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Letter to Neuroscience

DEGENERATION OF PURKINJE CELLS IN PARASAGITTAL ZONES OF THE CEREBELLAR VERMIS AFTER TREATMENT WITH IBOGAINE OR HARMALINE

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The indole alkaloids ibogaine and harmaline are β-carboline derivatives that cause both hallucinations and tremor. Reports that ibogaine may have potent anti-addictive properties have led to initiatives that it be tested for the treatment of opiate and cocaine addiction. In this study, ibogaine-treated rats were analysed for evidence of neurotoxic effects because human clinical trials of ibogaine have been proposed. We recently found that ibogaine induces a marked glial reaction in the cerebellum with activated astrocytes and microglia aligned in parasagittal stripes within the vermis.[28] Based on those findings, the present study was conducted to investigate whether ibogaine may cause neuronal injury or degeneration. The results demonstrate that, after treatment with ibogaine or harmaline, a subset of Purkinje cells in the vermis degenerates. We observed a loss of the neuronal proteins microtubule-associated protein 2 and calbindin co-extensive with loss of Nissl-stained Purkinje cell bodies. Argyrophilic staining of Purkinje cell bodies, dendrites and axons was obtained with the Gallyas reduced silver method for degenerating neurons. Degenerating neurons were confined to narrow parasagittal stripes within the vermis. We conclude that both ibogaine and harmaline have selective neurotoxic effects which lead to degeneration of Purkinje cells in the cerebellar vermis. The longitudinal stripes of neuronal damage may be related to the parasagittal organization of the olivocerebellar climbing fiber projection. Since these drugs produce sustained activation of inferior olivary neurons, we hypothesize that release of an excitatory amino acid from climbing fiber synaptic terminals may lead to excitotoxic degeneration of Purkinje cells.

Ibogaine, the principal alkaloid in roots of the African shrub *Tabernanthe iboga*, is an indole-alkylamine which is similar to harmaline in chemical structure. Both compounds produce CNS stimulation, hallucinations and generalized tremor.[16,31,37] Ibogaine has been used by native hunters of Gabon in west Africa as a stimulant and is given for hallucinogenic and psychotomimetic effects in tribal religious rites.[10,16,31] Recently, ibogaine has been proposed for use in the treatment of drug addiction based on claims that it may decrease narcotic dependence and symptoms of opiate withdrawal [Lotsof, H.S.: U.S. Patent no. 4,449,096 (1985) and no. 4,587,243 (1986)]. In support of that claim, animal studies have shown that administration of ibogaine reduces morphine self-administration in rats[15] and ameliorates symptoms associated with morphine withdrawal.[11,14]

When administered to rats[14,15,32,34] ibogaine causes a marked high-frequency tremor similar to that produced by harmaline.[37] Electrophysiologic studies of harmaline demonstrate that it activates neurons in the inferior olive and produces bursts of rhythmic complex spikes in Purkinje cells via the olivocerebellar climbing fiber projection.[8,21] Ibogaline, an alkaloid from the *Tabernanthe iboga* plant that is closely related to ibogaine, causes tremor and olivo-cerebellar activation[9] suggesting that these related plant alkaloids have similar mechanisms of action.

We recently observed that administration of ibogaine to rats produces activation of microglia and astrocytes in the cerebellar vermis.[28] The activated glial cells are not randomly distributed, but form radial, parasagittal bands. Since glial activation is suggestive of neuronal injury, we have employed immunocytochemical markers and silver stains in order to detect direct evidence of ibogaine-induced neurotoxicity in the cerebellum.

Morphologic signs of neuronal injury were analysed in cerebellum from rats that were injected with ibogaine (i.p.) and killed later by aldehyde perfusion ($n = 50$). Sprague–Dawley rats (Harlan SD, Inc., Indianapolis; males, 175–200 g) were

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Abbreviation: MAP2, microtubule associated protein 2.

housed in individual cages on a 12-h light–dark cycle with free access to food and water. For comparison with ibogaine, another group of rats ($n = 11$) was injected with harmaline. At two- to 15-day survival times, treated rats and untreated controls were perfused through the heart with 4% paraformaldehyde and sections of cerebellum processed for immunocytochemistry as described previously.[24,28]

For immunocytochemistry, cerebellar sections were incubated with primary antisera directed against microtubule associated protein 2 (MAP2) or calbindin D_{28} (from P. Emson). Several antisera against neuronal proteins that did not show obvious changes, including Tau (Sternberger SMI 51); neurofilament proteins (phosphorylated and non-phosphorylated: Sternberger SMI 31, 32 and 35); and the 68,000 mol. wt neurofilament protein (Sigma no. 68 NF) were also screened.

Several minutes after one dose of ibogaine, rats exhibited truncal ataxia and a high-frequency tremor of the head and trunk. Some rats had vigorous extensor movements of limbs that propelled them off the floor of the cage. After the initial phase of excitation, truncal hypotonia with flaccid limbs was seen during the first hour; the animals typically lay motionless on the floor of the cage with their eyes open and they responded to tactile stimuli. The tail had a characteristic extended position. Tremor and ataxia gradually resolved over 6–8 h and spontaneous activity increased. Locomotion appeared normal the next day but quantitative assessment of motor control was not conducted. A similar tremor was present in rats that received harmaline, but hypotonia and ataxia were less pronounced. Moderate interanimal variation was noted in the degree of drug-induced behavioral changes.

In control rats, neuronal cell bodies and dendrites throughout the cerebellar cortex were immunostained with the antibody to MAP2. MAP2 immunoreactivity was prominent in Purkinje cell dendrites throughout the molecular layer but was less intense in cell bodies, as previously described.[7,25] MAP2 reveals profiles of Purkinje cell perikarya with ascending dendrites radiating outward toward the pial surface (Fig. 1). Basket and stellate perikarya were also strongly immunoreactive. The granule layer was densely labeled with MAP2 making it difficult to distinguish granule cell bodies and their dendrites from other neurons. The cerebellar white matter was devoid of MAP2 consistent with the selective localization of this protein in perikarya and dendrites, but not axons.[7]

After ibogaine treatment, the molecular layer of the cerebellar vermis exhibited distinct radial stripes of decreased MAP2 immunoreactivity alongside densely stained MAP2-positive zones. The thin, unstained bands range from one to five Purkinje cells in width (Fig. 1). The pale, radial zones in the molecular layer which lack MAP2 were in register with Purkinje cell bodies that did not show MAP2 immunoreactivity. These MAP2-negative bands resulted from loss of immunoreactivity in dendrites of vermal Purkinje cells. Rarely, a thickened radial dendrite with increased MAP2 immunoreactivity arose from a dysmorphic Purkinje cell body. In the vicinity of unstained (or absent) Purkinje cells, somata of basket cells retained MAP2 staining and appeared to be unaffected.

Calbindin, a cytoplasmic calcium-binding protein, is differentially distributed in particular neuron types throughout the central nervous system.[2,6] Purkinje cell bodies and dendrites were strongly reactive with calbindin antibodies[6] while other cerebellar neurons were unstained (Fig. 2). Calbindin was also present in Purkinje cell axons seen in the granule layer and white matter. Purkinje cell bodies stained with calbindin formed a continuous monocellular sheet along the outer margin of the granule layer.

After ibogaine treatment, pale radial stripes devoid of calbindin immunoreactivity (Fig. 2) were found adjacent to normally stained regions of the vermis. These unstained longitudinal stripes are similar in size and orientation to the stripes that lack MAP2 immunoreactivity. The parasagittal bands of absent calbindin staining revealed a sharply circumscribed loss of Purkinje cells, a result also seen after harmaline treatment (Fig. 3). Occasional dysmorphic Purkinje cell dendrites displayed increased calbindin immunoreactivity; these thickened radial dendrites arose from darkly stained, pyknotic Purkinje cell bodies that were adjacent to large vacuoles. In several cases, darkly immunoreactive Purkinje cell bodies, with an irregular crescent shape, formed the edge of vacuoles. Such vacuoles, not seen in control rats, indicate a degenerative process. Another pathologic feature of ibogaine-treated rats was that some abnormal Purkinje cells emit descending axons with a spheroidal, calbindin-positive swelling along their course. These axonal enlargements in the outer part of the granule layer are reminiscent of axonal clubs that Cajal described on damaged Purkinje cell axons.[5] Similar dysmorphic axons, called "torpedoes" in neuropathology, are commonly associated with degenerating Purkinje cells[1] and are abundant in the "hyperspiny Purkinje cell" mutant mouse.[35]

The Gallyas reduced silver method[12] for degenerating neurons revealed densely stained, argyrophilic Purkinje cells in the vermis of ibogaine-treated rats (Fig. 4). Granular silver-stained dendritic fragments formed radial columns above the darkly stained somata (Fig 4B). Control rats were devoid of significant silver-stained cells. In treated rats, degenerating Purkinje cell bodies were intensely silver stained and were often associated with vacuoles (Fig. 4C). The degenerating Purkinje cells were distributed in thin parasagittal stripes corresponding to the zones lacking MAP2 and calbindin immunoreactivity. Axons from these degenerating cells descended into the white matter where they were distinguished by a

high affinity for silver (Fig. 4C). Clusters of degenerating axons formed compact bundles alternating with normal fibers in the white matter. Numerous silver-positive axon terminals were found in the fastigial nucleus and in the lateral vestibular nucleus, both of which receive direct projections from vermal Purkinje cells.

Purkinje cell bodies formed a continuous, uninterrupted layer in Nissl-stained sections from control rats (Fig. 4D). After treatment with ibogaine or harmaline, the Purkinje cell layer exhibited discontinuities marked by absent Purkinje cell bodies (Fig. 4E) adjacent to zones with normal appearing cell bodies. In semi-adjacent sections, the zones lacking Purkinje cells matched the distribution of calbindin-negative stripes. At sites where closely adjacent Purkinje cells were lost, we occasionally observed fragmented remnants of Purkinje cell bodies. However, the material used in this study (30-μm sections processed for immunocytochemical staining) did not afford high-resolution cytologic evaluation. Cytophathologic changes in tissue prepared for fine structural analysis of perikaryal morphology will be reported separately.

Control sections stained with markers for phosphorylated neurofilament proteins revealed numerous intensely stained axons in the cerebellar cortex; basket cell axons surrounding Purkinje cell bodies

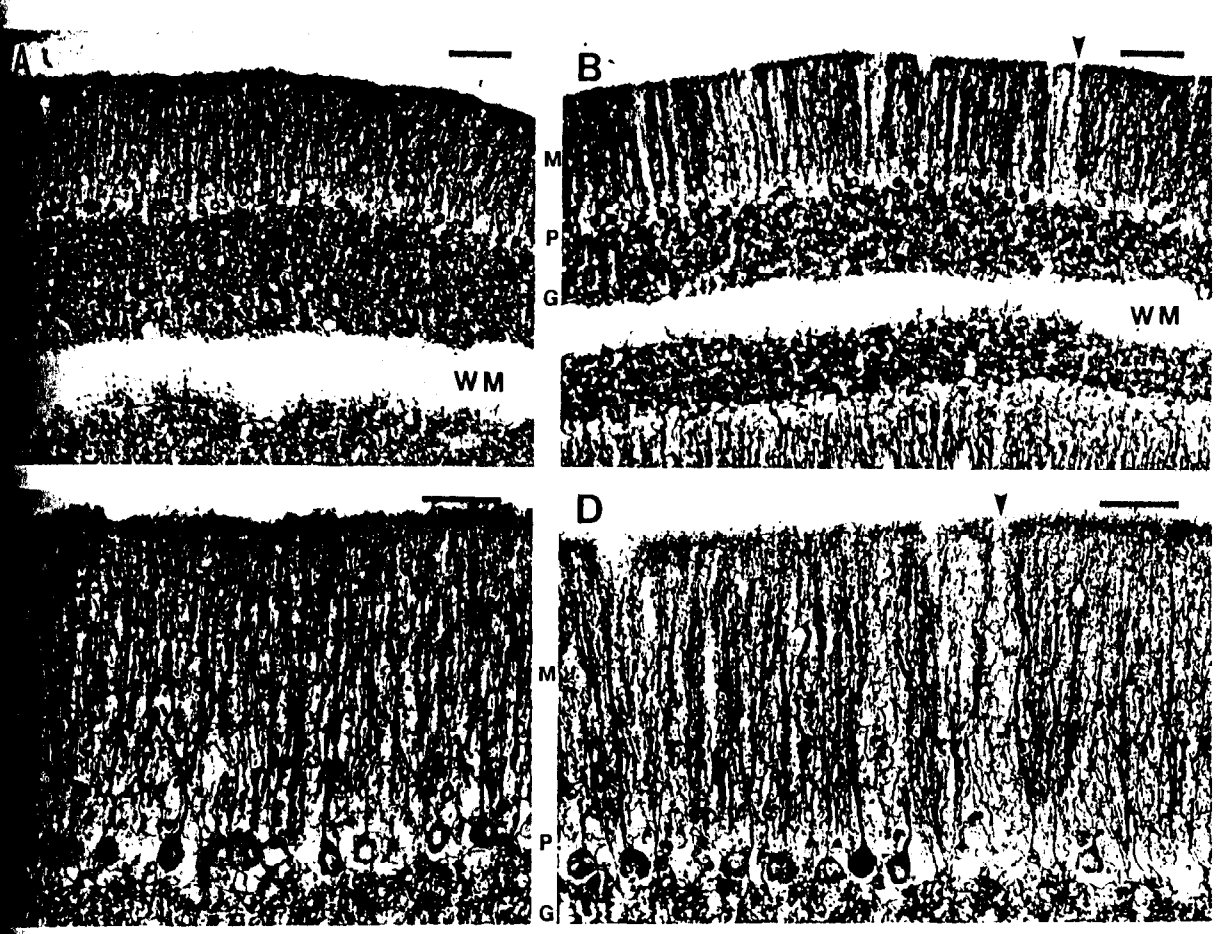

Fig. 1. MAP2 in neurons of cerebellar cortex (vermis, coronal sections) shown at low (A, B) and higher magnification (C, D). (A, C) untreated controls; (B, D) rats treated with ibogaine (100 mg/kg daily × 3; one week survival). Neuronal cell bodies and dendrites are uniformly stained in controls, but the white matter (WM) is pale since axons do not contain MAP2. After ibogaine treatment, several thin, unstained radial bands (arrowhead) can be seen in the molecular (M) and Purkinje cell (P) layers (B). As shown at higher magnification (D), the pale bands result from loss of MAP2 in small clusters of Purkinje cell bodies (one to five cells) and their ascending dendrites. Scale bars = 100 μm (A, B); 50 μm (C, D). At one week survival, rats were anesthetized with pentobarbital and perfused through the heart with phosphate-buffered saline followed by cold 4% paraformaldehyde (pH 7.4). Brains were immersed in 4% paraformaldehyde, frozen sections cut in the coronal plane and processed for immunocytochemistry as described previously.[28] To reduce background staining, floating sections were placed in a blocking solution (2% normal serum in phosphate-buffered saline with 0.3% Triton and 5% powdered milk) for 30 min prior to incubation in primary antisera at 4°C. Primary antibodies were visualized using Vectastain ABC-Elite reagents (Vector Labs, CA). The primary antibody was a mouse monoclonal against MAP2 (diluted 1:8000; no. SMI 52-Sternberger Monoclonals Inc.). G, granule layer.

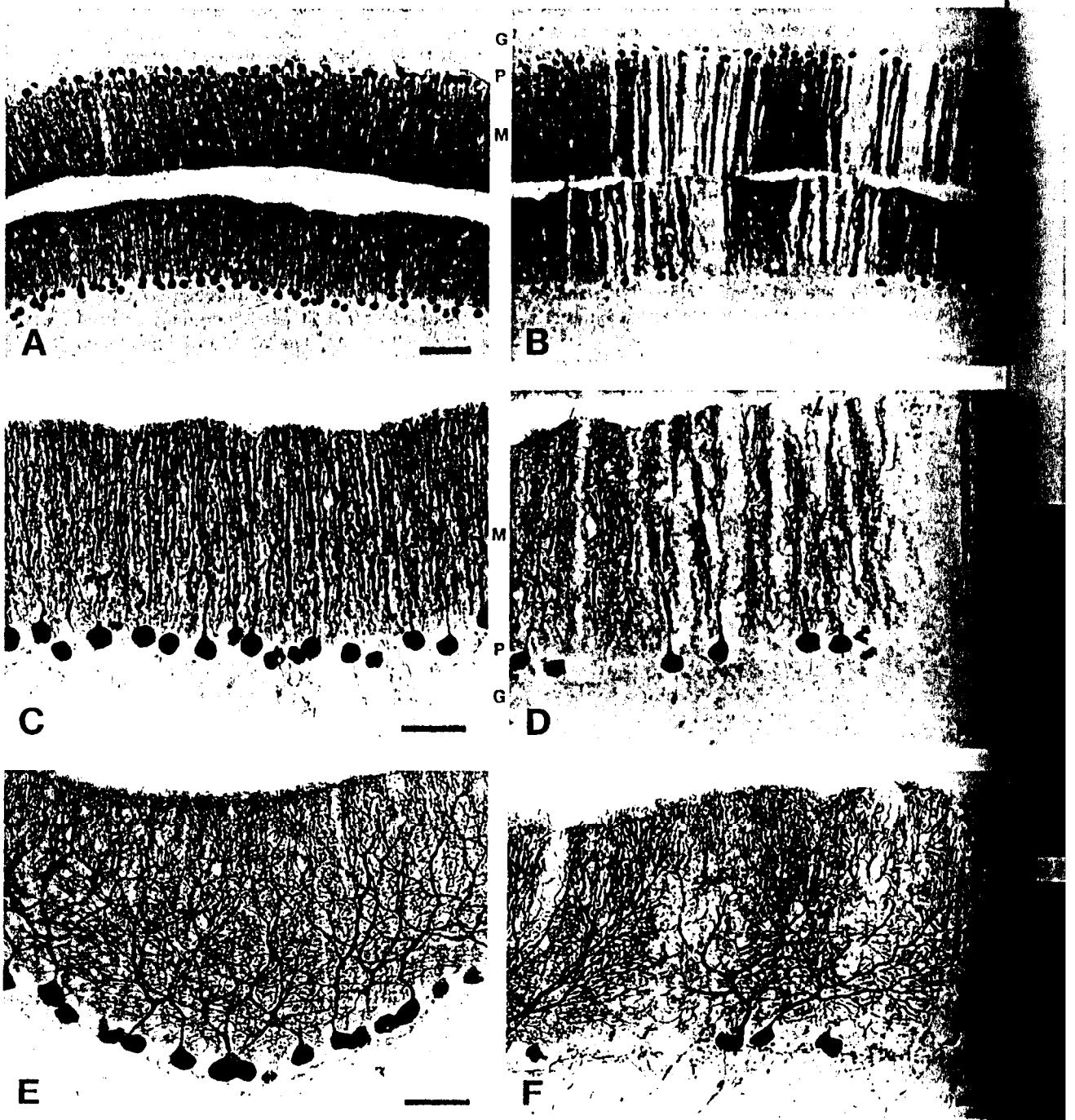

Fig. 2. Loss of calbindin immunoreactivity in the cerebellar vermis after ibogaine treatment. (A, C, E) Untreated controls; (B, D, F) rats treated with ibogaine (1 dose of 100 mg/kg; two weeks survival). A and B shown at low magnification for overview of vermis. (A, C, E) Purkinje cell bodies in control rats are densely immunoreactive with anti-calbindin and form an uninterrupted layer of single somata with their dendrites ascending through the molecular layer. (B, D, F) Two weeks after one dose of ibogaine, Purkinje cell bodies and dendrites exhibit a total loss of detectable calbindin demonstrated by parasagittal stripes of sharply demarcated, pale, radial bands in the vermis. Portions of shrunken Purkinje cell bodies and dendrite fragments are seen within some pale zones. In treated rats, axons with dysmorphic globular swellings extend beneath some Purkinje cell bodies, especially near zones of calbindin loss. (A–D) Coronal sections; (E, F) sagittal plane. Scale bars = 100 μm (A, B); 50 μm (C–F). Animals and tissue prepared as in Fig. 1. Abbreviations as in Fig. 1. Antiserum to Calbindin D_{28} was diluted 1:8000 (gift of Dr P. Emson; rabbit polyclonal antibody no. R-17).

were especially prominent. In treated rats, abnormal neurofilament staining was not observed nor were there accumulations of phosphorylated neurofilament proteins in damaged Purkinje cells. The lack of neurofilament changes is consistent with primary drug-induced damage to cell bodies rather than to axons. Purkinje cells, the only neurons to display morphologic alterations, showed the same cytologic effects at all survival times examined, after single or multiple doses. The effects of ibogaine and harmaline are qualitatively indistinguishable; yet, a dose–response analysis will be conducted to compare their relative potencies and to determine whether the toxic effect is dose related. The inferior olivary nucleus, examined with the same markers used in the cerebellum, did not reveal gliosis or neuronal damage in ibogaine or harmaline-treated rats.

The results of the present study demonstrate that administration of ibogaine and harmaline, at the doses employed, leads to degeneration of a subset of Purkinje cells in the cerebellar vermis. Large doses of ibogaine were used in the present study to reveal any neurotoxic effects that might be present. The doses used are four-times greater than the proposed human dose of approximately 25 mg/kg. A systematic dose response analysis is needed to assess the dose-relatedness of the neurotoxicity. Additionally, extrapolation of doses from rat to human should be evaluated with caution since drug metabolism and sensitivity may differ among species. Nonetheless, the presence of neurotoxicity in rats warrants caution before proceeding with administration of ibogaine to humans.

The distribution of decreased immunostaining for MAP2 and calbindin accompanied by the loss of Nissl-stained cell bodies following administration of ibogaine indicates structural damage to Purkinje cells. The vacuoles adjacent to pyknotic Purkinje cells may result from swelling and loss of large cell bodies. Argyrophilic Purkinje cell bodies and dendrites impregnated with the reduced silver method provide positive evidence for degeneration of these neurons.

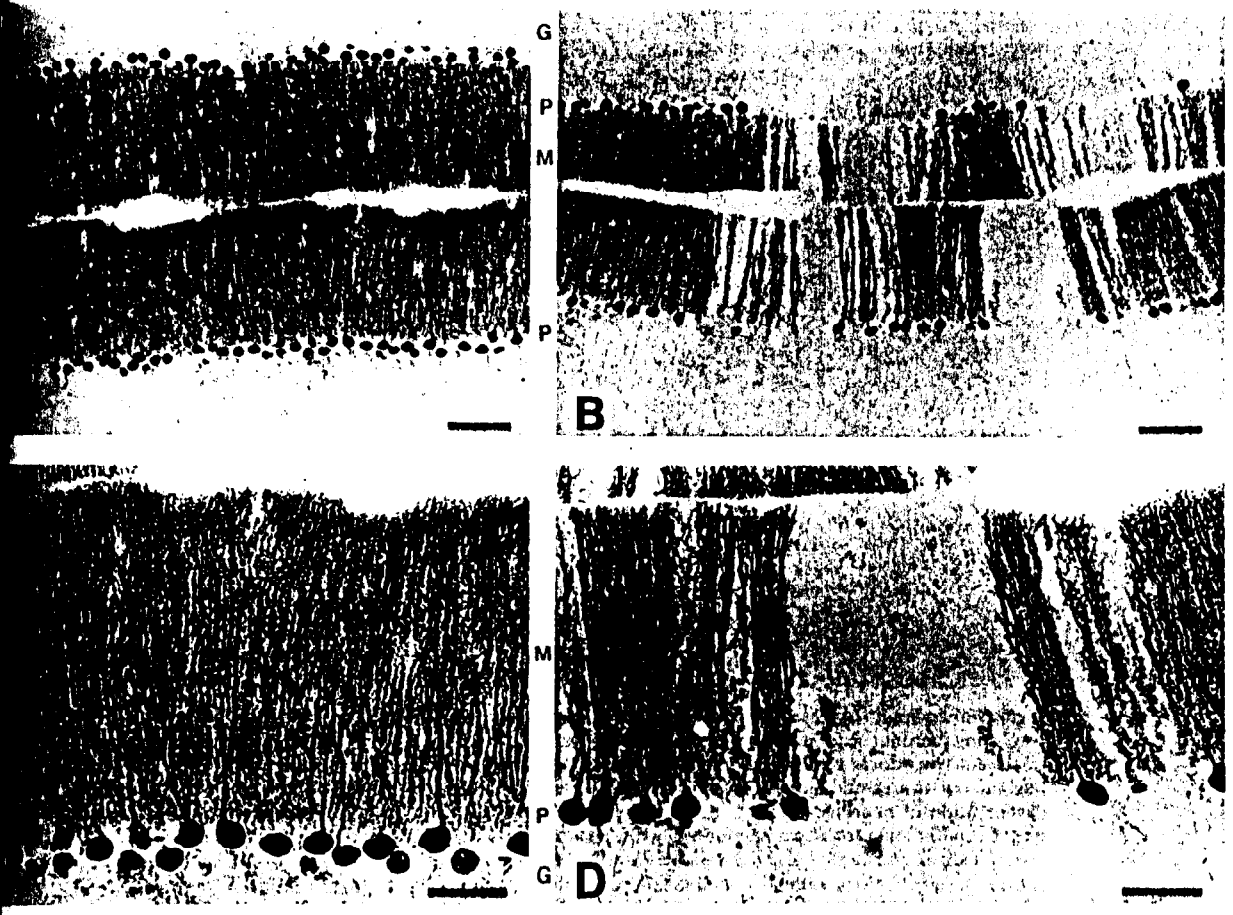

Fig. 3. Loss of calbindin in Purkinje cells after harmaline treatment (low and high magnification) (one dose of 40 mg/kg or three doses of 25 mg/kg 24 h apart). Coronal sections of cerebellar vermis prepared with antibody to calbindin (methods as in Figs 1 and 2). (A, C) Vermis from control rats; (B, D) calbindin staining 10 days after treatment with harmaline (25 mg/kg daily × three days). Harmaline treatment (B, D) produced radial, parasagittal zones with total loss of calbindin-stained Purkinje cell bodies and dendrites. The distribution of this cytopathologic change is indistinguishable from that seen after ibogaine treatment. Scale bars = 100 μm (A, B); 50 μm (C, D). Abbreviations as in Fig. 1.

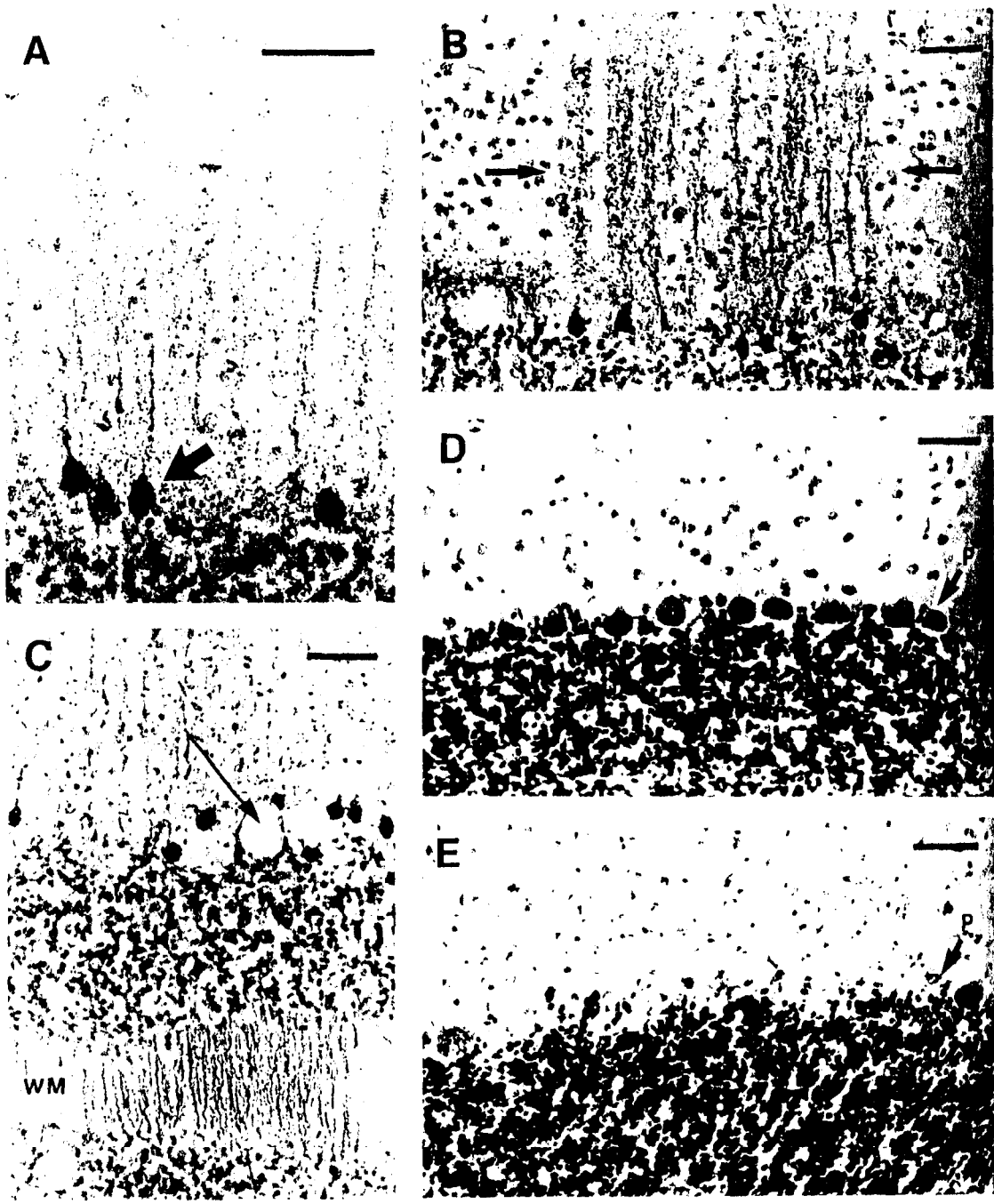

Fig. 4. Silver-stain. (A–C) Silver-stain shows degenerating Purkinje cells after treatment with ibogaine. A–C show coronal sections from the cerebellar vermis of rats that survived for two days after ibogaine treatment (two doses of 100 mg/kg, 8 h apart). (A, B) Darkly stained, argyrophilic Purkinje cell bodies (e.g. arrow in A) and granular ascending dendrites in the molecular layer (arrows in B) indicate degeneration of neurons. B and C demonstrate pale vacuoles (arrow in C) in the Purkinje cell layer, a frequent pathologic accompaniment of ibogaine induced neurodegeneration. Pyknotic Purkinje cell bodies are typically found at the border of vacuoles. (C) A sheet of degenerating axons in the white matter (WM), is often seen in register with silver-stained, degenerating Purkinje cells. To detect degenerating cerebellar neurons and their projections, frozen sections were prepared using the ammoniacal reduced silver impregnation method of Gallyas.[12,26] Following perfusion with 4% PAF, tissue was immersed in PAF for three to seven days and sectioned at 40 μm prior to silver nitrate impregnation. Scale bars = 50 μm (A–C). Alternate sections were stained with Cresyl Violet for examination of cell bodies. Nissl-stain: (D, E) Nissl preparations of the Purkinje cell layer in control (D) and ibogaine treated (E) rats five days after one dose of ibogaine (100 mg/kg). A selective loss of Purkinje cell bodies is seen in the ibogaine treated rat (E), whereas the control (D) exhibits a continuous row of rounded Purkinje cell perikarya. (P and arrow indicate Purkinje cell layer.) Scale bars = 50 μm (D, E).

That Purkinje cells degenerate is further supported by finding silver-impregnated axons in the cerebellar white matter and degenerating axon terminals in the fastigial and lateral vestibular nuclei, both of which receive Purkinje cell afferents. The parasagittal stripes of degenerating Purkinje cells observed in the present study match the pattern of glial activation previously reported.[28] The spatial relationship of gliosis and neuronal degeneration shows that microglia and astrocytes are activated in the immediate vicinity of degenerating Purkinje cells, perhaps in response to a chemical signal released by injured neurons.

The functional organization of the olivocerebellar system is largely in the sagittal plane based on evidence that the inferior olivary nucleus projects topographically to longitudinal zones in the cerebellum.[4,17,29] Moreover, stimulation of the inferior olive with harmaline excites Purkinje cells in narrow rostrocaudal rows.[30] After ibogaine treatment, the distribution of activated glia and of degenerating Purkinje cells in thin parasagittal stripes may be related to the topographic organization of the olivocerebellar climbing fiber projection. The present data provide anatomic evidence for cerebellar microzones that are one to five Purkinje cells wide and support previous electrophysiologic evidence showing narrow parasagittal microzones that are only a few Purkinje cells across.[30] The parasagittal zones were initially defined as being several hundred micrometers wide[4,17,29] based on studies of anatomic connections, yet different chemical markers for Purkinje cells have revealed narrow parasagittal bands of varying widths.[18] Ongoing studies should reveal whether the bands of degeneration are coincident with other chemical markers of Purkinje cell heterogeneity.

The mechanisms by which harmaline and related β-carbolines cause tremor have been investigated extensively. Harmaline induces rhythmic bursts of activity in inferior olivary neurons, which excite Purkinje cells via climbing fiber synapses leading to repetitive burst firing of Purkinje cells.[8,19,21,23] Thus, harmaline evokes intense, sustained activation of Purkinje cells by producing rhythmic climbing fiber activity that may continue for hours.[21] While the electrophysiologic effects of ibogaine have not been directly studied, ibogaline, a closely related alkaloid, is similar to harmaline in evoking a nearly identical pattern of sustained burst responses in olivary neurons and Purkinje cells.[9] Therefore, ibogaine is likely to have the same physiologic actions as does harmaline.

The results of this study, combined with the known physiology of olivocerebellar organization, lead us to propose the hypothesis that ibogaine and harmaline may evoke excitotoxic degeneration of Purkinje cells via activation of the inferior olivary nucleus. Harmaline and related iboga alkaloids[33,37] that strongly activate the inferior olive[8] may result in prolonged release of an excitatory neurotransmitter at climbing fiber synapses on Purkinje cells. The neurotransmitter of climbing fibers has not yet been established[36] but has many properties of an excitatory amino acid. The olivocerebellar climbing fibers form repeated synapses on Purkinje cell dendrites providing a high degree of security for synaptic transmission, yet this arrangement may also confer a great potential for inducing excitotoxicity. Repetitive action potentials in Purkinje cell dendrites causing influx of calcium,[20] combined with mobilization of intracellular calcium stores,[13] may allow excessive calcium to accumulate in the cytosol leading to calcium-mediated cytotoxicity.[3,27] The hypothesis that climbing fibers mediate the neurotoxicity is currently being tested by administering ibogaine to rats after producing a chemical lesion of the inferior olive using 3-acetylpyridine.[22]

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