SUPPLEMENTARY MATERIAL

Supplementary Methods & Materials

Animals

Nos1$^{-/-}$ knock-out mice were kindly provided by Andreas Reif (36-53 weeks old, 10 *Nos1*$^{-/-}$ females and 12 *Nos1*$^{+/+}$ females); C57BL/6 NCr mice (6-8 weeks old, 302 male, 55 female, 1 pregnant female on gestational day 18; 50-55 weeks old, 50 male), from Charles River (Sulzfeld, Germany); adult Sprague-Dawley rats (6-8 weeks old, 275–325 g), from either Charles River (Sulzfeld, Germany, n=25) or the animal service of the Autonomous University of Madrid (n=7). Mice and rats were kept separately, at a constant dark-light cycle of 12 h and received food and water ad libitum. All experiments were conducted in accordance with: the *Guide for the Care and Use of Laboratory Animals,* the local institutional guidelines for the use of experimental animals (University of Würzburg, Germany, approved by the local governmental authorities Regierung von Unterfranken, Würzburg, Germany), the institutional Ethics Committee of the Universidad Autónoma de Madrid, Spain, the European Guidelines for the use and care of animals for research, the European Communities Council Directive of 24 November 1986 (86/609/EEC), the Spanish Real Decreto of 10 October 2005 (RD 1201/2005), and followed the ARRIVE guidelines including blinding of raters and operators, randomization of animals and pre-specified sample size and power calculations (http://www.nc3rs.org.uk/arrive-guidelines).

Compounds

PSD-95 interfering drugs, Tat-NR2B9c monomer/Tat-N-dimer and ZL006, were obtained from Peps 4LS GmbH (Heidelberg, Germany) and Sigma-Aldrich Chemie GmbH (Munich, Germany), respectively. The NOS inhibitor, L-N^G-Nitroarginine Methyl Ester (L-NAME), was purchased from Sigma-Aldrich (Madrid, Spain and Munich, Germany).

MCAO model of ischemic stroke

Cerebral ischemia was induced by 60 min of transient middle cerebral artery occlusion (tMCAO) in mice and 90 min tMCAO in rats [1,2]. Mice and rats were anesthetized with 2-2.5% isoflurane (CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany) in oxygen. To occlude the origin of the right MCA a standardized silicon rubber-coated 6.0 nylon monofilament (60SPPK10; Doccol Corporation, Sharon, MA, USA) was inserted in mice and a 4.0 nylon monofilament (40SP, Doccol Corporation, Sharon, MA, USA) in rats. For permanent MCA occlusion the occluding monofilament was left *in situ*. Neurological deficits in mice and rats were scored according to Bederson[3] and with the grip test[4], either 6 h, 24 h or 72 h after ischemia. Then the animals were sacrificed, the brains were quickly removed and cut in three (mice) or five (rats) 2 mm thick coronal sections using a mouse/rat brain slice matrix (Harvard Apparatus, Holliston, MA, USA). The slices were stained for 20 min at 37°C with 2% 2,3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich Chemie GmbH, Munich, Germany) in PBS to visualize the infarctions[5]. Indirect, i.e. corrected for brain edema, infarct volumes were calculated by volumetry (ImageJ 1.49f software, National Institutes of Health) according to the following equation: $V_{indirect}$ (mm^3) = $V_{infarct} \times (1-(V_{ih} - V_{ch})/V_{ch})$, whereas the term $(V_{ih} - V_{ch})$ represents the volume difference between the ischemic hemisphere and the control hemisphere and $(V_{ih} - V_{ch})/V_{ch}$ expresses this difference as a percentage of the control hemisphere.

Exclusion Criteria

Animals were excluded from the analysis, if they died before the predefined experimental end point, if an intracerebral hemorrhage occurred, or the animal lost more than 20% of body weight and were scored 5 in the Bederson score.

Supplementary Table 1: Excluded animals for each experimental set up.

Inhibitor	Animals	Ischemia Model	Experiment Duration	Excluded Animals
Tat-Src	C57BL/6	pMCAO	24 h	4 of 11
Vector	Male			1 of 11
NR2B	6-8 weeks			2 of 11
Dimer				3 of 11
ZI006				5 of 11
Tat-Src	C57BL/6	tMCAO	6 h	0 of 11
Vector	Male			0 of 11
NR2B	6-8 weeks			0 of 11
Dimer				0 of 11
ZI006				0 of 11
Tat-Src	C57BL/6	tMCAO	24 h	3 of 13
Tat-Src High dose	Male			2 of 13
Vector	6-8 weeks			1 of 13
NR2B				4 of 13
NR2B High dose				1 of 13
Dimer				4 of 13
ZI006				1 of 13
Tat-Src	C57BL/6	tMCAO	72 h	6 of 15
Vector	Male			5 of 15
NR2B	6-8 weeks			7 of 15
Dimer				5 of 15
ZI006				6 of 15
Tat-Src	C57BL/6	tMCAO	24 h	3 of 11
Vector	Female			1 of 11
NR2B	6-8 weeks			2 of 11
Dimer				2 of 11
ZI006				1 of 11
Tat-Src	C57BL/6	tMCAO	24 h	4 of 10
Vector	Male			2 of 10
NR2B	50-55 weeks			4 of 10
Dimer				2 of 10
ZI006				2 of 10
+/+	Nos ko	tMCAO	24 h	2 of 12
-/-				2 of 10
Tat-Src	SD rats	tMCAO	24 h	2 of 12
NR2B				3 of 13
Vector	C57BL/6	tMCAO	24 h	0 of 13
L-NAME	Male, 6-8 weeks			1 of 13
Vector	SD rats			0/7
L-NAME 0,3		OGD with	15 min + 45 min	
L-NAME 1,0		Reperfusion		
L-NAME 3,0				
Vector	C57BL/6	OGD	4 h	0/1
NR2B	G18			

Immunohistochemistry

Cryo-embedded brain slices were stained, as described[6], overnight with an antibody against HIV1 tat (mouse, abcam, ab63957, 1:200) in 1x PBS containing 10% goat serum (Sigma-Aldrich, Munich, Germany), 1% bovine serum albumin (Sigma-Aldrich, Munich, Germany), and 0.2% Triton X-100 (Sigma-Aldrich, Munich, Germany). Subsequently, slices were incubated with Cy2 conjugated goat anti-mouse IgG (Dianova, 115-225-003, 1:400), in PBS containing 1% bovine serum albumin. For staining of DNA (cell nuclei) a fluorescent Hoechst dye (Hoechst 33342, Sigma-Aldrich, Munich, Germany) was added for 10 min at a concentration of 2 ng/ml. Sections were embedded in Mowiol 4-88 (Sigma-Aldrich, Munich, Germany) and analyzed under a microscope (Nikon Eclipse 50i).
Hippocampal neuronal cell cultures were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany), washed three times with 10 mM phosphate-buffered saline (PBS), and incubated for 60 min at 4°C in 10 mM PBS containing 5% goat serum (Sigma-Aldrich, Munich, Germany) and 0.2% Triton X-100 (Sigma-Aldrich, Munich, Germany). Primary antibodies (rabbit antibody against cleaved caspase-3, 1:400, Cell Signaling and mouse anti-NeuN, 1:100; MAB377, Merck Millipore) were diluted in 10 mM PBS containing 1% BSA (Sigma-Aldrich, Munich, Germany) and incubated for 1 h at 21°C. The cover slips were washed with 10 mM PBS and then incubated with secondary antibodies (Alexa 594-labeled goat antibody against rabbit IgG, 1:400, life technologies and Dyelight 488-labeled goat antibody against mouse immunoglobulin G (IgG), 1:400, abcam) in the same manner. Finally, cultures were washed and then covered with Vectashield mounting medium (Vector, Burlingame, CA, USA). Images were obtained by immunofluorescence microscopy (Nikon Eclipse 50i). Negative controls for all histological experiments included omission of primary antibodies and gave no signals (not shown).

Western Blot

Western blot analysis was performed according to standard procedures[2]. The following antibodies were used: rabbit caspase-3 antibody, 1:1000, Cell Signaling (#9662) and mouse anti–b-actin, 1:500,000 (A5441, Sigma-Aldrich).

Oxygen-glucose deprivation of rat hippocampal slices and quantification of viability

In vitro damage caused by oxygen and glucose deprivation followed by re-oxygenation (OGD/Reox) and the protection elicited by L-N^G-nitro arginine methyl ester (L-NAME) was studied in acutely isolated rat hippocampal slices [7]. Briefly, adult male Sprague–Dawley rats (275–325 g) were decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.), forebrains removed and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4), containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 0.5, $NaHCO_3$ 26, $MgSO_4$ 10, KH_2PO_4 1.18, glucose 11 and sucrose 200. Thereafter, hippocampi were dissected, and slices (350 μm thick) were prepared using a McIlwain Tissue Chopper. Then, the slices were transferred to vials of sucrose-free dissection buffer, bubbled with 95% O_2/5% CO_2 in a water bath for 45 min at 34ºC, to allow tissue recovery from slicing trauma before starting the experiments (equilibration period). Slices corresponding to the control group were incubated 15 min in a Krebs solution with the following composition (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18 and glucose 11; this solution was equilibrated with 95% O_2/5% CO_2. Oxygen and glucose deprivation was induced by incubating the slices for a 15 min period in a glucose-free Krebs solution, equilibrated with a 95% N_2/5% CO_2 gas mixture; glucose was replaced by 2-deoxyglucose. After this OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose (re-oxygenation period). Experiments were performed at 37°C. A control and an OGD group were included in all

experiments; L-NAME (0.3, 1 and 3 mM) was added after OGD, i.e. during the re-oxygenation period.
Hippocampal cell viability was determined through the ability of the cells to reduce MTT[8]. Hippocampal slices were collected immediately after the re-oxygenation period and were incubated with MTT (0.5 mg/ml) in Krebs bicarbonate solution for 40 min at 37ºC. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in order to produce a precipitated formazan derivative. The formazan produced was solubilized by adding 200 µl DMSO, resulting in a colored compound whose optical density was measured spectrophotometrically at 540 nm using a microplate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). Absorbance values obtained in control slices was taken as 100% viability.

Oxygen-glucose deprivation of mice hippocampal neuronal cell cultures and quantification of cell death

Neuronal cell cultures 9 were obtained from C57BL/6 mice embryos (E18). Briefly, pregnant mice were killed by cervical dislocation and embryos were removed and transferred into Hank's buffered salt solution (HBSS). After the preparation of hippocampi, tissue was collected in a tube containing 5 ml of 0.25% trypsin in HBSS. After 5 min of incubation at 37°C, the tissue was washed twice with HBSS. Thereafter, the tissue was dissociated in 1 ml of PNGM neuronal medium supplemented with 2 mM L-glutamine, 50 µg/ml gentamicin, 37 ng/ml amphotericin and 2% NSF-1 (all Lonza, Cologne, Germany) by triturating with fire-polished Pasteur pipettes of decreasing tip diameter. Neurons were plated at a density of 80,000 cells/cm^2 on poly-D-lysine (Sigma-Aldrich, Munich, Germany) –coated coverslips in four-well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Before the experiments, all cell cultures were incubated at 37°C and 5% CO_2 and maintained in culture for up to 7 to 12 days. To induce in vitro ischemic conditions, O_2 was restricted per incubation at 37°C in a humidified incubator with 5% CO_2, 3% O_2, and 92% N_2 and glucose restriction. Tat-NR2B9c or vehicle was added to the cell cultures 1 h prior to the induction of ischemic conditions. Cell viability was assessed by antibody staining for activated caspase-3 as described above. For quantification of neuronal cell death after OGD three visual fields with the size of 0.07 mm^2 were counted for neuronal cells stained with NeuN and the percentage of caspase-3 co-labeled cells were calculated.

Statistical analysis

All statistical analysis was performed using the GraphPad Prism 5 software for Windows (Version 5.02). All data were tested for Gaussian distribution using the Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefor for P values), or, for $n > 10$, the D'Agostino and Pearson omnibus normality test. If the data passed the normality tests, the parametric, unpaired t-tests, or, for more than two experimental groups, a one-way ANOVA was conducted. If the data did not pass the normality tests, the Mann-Whitney, or, for more than two experimental groups, the Kruskal-Wallis test was used. Following one-way ANOVA, Bonferroni's multiple comparison was used, and, after the Kruskal-Wallis test, Dunn's multiple comparison was performed

Power analysis and funnel plot.

We conducted a *post hoc* analysis of power in all earlier published studies using PSD-95i in animal stroke models. Eight studies and our own new data were analyzed for their power to detect a difference of 40% in infarct size. This threshold of 40% difference was based on post-hoc analysis of failed clinical trials where preclinical studies showed a 30-40% difference [10-

[12]. Power was calculated using Russ Lenth's power software, an alpha of 0.05, an effect of 0.4 and a pooled variance [(Lenth, R.V 2006-9, java Applets for Power and Sample Size [Computer Software], Retrieved 02-17-2014, from http://www.stat.uiowa.edu/~rlenth/Power)]. From each individual study, the coefficient of variation (CV) was calculated for WT and treated animal groups. For each paper, different groups with respect to sort of inhibitor, ischemic duration and animal species used were separately analyzed.
The data on infarct size that were used for the power analysis were also assessed for the possibility of publication bias by visually evaluating the possible asymmetry in funnel plots [13]. Funnel plots were made using the free software of the Cochrane Collaboration, Review Manager 5 (version 5.2.11, downloaded from http://tech.cochrane.org/revman/download at April 9, 2014).

Supplementary Tables

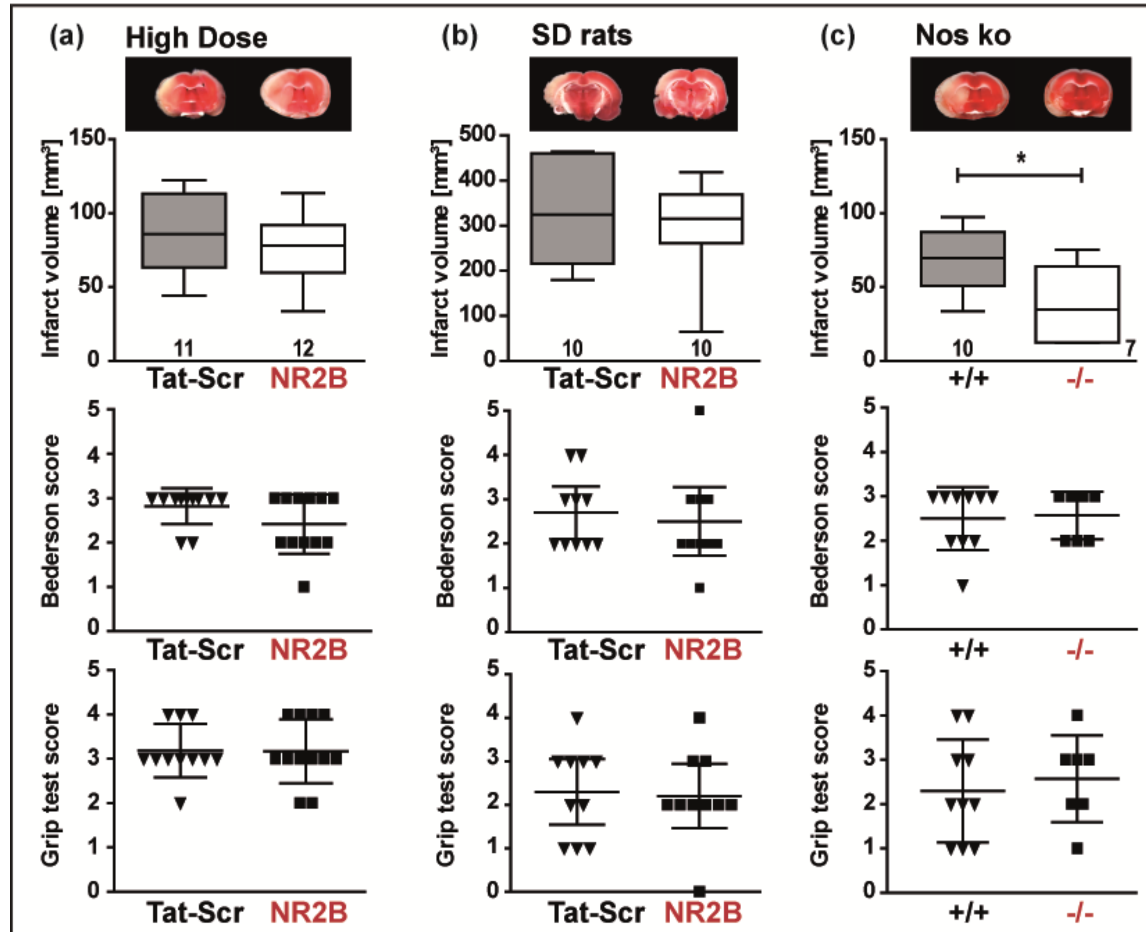
Supplementary Table 2. . Frequent lack of power[19] in previous studies on the role of PSD-95 inhibitors in experimental stroke

#	Ref.	Inhibitor	Animals	Model ischemia	Duration ischemia	Time infarct size	Time inhibitor	Infarct size: Inhibitor N	Inhibitor Mean	Inhibitor SD	Control N	Control Mean	Control SD	Pooled variance	Power for 40%	Found difference	Power for found difference
1	**Aarts *et al.* (2002)[14]**	Tat-NR2B9c (3nmol/gr)	SD rats	tMCAO	90min	24h	45min pre	6	81.0	61.2	6	178.0	24.0	0.54	21.2	54.5	35.0
2							60 min	9	45.0	12.6	10	136.0	44.9	0.31	75.9	66.9	99.4
3	**Bach *et al.* (2012)[15]**	Tat-N-Dimer (3nmol/gr)	C57BL6 mice	pMCAO	permanent	6h	30 min	17	11.3	7.4	16	19.0	7.2	0.54	54.0	40.5	55.0
4						48h	30 min	17	15.0	8.2	16	23.9	8.4	0.46	60.4	37.2	60.1
5		Tat-NR2B9c (3nmol/gr)				6h	30 min	18	15.3	7.6	16	19.0	7.2	0.45	71.4	19.5	23.4
6						48h	30 min	17	22.7	8.6	16	23.9	8.4	0.37	85.8	5.0	6.7
7	**Bell *et al.* (2013)[16]**	Tat-NR2B9c (3nmol/gr)	SD rats	PVO	permanent	24h	15 min pre	8	1.2	2.3	8	9.9	4.8	1.38	8.4	87.9	22.2
8	**Bråtane *et al.* (2011)[17]**	Tat-NR2B9c (7.5mg/kg)	Wistar rats	pMCAO	permanent	24h	60 min	8	182.9	78.7	8	236.9	48.7	0.33	61.3	30.7	40.8
9	**Cook *et al.* (2012a)[18]**	Tat-NR2B9c (2.6mg/kg)	Macaca fascicularis	tMCAO	3.5h	48h	3h	12	16.3	3.5	12	20.2	21.1	0.75	23.7	19.3	9.2
10						14d	3h	12	10.2	3.5	12	13.8	7.6	0.45	53.3	26.1	26.6
11					4.5h	48h	60min	6	23.9	6.9	6	30.5	4.9	0.23	76.6	21.6	30.3
12						7d	60min	6	20.6	4.2	6	28.2	3.7	0.17	95.5	27.0	69.8
13					90min	24h	60min	10	21.8	12	10	34.7	11.4	0.51	38.3	37.2	33.9
14						30d	60min	10	21	7.6	10	34.1	16.1	0.35	68.2	38.4	64.7
15	**Cook *et al.* (2012b)[19]**	Tat-NR2B9c (2.6mg/kg)	Macaca fascicularis	Embolic	permanent	24h	60min	9	25.1	4.2	8	63.3	42.5	0.47	36.9	60.3	68.8
16						30d	60min	9	7.5	2.0	8	22.9	2.7	0.21	95.5	67.4	100
17	**Sun *et al.* (2008)[20]**	Tat-NR2B9c (3nmol/g)	SD rats	tMCAO	90min	24h	3h	8	78.0	62.2	11	170.5	64	0.59	28.3	54.3	46.6
18						6d	3h	9	21.5	26.1	21	5	51.8	0.80	23.0	77.4	65.4
19				pMCAO	permanent	24h	1h	8	137..0	92..5	14	215..0	51..6	0..44	49..0	36..3	41..9
20			SD rats	PVO	permanent	24h	1h	8	4.0	3.9	8	9.2	8.5	0.96	12.2	56.5	19.7
21	**Teves et al (2015)**	Tat-NR2B9C 3nmol/g	C57Bl6 mice	tMCAO	30	24	At R	10	90.5	16.7	10	85.8	16.2	0.19	99.5	-5.5	9.5
22		10nmol/g						8	65.0	9.4	10	85.8	16.2	0.17	99.6	24.2	79.3
23		3nmol/g			60	24	At R	20	100.3	7.6	20	100.1	7.6	0.08	100	-0.2	5.0
24		10nmol/g						20	74.1	8.1	20	100.1	7.6	0.09	100	26.0	100
25	**Zhou *et al.* (2010)[21]**	ZL006 (1.5mg/kg)	SD rats	tMCAO rats	120	24h	1h after R	8	15.1	5.4	8	30.7	4.5	0.27	78.1	50.8	93.4
26			C57BL6 mice	tMCAO mice	90	24h	1h after R	8	11.0	3.9	8	32.2	2.4	0.32	63.5	65.8	96.6
27							3h after R	8	15.3	5.7	8	32.2*	2.4*	0.33	62.2	52.5	84.5
28							5h after R	8	24.1	6.4	8	32.2*	2.4*	0.27	77.8	25.2	40.5
29	**Current study**	Tat-NR2B9C (3nmol/g)**	Male mice	pMCAO	permanent	24h	1h after R	9	111..6	9.1	10	104.9	18.1	0.14	100	-6.4	15.9
30		Tat-N-Dimer (3nmol/g)**						8	113.4	11.6	10	104.9	18.1	0.15	100	-8.1	19.6
31		Zl006 (1.5mg/kg)**						5	106.2	17.9	10	104.9	18.1	0.17	96.9	-1.2	5.2
32		Tat-NR2B9C	Male mice	tMCAO	1h	6h	1h after R	11	76.2	29.3	11	84.0	28.7	0.36	88.3	9.3	8.8
33		Tat-N-Dimer						11	82.5	30.3	11	84.0	28.7	0.35	89.8	1.8	5.1

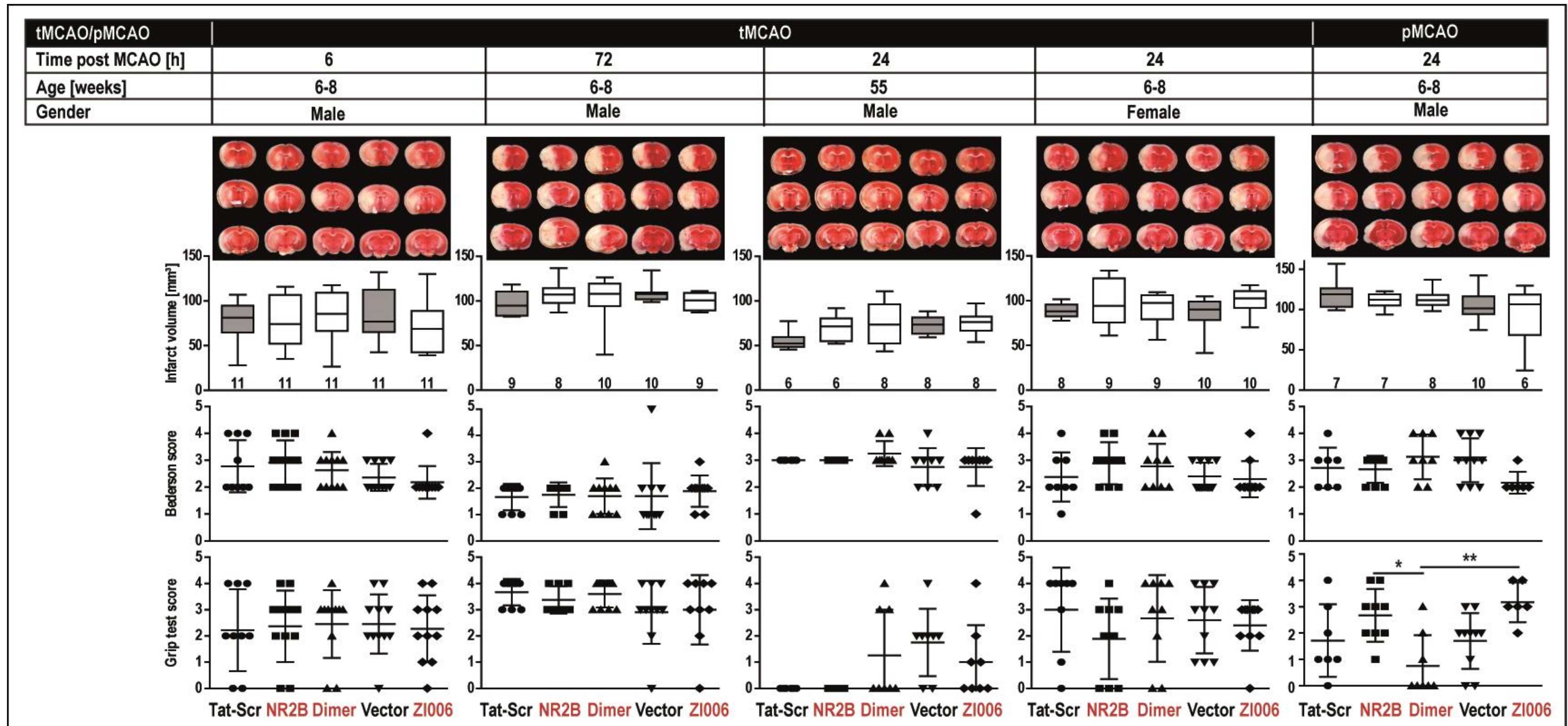
34		Zl006						11	69.9	27.9	11	84.0	28.7	0.37	86.7	16.8	17.3
35		Tat-NR2B9C						9	101.7	15.2	12	87.3	20.1	0.20	99.0	-16.5	42.6
36		Tat-N-Dimer				24h		9	92.2	13.2	12	87.3	20.1	0.20	99.1	-5.6	9.4
37		Zl006						13	107.6	16.1	12	87.3	20.1	0.19	99.9	-23.3	82.4
38		Tat-NR2B9C (10nmol/g)						11	75.3	22.9	12	87.3	20.1	0.27	92.6	13.7	21.5
39		Tat-NR2B9C						8	107.7	14.8	10	108.7	9.9	0.11	100	0.9	5.3
40		Tat-N-Dimer				72h		10	101.4	25.4	10	108.7	9.9	0.19	99.8	6.7	11.8
41		Zl006						9	99.2	9.6	10	108.7	9.9	0.09	100	8.7	48.1
42		Tat-NR2B9C						9	99.5	26.1	10	85.8	19.3	0.24	97.8	-16.0	27.1
43		Tat-N-Dimer	Female mice	tMCAO	1h	24h	1h after R	9	91.6	18.3	10	85.8	19.3	0.21	99.3	-6.8	10.0
44		Zl006						10	99.8	14.2	10	85.8	19.3	0.19	99.8	-16.3	45.1
45		Tat-NR2B9C						6	69.9	14.6	7	73.0	10.0	0.17	98.4	4.2	73.7
46		Tat-N-Dimer	Male middle - aged mice	tMCAO	1h	24h	1h after R	8	74.9	23.8	7	73.0	10.0	0.24	95.5	-2.6	5.5
47		Zl006						8	75.5	12.9	7	73.0	10.0	0.15	99.8	-3.4	7.2
48		Tat-NR2B9C	Rats	tMCAO	1.5	24h	at R	8	319.6	64.4	7	296.5	100.2	0.27	89.5	-7.8	7.9

To assess the power of already published studies, original data were extracted from the papers. Means, standard deviations (SD) and numbers of animals were extracted from the text or figures using the analysis tools from the Adobe Acrobat X Pro software. If only the standard error of the mean (SEM) was reported, the SD was calculated using the formula $SD = \sqrt{n} \cdot SEM$. For every group, the pooled variances of controls and inhibitor were caclculated (CV=SD/mean, pooled variance=$\sqrt{(CV^2*(n-1))/(n-1)}$ To calculate the power for detecting a 40% difference in infarct size, the two-sample t test (pooled or Satterthwaite) from Russ Lenth's power and sample size software (http://homepage.stat.uiowa.edu/~rlenth/Power/index.html) was used (alfa 0.05, true difference 0.4, equal sigmas (pooled variance) and n-numbers independent for treated and non-treated groups). Times of administration of the inhibitor are post ischemic start, unless mentioned otherwise. I, ischemia; PVO, pial vessels occlusion; R, reperfusion; tMCAO, transient middle cerebral artery occlusion; *, the control group for the three tMCAO groups in mice from Zhou *et al.* (2010)[21] received vehicle 1h after reperfusion; **, the dosages in the current study were the same in all groups. The numbers, 1-43, preceding each row corresponds to the numbers in panel a of Fig. 2.

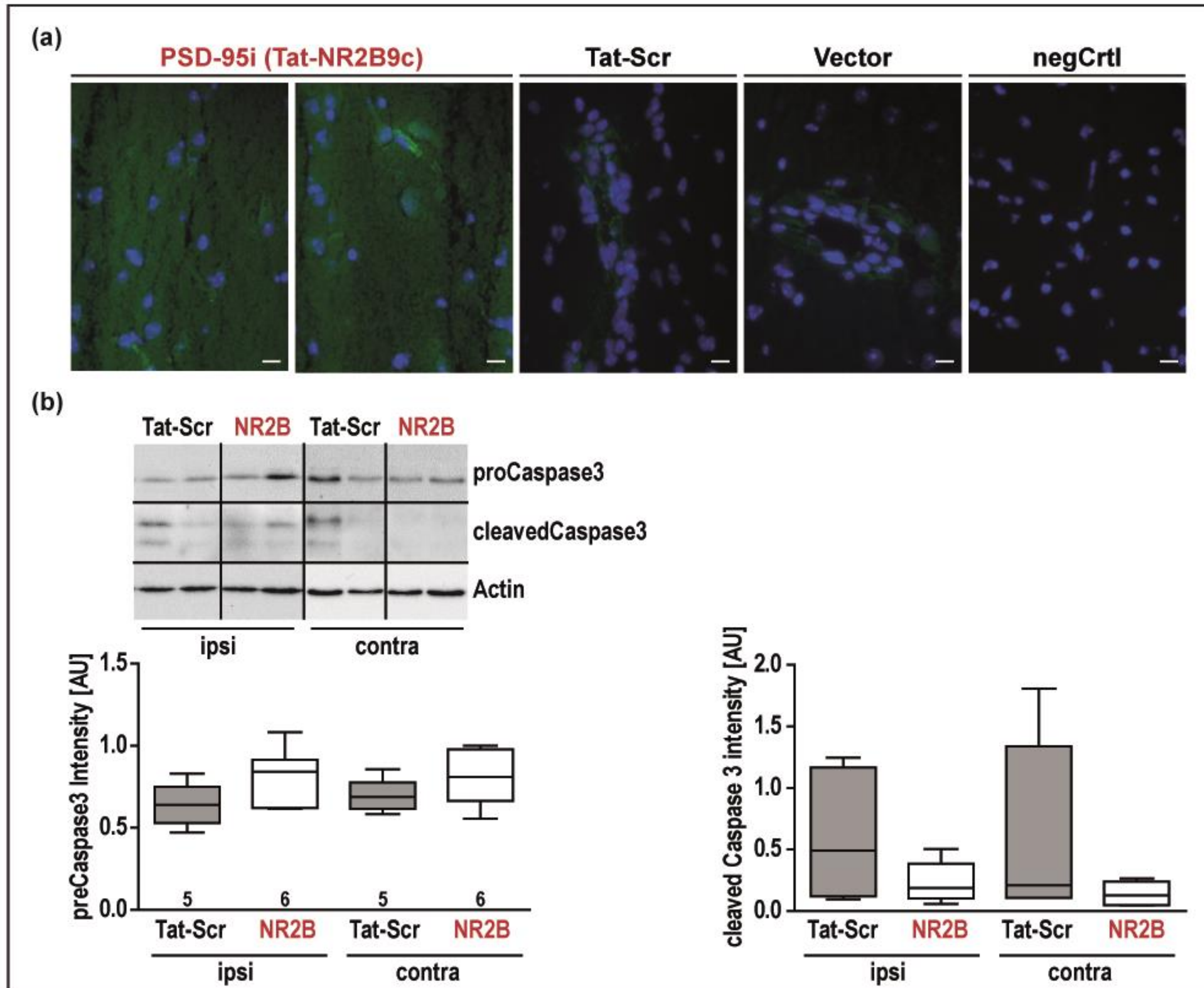
Supplementary Figures and Legends



Supplementary Figure 1. PSD-95 inhibitors had no effect on functional stroke outcome in higher concentrations in mice or in rats, while *NOS1*$^{-/-}$ mice have smaller infarcts **(a)** 6-8 week old C57BL/6 mice treated with 10 nmol/g NR2B or control peptide at reperfusion 60 min after MCAO showed no difference in infarct volume (box plots under representative TTC stained brain slice showing 5- 95 percentile and animal number; Unpaired t test $P_{infarct} = 0.310$) or behavior (scatter dot plots with mean and SD; Mann-Whitney test; $P_{Bederson} = 0.180$ and $P_{Grip} = 0.973$. **(b)** Sprague Dawley rats treated with 3 nmol/g NR2B or control peptide at reperfusion 90 min after MCAO showed also no amelioration of stroke outcome (box plots under representative TTC stained brain slice showing 5-95 percentile and animal number, scatter dot plots with mean and SD; Mann Whitney test: $P_{Infarct} = 0.631$; $P_{Bederson} = 0.538$; $P_{Grip} = 0.874$). **(c)** NOS1 ko mice following 60 min of tMCAO have smaller infarcts (box plots under representative TTC stained brain slice showing 5-95 percentile and animal number; Unpaired t test $P_{Infarct} = 0.020$) but show no improvement on coordination and motoric function 24 h after treatment (scatter dot plots with mean and SD; Mann Whitney test: $P_{Bederson} = 1.0$; $P_{Grip} = 0.621$).



Supplementary Figure 2. Lack of efficacy of PSD-95 inhibitors in mice is independent of age, gender and time post-stroke. C57BL/6 mice (8-10 weeks old males or females and >55 weeks old males as indicated above the representative TTC stained brain slices of each group) were subjected to 60 min tMCAO or pMCAO and treated with 3 nmol/g NR2B, Dimer, 1.5 mg/kg Zl006, a control peptide (Tat-Scr) or potassium chloride (vehicle). All substances were injected intravenously at reperfusion 1 h post tMCAO or pMCAO. At 6 h, 24 h or 72 h after tMCAO or pMCAO infarct volume was calculated from TTC-stained brain slices (top row; box plots showing 5-95 percentile and animal number) and behavior was assessed with the Bederson score and Grip test (middle and bottom row; scatter dot plots with mean and SD). All different experiments were analyzed using ANOVA or when not normal distributed with Kruskal-Wallis test und all subgroups were compared with the post-hoc tests Bonferroni's Multiple Comparison Test or Dunn's multiple comparison test, respectively. Only within the pMCAO experiment the Dimer subgroup was significantly worser in the Grip test score when compared with the NR2B ($P_{KruskalWallis} = 0.006$; * $P_{Dunn´s} < 0.05$) or Zl006 NR2B ($P_{KruskalWallis} = 0.006$; ** $P_{Dunn´s} < 0.01$) treated subgroups; not significant difference was found when compared with the control subgroups.



Supplementary Figure 3. Immunohistochemical localization of the Tat-construct and Western Blot analysis of caspase-3 and AIF after stroke **(a)** Immunohistochemical localization of the Tat-construct in brain sections 24 h after tMCAO using an anti-HIV1 tat antibody. For negative control (neg Ctrl) the HIV1 tat antibody was omitted. Bar, 10 µm. **(b)** Representative Western Blot showing procaspase-3, cleaved caspase-3 and loading control actin in ipsi- and contralateral brain lysates of 2 animals for each subgroup. Statistical analysis of both caspase forms showed no significant differences between the Tat-Scr or NR2B treated groups or between the hemispheres (box plots showing 5-95 percentile and animal numbers; Kruskal-Wallis test with Dunn's multiple comparison test ($P_{procasp3} = 0.248$ and $P_{cleavedcasp3} = 0.354$).

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