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# Importance of nitric oxide for local increases of blood flow in rat cerebellar cortex during electrical stimulation

**(endothelium-derived relaxing factor/cerebral circulation/glutamate)**

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**ABSTRACT** **The endothelium-derived relaxing factor, probably nitric oxide (NO), is a potent vasodilator that regulates the vascular tone in several vascular beds, including the brain. We explored the possibility that NO might be of importance for the increase of cerebral blood flow (CBF) associated with activity of the well-defined neuronal circuits of the rat cerebellar cortex. Laser-Doppler flowmetry was used to measure increases of cerebellar blood flow evoked by trains of electrical stimulations of the dorsal surface. The evoked increases of CBF were frequency-dependent, being larger on than off the parallel fiber tracts, suggesting that conduction along parallel fibers and synaptic activation of target cells were important for the increase of CBF. This was verified experimentally since the evoked CBF increases were abolished by tetrodotoxin and reduced by 10 mM $Mg^{2+}$ and selective antagonists for non-*N*-methyl-D-aspartate receptors. The cerebellar cortex contains high levels of NO synthase. This raised the possibility that NO was involved in the increase of CBF associated with neuronal activation. NO synthase inhibition by topical application of $N^G$-nitro-L-arginine attenuated the evoked CBF increase by about 50%. This effect was partially reversed by pretreatment with L-arginine, the natural substrate for the enzyme, while $N^G$-nitro-D-arginine, the inactive enantiomer, had no effect on the evoked CBF increases. Simultaneous blockade of non-*N*-methyl-D-aspartate receptors and NO synthase had no further suppressing effect on the blood flow increase than either substance alone, suggesting that the NO-dependent flow rise was dependent on postsynaptic mechanisms. These findings are consistent with the idea that local synthesis of NO is involved in the transduction mechanism between neuronal activity and increased CBF.**

Nitric oxide (NO) is involved in a variety of biological actions as an intercellular messenger (1–4) in the brain between nerve cells within a small volume of tissue and possibly also between nerve cells and local blood vessels (5). Recent studies have shown that NO synthesis is important for the rises of cerebral blood flow (CBF) associated with hypercapnia (6–11) and increased synaptic activity (7, 12–14), though some results have been contradictory (15, 16). One of the problems in studying functional activation is that the CBF changes are studied in the cerebral cortex (7, 11–13, 15–19), despite of the fact that the cortical neuronal circuitry does not readily lend itself to stimulation of defined inputs and recordings of physiological variables elicited by monosynaptic responses.

We used the cerebellar cortex to study the coupling mechanism between neuronal activity and CBF for practical and theoretical reasons. The neuronal circuits of the cerebellar cortex are well-defined and the basic electrophysiology is well-established (20). Local stimulation of the cerebellar surface evokes action potentials in parallel fibers (PFs) that triggers glutamate release (21) and monosynaptic excitatory postsynaptic potentials in target cells that are mediated via non-*N*-methyl-D-aspartate (NMDA) receptors (22, 23). GABAergic (where GABA = γ-aminobutyric acid) inhibitory interneurons may in addition be activated via NMDA receptors (24). The PFs themselves, glial cells, and interneurons contain the NO synthase (NOS) immunoreactivity of the cerebellar cortex (25–27). Local NO synthesis from pre- and postsynaptic sources might therefore influence the microcirculation during periods of neuronal activation. This idea was tested using a specific, irreversible inhibitor of the constitutive form of NOS, $N^G$-nitro-L-arginine (NOLAG), and specific antagonists of NMDA and non-NMDA receptors. Our study indicates that physiological increases of CBF associated with synaptic activity in neuronal networks are partly dependent on neuronal synthesis of NO.

## MATERIALS AND METHODS

Fifty-three male Wistar rats (300–350 g) were anesthetized with halothane (Vapor; Dräger) (4% induction, 1.5% during surgery, and 0.7% maintenance) in 30% $O_2$/70% $N_2O$. Catheters were inserted in a femoral artery and a femoral vein, and the trachea was cannulated. After relaxation with 5–15 mg of suxamethonium i.p., rats were ventilated with a volume respirator and placed in a headholder, and the occipital bone and the dura were removed over the cerebellar cortex. The craniotomy site was continuously superfused with artificial cerebrospinal fluid (CSF) at 37°C, aerated with 95% $O_2$/5% $CO_2$ (composition in mM: NaCl, 120.00; KCl, 2.8; $NaHCO_3$, 22.00; $CaCl_2$, 1.45; $Na_2HPO_4$, 1.00; $MgCl_2$, 0.876).

CBF was continuously monitored by two laser-Doppler probes at fixed positions 0.5 mm above the pial surface (PF 403; outer diameter, 450 μm; fiber separation, 150 μm; wavelength, 670 nm; maximal intensity, 1 mW; Periflux 4001 Master, Perimed AB, JärFälla, Sweden). The two laser-Doppler probes and a bipolar stimulating electrode were positioned on the surface of the cerebellar cortex. One laser-Doppler probe was positioned 500–600 μm lateral to the stimulating electrode, in the direction of a PF beam (OnB), while the second probe was positioned 500–600 μm anterior to the stimulating electrode, 90° off beam (OffB). The experimental design is depicted in Fig. 1.

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Abbreviations: CBF, cerebral blood flow; NOLAG, $N^G$-nitro-L-arginine; NODAG, $N^G$-nitro-D-arginine; CSF, cerebrospinal fluid; NMDA, *N*-methyl-D-aspartate; PF, parallel fiber; NOS, nitric oxide synthase; TTX, tetrodotoxin; SNP, sodium nitroprusside; APH, amino-7-phosphonoheptanoate; CNQX, 6-cyano-7-nitroquinoxaline; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; OnB, in the direction of a PF beam; OffB, perpendicular to a PF beam (90° off-beam).

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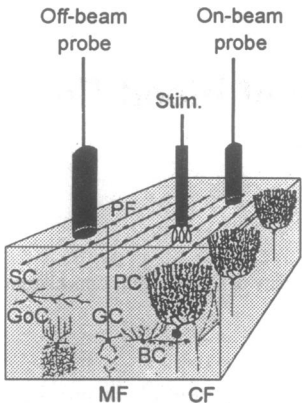


FIG. 1. Schematic drawing of sagittal view of rat cerebellar folium with two laser-Doppler probes and a bipolar stimulating electrode (Stim.), consisting of two twisted platinum/iridium wires, separated by 100 μm, positioned on the surface. Positions of OnB and OffB probes are indicated. Major cell types and input pathways of cerebellar cortex are as follows: SC, stellate cells; PC, Purkinje cells; BC, basket cells; GC, granular cells; GoC, Golgi cells; PF, PFs; MF, mossy fibers; CF, climbing fibers.

The arginine analog NOLAG, L-arginine, tetrodotoxin (TTX), adenosine, sodium nitroprusside (SNP), and amino-7-phosphonoheptanoate (APH) were purchased from Sigma. $N^G$-Nitro-D-arginine (NODAG) was from Adams Chemical (Round Lake, IL). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and NBQX [2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline] were generous gifts from Novo-Nordisk (Copenhagen). For i.v. infusion, NOLAG was dissolved in saline and ultrasonicated for 5–10 min. For topical application, drugs were dissolved in artificial CSF, ultrasonicated when necessary, and kept at 37°C while bubbling with 95% $O_2$/5% $CO_2$. The pH of the superfusing medium was 7.30–7.40. $MgCl_2$ at 10 mM was added to calcium-free CSF to obtain $Mg^{2+}$-CSF, while NaCl was reduced to maintain isoosmolarity.

In a few rats the field potential was monitored to define the location of the laser probes with respect to the PFs using a single-barreled microelectrode with a tip diameter of 2.5–5 μm (pointed tip), filled with 1 M NaCl. The electrical stimulation was delivered from a Grass SK4 stimulator through a pair of twisted Teflon-coated platinum/iridium wires, separated by 100 μm. For studies of CBF we used 100-μs-long pulses in trains of 15 s duration with frequencies of 20, 30, and 75 Hz at 100 V. The reliability of the electrical stimulation was tested at all frequencies once in every rat before drug treatment. The reactivity of the cerebellar microcirculation was also tested during hypercapnia by adding 5% $CO_2$ to the inhaled air before and after stimulation and before and after the application of drugs. CBF readings were taken after 5–10 min of hypercapnia. At end of experiments, adenosine (1 mM) was applied to verify that the two laser-Doppler probes measured CBF with the same sensitivity after stimulation and drug application. In seven rats SNP (300 μM) was applied as well. The time interval between tests ranged from 15 to 30 min. All changes of CBF were calculated as percent of the baseline value immediately preceding the test as described (11).

To verify the neural origin of the evoked increase of CBF, we measured CBF before and after having added TTX for 15–20 min to the superfusing medium. The role of $Ca^{2+}$-mediated neuronal processes was made evident by the application of $Ca^{2+}$-free CSF containing 10 mM $Mg^{2+}$. The influence of excitatory synaptic activity was examined by blocking NMDA receptors with APH (10 μM) or non-NMDA receptors with CNQX (10 μM) or NBQX (10 μM). Drugs were added to the CSF for 15–30 min before stimulation.

The influence of synthesis of NO was examined in one group of rats in which NOLAG was given at dose of 30 mg/kg in 2 ml of saline as a slow i.v. infusion over 6 min. In a second group, NOLAG (1 mM) was applied topically for 30–60 min before stimulation. Inhibition of NOS activity was verified in every single rat by measuring CBF during a second period of hypercapnia, since NOLAG attenuates the hypercapnic flow rise in rat cerebellar cortex (11). In a third group of rats, 10 mM L-arginine was applied topically before and during topical NOLAG application to test the interaction of a large dose of the natural substrate with the inhibitor. In a fourth group of rats, the stereospecificity of the inhibitor was demonstrated by topical application of NODAG (1 mM). Finally, NOLAG (1 mM) and NBQX (10 μM) were applied topically at the same time in a separate group of rats to uncover whether the effect of the two drugs on the CBF increase was additive.

For each drug, frequency, and OnB/OffB combination, the logarithmically transformed results were compared by paired *t* tests after having pooled the variance estimates across drug groups. Values were considered statistically significant at $P \leq 0.05$. Values are expressed as mean ± SEM; *n* indicates number of rats.

## RESULTS

The experimental arrangement is shown in Fig. 1. Stimulation of the cerebellar cortex at 20, 30, and 75 Hz evoked frequency-dependent increases of local CBF (Figs. 2 and 3). The magnitude of the CBF rise was 31–38% higher OnB than OffB at all frequencies. This was not due to different reactivities of the microcirculation at the two recording sites since the rise of CBF in response to hypercapnia or topical application of adenosine or SNP was the same OnB and OffB (Table 1). The limited spatial resolution and the fact that the CBF increases are only secondary indicators of neuronal excitation make it difficult to determine accurately the direction and origin of the substances causing the vasodilatation. On the other hand, the CBF increase was consistently larger OnB than OffB, suggesting that PF activity was important for the CBF increase. The time to peak of the CBF rise lasted only a few seconds (Fig. 2). This suggests rapid release of the vasoactive substances responsible for the evoked CBF increase.

CBF values before and after application of drugs are given in Table 2 for stimulation at 75 Hz (data for stimulation at 20 and 30 Hz are not shown). Topical TTX at 20 μM ($n = 5$) almost abolished the CBF increase elicited by stimulation (Figs. 2 and 3). Topical $Mg^{2+}$ (10 mM) in $Ca^{2+}$-free CSF ($n = 5$) attenuated the CBF increase by 43–52% OnB, but the effect was not statistically significant OffB. This suggests that $Ca^{2+}$-mediated neuronal processes make important contributions to the evoked CBF increases, at least OnB.

To examine the contribution of glutamate receptors, APH, NBQX, or CNQX (all at 10 μM) was applied topically for 15–30 min before stimulation. NBQX ($n = 6$) decreased the OnB CBF increase by 58–71%, while the OffB response was attenuated by 42–53% (Table 2, Fig. 3). CNQX ($n = 4$), another non-NMDA receptor antagonist, diminished the OnB CBF increase by 26–46% and the OffB response by 26–51%. APH had no effect on the evoked CBF increases ($n = 3$) (Table 2).

NO-dependent increases of CBF were studied by the use of NOLAG injected i.v. (30 mg/kg) ($n = 7$) or applied topically (1 mM) ($n = 10$). NOLAG i.v. had no effect on the evoked CBF increases, although its biological effect was clear from the rise in arterial blood pressure and an attenuation of the hypercapnic CBF increase. In contrast, topically applied NOLAG, which also reduced the hypercapnic vasodilation, attenuated the evoked CBF rises by 38–62% (Figs. 2 and 3). These results indicate that about half of the increases

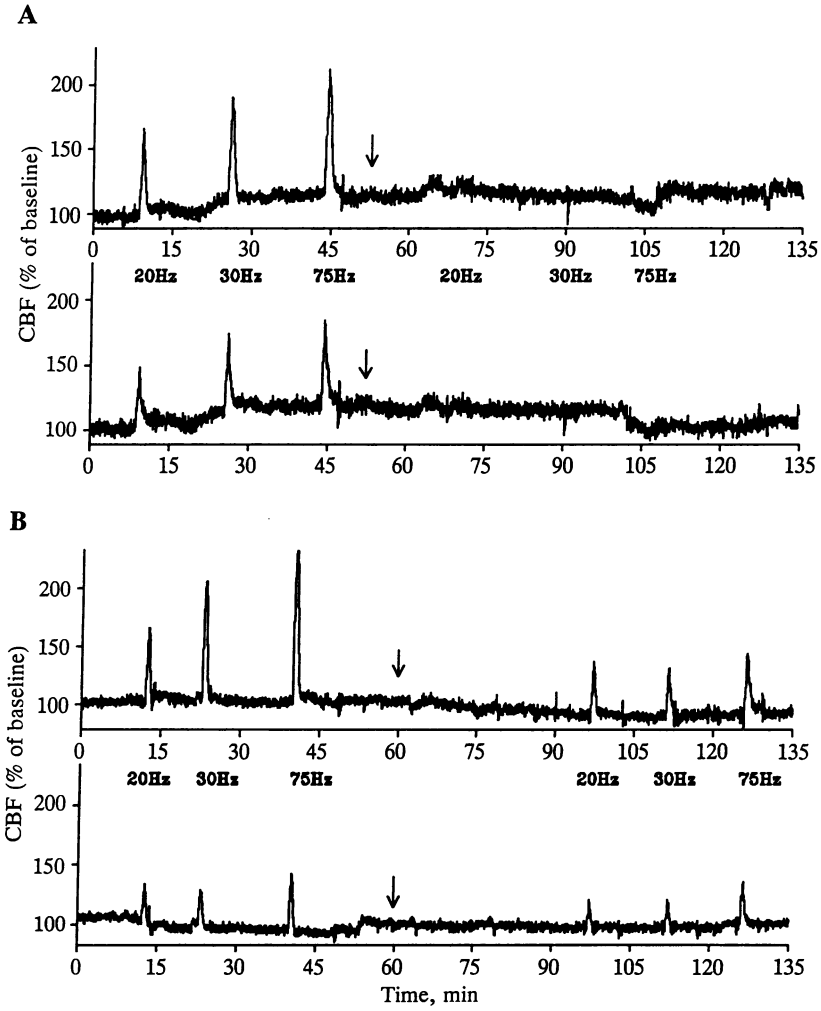


FIG. 2. Original records of CBF increases in response to electrical stimulation of the cerebellar cortex at PF beam (OnB) and 90° off-beam (OffB) measured by laser-Doppler flowmetry. The ordinate indicates CBF as a percentage of values prior to drug administration. Arrows indicate time of application of drug to superfusing CSF. (*A*) Abolition of the frequency-dependent CBF increases by TTX (20 μM), which indicates that the vascular reactions are of neural origin. (*B*) NO synthesis was inhibited by topical application of NOLAG (1 mM). This suppressed the CBF increases markedly both OnB and OffB, suggesting a role for NO in cerebellar vasodilatation.

of CBF in association with cerebellar neuronal activity are influenced by NOS activity. To document the specificity of the reaction, L-arginine (10 mM) ($n = 4$) was applied topically 15–20 min before and during topical application of NOLAG. Under these conditions, the response to stimulation and to hypercapnia remained constant. The D-isomer of NOLAG, NODAG, had no influence on the evoked CBF increases when topically applied or on the rise of CBF during hypercapnia ($n = 4$). There was no further reduction of the evoked CBF increase when NOLAG (1 mM) and NBQX (10 μM) were applied together, simultaneously or consecutively. This suggests that the effect of the two substances on cerebellar blood flow is sequential, probably dependent on postsynaptic mechanisms.

Finally, it was noted that the hypercapnic rise of CBF remained constant during topical application of 20 μM TTX, 10 mM $Mg^{2+}$ in $Ca^{2+}$-free medium, 10 μM NBQX, 10 μM CNQX, 10 μM APH, 10 mM L-arginine with 1 mM NOLAG, and 1 mM NODAG.

## DISCUSSION

This study examined the utility of the cerebellar cortex as a preparation in which the coupling between nerve cell activity and blood flow can be studied and sought to establish whether local synthesis of NO influenced the vasodilation elicited by activity in the neuronal networks of the cerebellum. Each of these subjects will now be considered in turn.

Stimulation of the cerebellar surface excites PFs to a depth of ≈100 μm (20). The PFs make synaptic contacts with Purkinje cells and inhibitory interneurons, while climbing fibers are believed not to be activated by stimulation. Thus, stimulation of the cerebellar cortex excites essentially only two cell types via a monosynaptic input (20). The cerebellar cortex cannot generate epileptic activity, or other autocatalytic neuronal events, and the elicitation of a spreading depression requires conditioning by changing the ionic composition of the interstitial fluid (28). These intrinsic properties of the cerebellum were essential for our experiments since it was possible to control the stimulus–response paradigm even at high levels of electrical stimulation.

The laser-Doppler probes were positioned in the direction of a PF beam and perpendicular to the beam, anterior to the stimulating electrodes. This orientation of the laser probes was used to examine CBF increases at one position at which the synaptic activity was supposed to be maximal and at a second position in which the CBF increase was supposed to be unrelated to excitatory synaptic activity. The evoked increases of CBF were larger OnB than OffB, but there was a definite CBF increase at both recording sites, though there are no excitatory neural structures perpendicular to the PF beam. A similar result was reported in an optical imaging

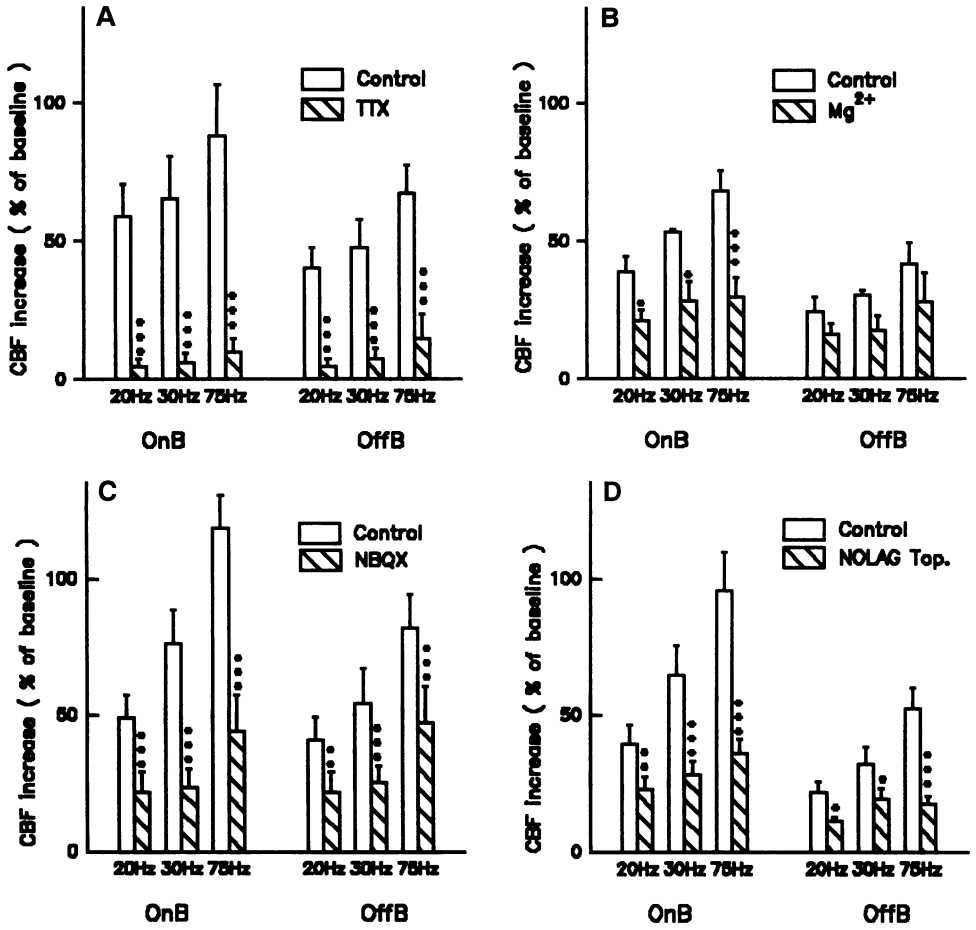


FIG. 3. Effect of TTX, $Mg^{2+}$, non-NMDA receptor blockade by NBQX, and NOS inhibition by NOLAG on rise of blood flow elicited by electrical stimulation of rat cerebellar cortex. Open bars, CBF increase prior to drug application; hatched bars, CBF increases at 30 min after topical application of: 20 μM TTX (*A*), 10 mM $Mg^{2+}$ in $Ca^{2+}$-free CSF (*B*), 10 μM NBQX (*C*), and 1 mM NOLAG (*D*). Error bars indicate SEM. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$ significantly different from control values.

study of voltage-sensitive dyes, which showed that small blood vessels perpendicular to the PF beam, extending for 500–600 μm, dilated in response to PF stimulation (29). It is possible that differences of volume transmission of vasoactive substances along, and perpendicular to the PF tract explain the different vascular reactions OnB and OffB (30).

OnB and OffB vascular responses were suppressed by TTX, a sodium channel blocker. This suggests that action potentials are essential for the elicitation of the CBF increases and excludes the idea that direct stimulation of the distal dendrites of the Purkinje cells or of glial cells is a main factor in eliciting the local CBF increases. $Mg^{2+}$ blocked part of the CBF increase OnB, suggesting that $Ca^{2+}$-mediated neuronal processes play a role in the vasodilation. This is important since the activity of the constitutive NOS is regulated by intracellular $Ca^{2+}$, $[Ca^{2+}]_i$ (4). Further studies will be needed to address this issue and to examine the

Table 1. CBF increases in response to stimulation, hypercapnia, adenosine, and SNP in controls

| Group | No. of rats | CBF rise, % above baseline OnB | OffB |
|---|---|---|---|
| Stimulation | | | |
| 20 Hz | 53 | 45 ± 3* | 30 ± 2 |
| 30 Hz | 53 | 65 ± 4* | 41 ± 3 |
| 75 Hz | 53 | 92 ± 5* | 60 ± 4 |
| Hypercapnia | | | |
| Before PF stimulation | 44 | 119 ± 6 | 126 ± 6 |
| After PF stimulation | 44 | 113 ± 7 | 133 ± 8 |
| Adenosine | 48 | 102 ± 9 | 94 ± 9 |
| SNP | 7 | 58 ± 9 | 53 ± 9 |

Values are mean ± SEM. The CBF increases OnB in response to PF stimulation were significantly higher than CBF increases OffB at all frequences (*, $P < 0.01$).

Table 2. CBF increases in percentage of baseline at 75 Hz stimulation

| Drug | No. of rats | OnB Control | OnB Test | OffB Control | OffB Test |
|---|---|---|---|---|---|
| TTX | 5 | 88 ± 19 | 10 ± 5*** | 67 ± 1 | 15 ± 9*** |
| $Mg^{2+}$ | 5 | 68 ± 10 | 30 ± 7* | 42 ± 7 | 28 ± 10 |
| NBQX | 6 | 118 ± 24 | 44 ± 14*** | 82 ± 12 | 47 ± 11*** |
| CNQX | 4 | 109 ± 16 | 60 ± 19* | 77 ± 18 | 41 ± 17** |
| APH | 3 | 110 ± 16 | 95 ± 13 | 75 ± 18 | 57 ± 4 |
| NOLAG | | | | | |
| i.v. | 7 | 63 ± 8 | 70 ± 11 | 48 ± 9 | 40 ± 9 |
| topical | 10 | 96 ± 14 | 36 ± 5*** | 52 ± 8 | 17 ± 3*** |
| L-Arg + NOLAG, topical | 4 | 90 ± 20 | 70 ± 9 | 47 ± 10 | 47 ± 14 |
| NBQX + NOLAG, topical | 5 | 82 ± 10 | 36 ± 4*** | 45 ± 10 | 23 ± 6* |
| NODAG | 4 | 113 ± 23 | 109 ± 18 | 82 ± 18 | 90 ± 12 |

Values are mean ± SEM. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$ as compared to control and test at corresponding frequency of stimulation.

mechanisms of increase of $[Ca^{2+}]_i$ that are important for cerebellar vasodilation. PFs release glutamate at the synapses with the target neurons (21), which contain mainly non-NMDA receptor channels in the postsynaptic membrane (22, 23). A large part of the evoked increase of CBF was linked to activation of non-NMDA receptors as indicated by a 40–50% block by NBQX and CNQX (31). NBQX was more effective in blocking the response than CNQX, in accord with the higher potency of NBQX in blocking non-NMDA receptors (32). The NMDA receptor antagonist APH did not attenuate the CBF increases even at high frequencies (24). Thus, the evoked CBF increase is associated with activity of non-NMDA, but not NMDA, receptor channels.

Purkinje cells are deficient in NOS, but granule, basket, stellate, and cerebellar glial cells appear positive for NOS (3, 25–27, 33, 34). NO has no influence on the excitatory postsynaptic potential elicited by PFs or the membrane properties of the Purkinje cells (35). Thus, decreased postsynaptic activity does not account for the effect of topically applied NOLAG, which suppressed the evoked CBF increase by up to 70%. The effect was neutralized by pretreatment with L-arginine, and the D-isomer of nitroarginine had no effect on the evoked CBF increases. This suggests that the effect of NOLAG was specific, due to interaction at the level of NOS. In contrast, i.v. NOLAG, which attenuates the hypercapnic rise of CBF, had no effect on the evoked CBF rises. We have previously found that the hypercapnic cerebrovasodilation was markedly inhibited by NOLAG i.v., while topically applied NOLAG was much less effective (11). This could suggest that structures close to the bloodstream, probably endothelial cells, supply the NO that modulates CBF during hypercapnia (11). The reverse pattern of efficiency of NOLAG was observed here during local electrical stimulation. This may suggest that local neural synthesis of NO, not endothelial NO, accounts for the NO-dependent part of the rise of CBF. There was no further effect on CBF when NOS and non-NMDA glutamate receptors were blocked at the same time. This raises the possibility that the two substances block NO synthesis along the same mechanism and thus occlude each other's action.

In summary, the studies of the cerebellar cortex showed that NO is necessary for the full CBF response to electrical stimulation of excitatory, mainly glutamatergic, synapses. These observations support the hypothesis that NO may serve as messenger molecule between nerve cells and blood vessels (5, 12).

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