CI) for virus types, timepoints of virus administration, animal species use and relative vs. absolute measures of the infarct volumes. Dashed line represents overall effect size (-1.82) of all studies included in meta-analysis.

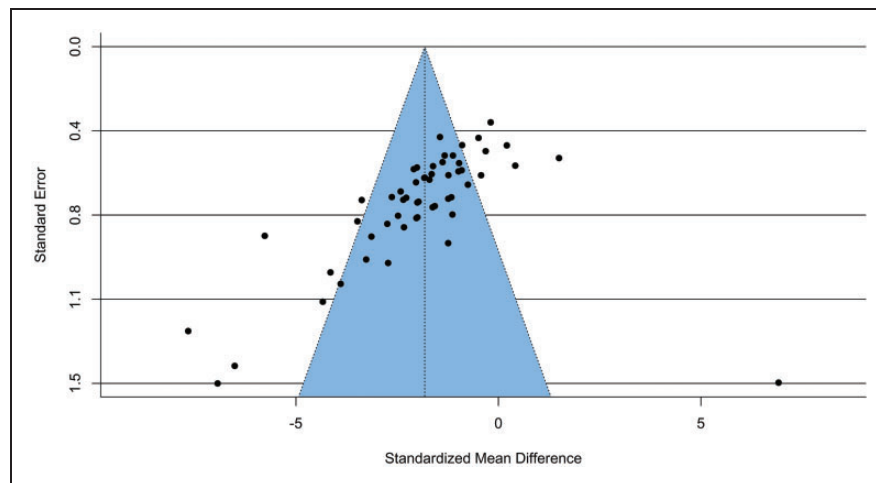


Figure 8. Funnel plot of the infarct volumes. The blue shaded area represents 95% confidence interval.

was confirmed by Egger's regression test ($P < 0.0001$), while trim and fill analysis predicted no missing studies. In addition, the metaregression analysis was performed between study effect size and scores obtained from the risk of bias ($P = 0.2874$, Supplementary Figure 4) study relevance assessment ($P = 0.2150$, Supplementary Figure 5) and study quality score ($P = 0.7408$, Supplementary Figure 6), which showed no correlation between these parameters.

Discussion

In this systematic review, we analyzed 87 articles published until the end of 2020 that utilized viral vector

mediated gene therapies for ischemic cerebral stroke in animal models. We analyzed the design and methodology of preclinical interventions in detail with the aim to provide a comprehensive overview of the current trends in virus-mediated gene therapy preclinical approaches. Moreover, a subset of 55 studies were used for meta-analysis to provide insight into the general efficacy of this methodology.

The preferred choice of preclinical stroke model

The analyzed studies exhibited a variety of experimental approaches. The standardization of preclinical studies is already recognized as a necessity to allow for

reproducibility and comparability of results obtained from different research groups, and necessary steps have been taken to create guidelines such as STAIR and RIGOR.^{42–45} However, what was analyzed in this systematic review as a historical collection is far from being unified. The use of mouse and rat models prevailed, and only one study evaluated the same treatment in both species.⁴⁶ Moreover, various mouse and rat strains with different genetic backgrounds and different susceptibility to ischemic stroke or outcome analysis were used.⁴⁷ In humans, as in rodents, stroke risks and outcomes are related to sex, age, and comorbidities, but the use of aged and female animal models was uncommon. Our search identified two studies that conducted experiments on female mice, as well as two other that used aged animals.^{29,48–50} Moreover, the experimental stroke models differ regarding their clinical relevance and their variability. The current consensus is that MCAO provides the best choice despite its high variability, and indeed the preferred experimental model in the included studies was transient proximal MCAO.

Preferred practices of AAVs and LVs mediated gene interventions for ischemic stroke

The delivery of genes using AAV and LV vectors into the brain was achieved by their postulated broad tropism, minimal immunogenicity, and ability to infect dividing and nondividing cells. The systematic search identified studies that confirmed specific transduction of neurons as nondividing cells,^{50–54} as well as dividing cells such as astrocytes,^{50,51} oligodendrocytes,⁵⁵ microglial^{52,56} or endothelial cells^{50,51} for both LVs and AAVs.

Dosages of viral vectors injected into the animals differed in the analyzed studies. Contrary to our expectations, there was no difference in the brain injection volumes between rat and mouse models. Even though the nature of parenchymal tissue and ventricular system of mice and rats permit different injection volumes, around 2 μ l for parenchyma and up to 30 μ l for ventricles,⁵⁷ we detected no preference in this regard. As expected, titers of LVs were lower and ranged from 10^6 to 10^{10} TU/mL, compared to AAVs titers, which ranged from 10^9 to 10^{13} TU/mL. Therefore, one would expect higher LV injection volumes compared to AAV, but the doses of LV were consistently lower than those of AAV. The above could reflect the findings that the VSV-G, a commonly used LV envelope protein, is known for its toxicity to recipient cells when used in high concentrations.⁵⁸ The important advantage of LVs is their higher cloning capacity. However, Mancini and Horvath (2018)⁵⁹ proclaim AAV as the preferable choice for brain gene

delivery due to higher cell specificity and production titers, allowing the application of lower volumes with few negative side effects on the transduction area.

Currently used AAVs and LVs have modified envelope/capsid proteins which reduce the viral immunogenicity. However, none of the included studies specifically investigated immune responses to viral injections into the brain. Moreover, no side effects were reported, also indicating that the use of viral vector did not interfere with the favorable gene effects.

Following virus administration, immunohistochemistry was the preferred method for validation of viral transduction efficacy and cell targeting. It enabled an assessment of the area affected by the virus, detection of cell-specific transduction as well as quantification measurements. However, 80.5% (70/87) of the studies reported transduction efficacy without quantifying the results, providing only visual confirmation of the transduced area and 12.6% (11/87) of the studies did not perform any validation. The methods of transduction assessment were mostly performed within the first two weeks after viral injection, while some studies used time points as far as 8 weeks after the procedure. This again confirms that long-term stable expression of the gene can be achieved, and argues in favor of the feasibility of post-stroke viral applications as detectable expression levels can be achieved within a short time and last considerably.

Effect size analysis

Considering the broad spectrum of genes used in the analyzed studies, methods for assessment of their effect on stroke were diverse. However, as the gold standard to assess outcomes following stroke, infarct volume was considered as a primary outcome measure.⁶⁰

A subset of 55 studies that provided the infarct volume and corresponding group size was used for the meta-analysis. The analysis confirmed the general efficacy of the method as the treatment was favored and the mean effect size was (-1.82 [-2.25, -1.38]). It should be noted that the heterogeneity of the studies was high ($I^2=84.2\%$), which is not at all surprising as we analyzed a mixture of diverse virus-mediated interventions using various genes and a variety of preclinical ischemic stroke models. Subsequently, the obtained results serve for orientation purposes, nevertheless clearly support the validity of the experimental approach reviewed here – preclinical ischemic stroke interventions based on virus-mediated gene therapy.

The calculated effect size was similar to the therapeutic potential of other approaches in the field, such as the delivery of therapeutic molecules via extracellular vesicles (-1.95 [-2.72, -1.18])⁶¹ or intraparenchymal

transplantation of neural stem/progenitor cells (-1.11 [-1.5, -0.73]).⁶²

Due to the high heterogeneity of the analyzed studies, we hoped to learn more about determinants of the effect size by subgroup analysis. However, when compared according to effect size, viral type (AAV vs. LV), the timing of viral application (before vs. after stroke), animal species (mice vs. rats), or ways of reporting (absolute vs. relative) did not show any statistically significant differences. Similarly, metaregression analysis on the correlation between virus dose and the effect size was not statistically significant. On the other hand, the diversity of the approaches can be considered as an advantage when addressing virus-mediated gene therapy interventions. The quantitative synthesis indicated that the method was indeed functional and resulted in a measurable effect. This certainly confirmed the potential of the method in conveying the desired impact in the preclinical setting. Moreover, it indicated the validity of major postulates for gene therapy to be translated into clinical practice.

Study quality assessment

Another possible reason for heterogeneity of the reported infarct sizes could be a difference in study qualities. As shown in the above-described analysis of the applied methodologies, many of the selected publications did not report all the details on their performed experiments. To provide quality assessment of the selected studies three measures were applied: publication bias, which referred to the subset of studies used for meta-analysis, and risk of bias and study relevance tools, which characterized every individual study.

The publication bias was present as shown by the funnel plot and Egger's regression test. The study distribution was highly asymmetric, and the studies with lower standard errors reported lower effect sizes. A frequent reason for these asymmetries in other meta-analyses is the overall under-reporting of the negative or statistically non-significant results. In our analysis, although the overall heterogeneity of the studies contributes to the shape of the funnel plot, there is a reasonable impression that the studies performed with the higher scientific rigor would report lower effects. This not only exemplifies the importance of scientific rigor in evaluating preclinical interventions, but also indicates possible reasons for the translational failures of therapeutic candidates for ischemic stroke.

To address the quality of each individual study, risk of bias and study relevance were assessed. The Risk of Bias Tool was designed according to the SYRCLE recommendations and modified to fit the preclinical stroke studies. Concerning the study relevance assessment, other systematic reviews used similar tools

referring to them with various names according to their specificities. Here, the applied study relevance tool was specifically designed to address the translational relevance of the analyzed preclinical stroke studies. Both tools, risk of bias and study relevance, illustrated how the analyzed studies did not fulfill the applied criteria, indicating the extent of the challenges facing preclinical stroke research in terms of appropriate study design and its reporting. However, the meta-regression analysis did not show a correlation of risk of bias and study relevance with the measured effect size.

The breakthrough potential of gene therapy applications in ischemic stroke

AAV and LV vectors have broad tropism, however, they could be specific in the sense of target cells depending on the viral capsid or envelope, whose components can be modified accordingly. Additionally, the choice of gene promoter can selectively drive the expression in the targeted cells. Despite these advantages, only 6 (10.7%; 6/56) of the analyzed studies utilized cell-specific promoters to direct expression in neurons,⁶³ astrocytes,^{29,64,65} or microglial⁵⁶ cells. For example, Andsberg et al. (2002)⁶³ used AAVs to deliver *Bdnf* or *Ngf* genes under the neuron-specific enolase (Nse) promoter. They detected neuron-specific expression of these genes and reported neuroprotective effects after stroke induction. Similarly, astrocyte glial fibrillary acidic protein (Gfap) promoter was used to drive the expression of genes *Igf1*, *Atg7* and *Pax6*.^{29,64} Improved neural survival was accompanied by reduced infarct volume in the mentioned studies. Furthermore, the same principle was exploited by Mamun et al. (2020)⁵⁶ to promote the expression of *Irf4* or *Irf5* genes in microglial cells. They found that overexpression of *Irf4* has neuroprotective effects, in contrast to *Irf5* that promoted M1 microglial polarization and lead to an increase in infarct volume.

Another approach to increasing cell specificity would be to combine multiple elements including the use of the dedicated virus capsid or envelope, specific promoter, micro RNA target sequence (miRT) detargeting, and the tetracycline-dependent self-regulating (Tet) systems. As miRNAs act as a post-transcriptional regulator, engineering of their target sequences enables a cell-specific inhibition of gene expression (e.g. in neurons, if the astrocyte-specific expression is required), while the use of the Tet system provides an opportunity for indirect miRT detargeting. This type of approach was applied in brain astrocyte-specific gene silencing.⁶⁶ Lentiviral vectors pseudotyped with MOK-G were used to deliver shRNA under TRE (Tet responsive element) promoter and tTA/S2 transactivator flanked with miR124T and

miR9**T* under astrocyte-specific promoter, which led to increased astrocyte-specific gene expression.⁶⁶ Analogous strategies can be employed for targeting different cell types within the CNS and therefore represent an important opportunity for an upgrade of virus-mediated stroke therapy compared to other therapeutic approaches.

The translational potential of the reviewed studies depends on the timing of the applied intervention. The LVs and AAVs are considered long-term expression vectors that can integrate into the host genome. The expression depends on gene promoter properties and the environment of the host cell. Therefore, it is important to consider the timespan of gene expression following cell transduction. It is encouraging that 64.5% (49/76) of the included publications confirmed gene expression within the first few days after injection, indicating that the desired effect could be achieved in a relatively short time. This is of particular importance in the case of ischemic stroke, which is an acute event, and an early onset of the therapeutic effect is important. Nevertheless, pre-stroke administration is a valid experimental strategy to obtain effective levels of a gene during both phases, ischemic damage and subsequent repair. Most of the reviewed studies (78.6%; 66/84) indeed applied viral vectors before stroke induction to assure effective gene levels and reveal therapeutic effects of the gene candidates. However, from a clinical perspective, this approach is questionable as a stroke is not predictable. Alternatively, 21.4% (18/84) studies provided a good example of the feasibility of virus administration after stroke induction.^{40,67,68} The gene expression could be detected even as early as one day after viral injection^{32,37,69} providing the opportunity to target various processes including apoptosis,⁶⁷ neuroinflammation⁶⁸ or neuronal remodeling.⁴⁰ In the context of future clinical application, this enables two strategies in the form of prophylactic or therapeutic injection. Regarding the duration of the achieved effect, there are reports claiming that gene expression can be detected even 55 days after viral application.^{28,29,36,70,71} Taken together, long-term expression sets up foundations for one-time single-dose administration of therapeutic agent in clinical applications.

As the brain is within the skull and bounded by the blood-brain barrier, even if only a single administration event would be needed, the question of the appropriate route of administration remains. The preferred practice in the analyzed studies was a stereotaxic injection in the cortex and striatum. The effectiveness of this approach is well documented in the analyzed publications, but still, they might not be appropriate for the substantially larger human brains. An important aspect is a determination of viral vector volume needed for achieving the same therapeutic effect in human patients.

The successful upscaling could be achieved by using large animal models that better mimic human anatomical features compared to rodent models. However, we still lack standardized experimental models to induce and evaluate ischemic lesions in the brains of large animals, as was shown in the study on monkeys.⁴¹ Therefore, to circumvent the low volume/dosage problem, some articles reported using multiple subsequent stereotaxic injections. Within this approach, there were two distinct options: injection at one site but different depths⁷² or injection at multiple sites in different brain regions.⁷³ As another alternative approach, Zeng et al. (2019)⁴⁸ used the retroorbital intravenous injection of AAV9 serotype to achieve brain-wide expression of *Trim9* gene. They employed the AAV9 serotype, which has been proven to successfully cross the blood-brain barrier enabling a non-invasive systemic therapy. Moreover, Wang et al. (2018)⁶⁷ applied the viruses via mouse tail vein injection. Both studies reported reduced infarct volumes and improved neurological scores following stroke, providing additional confirmation of AAV9 serotype capability to cross the BBB. A similar approach was adopted by Massaro et al. (2020),⁷⁴ who utilized systemic delivery via the superficial temporal vein injection of AAV9 carrying hGBA (human glucosylceramidase beta) under *Syn1* promoter for treatment of mouse model of neuronopathic Gaucher disease. *Syn1* promoter restricts gene expression to a specific cell population within the brain. These approaches could improve the chances of use of AAV9 in clinical practice, as a non-specific expression in non-target tissues could be avoided.⁸ Regardless of these positive aspects of AAV9, there are several concerns as cell-specific transduction can differ depending on the age of the animal models.⁷⁵ Additionally, long-term side-effects of systemic virus administration should be taken into account and thoroughly investigated. An alternative method for viral vector delivery, utilized by Gan et al. (2021),⁷⁶ is an intramuscular injection, a non-invasive peripheral approach that allows retrograde axonal transport to the cortico-spinal tract. After post-stroke intramuscular application of AAV serotype 5 to overexpress tPA as a neurorestorative agent, successful transduction and expression of the transferred gene resulted in enhanced axonal remodeling and improved motor behavioral recovery. It is to be mentioned that there are emerging technologies which bypass the need for the virus itself to be given to the animal, but the transduction product is delivered to the animals by exosomes, further enhancing translational potential of gene therapy interventions.⁷⁷

Introducing the primate experimental model to evaluate recently claimed *in situ* astrocyte-to-neuron conversion technology using the NeuroD1 transcription

factor represents novel evidence in this field. Following Chen et al. (2020),⁴⁰ who investigated this method in a rodent model, Ge et al. (2002)⁴¹ utilized this approach in rhesus macaques and confirmed that astrocyte-to-neuron conversion technology both generates new neurons and alters the microenvironment for enhanced neuroprotection. Although the non-human primate (NHP) model was used in only one of the included studies, it represents a good direction through which several translational concerns could be addressed. The mentioned preclinical approach was enhanced by the usage of NHP models, post-stroke virus delivery, and a long-term outcome evaluation. Though more complex, NHP models have higher extrapolation potential than rodents, as they are phylogenetically closer to humans and possess strong similarities in brain volume and structure. Besides having higher white matter content than rodents, implicated in higher brain plasticity and function, and more complex vascular anatomy, NHP models have a crucial role in the elucidation of long-term physiological and behavioral outcomes. Finally, the described approach introduces an important step between preclinical animal studies and human clinical trials.⁷⁸

Studies investigating the effect of *Igf1*^{27–29} gene on stroke outcome serve as another positive example as they were conducted on both female and male mice and rats. They investigated the effects of gene delivery before and after stroke induction and evaluated the effect in transient as well as permanent models of ischemic stroke. We noticed similar characteristics in the group of studies investigating the therapeutic effect of *Ntn1*^{30–34} *Ang1*^{35–37} and *Vegf*^{36–39} genes. As there is a slight controversy on whether one type of intervention should be tested in different experimental settings by the same group, we want to suggest that studies should be done by several groups of researchers as well. Confirmation of results by more than one research group gives additional proof of principle as well as a solid ground for clinical translation.

Limitations of the study and recommendations for the future

We analyzed virus-mediated gene transfer only in the context of preclinical models of ischemic stroke. Other viral applications for brain diseases were not considered here but could be even more technologically advanced and cross-applicable to ischemic stroke. Moreover, many analyzed studies are part of the historical collection and might have therefore missed experimental details relevant for the interpretation of the achieved outcomes.

Subsequently, future studies should aim to increase scientific rigor and translational relevance. The most

recent guidelines (e.g. STAIR⁴⁴ and RIGOR⁴⁵) should be respected and followed. To represent the patient population more accurately, future studies should consider including female and older animals, possibly with stroke-related comorbidities. Further, studies could prefer viral vector delivery following stroke induction while the design behind therapeutical construct should be described in more detail. More relevant and clinically applicable approaches would include validation over a longer period as these would provide more detail about the recovery process. As a final step, the most promising results should be tested on large animal models. Conclusively, maximal precision of reporting on study design, animal handling, experiment execution, data acquisition, and analysis should be assured. An overall increase of the scientific rigor and adherence to the agreed guidelines when performing preclinical studies is essential for reproducibility and comparability of future studies.

Conclusions

This systematic review is the first to summarize all available data on AAV and LV vector-mediated gene delivery for preclinical in vivo ischemic stroke models. We provided qualitative and quantitative synthesis on an experimental design involving viral vector delivery serving as a basis for the future translation of viral-mediated stroke therapy. The preferred practice was transient MCAO in rodents (mice or rats equally) and application of viruses via stereotaxic injection in the cortex and striatum, however predominantly before ischemic lesion. AAVs and LVs were used equally although the virus loads of AAVs were higher, preferred were serotypes having broad cell tropism and the use of constitutive promoters. The meta-analysis based on infarction volume and group sizes confirmed the efficacy of the approach. However, the quality assessment of the selected studies indicated publication bias, presence of risks of bias and setbacks in study relevance. Finally, we stress the importance of standardized approaches to increase the reproducibility and translational value of this promising technology to provide for novel stroke therapies in the future.

Availability of data and material

The dataset presented in this study (detailed coding of the contents of the selected publications) can be found in the online repository;

Skukan L, Brezak M, Gajovic S. Analysis of the research studies selected for a systematic review of preclinical in vivo studies on lentivirus- or AAV-mediated gene therapy interventions in ischemic stroke. *figshare* Dataset. 2021; <https://doi.org/10.6084/m9.figshare.14955180>

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the European Union through the European Regional Development Fund, under grant agreement No. KK.01.1.1.04.0085, project “Genomic engineering and gene regulation in cell lines and model organisms by CRISPR/Cas9 technology – CasMouse”; This study was supported by the European Union through the European Regional Development Fund, under Grant Agreement No. KK.01.1.1.07.0071, project “Sinergy of molecular markers and multimodal in vivo imaging during preclinical assessment of the consequences of the ischemic stroke (SineMozak)”; This study was supported by the European Union through the European Regional Development Fund, as the Scientific Centre of Excellence for Basic, Clinical and Translational Neuroscience under Grant Agreement No. KK.01.1.1.01.0007, project “Experimental and clinical research of hypoxic-ischemic damage in perinatal and adult brain”; This study was supported by Croatian Science Foundation project RepairStroke IP-06-2016-1892. The work of doctoral student Rok Ister has been fully supported by the “Young researchers’ career development project – training of doctoral students” of the Croatian Science Foundation funded by the European Union from the European Social Fund.

Acknowledgements

We acknowledge the help of Anni Hasratian and Monika Berecki in language editing of the manuscript.

Declaration of conflicting interests

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

Authors’ contributions

All authors contributed to the systematic review conception and design; MB, LS and SG performed the literature search, selected the publications, coded their content, and created the resulting dataset. MB, LS, RI and SG selected the subset of publications for meta-analysis and performed the quantitative synthesis. All authors participated in the analysis of the dataset, and interpretation of the data. MB, LS and SG drafted the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. MB and LS are equally contributing authors.

ORCID iDs

Matea Brezak  <https://orcid.org/0000-0001-8382-4782>
Srećko Gajović  <https://orcid.org/0000-0001-8668-5239>

References

1. Rehni AK, Liu A, Perez-Pinzon MA, et al. Diabetic aggravation of stroke and animal models. *Exp Neurol* 2017; 292: 63–79.

2. Nogles TE and Galuska MA. *Middle cerebral artery stroke*. Treasure Island, FL: StatPearls Publishing, 2020.
3. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, et al. Guidelines for the early management of patients with acute ischemic stroke: 2019 update to the 2018 guidelines for the early management of acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke. *Stroke* 2019; 50: e344–e418.
4. Lin L and Hu K. Tissue plasminogen activator: side effects and signaling. *J Drug Des Res* 2014; 1: 1001.
5. Gorup D, Škokić S, Kriz J, et al. Tlr2 deficiency is associated with enhanced elements of neuronal repair and caspase 3 activation following brain ischemia. *Sci Rep* 2019; 9: 2821.
6. Choong C-J, Baba K and Mochizuki H. Gene therapy for neurological disorders. *Expert Opin Biol Ther* 2016; 16: 143–159.
7. Jayant RD, Sosa D, Kaushik A, et al. Current status of non-viral gene therapy for CNS disorders. *Expert Opin Drug Deliv* 2016; 13: 1433–1445.
8. Hudry E and Vandenberghe LH. Therapeutic AAV gene transfer to the nervous system: a clinical reality. *Neuron* 2019; 101: 839–862.
9. Kanninen KM, Pomeschik Y, Leinonen H, et al. Applications of the Keap1–Nrf2 system for gene and cell therapy. *Free Radic Biol Med* 2015; 88: 350–361.
10. Lykken EA, Shyng C, Edwards RJ, et al. Recent progress and considerations for AAV gene therapies targeting the central nervous system. *J Neurodev Disord* 2018; 10: 16.
11. Hughes S, Parr-Brownlie L, Bosch-Bouju C, et al. Lentiviral vectors as tools to understand central nervous system biology in mammalian model organisms. *Front Mol Neurosci* 2015; 8: 14.
12. Collins DE, Reuter JD, Rush HG, et al. Viral vector biosafety in laboratory animal research. *Comp Med* 2017; 67: 215–221.
13. Cronin J, Zhang X-Y and Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* 2005; 5: 387–398.
14. Castle MJ, Turunen HT, Vandenberghe LH, et al. Controlling AAV tropism in the nervous system with natural and engineered capsids. *Methods Mol Biol* 2016; 1382: 133–149.
15. Kobiler O, Drayman N, Butin-Israeli V, et al. Virus strategies for passing the nuclear envelope barrier. *Nucleus* 2012; 3: 526–539.
16. Gan Y, Jing Z, Stetler RA, et al. Gene delivery with viral vectors for cerebrovascular diseases. *Front Biosci (Elite Ed)* 2013; 5: 188–203.
17. Gan Y. Gene delivery with viral vectors for cerebrovascular diseases. *Front Biosci* 2013; E5: 188–203.
18. Nicolson SC and Samulski RJ. Recombinant adeno-associated virus utilizes host cell nuclear import machinery to enter the nucleus. *J Virol* 2014; 88: 4132–4144.
19. Lowes L, Al-Zaidy S, Shell R, et al. AVXS-101 phase 1 gene therapy clinical trial in SMA type 1: correlation between CHOP-INTEND and motor milestone achievements (S13.002). *Neurology* 2017; 88: s13.002.

20. Shahryari A, Saghaeian Jazi M, Mohammadi S, et al. Development and clinical translation of approved gene therapy products for genetic disorders. *Front Genet* 2019; 10: 868.
21. de Vries RBM, Hooijmans CR, Langendam MW, et al. A protocol format for the preparation, registration and publication of systematic reviews of animal intervention studies. *Evidence-Based Preclin Med* 2015; 2: 1-9 e00007.
22. Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *Bmj* 2009; 339: b2700.
23. Rohatgi A. Webplotdigitizer: Version 4.4. 2020.
24. R Core Team. R: a language and environment for statistical computing. 2021.
25. Viechtbauer W. Conducting meta-analyses in {R} with the {metafor} package. *J Stat Softw* 2010; 36: 1–48.
26. Ripley AJ, Jeffers MS, McDonald MW, et al. Neuroprotection by remote ischemic conditioning in rodent models of focal ischemia: a systematic review and meta-analysis. *Transl Stroke Res* 2021; 12: 461–473.
27. Zhu W, Fan Y, Frenzel T, et al. Insulin growth factor-1 gene transfer enhances neurovascular remodeling and improves long-term stroke outcome in mice. *Stroke* 2008; 39: 1254–1261.
28. Zhu W, Fan Y, Hao Q, et al. Postischemic IGF-1 gene transfer promotes neurovascular regeneration after experimental stroke. *J Cereb Blood Flow Metab* 2009; 29: 1528–1537.
29. Okoreeh AK, Bake S and Sohrabji F. Astrocyte-specific insulin-like growth factor-1 gene transfer in aging female rats improves stroke outcomes. *Glia* 2017; 65: 1043–1058.
30. Lu H, Wang Y, He X, et al. Netrin-1 hyperexpression in mouse brain promotes angiogenesis and long-term neurological recovery after transient focal ischemia. *Stroke* 2012; 43: 838–843.
31. Lu H, Wang Y, Yuan F, et al. Overexpression of netrin-1 improves neurological outcomes in mice following transient Middle cerebral artery occlusion. *Front Med* 2011; 5: 86–93.
32. Sun H, Le T, Chang TTJJ, et al. AAV-mediated netrin-1 overexpression increases peri-infarct blood vessel density and improves motor function recovery after experimental stroke. *Neurobiol Dis* 2011; 44: 73–83.
33. He X, Li Y, Lu H, et al. Netrin-1 overexpression promotes white matter repairing and remodeling after focal cerebral ischemia in mice. *J Cereb Blood Flow Metab* 2013; 33: 1921–1927.
34. Zheng M, Chen R, Chen H, et al. Netrin-1 promotes synaptic formation and axonal regeneration via JNK1/c-Jun pathway after the Middle cerebral artery occlusion. *Front Cell Neurosci* 2018; 12: 13.
35. Meng Z, Li M, He Q, et al. Ectopic expression of human angiopoietin-1 promotes functional recovery and neurogenesis after focal cerebral ischemia. *Neuroscience* 2014; 267: 135–146.
36. Zhao Y, Li Z, Wang R, et al. Angiopoietin 1 counteracts vascular endothelial growth factor-induced blood-brain barrier permeability and alleviates ischemic injury in the early stages of transient focal cerebral ischemia in rats. *Neurol Res* 2010; 32: 748–755.
37. Shen F, Walker EJ, Jiang L, et al. Coexpression of angiopoietin-1 with VEGF increases the structural integrity of the blood-brain barrier and reduces atrophy volume. *J Cereb Blood Flow Metab* 2011; 31: 2343–2351.
38. Shen F, Su H, Fan Y, et al. Adeno-associated viral-vector-mediated hypoxia-inducible vascular endothelial growth factor gene expression attenuates ischemic brain injury after focal cerebral ischemia in mice. *Stroke* 2006; 37: 2601–2606.
39. Shen F, Fan Y, Su H, et al. Adeno-associated viral vector-mediated hypoxia-regulated VEGF gene transfer promotes angiogenesis following focal cerebral ischemia in mice. *Gene Ther* 2008; 15: 30–39.
40. Chen Y-C, Ma N-X, Pei Z-F, et al. A NeuroD1 AAV-based gene therapy for functional brain repair after ischemic injury through in vivo astrocyte-to-neuron conversion. *Mol Ther* 2020; 28: 217–234.
41. Ge LJ, Yang FH, Li W, et al. In vivo neuroregeneration to treat ischemic stroke through NeuroD1 AAV-Based gene therapy in adult non-human primates. *Front Cell Dev Biol* 2020; 8: 1–21.
42. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke* 1999; 30: 2752–2758.
43. Fisher M, Feuerstein G, Howells DW, et al.; STAIR Group. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke* 2009; 40: 2244–2250.
44. Savitz SI, Baron J-C and Fisher M, STAIR X Consortium. Stroke treatment academic industry roundtable X: brain cytoprotection therapies in the reperfusion era. *Stroke* 2019; 50: 1026–1031.
45. Lapchak PA, Zhang JH and Noble-Haesslein LJ. RIGOR guidelines: escalating STAIR and STEPS for effective translational research. *Transl Stroke Res* 2013; 4: 279–285.
46. Wang P, Xu T-Y, Guan Y-F, et al. Nicotinamide phosphoribosyltransferase protects against ischemic stroke through SIRT1-dependent adenosine monophosphate-activated kinase pathway. *Ann Neurol* 2011; 69: 360–374.
47. Fluri F, Schuhmann M and Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. *Drug Des Devel Ther* 2015; 9: 3445–3454.
48. Zeng J, Wang Y, Luo Z, et al. TRIM9-Mediated resolution of neuroinflammation confers neuroprotection upon ischemic stroke in mice. *Cell Rep* 2019; 27: 549–560.e6.
49. Liu L, Yuan H, Denton K, et al. Calcium/calmodulin-dependent protein kinase kinase is neuroprotective in stroke in aged mice. *Eur J Neurosci* 2016; 44: 2139–2146.
50. Miao J, Shen L-H, Tang Y-H, et al. Overexpression of adiponectin improves neurobehavioral outcomes after focal cerebral ischemia in aged mice. *CNS Neurosci Ther* 2013; 19: 969–977.
51. Li Y, Huang J, He X, et al. Postacute stromal cell-derived factor-1 α expression promotes neurovascular recovery in ischemic mice. *Stroke* 2014; 45: 1822–1829.

52. Rajput PS, Lyden PD, Chen B, et al. Protease activated receptor-1 mediates cytotoxicity during ischemia using in vivo and in vitro models. *Neuroscience* 2014; 281: 229–240.
53. Hu Q, Chen C, Khatibi NH, et al. Lentivirus-mediated transfer of MMP-9 shRNA provides neuroprotection following focal ischemic brain injury in rats. *Brain Res* 2011; 1367: 347–359.
54. Cao J-Y, Lin Y, Han Y-F, et al. Expression of nerve growth factor carried by pseudotyped lentivirus improves neuron survival and cognitive functional recovery of post-ischemia in rats. *CNS Neurosci Ther* 2018; 24: 508–518.
55. Cai H, Mu Z, Jiang Z, et al. Hypoxia-controlled matrix metalloproteinase-9 hyperexpression promotes behavioral recovery after ischemia. *Neurosci Bull* 2015; 31: 550–560.
56. Al Mamun A, Chauhan A, Qi S, et al. Microglial IRF5-IRF4 regulatory axis regulates neuroinflammation after cerebral ischemia and impacts stroke outcomes. *Proc Natl Acad Sci USA* 2020; 117: 1742–1752.
57. Puntel M, Kroeger KM, Sanderson NSR, et al. Gene transfer into rat brain using adenoviral vectors. *Curr Protoc Neurosci* 2010; Chapter 4: Unit 4.24.
58. Joglekar AV and Sandoval S. Pseudotyped lentiviral vectors: one vector, many guises. *Hum Gene Ther Methods* 2017; 28: 291–301.
59. Mancini G and Horvath TL. Viral vectors for studying brain mechanisms that control energy homeostasis. *Cell Metab* 2018; 27: 1168–1175.
60. Turner RC, DiPasquale K, Logsdon AF, et al. The role for infarct volume as a surrogate measure of functional outcome following ischemic stroke. *J Syst Integr Neurosci* 2016; 2: 10.
61. Thomas JM, Cunningham CJ, Lawrence CB, et al. Therapeutic potential of extracellular vesicles in preclinical stroke models: a systematic review and Meta-analysis. *BMJ Open Sci* 2020; 4: e100047.
62. Huang H, Qian K, Han X, et al. Intraparenchymal neural stem/progenitor cell transplantation for ischemic stroke animals: a meta-analysis and systematic review. *Stem Cells Int* 2018; 2018: 4826407–4826410.
63. Andsberg G, Kokaia Z, Klein RL, et al. Neuropathological and behavioral consequences of adeno-associated viral vector-mediated continuous intrastriatal neurotrophin delivery in a focal ischemia model in rats. *Neurobiol Dis* 2002; 9: 187–204.
64. Liu X, Tian F, Wang S, et al. Astrocyte autophagy flux protects neurons against oxygen-glucose deprivation and ischemic/reperfusion injury. *Rejuvenation Res* 2018; 21: 405–415.
65. Mo J-L, Liu Q, Kou Z-W, et al. MicroRNA-365 modulates astrocyte conversion into neuron in adult rat brain after stroke by targeting Pax6. *Glia* 2018; 66: 1346–1362.
66. Merienne N, Delzor A, Viret A, et al. Gene transfer engineering for astrocyte-specific silencing in the CNS. *Gene Ther* 2015; 22: 830–839.
67. Wang L, Wang Z, Shi J, et al. Inhibition of proprotein convertase subtilisin/kexin type 9 attenuates neuronal apoptosis following focal cerebral ischemia via apolipoprotein E receptor 2 downregulation in hyperlipidemic mice. *Int J Mol Med* 2018; 42: 2098–2106.
68. Zhao H, Wang J, Gao L, et al. MiRNA-424 protects against permanent focal cerebral ischemia injury in mice involving suppressing microglia activation. *Stroke* 2013; 44: 1706–1713.
69. Xie R, Cheng M, Li M, et al. Akt isoforms differentially protect against stroke-induced neuronal injury by regulating mTOR activities. *J Cereb Blood Flow Metab* 2013; 33: 1875–1885.
70. Cao G, Xiao M, Sun F, et al. Cloning of a novel apaf-1-interacting protein: a potent suppressor of apoptosis and ischemic neuronal cell death. *J Neurosci* 2004; 24: 6189–6201.
71. Choi D-H, Kim J-H, Lee K-H, et al. Role of neuronal NADPH oxidase 1 in the peri-infarct regions after stroke. *PLoS One* 2015; 10: e0116814.
72. Lai Y, Lin P, Chen M, et al. Restoration of L-OPA1 alleviates acute ischemic stroke injury in rats via inhibiting neuronal apoptosis and preserving mitochondrial function. *Redox Biol* 2020; 34: 101503.
73. Matlik K, Anttila JE, Kuan-Yin T, et al. Poststroke delivery of MANF promotes functional recovery in rats. *Sci Adv* 2018; 4: eaap8957.
74. Massaro G, Hughes MP, Whaler SM, et al. Systemic AAV9 gene therapy using the synapsin I promoter rescues a mouse model of neuronopathic Gaucher disease but with limited cross-correction potential to astrocytes. *Hum Mol Genet* 2020; 29: 1933–1949.
75. Saraiva J, Nobre RJ and Pereira de Almeida L. Gene therapy for the CNS using AAVs: the impact of systemic delivery by AAV9. *J Control Release* 2016; 241: 94–109.
76. Gan X, Chopp M, Xin H, et al. Targeted tPA overexpression in denervated spinal motor neurons promotes stroke recovery in mice. *J Cereb Blood Flow Metab* 2021; 41: 92–104.
77. Xin H, Liu Z, Buller B, et al. MiR-17-92 enriched exosomes derived from multipotent mesenchymal stromal cells enhance axon-myelin remodeling and motor electrophysiological recovery after stroke. *J Cereb Blood Flow Metab* 2021; 41: 1131–1144.
78. Herrmann AM, Meckel S, Gounis MJ, et al. Large animals in neurointerventional research: a systematic review on models, techniques and their application in endovascular procedures for stroke, aneurysms and vascular malformations. *J Cereb Blood Flow Metab* 2019; 39: 375–394.