Attenuation of activity-induced increases in cerebellar blood flow by lesion of the inferior olive

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Zhang, Yi, Colleen Forster, Teresa A. Milner, and Costantino Iadecola. Attenuation of activity-induced increases in cerebellar blood flow by lesion of the inferior olive. Am J Physiol Heart Circ Physiol 285: H1177–H1182, 2003.—We sought to define the contribution of the climbing fibers (CF), one of the major inputs to Purkinje neurons, to the increase in cerebellar blood flow (BFcrb) produced by activation of the cerebellar cortex. The neurotoxin 3-acetylpyridine was used to lesion the inferior olive, the site from which the CF originate. Crus II, an area of the cerebellar cortex that receives sensory afferents from the perioral region, was activated by low-intensity stimulation of the upper lip (5–25 V and 4–16 Hz) in sham-lesioned and lesioned mice. BFcrb was recorded in crus II using a laser-Doppler flow probe. The increase in BFcrb produced by harmaline, an alkaloid that activates the CF, was abolished in lesioned mice (P > 0.05 vs. BFcrb before harmaline, n = 6), attesting to the effectiveness of the lesion. In sham-lesioned animals, upper lip stimulation increased BFcrb in crus II by 25 ± 2% (25 V and 10 Hz, n = 6). The rise in BFcrb was attenuated by 63 ± 7% (25 V and 10 Hz) in lesioned mice (P < 0.05, n = 6). In contrast, the increase in BFcrb produced by hypercapnia was not affected (P > 0.05). These data suggest that CF are responsible for a substantial fraction of the increase in BFcrb produced by crus II activation. Thus the hemodynamic response evoked by functional activation of the cerebellar cortex reflects, in large part, CF activity.

Furthermore, the increase in BFcrb evoked by crus II activation, but not the associated field potentials, is markedly reduced in nNOS-deficient mice (25). These findings support the hypothesis that the hyperemic response is mediated by glutamate receptor-induced neural production of the potent vasodilator NO, which, in turn, is responsible for the increase in BFcrb.

The neural inputs responsible for the increase in BFcrb evoked by crus II activation have not been elucidated. Trigeminal afferents from the perioral region activate Purkinje cells in crus II via two major pathways (7, 11, 20); one reaches Purkinje cells through the mossy fibers and the granule neurons and their axons, the parallel fibers (PF), and the other originates in the inferior olive and reaches the Purkinje cells through the climbing fibers (CF). Therefore, in crus II, as in other regions of the cerebellar cortex, the CF and PF are the two major inputs to Purkinje cells (11, 20).

The relative contribution of these two pathways to the increase in BFcrb produced by cerebellar activation has not been established. This issue is relevant to functional imaging studies in which the hemodynamic changes evoked by neural activity are used to gain insight into the neural processes in the cerebellum during a wide variety of motor and cognitive tasks (4, 17, 19). Therefore, it would be important to define the pathways responsible for the hemodynamic changes produced by activation of the cerebellar cortex. Accordingly, in this study, we produced lesions of the inferior olivary complex using the neurotoxin 3-acetylpyridine (3-AP) to examine the role of the CF in the increase in BFcrb evoked by crus II activation.

METHODS

General Surgical Procedures

Studies were performed with approval of the Institutional Animal Care and Use Committee. Two- to three-month-old male C57BL6/J mice (Jackson) were anesthetized with 5% halothane in 100% O2. After induction of anesthesia, the concentration of halothane was reduced to 1–2%. Catheters were inserted in the femoral artery (PE-10) and in the trachea (PE-90). Animals were then placed in a stereotaxic frame (Kopf Instruments) and ventilated with an O2-N2

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mixture by a mechanical respirator (model SAR-830, CWE, Ardmore, PA). The O2 concentration in the mixture was adjusted to maintain arterial Po2 at 120–150 mmHg (Table 1). End-tidal CO2 was continuously monitored using a CO2 analyzer (Capstar-100, CWE) (23). Body temperature was maintained at 37 ± 0.5°C using a heating lamp thermostatically controlled by a rectal probe (model 75A-TA, Yellow Springs Instruments). The arterial catheter was used for continuous recording of arterial pressure and heart rate by a computerized data-acquisition system (PowerLab, ADInstruments) and for blood sampling. At the end of the surgical procedures, the halothane concentration was reduced to 1%. Because mice were not paralyzed, the adequacy of the level of anesthesia was assessed by testing corneal reflexes and motor responses to tail pinch. Throughout the experiment, two or three samples (50 µl) of arterial blood were collected for blood gas analysis. Such blood removal did not affect arterial pressure.

Monitoring of BF crb

Techniques used for monitoring BF crb in anesthetized mice have been described previously (23). A small hole (3 × 3 mm) was drilled in the occipital region to expose crus II, and the dura was carefully removed. The cranial window was contoured with a modified Ringer solution (pH 7.3–7.4, 37°C; see Ref. 8 for composition). BF crb was monitored using a laser-Doppler flowmeter (model BPM 403A, VasaMedic). The flow probe (0.5 mm tip diameter) was mounted on a micromanipulator (Kopf) and positioned 0.5 mm above the pial surface. The analog output of the flowmeter was amplified (DC amplifier, model 7P1, Grass Instruments) and fed into a data-acquisition system (PowerLab). Changes in BF crb were calculated as a percentage of baseline flow. The value for zero flow was determined at the end of the experiment after the heart was stopped with an overdose of halothane.

Lesions of the Inferior Olive by 3-AP

For lesions of the olive, the nicotinamide analog 3-AP was used (6, 12, 14, 15). 3-AP is a neurotoxin that antagonizes the incorporation of nicotinamide into NAD and, when administered systemically, damages selected neuronal groups, including those of the inferior olive, hypoglossal nucleus, hippocampus, and amygdala (6, 12). Coadministration of harmaline, an alkaloid that activates inferior olivary neurons, followed by nicamamide restricts the neurotoxicity mainly to neurons of the inferior olivary complex (12, 14). Administration of 3-AP (75 mg/kg ip) was followed 3 h later by harmaline (15 mg/kg ip) and 1.5 h later by nicamamide (300 mg/kg ip) (15). After harmaline administration, mice developed fine body tremors, followed by mild motor incoordination. However, as shown previously, these deficits did not affect the ability of the mice to thrive (15). After 1 wk, mice were anesthetized and instrumented for measurement of BF crb. Mice that received intraperitoneal injection of vehicle (saline) served as sham-lesioned controls. For histological verification of lesions after the BF crb experiment, mice were perfused transcardiacally with 4% paraformaldehyde in phosphate buffer. Brains were removed, postfixed, and embedded in paraffin as previously described (23). Sections (7 µm thick) were cut through the lower brain stem, mounted on microscope slides, and stained with hematoxylin and eosin. Slides were examined under a Nikon Eclipse microscope equipped with a digital camera (Photometrics) to assess the degree of destruction of olivary neurons. Digital images (Fig. 1) were adjusted with PhotoShop 6 (Adobe Systems) for optimal brightness and contrast. Processed images were assembled in Quark X-press 4.1 (Adobe).

Table 1. Arterial pressure and blood gases in the mice in which BF crb was measured

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>PaCO2, mmHg</th>
<th>PaO2, mmHg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harmaline</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sham</td>
<td>34.3 ± 1.6</td>
<td>129 ± 9</td>
<td>7.41 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>3-AP</td>
<td>35.2 ± 0.8</td>
<td>136 ± 5</td>
<td>7.38 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Crus II stimulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>72 ± 3</td>
<td>35.4 ± 1.4</td>
<td>132 ± 6</td>
<td>7.37 ± 0.04</td>
</tr>
<tr>
<td>3-AP</td>
<td>75 ± 3</td>
<td>34.1 ± 1.2</td>
<td>131 ± 8</td>
<td>7.39 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 mice. See Fig. 1 for mean arterial pressure (MAP) with harmaline. BF crb, cerebellar blood flow; PaCO2 and PaO2, arterial PCO2 and PO2; 3-AP, 3-acetylpyridine.

Experimental Protocol

After surgical procedures were completed, the superfusion with Ringer solution was started, and blood gases were adjusted. Experimental manipulations started when hemodynamic and respiratory parameters reached a steady state (Table 1).

Effect of harmaline on BF crb. Harmaline activates the CF by antagonizing serotonergic inputs to the inferior olive, the site from which the CF originate (10, 20). Methods for assessment of the effect of harmaline on BF crb have been described in detail previously (24, 25). Briefly, harmaline (20 mg/kg ip) was injected, and the BF crb increase was recorded for the following 90 min. In some mice, harmaline produced fine tremors restricted to the facial whiskers (24). The reactivity of the preparation to hypercapnia was tested before administration of harmaline. Moderate hypercapnia (PCO2 = 40–45 mmHg) was produced by introducing CO2 into the circuit of the ventilator as previously described (24, 25).

Effect of upper lip stimulation on BF crb in crus II. The electrodes for stimulation of the upper lip were inserted, and the animal was allowed to stabilize for 30 min. To activate crus II, the upper lip was stimulated for periods of 30–40 s with increasing current intensities (5–25 V and 10 Hz) or frequencies (4–16 Hz and 25 V), and the evoked increase in BF crb was monitored in the ipsilateral crus II. Crus II activation produces increases in BF crb that reach a plateau after ~30 s of stimulation (22). The BF crb increase was measured at the level of the plateau. In these experiments, the reactivity of the preparation to hypercapnia was also tested.

Data Analysis

Values are means ± SE. Multiple comparisons were evaluated by analysis of variance and Tukey’s test (Systat). Two-group comparisons were evaluated by the two-tailed Student’s t-test. Differences were considered significant for P < 0.05.

RESULTS

Effect of 3-AP on Inferior Olivary Neurons

Administration of 3-AP resulted in severe depletion of small neurons and gliosis throughout the rostrocaudal extent of the inferior olive (Fig. 1, A–D). In addition, large neurons in the reticular formation adjacent to the olive were affected. In contrast, cerebellar granule neurons, the site of origin of the PF, did not appear depleted by the treatment. Furthermore, neuronal cell
bodies in the region of the trigeminal complex that receives afferents from the perioral area (16) did not differ in morphology, number, or distribution between sham-lesioned and 3-AP-lesioned mice (Fig. 1, E and F). Therefore, 3-AP produced degeneration of neurons in the inferior olive but not in the granular cell layer and trigeminal complex.

**Effect of Harmaline on BF\textsubscript{crb} in Sham- and 3-AP-Lesioned Mice**

To examine the functional consequences of CF lesions, the effect of harmaline on BF\textsubscript{crb} in sham-lesioned and 3-AP-lesioned mice was studied. In sham-lesioned mice (n = 6), harmaline produced marked and time-dependent increases in BF\textsubscript{crb} (Fig. 2B). The increases in BF\textsubscript{crb} were independent of changes in mean arterial pressure (Fig. 2A) or blood gases (Table 1). The increases in BF\textsubscript{crb} were abolished in 3-AP-lesioned mice (Fig. 2B; n = 6, P > 0.05 vs. BF\textsubscript{crb} before harmaline, by analysis of variance and Tukey’s test). In contrast, the increases in BF\textsubscript{crb} produced by mild hypercapnia were not affected in 3-AP-lesioned mice (Fig. 3; n = 6, P > 0.05 by t-test).

**Effect of Crus II Activation on BF\textsubscript{crb} in Sham- and 3-AP-Lesioned Mice**

Low-intensity electrical stimulation of the upper lip elicited increases in BF\textsubscript{crb} in crus II that were greatest
at 25 V and 10 Hz (Fig. 4; n = 6, P < 0.05). The magnitude of the BF_crb increases was comparable to that previously reported from this laboratory (23). Crus II activation in 3-AP-lesioned mice produced increases in BF_crb that were 60–65% smaller than those observed in sham-lesioned mice (63 ± 7% at 25 V and 10 Hz, n = 6, P < 0.05; Fig. 4). In contrast, the increase in BF_crb produced by hypercapnia was not attenuated in 3-AP-lesioned mice (Fig. 5; P > 0.05, n = 6).

DISCUSSION

We produced lesions of the inferior olive to examine the contribution of the CF to the increase in BF_crb produced by crus II activation. Administration of 3-AP resulted in marked neuronal depletion in the inferior olivary complex and abolished the increase in BF_crb produced by CF activation with harmaline. The effects of 3-AP treatment on the increase in BF_crb produced in
cru II by stimulation of the upper lip were then studied. It was found that 3-AP attenuates the BFcrb response to crus II activation substantially, whereas the increase in BFcrb produced by hypercapnia is not affected. The findings suggest that neural inputs reaching the Purkinje cells through the CF contribute to the increase in BFcrb produced by crus II activation.

The attenuation in functional hyperemia in 3-AP-treated mice cannot result from differences in arterial pressure or blood gases, because these variables were carefully controlled and did not differ among the groups of mice studied. Furthermore, the effect cannot be a consequence of a nonspecific alteration in cerebrovascular reactivity resulting from 3-AP, because the increase in BFcrb produced by systemic hypercapnia was not affected. However, we cannot rule out the possibility that other aspects of cerebrovascular regulation, e.g., endothelium-dependent vasodilation, are altered by 3-AP. 3-AP has also been reported to produce lesions of extraolivary sites in the brain stem (6, 12). However, it is unlikely that the attenuation in functional hyperemia was due to lesion of the trigeminal input to the inferior olive, because no damage was observed histologically in the subregion of the trigeminal complex that receives afferents from the perioral area (7). Therefore, the reduction of the BFcrb response in 3-AP-treated mice cannot be attributed to changes in systemic variables, nonspecific alterations in vascular reactivity, or lesions of the trigeminal pathway conveying the stimulus to the brain stem.

The major inputs to the Purkinje cells are the PF and the CF (20). Previous studies with optical imaging and electrophysiological recordings have indicated that CF activity is the major determinant of neural signals evoked in crus II by perioral stimulation (3, 5). The results of the present study expand on these findings by demonstrating that the hemodynamic response is markedly attenuated by lesion of the CF. Studies using functional brain imaging have found that motor, sensory, and cognitive tasks produce hemodynamic changes in the cerebellar cortex (4, 19). The present results suggest that a major contributor to these hemodynamic signals is the activity of the inferior olive-CF pathway. However, the BFcrb increase is not completely abolished by CF lesion, raising the possibility that PF also contribute to the hemodynamic response. Although we cannot rule out the possibility that 3-AP did not eliminate the CF entirely, our finding that 3-AP abolished the harmaline-induced increase in BFcrb attests to the completeness of the CF lesion. Irrespective of the contribution of the PF, the data suggest that the CF are responsible for a substantial component of the BFcrb increase. Harmaline has been reported to produce direct vasodilation through endothelium-dependent mechanisms (18). However, the observation that 3-AP treatment nearly abolishes the BFcrb increase produced by harmaline demonstrates that such a direct vascular effect does not play a major role in our experimental preparation.

The mediators by which CF contribute to the hemodynamic response evoked by crus II activation are not known. However, several lines of evidence suggest that neuronal NO is involved: 1) the increase in BFcrb produced by chemical (harmaline) or electrical stimulation of the CF is attenuated by pharmacological inhibition of nNOS (1, 24); 2) null mice lacking nNOS have a reduced hemodynamic response to CF activation (25); and 3) the increase in BFcrb produced by crus II activation is attenuated by nNOS inhibitors and is reduced in nNOS-null mice (22, 25). These findings collectively suggest that neurally derived NO is responsible for the component of the vasodilation initiated by the CF. This conclusion is also supported by studies demonstrating that activation of the CF by harmaline releases NO and increases cGMP (15, 21). However, NO is also involved in the residual component of the increase in BFcrb mediated by the PF (9).

In conclusion, we have demonstrated that CF lesion abolishes the increase in BFcrb produced by harmaline and attenuates markedly the increase in BFcrb evoked by crus II activation. The data indicate that CF activity is an important determinant of BFcrb increases produced by crus II activation. Inasmuch as crus II is representative of the rest of the cerebellar cortex, the functional hyperemic response evoked by somatosensory activation of the cerebellar cortex reflects, in large part, the CF input to Purkinje cells. These findings help in the interpretation of functional imaging studies in which evoked hemodynamic responses are used to explore the function of the cerebellar cortex.

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DISCLOSURES
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