Aus dem Institut für Experimentelle Neurologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

A possible role of the Na^+/K^+-ATPase in the pathomechanism of spreading ischemia.

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Doctor medicinae (Dr. med.)

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Abstract

Spreading depolarization (SD) is characterized by a sustained neuronal depolarization and near-complete breakdown of the ion gradients across the cellular membranes. The recovery from SD is energy-dependent. Accordingly the local parenchymal ATP concentration falls by 45%. In healthy tissue the increased energy demand is satisfied by a marked rise in cerebral blood flow (CBF), but this hemodynamic response can become inverted under pathologic conditions like subarachnoid hemorrhage, cerebral ischemia or traumatic brain injury. Under this condition SD induces long-lasting vasoconstriction, which spreads together with the depolarization wave in the tissue. In an experimental animal model the resulting spreading ischemia (SI) led to cortical infarcts. SI occurs when SD runs in tissue with increased baseline extracellular potassium ($[K^+]_o$) and depletion of nitric oxide (NO).

In cell culture chronically increased $[K^+]_o$ reduces the activity of the sodium-potassium ATPase (NaKA). Therefore, we tested *in vivo* if direct inhibition of NaKA with ouabain induces SI in rats when the NO concentration is simultaneously decreased.
First we confirmed that chronically increased $[K^+]_o$ also reduces NaKA activity *in vivo*. Notably the α_2/α_3 isoforms were selectively affected. We then found that ouabain, in a concentration selectively inhibiting the α_2/α_3 isoforms, induced SI when NO was simultaneously depleted.

What could be the mechanism underlying this effect of α_2/α_3 NaKA inhibition? The α_2/α_3 isoforms are colocalized with the Na^+/Ca^{2+}-exchanger (NCX) at cell membrane sites adjacent to the (sarco-)endoplasmic reticulum (SER). Inhibition of the α_2/α_3 isoforms decreases the local sodium gradient across the cell membrane, which consequently reduces the amount of Ca^{2+} transported out of the cell by the NCX. The surplus Ca^{2+} is then stored in the SER and might directly increase calcium dependent contractility of vascular smooth muscle cells and pericytes. Their contractility is additionally enhanced by increased Ca^{2+} release from astrocytic SER and consequent release of vasoconstrictive substances from astrocytes. These processes appear to play a specific role for the duration of SI because thapsigargin, depleting Ca^{2+} from the SER, significantly shortened SI.

These results are clinically relevant because we and others showed that SD and SI occur in patients with the above-mentioned diseases and are associated with unfavorable outcome. Better understanding of the pathomechanisms underlying SI may lead to the development of new diagnostic and therapeutic strategies for clinical conditions associated with SD and SI.

Abstract (German)

Spreading Depolarization (SD) zeichnet sich durch einen fast vollständigen Zusammenbruch des neuronalen Membranpotentials aus, der durch massive Ionenverschiebungen zwischen Intra- und Extrazellulärraum verursacht wird. Die Wiederherstellung der physiologischen Ionenkonzentrationen führt bereits nach einer einzigen SD zur Reduktion der ATP-Konzentration im betroffenen Hirngewebe auf etwa 55% des Ausgangsniveaus. Dieser erhöhte Energiebedarf wird im gesunden Hirngewebe durch einen Blutflussanstieg ausgeglichen.

Unter pathologischen Umständen, z.B. nach einer Subarachnoidalblutung, einem Hirninfarkt, oder -trauma, kann es zur Umkehr dieser Blutflussantwort kommen. Dann induziert SD eine anhaltende Vasokonstriktion, die zu einer wandernden Mangeldurchblutung führt (englisch = Spreading Ischemia (SI)) und im Tiermodell kortikale Infarkte verursacht. Experimentell wird SI beobachtet, wenn die extrazelluläre Basis-K^+-Konzentration ($[K^+]_o$) vor der SD erhöht und gleichzeitig die NO-Konzentration ([NO]) erniedrigt ist.

In der Zellkultur reduziert chronische Erhöhung der $[K^+]_o$ die Aktivität der Natrium-Kalium-ATPase (NaKA). Daher haben wir in der vorliegenden Arbeit untersucht, ob eine direkte Hemmung der NaKA mit Ouabain in Kombination mit [NO]-Erniedrigung bei Ratten zu SI führt.
Zunächst konnten wir auch *in-vivo* bestätigen, dass chronisch erhöhte $[K^+]_o$ die Aktivität der NaKA reduziert, und zwar vorrangig die der α_2/α_3 Isoformen. Dass dieser Mechanismus zur SI beitragen könnte, belegten wir dadurch, dass wir SI auch durch direkte Inhibition der α_2/α_3 Isoformen mit Ouabain bei gleichzeitiger [NO]-Verminderung induzieren konnten.

Auf welche Weise führt eine Hemmung der α_2/α_3 NaKA zu verstärkter SD-induzierter Vasokonstriktion während SI? Die α_2/α_3 Isoformen der NaKA werden gemeinsam mit dem Na^+/Ca^{2+}-Austauscher (NCX) in Bereichen der Plasmamembran exprimiert, die in unmittelbarer Nachbarschaft zum (sarco-)endoplasmatischen Reticulum (SER) liegen. Inhibition der α_2/α_3 Isoformen führt zum Abfall des lokalen Na^+-Gradienten über die Zellmembran. Dadurch kann der NCX weniger Ca^{2+} aus der Zelle heraustransportieren, welches stattdessen vermehrt in das SER gepumpt wird. Daraus resultiert eine direkt verstärkte Ca^{2+}-abhängige Kontraktilität von glatten Gefäßmuskelzellen und Perizyten. Außerdem nimmt ihre Kontraktilität auch indirekt als Folge verstärkter Ca^{2+}-Ausschüttung aus dem astrozytären SER mit nachfolgender Freisetzung vasokonstriktiver Substanzen aus Astrozyten zu. Diese Prozesse scheinen in besonderer Weise eine Rolle für die Dauer der SI zu spielen. So konnten wir SI deutlich verkürzen, indem wir mit Thapsigargin die Ca^{2+}-Konzentration im SER vor der SI-Induktion reduziert haben.

Die Ergebnisse dieser Arbeit sind klinisch relevant, da wir und andere zeigen konnten, dass SD und SI bei den oben erwähnten neurologischen Krankheitsbildern im Patienten auftreten und mit schlechterem Outcome assoziiert sind. Die Entschlüsselung der zugrundeliegenden Pathomechanismen kann zum besseren Verständnis dieser Krankheiten und zur Entwicklung neuer diagnostischer und therapeutischer Strategien beitragen.

Affidavit

I, Stoigniew Sebastian Major certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "A possible role of the Na^+/K^+-ATPase in the pathomechanism of spreading ischemia". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

Detailed Declaration of Contribution

Sebastian Major had the following share in the following publication:

> Publication: Sebastian Major, Gabor C. Petzold, Clemens Reiffurth, Olaf Windmüller, Marco Foddis, Ute Lindauer, Eun-Jeung Kang and Jens P Dreier, **A role of the sodium pump in spreading ischemia in rats**, Journal of Cerebral Blood Flow & Metabolism, 2016. http://dx.doi.org/10.1177/0271678X16639059.
> Contribution in detail: Sebastian Major worked on design, performed and analyzed all experiments in groups 4-10, 12-15, 23 and 24 (all together 89 *in-vivo* experiments). Further, he analysed the experiments in groups 21 and 22, interpreted the data, wrote the initial version of the manuscript, prepared the manuscript for submission and took part in revising the study according to the reviewer's comments.

Signature, date and stamp of the supervising University teacher

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	6	NEURON	0896-6273	77446	15.054	16.839	3.221	399	8.2	0.22320	8.682
	7	MOL PSYCHIATR	1359-4184	14510	14.496	13.834	3.152	151	5.4	0.04317	5.007
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	9	ACTA NEUROPATHOL	0001-6322	13098	10.762	9.762	2.500	116	6.7	0.03329	3.483
	10	BIOL PSYCHIAT	0006-3223	40812	10.255	10.359	2.667	210	7.7	0.07948	3.553
	11	PROG NEUROBIOL	0301-0082	11430	9.992	10.487	2.839	62	9.4	0.02014	3.553
	12	ANN NEUROL	0364-5134	32934	9.977	10.792	1.970	167	9.9	0.06054	4.176
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	15	NEUROSCI BIOBEHAV R	0149-7634	16868	8.802	10.528	1.657	198	6.4	0.04123	3.596
	16	CEREB CORTEX	1047-3211	26191	8.665	8.335	2.043	282	6.7	0.06927	3.290
	17	SLEEP MED REV	1087-0792	3919	8.513	9.258	2.062	48	6.6	0.00959	3.247
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	19	NEUROPSYCHOPHARMACOL	0893-133X	22005	7.048	8.168	1.582	292	6.4	0.05006	2.643
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☐	22	CURR OPIN NEUROBIOL	0959-4388	12732	6.628	7.284	1.604	164	8.2	0.03380	3.559
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☐	25	J NEUROSCI	0270-6474	173265	6.344	7.348	1.188	1486	8.0	0.38326	2.862
☐	26	GLIA	0894-1491	11659	6.031	5.511	1.980	147	7.4	0.02283	1.782
☐	27	HUM BRAIN MAPP	1065-9471	16505	5.969	6.687	1.176	454	6.0	0.04232	2.288
☐	28	BRAIN BEHAV IMMUN	0889-1591	8218	5.889	5.895	1.278	205	4.7	0.02239	1.728
☐	29	J PSYCHIATR NEUROSCI	1180-4882	2491	5.861	6.789	0.902	41	6.0	0.00622	2.175
☐	30	BRAIN STRUCT FUNCT	1863-2653	2478	5.618	6.935	1.354	147	3.8	0.01071	2.435
☐	31	MOL AUTISM	2040-2392	577	5.413	5.821	0.442	52	2.4	0.00308	2.045
☐	32	J NEUROINFLAMM	1742-2094	5318	5.408	5.632	0.605	215	3.2	0.01820	1.524
☑	33	J CEREBR BLOOD F MET	0271-678X	15903	5.407	5.455	0.974	232	8.0	0.02926	1.785
☐	34	CURR OPIN NEUROL	1350-7540	4789	5.307	5.004	0.828	87	6.0	0.01265	1.732
☐	35	PAIN	0304-3959	31705	5.213	6.241	1.239	284	9.6	0.05060	2.160
☐	36	MOL NEUROBIOL	0893-7648	4012	5.137	5.460	1.209	206	4.0	0.01026	1.509
☐	37	CORTEX	0010-9452	6768	5.128	5.014	0.966	178	5.8	0.01690	1.623
☐	38	NEUROPHARMACOLOGY	0028-3908	16524	5.106	4.823	1.182	417	6.0	0.03722	1.454
☐	39	NEUROBIOL DIS	0969-9961	12714	5.078	5.298	1.142	275	5.4	0.03304	1.662
☐	40	NEUROTHERAPEUTICS	1933-7213	2584	5.054	5.821	1.319	72	4.5	0.00909	1.829

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A role of the sodium pump in spreading ischemia in rats

Sebastian Major, Gabor C. Petzold, Clemens Reiffurth, Olaf Windmüller, Marco Foddis, Ute Lindauer, Eun-Jeung Kang, Jens P. Dreier

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Supplemental Methods

NaKA assay

Enzymatic activity of NaKA (EC 3.6.1.37) was determined using a spectrophotometric enzyme assay (pyruvate kinase–lactate dehydrogenase), coupling the generation of ADP and oxidation of NADH.[1] The homogenization buffer contained in mmol/L: sucrose 250; EGTA 1.25; Tris 10. The reaction buffer contained in mmol/L: EGTA 1; Tris 125; NaCl 120; KCl 12.5; NaN_3 5; $MgCl_2$ 5; ATP-$MgCl_2$ 5; β-NADH 0.25; phosphoenolpyruvate 2.5. Brain tissue was placed on ice, weighed and homogenized (1/10w/v) in ice-cold homogenization buffer in a cooled Potter Braun S homogenizer. The reaction buffer was preincubated at 37°C for 3min in the temperature-controlled cuvette compartment of a continuously recording spectrophotometer (Shimadzu UV-1202, Shimadzu Scientific Instruments Inc., Columbia, USA). The reaction was started by adding 10u/ml pyruvate kinase (type III), 10u/ml lactic dehydrogenase (type XI) and a sample of the homogenized brain tissue to the reaction buffer. Oxidation of β-NADH in the cuvette was continuously monitored at 340nm using the spectrophotometer connected to a PC (PC-1201 software, Shimadzu Scientific Instruments Inc., Columbia, USA). Activity of all ATPases in the homogenate was calculated from the slope of the linear portion of the tracing, the β-NADH extinction coefficient, the volume of the reaction mix and the protein content in the brain tissue sample (with bovine serum albumin as standard).

To specifically determine total NaKA activity, the NaKA inhibitor ouabain (10mmol/L) was added to the reaction mix in a second cuvette. Activity of the NaKA represented the portion of total ATPase activity suppressible by 10mmol/L ouabain. Neurons and glial cells express distinct isoforms of NaKA (i.e., α_2 and α_3). In rats these isoforms can be distinguished from the α_1 isoform by their higher affinity to ouabain.[2] Therefore, in a third cuvette, we applied ouabain at 10μmol/L to the reaction mix in order to determine the activity of the α_2/α_3 isoforms. NaKA activity attributable to the α_2/α_3 isoforms was calculated by subtracting the portion suppressible by 10μmol/L ouabain from the fraction suppressible by 10mmol/L ouabain (i.e., total NaKA activity).[3]

Hb preparation

Hb was freshly prepared from citrate blood of Wistar rats as described previously.[4] Blood was centrifuged (3000G, 5min at 4^{o}C) and plasma discarded. Cells were washed twice with three to four volumes of cold 0.9% NaCl. The buffy coat was removed. Red blood cells were lysed by sonication. The resulting suspension was centrifuged (15,000G, 10min at 4^{o}C) and the pellet removed. The Hb-containing supernatant was transferred by gel chromatography (Bio-Gel P-6, Bio Rad, Richmond, VA, USA) to the ACSF. The total concentration of Hb in the ACSF was measured as cyanmetHb using a spectrophotometer (Shimadzu UV-1202, Shimadzu Scientific Instruments Inc., Columbia, USA) at 546nm wavelength.

Neocortical brain slice experiments

Twelve male Wistar rats (150-200g) were decapitated under ether anesthesia. Coronal neocortical slices (400µm) were obtained using a vibratome (WPI, Berlin, Germany) as previously described[5] and perfused with prewarmed carbogenated ACSF containing in mmol/L: NaCl 126.0; KCl 3.0; $CaCl_2$ 2.0; $MgSO_4$ 2.0; $NaHCO_3$ 26.0; NaH_2PO_4 1.25; glucose 10.0 at pH 7.40 in an interface-type recording chamber. SD was triggered by microinjection of 3mol/L KCl in layers II/III of rat neocortex using a glass capillary (~6µm tip diameter) connected to a pressure ejection system (Ionophor 3; Science Products, Hofheim, Germany). DC potential amplitude, duration at half-maximal amplitude ($T_{½max}$) and $[K^+]_o$ were recorded by two K^+-sensitive microelectrodes in layers II/III and digitized with a DASH-8u recorder (Astro-Med, West Warwick, RI, USA). Intrinsic optical signals (IOS) were monitored by transilluminating slices and recorded using a microscope-mounted CCD-camera. The control image in a series, captured before SD, was subtracted from each subsequent image, revealing changes in light transmittance (LT) over time. Regions of interest were selected to quantify and compare LT changes. SD velocity was determined by the propagation of the transient LT decrease.

Isolation and cannulation of the rat middle cerebral artery (MCA)

Male Wistar rats (n = 10; 250-350 g) were anesthetised with isoflurane and decapitated. All experiments were approved by the Governmental Animal Care and Use Committee (LAGetSi, T 0032/99). The brain was rapidly removed from the skull and put in cold (4°C) 3-(N-morpholino)propanesulfonic acid (MOPS) buffered saline solution with 1% dialysed bovine serum albumin containing in mmol/L: NaCl 144.0; KCl 3.0; $CaCl_2$ 2.5; $MgSO_4$ 1.5; NaH_2PO_4 1.21; EDTA 0.02; pyruvate 2.0; MOPS 2.0; glucose 5.0 at pH 7.40. For a detailed description of MCA isolation/cannulation see Lindauer and colleagues.[6] Briefly, approximately 1cm of the MCA was carefully dissected from the brain and cannulated on glass micropipettes. The vessel was continuously perfused with MOPS buffered saline solution at a transmural pressure of 80mmHg with a temperature of 37°C. The extraluminal bath contained MOPS buffered saline solution at a temperature of 37°C without bovine serum albumin and was continuously exchanged at a rate of 20ml/min. The vessel chamber was placed on an inverted microscope equipped with a videocamera. A monitor was used for online analysis of the luminal diameter. After preparation, the artery was allowed to equilibrate for one hour. During the entire experiment, temperature, perfusion inflow pressure, and flow rate were kept constant. All pharmacologically active substances were added to the extraluminal bath.

After development of spontaneous tone (at least 20% reduction of resting diameter compared to diameters measured immediately after pressurising), experiments started with

isolated increase of the extraluminal K^+ concentration ($[K^+]_e$) to 20mmol/L (= hypertonic solution) to test the arterial smooth muscle function. Arteries were excluded if they did not show K^+-induced vasodilation of at least 30%. Thereafter, $[K^+]_e$ was again lowered to 3mmol/L. In both groups 25 (n=5) and 26 (n=5), we applied MOPS buffered saline solution with an ion composition matching the extracellular changes as previously measured during SD/SI (= $cocktail_{SD}$) containing in mmol/L: NaCl 60.0; KCl 50.0; $CaCl_2$ 0.1; $MgSO_4$ 0.7; NaH_2PO_4 1.21; EDTA 0.02; pyruvate 2.0; MOPS 2.0; glucose 5.0; pH 6.90.[7] Then, $cocktail_{SD}$ was washed out again. Subsequently, L-NNA (Sigma-Aldrich, Deisenhofen, Germany) at 10µmol/L was washed in and, after equilibration, $cocktail_{SD}$ was co-applied with L-NNA. Experiments of group 26 were similar to those of group 25 but L-NNA was co-applied with ouabain at 5 µM.

Supplemental References

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Supplemental Figure legend

Figure 1: Extraluminal application of ionic cocktail matching previously measured ion changes during SD ($cocktail_{SD}$) led to marked dilation in absence and constriction in presence of NOS inhibition similar to an earlier study.[7] Ouabain merely augmented the vasoconstrictor response to the ionic cocktail under NOS inhibition when the response was analyzed in relation with the dilator response to the cocktail in absence of NOS inhibition, which corrects for the differences in vascular reactivity between individual MCAs (**P*<0.05, ***P*<0.01, ****P*<0.001, n=5 in both groups, Two Way Repeated Measures ANOVA [One Factor Repetition] with Bonferroni post-hoc tests).

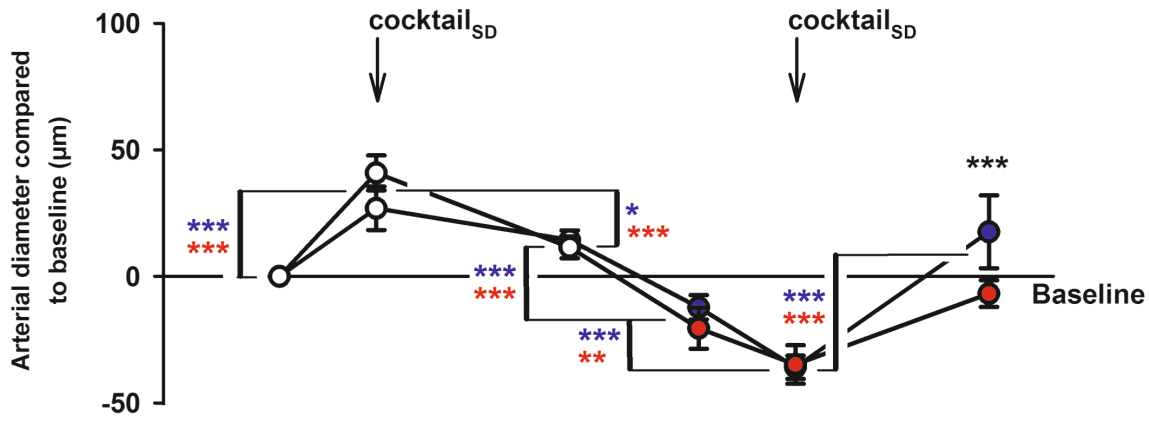
Supplemental Table

Supplemental Table 1. Thapsigargin did not prohibit SI but significantly shortened the durations of both hypoperfusion and negative DC shift compared to a vehicle control group

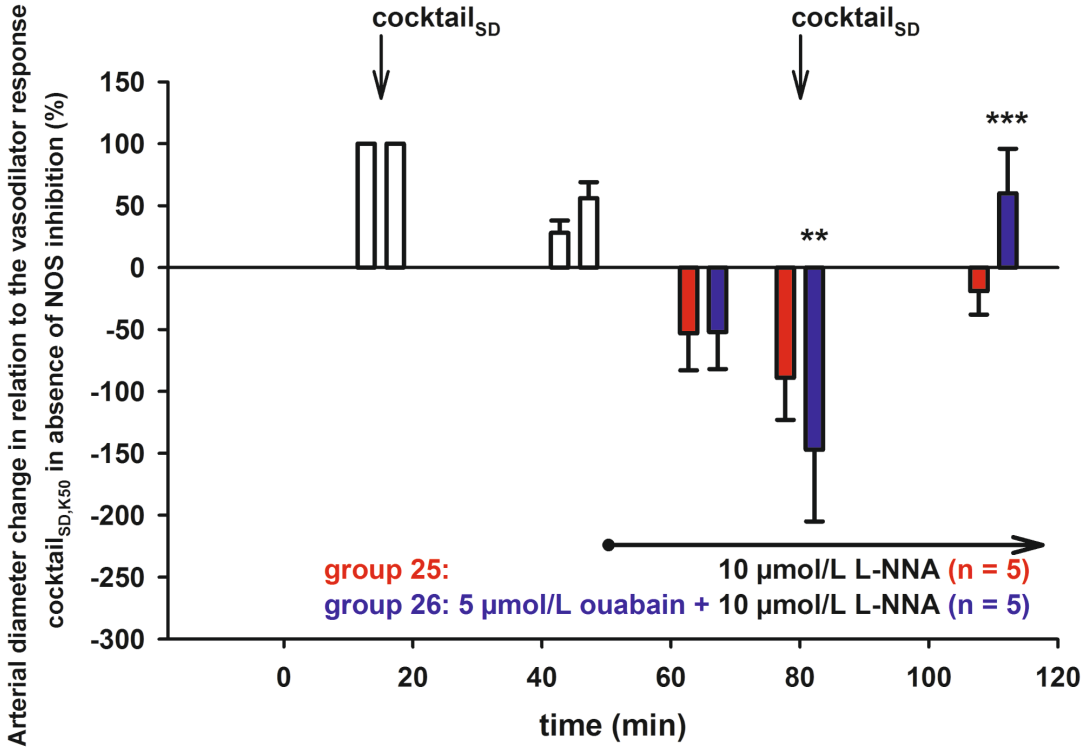
	$rCBF_{pre}$ (%)	$rCBF_{hypo}$ (%)	$rCBF_{hypo}$ dur (s)	CBF_{hyper} (%)	DC_{sa} amp (mV)	DC_{sa} dur (s)
L-NNA (1mmol/L) + thapsigargin (100nmol/L) + ouabain (50µmol/L) (group 23, n=6)	99 (86, 103)	46 (41, 53)	77* (60, 170)	177 (149, 196)	-3.3 (-2.3, -6.1)	261* (208, 369)
L-NNA (1mmol/L) + ouabain (50µmol/L) (group 24, n=6)	100 (93, 119)	37 (30, 39)	271 (159, 468)	197 (185, 244)	-4.1 (-2.2, -6.7)	810 (482, 824)

$rCBF_{pre}$ = rCBF level immediately before SD; $rCBF_{hypo}$ = lowest rCBF level during initial hypoperfusion in response to SD; $rCBF_{hypo}$ dur = duration of $rCBF_{hypo}$; $rCBF_{hyper}$ = highest rCBF level during transient hyperemia following SD; DC_{sa} amp = amplitude of subarachnoid DC shift during SD; DC_{sa} dur = duration of negative subarachnoid DC shift during SD. The two groups were compared with Mann-Whitney Rank Sum Tests (*P<0.05).

NOS inhibition converts middle cerebral artery dilation in response to cocktail$_{SD}$ into significant constriction



In relation to the vasodilator response in absence of NOS inhibition, the vasoconstrictor response to cocktail$_{SD}$ in presence of NOS inhibition is augmented by ouabain



Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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