Adrenergic Agents Inhibit Rapid Increases in Cerebellar Purkinje Cell Glutamic Acid Decarboxylase (GAD₆₇) mRNA Levels after Climbing Fiber Lesions or Reserpine Treatment

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Loss of the inferior olive-climbing fiber input to the cerebellar cortex after treatment with the neurotoxin 3-acetylpyridine (3-AP) has been reported to double the simple spike activity of the cerebellar Purkinje cell and eliminates complex spike activity. This is quickly followed by a three- to fourfold increase in Purkinje cell mRNA for the 67 kDa form of glutamic acid decarboxylase (GAD), a synthetic enzyme for the neurotransmitter GABA. Treatment with the indirectly acting sympathomimetic amphetamine or the direct acting β_2 adrenergic agonist clenbuterol inhibited the increase in GAD₆₇ mRNA, and this inhibition was blocked by pretreatment with the β receptor antagonist propranolol. The activity-enhancing effect of 3-AP treatment on cerebellar neurons was confirmed by extracellular recordings. Clenbuterol treatment prevented the increase in neuronal firing without altering lesion induction or the loss of

complex spikes, and propranolol treatment produced a partial reversal of the inhibitory effect of clenbuterol on the neuronal firing rate. These results suggest that β receptor-mediated effects on cerebellar neuronal activity may prevent the increase in mRNA levels, but that firing rate-independent β -mediated effects on genomic expression may also play a role. A role for noradrenergic systems in modulating GAD₆₇ mRNA is also supported by the finding that reducing endogenous cerebellar norepinephrine levels by treatment with reserpine increased Purkinje cell GAD₆₇ mRNA levels (250% of control), and this also was inhibited by clenbuterol treatment.

Key words: glutamic acid decarboxylase; Purkinje cell; climbing fiber lesions; cerebellum; GAD₆₇ mRNA; β -adrenergic receptors; clenbuterol; amphetamine; reserpine; norepinephrine; 3-acetylpyridine

The neurotransmitter GABA is an important inhibitory modulator of neural activity throughout the CNS. GABA is synthesized from its substrate, glutamate, in a single step by the pyridoxal phosphate (PLP)-dependent enzyme glutamic acid decarboxylase (GAD). Recent work indicates that two independent genes code for GAD, producing two forms of the enzyme, GAD₆₅ and GAD₆₇ (Erlander et al., 1991; Bu et al., 1992). The two forms differ in their affinity for PLP: GAD₆₇ is constitutively bound to PLP, whereas GAD₆₅ cycles through active (bound) and inactive (unbound) states (Porter and Martin, 1988; Kaufman et al., 1991; Martin et al., 1991). The mRNAs coding for both GAD enzymes are coexpressed by GABAergic neurons in most brain areas (Oertel et al., 1981; Kaufman et al., 1991), but in differing proportions in specific neuronal types (Esclapez et al., 1993, 1994).

The cerebellar Purkinje cell is a large GABAergic neuron that can express high levels of GAD₆₇ mRNA (Erlander et al., 1991; Esclapez et al., 1994). This cell receives a well described system of excitatory and inhibitory inputs, and it is possible to modify or mimic these inputs pharmacologically to alter Purkinje cell firing rates. Lesion of the inferior olive-climbing fiber input to the

Purkinje cell by treatment with the neurotoxin 3-acetylpyridine (3-AP) eliminates low-frequency Purkinje cell complex spike activity, and produces large increases in the simple spike-firing rate of the neuron within 3–5 hr after treatment (Desclin, 1974; Benedetti et al., 1984; Batini and Billard, 1985; Lopiano and Savio, 1986). Alternatively, Purkinje cell firing rates can be reduced by activation of intrinsic inhibitory neurons (e.g., stellate and basket cells) or systems such as the noradrenergic input arising from the locus coeruleus (Siggins et al., 1971; Freedman et al., 1977; Schultz et al., 1981; Moises et al., 1983, 1990; Bickford-Wimer et al., 1991; Tan and Collewyn, 1992). The noradrenergic effects appear to be mediated, in part, by activation of Purkinje cell β receptors and can be mimicked by treatment with adrenergic agonists (Siggins et al., 1971; Schultz et al., 1981; Moises et al., 1983, 1990; McEligott et al., 1986; Bickford-Wimer et al., 1991; Llano and Gerschenfeld, 1993).

The increase in Purkinje cell firing produced by 3-AP lesions of the climbing fiber input is followed by significant increases in GAD activity in the Purkinje cell terminals (Oltmans et al., 1985, 1986; Sukin et al., 1987; Litwak et al., 1990). Although the origin of the increase in GAD activity has not been established definitively, increased GAD₆₇ mRNA levels in Purkinje cell soma at 7 d after lesion have been reported (Litwak et al., 1991), suggesting the possibility that increased translation from the abundant message could account for the increased GAD activity. This hypothesis is supported by a recent report that Purkinje cell GAD₆₇ mRNA levels are significantly increased 8 hr after 3-AP treatment (Drengher and Oltmans, 1993). This represents a time when cell firing rates have been increased for 3–4 hr (Desclin and Escubi,

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1974), but before the time that increases in GAD activity can be measured (24–48 hr) (Sukin et al., 1987; Litwak et al., 1991). It appears that sustained increases in Purkinje cell firing produce a rapid induction of GAD message, which may induce the later increase in GAD activity.

The relatively brief (8 hr) period required to reliably produce increases in Purkinje cell GAD₆₇ mRNA levels was used in the current work to study the effects of specific pharmacological treatments on the increase in GAD₆₇ message, and to evaluate the possibility that the increased GAD₆₇ mRNA levels were the result of increased firing rates. As noted above, the noradrenergic input to the Purkinje cell inhibits Purkinje cell firing. Therefore, treatment with centrally acting adrenergic agents such as the β_2 agonist clenbuterol (Tondo et al., 1985) or the indirectly acting sympathomimetic amphetamine was combined with 3-AP toxin treatment, and the effects on GAD₆₇ mRNA levels were determined. In addition, in experiments with separate groups of animals the effects of selected treatments on cerebellar neuronal firing rates were assessed using extracellular recordings.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 gm, Zivic-Miller, Zelienople, PA) were administered the neurotoxin 3-AP (75 mg/kg, i.p.) prepared in isotonic saline to lesion the climbing fiber system. Nonlesion controls were administered isotonic saline. In studies involving climbing fiber lesions, animals were killed by decapitation 8 hr after 3-AP treatment. This time interval allows toxin-induced destruction of the olivary cell with the subsequent sustained increases in Purkinje cell firing rates for a period of ~4 hr (Desclin and Colin, 1980), and a significant increase in Purkinje cell GAD₆₇ mRNA levels (Drengler and Oltmans, 1993).

Animals were administered different adrenergic agonists and antagonists in conjunction with the 3-AP treatment. To directly stimulate Purkinje cell β receptors, the β_2 adrenergic agonist clenbuterol (10 mg/kg), which crosses the blood–brain barrier and interacts with Purkinje cell β_2 receptors (Tondo et al., 1985; Conway et al., 1987; Vos et al., 1987), was administered 40 min before treatment with 3-AP. To stimulate endogenous norepinephrine release and increase synaptic norepinephrine levels in the cerebellum, the indirectly acting sympathomimetic *d*-amphetamine sulfate (2 mg/kg) was administered 20 min before treatment with 3-AP (Carlsson et al., 1966; Azzaro and Rutledge, 1973). To test for β receptor specificity of effects, the nonselective β antagonist propranolol (9.5 mg/kg) was administered 20 min before treatment with either clenbuterol or amphetamine, and then again 4 hr later. All drugs were dissolved in isotonic saline. The drug doses used and the frequency of administration were selected based on preliminary experiments using values from the literature indicating both an effective CNS level and a duration of action sufficient to cover the period of study (Dring et al., 1970; Tondo et al., 1985; Ordway et al., 1987; Vos et al., 1987).

To assess the effect of activating noradrenergic systems on cerebellar GAD₆₇ mRNA levels in nonlesioned animals, amphetamine sulfate (2 mg/kg) was administered to normal animals every 12 hr, and the animals were killed 40 hr after the initial injection. To attenuate central noradrenergic effects, reserpine (2 mg/kg in vehicle) was administered twice (0 and 24 hr) to deplete endogenous norepinephrine (Alpers and Shore, 1969; Giachetti and Shore, 1978; Oltmans et al., 1980), and animals were killed 40 hr after the initial injection. The interaction of reserpine with other treatments on GAD₆₇ mRNA levels was also assessed. Reserpine (2 mg/kg) was administered, followed 16 hr later by either clenbuterol (10 mg/kg) or 3-AP (75 mg/kg). The animals were killed 24 hr after the initial reserpine injection.

In all studies, vehicle-treated control animals were included as separate groups to provide appropriate reference groups for comparison with the experimental treatments. Clenbuterol, *d*-amphetamine sulfate, propranolol, and 3-AP were obtained from Sigma (St. Louis, MO). Reserpine (Serpasil) was obtained from CIBA Pharmaceuticals (Suffren, NY). All drug treatments were administered intraperitoneally.

As described previously (Drengler and Oltmans, 1993), at the end of each experiment animals were killed by decapitation and the brains were removed, frozen, and stored at -70°C until used for determination of GAD₆₇ mRNA. Adjacent 12 μm coronal cerebellar sections from the area corresponding to coordinates -11.5 to -11.0 to Bregma (Paxinos

and Watson, 1982) were preselected for large Purkinje cell soma, dried to translucence at 35°C (3–5 min), and stored frozen (-20°C) until analysis.

In situ hybridization was performed according to the method of Cheslet et al. (1987) to determine GAD₆₇ mRNA levels. Antisense probes labeled with [^{35}S]uridine triphosphate were synthesized from linearized templates (plasmids courtesy of A. Tobin, UCLA) containing the feline GAD₆₇ gene cDNA attached to an SP6 promoter. To facilitate tissue penetration, the full-length transcript (2.2 kb) was reduced to fragments (~100–200 nucleotides long) using partial alkaline hydrolysis (Wuenshell et al., 1986).

Prehybridization and posthybridization treatments were performed as described by Litwak et al. (1990). Hybridization was performed by adding 3–6 ng of probe (4×10^8 dpm/mg) to each cerebellar section. The slides were hybridized in humid chambers at 50°C for 3.5 hr. After posthybridization washes, the sections were dipped in NTB2 emulsion to detect the presence of hybridized radioisotope-labeled probe. The emulsion was exposed for 10 d in the cold. Silver grains (signifying bound [^{35}S]GAD₆₇ antisense mRNA) were visualized using Kodak D-19 developer and fixer (Rochester, NY). To improve contrast and enhance identification of Purkinje cells, sections were stained with hematoxylin and eosin. The relative area of the Purkinje cell covered by grains was quantified using computer-assisted grain counting. The grain-counting apparatus consists of a microscope connected to a video camera, which is capable of capturing images to a monitor. A magnetic mouseboard makes selection of individual Purkinje cells possible for computer-size analysis. The silver grains are selected because of their increased level of “black density” over the gray image of the tissue. The area in pixels covered by silver grains divided by the number of pixels of total Purkinje cell area was reported as grain density. To ensure sufficient exposure time and to guard against saturation of the emulsion, test slides were developed and analyzed after 5, 7, and 10 d of exposure.

To determine the effect of drug treatment on GAD₆₇ mRNA, a row of 20 sequential Purkinje cells located most dorsal in the central vermis of cerebellar lobule 6 was analyzed. Tissue from different animals was matched to provide comparable levels of section. Reported values for each time point were derived from analysis of two adjacent sections per animal. Values were corrected for background by subtracting binding densities of radiolabeled sense probes.

Lesion analysis. In the doses used, 3-AP treatment reliably produces extensive destruction of the inferior olive-climbing fiber system (Desclin, 1974; Desclin and Escubi, 1974; Desclin and Colin, 1980) and early onset motor symptoms (hypotonia, ataxia, and limb splaying), which reliably reflect extensive olivary damage. In the current studies, histological confirmation of the lesion was precluded by the incompatibility of the brief period (8 hr) under evaluation with the period required to observe cell loss with standard histological techniques (Desclin, 1974; Desclin and Escubi, 1974). Therefore, to indirectly confirm lesion presence, animals were observed for the presence of motor signs noted above.

To obtain a more complete histological profile, in two separate groups of animals (5 animals/group), 3-AP or a clenbuterol + 3-AP combination was administered. After a 3 d survival period, the animals were killed by an overdose of sodium pentobarbital, perfused transcardially with saline, and neutral-buffered formalin, and the brainstem containing the inferior olive was placed in 20% sucrose for at least 24 hr. The brainstems were sectioned at 30 μm in a freezing microtome and stained with cresyl violet, and alternate sections were examined at $250\times$ magnification for the presence of spared cells in the inferior olive. The locations of surviving cells were assessed using the studies of Apps (1990) and Azizi and Woodward (1987) as guides. One animal treated with 3-AP alone died before the 3 d period was complete.

Electrophysiology. Nine rats were treated with isotonic saline followed 40 min later by 3-AP (75 mg/kg) (3-AP group). Six rats received 10 mg/kg clenbuterol followed 40 min later by 3-AP (3-AP + clenbuterol). Six rats received 9.5 mg/kg propranolol followed 20 min later by 10 mg/kg clenbuterol and 40 min later by 3-AP, and two additional rats were also administered a second propranolol injection 4 hr after the first treatment. These two treatments did not produce different effects, and the results were consolidated into a single group (3-AP + clenbuterol + propranolol). Control rats received saline treatment only (saline). Two hours after 3-AP injection, all rats were anesthetized with 1.9 gm/kg urethane and prepared surgically for extracellular recordings from lobules V and VI of the cerebellar cortex as described previously (Stratton et al., 1988). Body temperature was maintained with Deltaphase heating pads (Braintree Scientific, Braintree, MA) throughout the surgery and recording period. When a cell was isolated, sponta-

neous activity was recorded for 5–15 min and waveforms were examined to detect the presence of complex spike activity. One to six cells were recorded per rat in the period from 3 to 5.5 hr after 3-AP injections. In a few cells, recordings over long periods were made to assess the stability of firing rates in 3-AP-treated rats. Unit data were analyzed using R.C. Electronics Computerscope software (Santa Barbara, CA).

In normal animals, Purkinje cells are readily identified by their complex and simple spike activity. Although loss of the climbing fiber input (which is a consequence of 3-AP treatment) eliminates the characteristic complex spike of the Purkinje cell, cell layers can still be identified in electrode penetrations through the cerebellar cortex. The Purkinje cell region can still be identified by the intense electrical activity in this layer, which alternates with regions of electrical silence as the recording electrode is lowered. Most of the large cells encountered in the high-activity regions in normal animals are Purkinje cells, and it is likely that this is still the case after toxin treatment. Therefore, electrophysiological recordings were used to sample simple spike activity in putative Purkinje cells in toxin-treated rats. Only a few cells in these animals retained complex spike activity at the 3–5.5 hr post-treatment interval. As in other studies using 3-AP-treated rats (Montarolo et al., 1982; Batini and Billard, 1985; Batini et al., 1985), the possibility that some cells recorded were Golgi or basket cells cannot be unequivocally excluded. The important comparison in the present study, however, is between the drug treatments in animals with lesions, and there is no reason to expect that different proportions of Purkinje cells would be isolated in these groups.

Data analysis. Overall data analysis was done by ANOVA, and follow-up comparisons were made using Dunnett's test. In some cases, comparisons between specific treatment groups within individual experiments were made using a Student's *t* test. For the *in situ* hybridization experiments, to compare results across experiments grain density values within each experiment were converted to a percent of the saline-treated control mean for that experiment. These data, along with the specific mean control density value for each experiment, are reported in the tables as the mean $\pm$ SD.

RESULTS

Lesion confirmation

In all experiments, motor symptoms (e.g., ataxia and hypotonia) consistent with the presence of an effective olivary-climbing fiber lesion were reliably observed within 4–6 hr after treatment in 3-AP-treated animals (regardless of the presence of clenbuterol). In the group of animals evaluated after a 3 d post-treatment period, extensive loss of cells in the inferior olive was found (see below).

Lesion and drug effects on Purkinje cell GAD₆₇ mRNA

In agreement with previous findings (Drengler and Oltmans, 1993), at 8 hr after treatment 3-AP produced a large increase in Purkinje cell GAD₆₇ mRNA levels (342% of saline-treated control value; Table 1). When 3-AP treatment was preceded (40 min) by administration of the β_2 adrenergic agonist clenbuterol, there was no increase in GAD₆₇ mRNA levels (98% of control). Pre-treatment of the 3-AP + clenbuterol combination with the β adrenergic receptor blocker propranolol produced GAD₆₇

mRNA levels (379% of control) comparable with those seen with 3-AP treatment alone. Taken together, these data suggest that GAD₆₇ mRNA levels can be regulated through β receptors.

In Figure 1 representative dark-field photomicrographs of cerebellar emulsions labeled with the GAD₆₇ mRNA antisense probe are shown. Heavy labeling of Purkinje cells in 3-AP-treated animals (*B*) can be compared with the relatively light labeling in controls (*A*). Clenbuterol treatment prevented the 3-AP effect, substantially reducing grain density in the lesioned animals, although a few cells did show high grain densities (*C*), whereas propranolol reversed the clenbuterol effect, reinstating the heavy labeling densities found with 3-AP alone (*D*).

These results indicate that β_2 receptor activation prevents the 3-AP lesion-induced increase in Purkinje cell GAD₆₇ mRNA levels, and that the inhibition can be reversed by β receptor blockade. In the cerebellum, the primary catecholamine neurotransmitter is norepinephrine, which at physiological concentrations stimulates primarily β_1 receptors, with decreased potency for β_2 receptors (Goodman and Gilman, 1985). To assess the ability of the endogenous neurotransmitter to prevent the 3-AP effect, amphetamine, an indirectly acting sympathomimetic, was administered to increase synaptic norepinephrine levels in 3-AP-treated animals.

The 3-AP treatment again produced a large increase in Purkinje cell GAD₆₇ mRNA levels (479% of control), which was prevented by amphetamine treatment (132% of control; Table 2). When the β receptor blocker propranolol was added to the 3-AP + amphetamine combination, Purkinje cell GAD₆₇ mRNA levels were again increased (373% of control; not significantly different from 3-AP treatment alone). The effects of amphetamine on 3-AP stimulation of GAD₆₇ mRNA levels parallel those of the previous 3-AP + clenbuterol experiments.

To assess the possibility that adrenergic input to the cerebellum might help regulate Purkinje cell GAD₆₇ mRNA levels, separate groups of animals ($n = 8$ /group) were administered amphetamine or reserpine (to increase or reduce endogenous noradrenergic activity, respectively) over a 40 hr period. Treatment with amphetamine did not appear to alter basal GAD₆₇ mRNA levels ($108 \pm 32\%$ of control, $p > 0.05$). In contrast, reserpine treatment produced a significant increase in GAD₆₇ mRNA levels ($347 \pm 50\%$ of control, $p < 0.01$) (control grain density = 0.0215 ± 0.006), similar in magnitude to that seen after 3-AP treatment.

The reserpine effects were studied further by administration of a single reserpine injection followed 16 hr later by either clenbuterol or 3-AP, and animals were killed 8 hr after the second treatment (24 hr after initial reserpine treatment). With the shorter 24 hr treatment period, reserpine produced a slightly smaller but still significant increase in GAD₆₇ mRNA levels

Table 1. Purkinje cell GAD₆₇ mRNA levels (mean $\pm$ SD) after 3-AP (8 hr): effects of clenbuterol and clenbuterol + propranolol treatment

Treatment	GAD mRNA density (% of saline control) (<i>n</i>)
Saline (0.01331)**	100 $\pm$ 27 (5)
3-AP	342 $\pm$ 84* (7)
3-AP + clenbuterol	98 $\pm$ 34 (9)
3-AP + clenbuterol + propranolol	379 $\pm$ 151* (13)

* Differs significantly from saline and 3-AP + clenbuterol treatments ($p < 0.01$).

** GAD₆₇ mRNA grain density values in saline controls. Values calculated as pixels covered by silver grains divided by pixels of Purkinje cell area.

Table 2. Purkinje cell GAD₆₇ mRNA levels (mean $\pm$ SD) after 3-AP (8 hr): effects of amphetamine and amphetamine + propranolol treatment

Treatment	GAD mRNA density (% of saline control) (<i>n</i>)
Saline (0.00630)**	100 $\pm$ 20 (8)
3-AP	479 $\pm$ 102* (6)
3-AP + amphetamine	132 $\pm$ 45 (7)
3-AP + amphetamine + propranolol	373 $\pm$ 99* (7)

* Differs significantly from saline and 3-AP + amphetamine treatments ($p < 0.01$).

** GAD₆₇ mRNA grain density values in saline controls (see Table 1).

Figure 1. Dark-field photomicrographs of *in situ* labeling using the GAD₆₇ mRNA antisense probe in cerebellar Purkinje cells. Heavy labeling in a row of Purkinje cells from 3-AP-treated animals (*B*) can be compared with labeling in control animals (*A*) (arrows point to individual Purkinje cells). Clenbuterol treatment (*C*) substantially reduced labeling in 3-AP-treated animals, and most cells appeared similar to those in control animals, although some cells did appear to show some increase (top right arrow in *C*). Combining propranolol treatment with clenbuterol in 3-AP-treated animals (*D*) reinstated the heavy labeling seen with 3-AP alone.

(250% of control; Table 3). Clenbuterol treatment prevented the reserpine effect (152% of control, $p > 0.05$). Combining reserpine treatment with 3-AP did not further increase the change seen with 3-AP alone (382 vs 348% of control). Like amphetamine, clenbuterol treatment alone (112% of control) had no effect on the GAD₆₇ mRNA levels. Taken together, the results of these experiments (Tables 1–3) suggest a β receptor-mediated noradrenergic

contribution to the regulation of GAD₆₇ mRNA levels in the cerebellar Purkinje cell.

Clenbuterol effects on inferior olive lesions

It is possible that clenbuterol prevented increases in Purkinje cell GAD₆₇ mRNA not by a β receptor-mediated effect, but by preventing 3-AP lesion induction. To determine the effectiveness of

Table 3. Purkinje cell GAD₆₇ mRNA levels (mean $\pm$ SD) after reserpine treatment (24 hr): effects of clenbuterol or 3-AP treatment (8 hr)

Treatment	GAD mRNA density (% of saline control) (n)
Saline (0.03397)***	100 $\pm$ 59 (6)
Clenbuterol	112 $\pm$ 49 (5)
3-AP	348 $\pm$ 72* (6)
Reserpine	250 $\pm$ 93*** (6)
Reserpine + clenbuterol	152 $\pm$ 24 (6)
Reserpine + 3-AP	382 $\pm$ 72* (6)

* Differs significantly from saline control ($p < 0.01$).** Differs significantly from reserpine + clenbuterol treatment ($p < 0.05$).*** GAD₆₇ mRNA grain density values in saline controls (see Table 1).

3-AP in producing inferior olive-climbing fiber lesions in the presence of clenbuterol, a group of animals was treated with 3-AP or 3-AP + clenbuterol, and extent of lesion was assessed 3 d after lesion. All of these animals showed clear behavioral evidence of 3-AP treatment. The two animals rated as most severely affected received 3-AP alone. The clenbuterol-pretreated animals could not be reliably distinguished from the remaining rats treated only with 3-AP.

Tissue analysis revealed widespread cell loss throughout the inferior olive in all cases, but surviving neurons could also be found. Surviving cells were found most frequently in subnucleus c of the medial accessory olive (MAO) and in the most ventral and lateral region of the principal olive (PO). In clenbuterol-pretreated subjects, two animals had relatively high densities of surviving cells in MAO subnucleus c. This was defined as three or more sections in which 10 or more surviving cells could be seen. Two animals had moderate densities (i.e., 5–10 cells/section in 3 or more sections) in this area. The remaining animal had relatively light sparing with <5 cells evident/section. Applying these criteria in animals treated only with 3-AP, one animal was found to have a high density of spared cells in subnucleus c. One other animal showed a moderate density of sparing, and the remaining two animals showed a light density. In other regions of the inferior olive, including the PO, sparing was similar in both groups when present.

According to the work of Desclin and Escubi (1974), it may take up to 2 weeks for the full toxic effect of 3-AP in the inferior olive to be observed histologically as somal destruction. A much more rapid method of evaluating functional loss of inferior olive is by observing ataxic movements. In the cases reported here, 3-AP produced characteristic motor dysfunction (and, thus, olivary damage) regardless of drug pretreatment. This finding is supported further by the electrophysiology studies of Purkinje cells reported below.

3-AP and drug effects on Purkinje cell electrophysiology

To identify possible mechanisms by which treatment with adrenergic agonists might block the lesion-induced increase in GAD₆₇ mRNA, cerebellar neuronal activity was studied using extracellular recording techniques. Qualitatively, in all animals receiving 3-AP treatment (3-AP, 3-AP + clenbuterol, and 3-AP + clenbuterol + propranolol groups), only 18 of 74 cells were identified unequivocally as Purkinje cells, because they retained complex spike activity. Of these 18 cells, 13 were recorded

Table 4. Cerebellar neuronal cell firing rates after 3-AP, 3-AP + clenbuterol, or 3-AP + clenbuterol + propranolol treatment in cells with or without complex spike (CS) activity (mean $\pm$ SD)

Treatment	Firing rate (Hz)	
	No CS activity (n)	CS activity present (n)
Saline control	—	21.1 $\pm$ 8.2 (13)
3-AP	53.9 $\pm$ 19.8* (13)	21.7 $\pm$ 7.9 (7)
3-AP + clenbuterol	25.1 $\pm$ 19.0 (17)	19.9 $\pm$ 5.2 (3)
3-AP + clenbuterol + propranolol	34.4 $\pm$ 11.2** (34)	18.1 $\pm$ 8.4 (8)

* Differs significantly from saline control and 3-AP + clenbuterol (no CS) groups ($p < 0.01$).** Differs significantly from saline control ($p < 0.01$) and 3-AP + clenbuterol (no CS) group ($p < 0.05$).

early in the post-treatment period (<4 hr after treatment) before the time the full lesion effect of 3-AP may have been expressed. In the 18 cells with complex spike activity, the firing rates were similar to those found in control animals treated with saline only (19.8 vs 21.1 Hz, respectively), and did not differ significantly between the treated groups (Table 4). The difficulty experienced in locating cells with complex spikes in the presumed Purkinje cell layer indicates that the 3-AP treatment was effective in removing the climbing fiber input to the Purkinje cells.

In animals treated with 3-AP alone, the firing rates of cells in the high-activity layer not showing complex spike activity ($n = 13$) were significantly higher than both those of cells retaining complex activity and those from saline-treated controls (255% of control rate, $p < 0.001$ for both comparisons; Table 4). This confirms the effectiveness of the 3-AP treatment in increasing cerebellar neuronal activity in the high-activity layers containing Purkinje cells. Treatment of 3-AP animals with clenbuterol significantly modified this effect. In the 3-AP + clenbuterol group, firing rates were reduced compared with 3-AP only ($t = 4.02$, $p < 0.001$) and were not significantly higher than in saline controls (118% of control rate, $t = 0.71$, $p > 0.05$). Addition of propranolol to the 3-AP + clenbuterol regimen partially reversed the clenbuterol effect, producing firing rates significantly higher than in either saline controls (163% of control rate; $t = 3.89$, $p < 0.001$) or the 3-AP + clenbuterol group ($t = 2.20$, $p < 0.05$), but less than those in the 3-AP alone group ($t = 4.26$, $p < 0.001$). Thus, changes in electrophysiological responses of cells to drug treatments precede and predict changes in GAD₆₇ mRNA levels observed later.

DISCUSSION

The *in situ* hybridization results confirmed our earlier observations that lesions of the olivo–cerebellar climbing fiber system produced rapid increases in Purkinje cell GAD₆₇ mRNA (Drengler and Oltmans, 1993). The increases in GAD₆₇ message were accompanied by increased cerebellar neuronal firing rates in the high-activity layer, which contains predominantly Purkinje cells, suggesting a causal relationship between these events. The finding of increased GAD₆₇ mRNA specifically in the Purkinje cells also supports the idea that the Purkinje cells were the source of the increased electrical activity. Treatment of lesioned animals with the centrally acting β_2 agonist clenbuterol prevented both the increase in GAD₆₇ mRNA levels and the increase in cell firing rates, strengthening the correlation between firing rates and

GAD₆₇ mRNA increases. Blocking β receptors with the antagonist propranolol, although completely preventing the clenbuterol inhibitory effect on GAD₆₇ mRNA levels, only partially prevented its inhibitory effect on firing rates. This suggests that the effects of clenbuterol on firing rates may not be the only β receptor-mediated factor in the regulation of GAD₆₇ message, and may indicate that the activation of intracellular pathways with inhibitory effects on GAD₆₇ gene transcription is also involved (Segovia et al., 1991). Alternatively, it is possible that other complicating factors (e.g., a propranolol–general anesthetic interaction) in the electrophysiological experiments produced an inhibitory effect on firing rates not present in nonanesthetized animals used for the *in situ* hybridization studies.

In addition to the direct β_2 adrenergic agonist, treatment with the indirectly acting sympathomimetic amphetamine prevented the lesion-induced increase in GAD₆₇ mRNA levels. As with clenbuterol, this effect was reversed by propranolol, indicating that the endogenous cerebellar norepinephrine was able to modulate the lesion effect on the GAD₆₇ mRNA, and suggesting that the noradrenergic input to the cerebellum may serve a regulatory role in cerebellar GAD₆₇ mRNA production. Such a role is supported by the observations of Segovia et al. (1991), who reported that in GAD₆₇ gene transfection studies in C6 neuroglioma cells, cAMP analogs decrease GAD₆₇ mRNA levels. The mechanisms mediating these effects are not clear, because no cAMP-responsive elements have yet been discovered in the GAD₆₇ gene.

The mechanisms underlying the effects on cerebellar GAD₆₇ mRNA of these two adrenergic agents, the direct-acting clenbuterol and the indirect-acting amphetamine, are not clear. In the cerebellum, the dominant adrenergic receptor subtype is β_2 (90–98% of total β receptors) (Dolphin et al., 1979; Sutin and Minneman, 1985), which would provide a target for clenbuterol but would be much less favorable for the predominantly β_1 -selective norepinephrine released by amphetamine treatment. This may indicate that the released norepinephrine can efficiently activate the less abundant β_1 receptors to inhibit GAD₆₇ mRNA production or, regardless of its low affinity, that norepinephrine recruits sufficient β_2 receptors to induce this effect. Both β_1 and β_2 receptors couple to G_s to stimulate adenylyl cyclase (Sessler et al., 1989), thereby providing a common pathway for both clenbuterol and amphetamine/norepinephrine to prevent the 3-AP induction of GAD₆₇ mRNA.

The finding that clenbuterol treatment inhibited the increase in GAD₆₇ mRNA suggests that under the appropriate conditions, clenbuterol might have a therapeutic efficacy in modifying cerebellar activity, yet produce minimal effects at other sites in the CNS where the predominate β receptor type is β_1 (Dolphin et al., 1979). It is also interesting to note that regulation of GAD₆₇ mRNA levels under nonstimulated conditions may not be the same as under conditions of increased neural activity, because neither clenbuterol nor amphetamine appeared to affect baseline GAD₆₇ mRNA levels. This may mean that the noradrenergic system can modulate increased production of message, but that it does not influence constitutive expression.

The large postlesion increase in Purkinje cell GAD₆₇ mRNA produced intense labeling of the Purkinje cell layer in 3-AP-treated animals. Substantial labeling also appeared to be present in the molecular and granular cell layers of these animals, but was not a prominent feature in controls. Although grain density was not quantitated in these other areas, interneurons of both the molecular and the granular layers are GABAergic and might be

expected to have GAD mRNA present. The appearance of the increased labeling was not consistent, however, with the interpretation that it was derived from the interneurons of these layers. Rather, the labeling was most intense close to the Purkinje cell body and decreased in intensity with increasing distance from the soma. This suggests that the labeling may represent mRNA extending into the dendrites and proximal axons of the Purkinje cell itself. Such a possibility has been suggested by others (Davis et al., 1990; Kleiman et al., 1993; Esclapez et al., 1994).

Although loss of the Purkinje cell complex spike activity is believed to be primarily responsible for the increase in simple spike firing after olivary lesions, a lesion effect on noradrenergic release produced by the climbing fiber denervation might also contribute. There is evidence of presynaptic NMDA receptors in the cerebellum, which facilitate noradrenergic release (Wood, 1991). Climbing fiber activation could stimulate these receptors by release of glutamate or aspartate. Loss of the climbing fiber input would potentially reduce stimulation of these receptors, thereby decreasing norepinephrine release and removing its inhibitory effects on the Purkinje cell. The subsequent increase in Purkinje cell activity would be expected to increase GAD₆₇ mRNA levels. This hypothesis is consistent with the current findings that cerebellar norepinephrine depletion by reserpine treatment significantly increased Purkinje cell GAD₆₇ mRNA levels. A common mechanism may be suggested by which either destruction of climbing fiber input or reserpine depletion could decrease synaptic norepinephrine availability, preventing its inhibition of cell firing and thereby producing increased GAD₆₇ mRNA levels.

Behavioral effects of 3-AP treatment were noticeable by 4–6 hr after toxin treatment. Control animals could easily be distinguished from those receiving toxin by comparing movement characteristics (ataxia, limb splaying, and head swinging) of the two groups. Qualitative comparison of motor dysfunction between 3-AP- and 3-AP + clenbuterol-treated animals does not present as clear a picture. The few animals presenting the most severe movement abnormalities were always treated with 3-AP alone, and the animals demonstrating delayed and milder dysfunction were always 3-AP + clenbuterol-treated, but there was much overlap in the two treatment groups. Thus, in these acutely treated animals, for the brief period of time the animals were available for observation, it was not possible to classify 3-AP- or 3-AP + clenbuterol-treated animals unequivocally by observing their movements, even though both groups could be easily distinguished from controls. For the toxin- and drug-treated animals maintained for a longer survival period (3 d) for histological analysis, again, there were no definitive differences between 3-AP and 3-AP + clenbuterol groups in the severity of motor behaviors. This is not unexpected, however, because the time of clenbuterol presence in these animals would be limited, after which the lesion effect would be dominant. The effect on motor parameters of more sustained clenbuterol treatment in 3-AP-treated animals was not assessed in the current study.

In agreement with the behavioral observations, histological evaluation of the inferior olive in animals cotreated with 3-AP and clenbuterol revealed that the lesion magnitudes were not discriminably different from those obtained in tissue treated with 3-AP alone. This finding is also supported by the results of the electrophysiological analysis—of the 20 cells recorded from the 3-AP + clenbuterol group, 17 did not demonstrate complex spike activity. Because complex spikes are generated by the olivary climbing fiber input to the Purkinje cell, the histological and electrophysiological data indicate that 3-AP retained its cytotoxic

effect in the inferior olive even in the presence of clenbuterol. Thus, changes in GAD₆₇ message after 3-AP + clenbuterol treatment were apparently attributable to β receptor-mediated events and not to inhibition of the lesion-inducing effect of 3-AP.

In summary, the results of the current work indicate a rapid increase in cerebellar Purkinje cell GAD₆₇ mRNA associated with the loss of the climbing fiber input to the cerebellar cortex and the subsequent increase in presumed Purkinje cell firing rates. β receptor-mediated events can prevent this effect, but it is unclear whether this is a result of a direct modulation of cell firing rate or of unknown secondary mechanisms. Although exact mechanisms are not clear, there appears to be a role for the adrenergic input to the cerebellum in modulating the Purkinje cell GABAergic system.

REFERENCES

- Alpers HS, Shore PA (1969) Specific binding of reserpine-association with norepinephrine depletion. *Biochem Pharmacol* 18:1363–1372.
- Apps R (1990) Columnar organization of the inferior olive projection to the posterior lobe of the rat cerebellum. *J Comp Neurol* 302:236–254.
- Azizi SA, Woodward DJ (1987) Inferior olivary nuclear complex of the rat: morphology and comments on the principles of organization within the olivocerebellar system. *J Comp Neurol* 263:467–484.
- Azzaro AJ, Rutledge CO (1973) Selectivity of release of norepinephrine, dopamine and 5-hydroxytryptamine by amphetamine in various regions of rat brain. *Biochem Pharmacol* 22:2801–2813.
- Batini C, Billard JM (1985) Release of cerebellar inhibition by climbing fiber deafferentation. *Exp Brain Res* 57:370–380.
- Batini C, Billard JM, Daniel H (1985) Long term modification of cerebellar inhibition after inferior olive degeneration. *Exp Brain Res* 59:404–409.
- Benedetti F, Montarolo PG, Rabacchi S (1984) Inferior olive lesion induces long lasting functional modification of Purkinje cells. *Exp Brain Res* 55:368–371.
- Bickford-Wimer P, Pang K, Rose GM, Gerhardt GA (1991) Electrically-evoked release of norepinephrine in the rat cerebellum: an *in vivo* electrochemical and electrophysiological study. *Brain Res* 558:305–311.
- Bu D-F, Erlander M, Hitz B, Tillakaratne NJK, Kaufman D, Wagner-McPherson C, Evans G, Tobin A (1992) Two human glutamate decarboxylases, 65 kDa GAD and 67 kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci USA* 89:2115–2119.
- Carlsson A, Fuxe K, Hamberger B, Lindquist M (1966) Biochemical and histochemical studies on the effects of imipramine-like drugs and (+)-amphetamine on central and peripheral catecholamine neurons. *Acta Physiol Scand* 67:481–497.
- Chesselet M-F, Weiss L, Wuenschell C, Tobin A, Affolter H-U (1987) Comparative distribution of mRNAs for glutamic acid decarboxylase, tyrosine hydroxylase, and tachykinins in the basal ganglia: an *in situ* hybridization study in the rodent brain. *J Comp Neurol* 262:125–140.
- Conway PG, Tejani-Butt S, Brunswick DJ (1987) Interaction of beta adrenergic agonists and antagonists with brain beta adrenergic receptors *in vivo*. *J Pharmacol Exp Ther* 241:755–762.
- Davis L, Burger B, Banker GA, Steward O (1990) Dendritic transport: quantitative analysis of the time course of somatodendritic transport of recently synthesized RNA. *J Neurosci* 10:3056–3068.
- Desclin JC (1974) Histological evidence supporting the inferior olive as the major source of cerebellar climbing fibers in rat. *Brain Res* 77:365–384.
- Desclin JC, Colin F (1980) The olivocerebellar system. II. Some ultrastructural correlates of inferior olive destruction in the rat. *Brain Res* 187:29–46.
- Desclin JC, Escubi J (1974) Effects of 3-AP on CNS of rats, as demonstrated by silver methods. *Brain Res* 77:349–384.
- Dolphin AC, Hamont M, Bockart J (1979) The resolution of dopamine and β 1- and β 2-adrenergic-sensitive adenylate cyclase activities in homogenates of cat cerebellum, hippocampus and cerebral cortex. *Brain Res* 179:305–317.
- Drengler SM, Oltmans GA (1993) Rapid increases in cerebellar Purkinje cell glutamic acid decarboxylase (GAD67) mRNA after lesion-induced increases in cell firing. *Brain Res* 615:175–179.
- Dring LG, Smith RL, Williams RT (1970) The metabolic fate of amphetamine in man and other species. *Biochem J* 116:425–435.
- Erlander M, Tillakaratne N, Feldblum S, Patel N, Tobin A (1991) Two genes encode distinct glutamate decarboxylases. *Neuron* 7:91–100.
- Esclapez M, Tillakaratne NJ, Tobin AJ, Houser CR (1993) Comparative localization of mRNAs encoding two forms of glutamic acid decarboxylase with nonradioactive *in situ* hybridization methods. *J Comp Neurol* 331:339–362.
- Esclapez M, Tillakaratne NJK, Kaufman DL, Tobin AJ (1994) Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci* 14:1834–1855.
- Freedman R, Hoffer B, Woodward D, Puro D (1977) Interaction of norepinephrine with cerebellar activity evoked by mossy and climbing fibers. *Exp Neurol* 55:269–288.
- Giachetti A, Shore PA (1978) The reserpine receptor. *Life Sci* 23:89–92.
- Goodman L, Gilman A (1985) The pharmacological basis of therapeutics. New York: Macmillan.
- Kaufman D, Houser C, Tobin A (1991) Two forms of the γ -aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem* 56:720–723.
- Kleiman R, Banker G, Steward O (1993) Subcellular distribution of rRNA and poly(A) RNA in hippocampal neurons in culture. *Mol Brain Res* 20:305–312.
- Litwak J, Mercugliano M, Chesselet M-F, Oltmans GA (1990) Increased glutamic acid decarboxylase (GAD₆₇) mRNA and GAD activity in cerebellar Purkinje cells following lesion-induced increases in cell firing. *Neurosci Lett* 116:179–183.
- Litwak J, Yin Z, Beales M, Oltmans G (1991) Glutamic acid decarboxylase (GAD) activity and mRNA levels in cerebellar Purkinje cells after changes in cell firing. *Soc Neurosci Abstr* 17:628.
- Llano I, Gerschenfeld HM (1993) Beta-adrenergic enhancement of inhibitory synaptic activity in rat cerebellar stellate and Purkinje cells. *J Physiol (Lond)* 468:201–224.
- Lopiano L, Savio T (1986) Inferior olive lesion induces long-term modifications of cerebellar inhibition of Deiters nuclei. *Neurosci Res* 4:51–61.
- Martin D, Martin S, Wu S, Espina N (1991) Regulatory properties of brain glutamate decarboxylase (GAD): the apoenzyme of GAD is present principally as the smaller of two molecular forms of GAD in brain. *J Neurosci* 11:2725–2731.
- McElligott J, Ebner T, Bloedel J (1986) Reduction of cerebellar norepinephrine alters climbing fiber enhancement of mossy fiber input to the Purkinje cell. *Brain Res* 397:245–252.
- Moises HC, Waterhouse BD, Woodward DJ (1983) Locus Coeruleus stimulation potentiates local inhibitory processes in rat cerebellum. *Brain Res Bull* 10:795–804.
- Moises HC, Burne RA, Woodward DJ (1990) Modification of the visual response properties of cerebellar neurons by norepinephrine. *Brain Res* 514:259–275.
- Montarolo PG, Palestini M, Strata P (1982) The inhibitory effect of the olivocerebellar input on the cerebellar Purkinje cells in the rat. *J Physiol (Lond)* 332:187–202.
- Oertel W, Schmechel D, Mugnaini E, Tappaz M, Coupon I (1981) Immunocytochemical localization of glutamate decarboxylase in rat cerebellum with a new antiserum. *Neuroscience* 85:2715–2735.
- Oltmans GA, Olsauskas R, Comaty JE (1980) Hypothalamic catecholamine systems in genetically obese mice (obob): decreased sensitivity to reserpine treatment. *Neuropharmacology* 19:25–33.
- Oltmans G, Lorden J, Beales M (1985) Lesions of inferior olive increases glutamic acid decarboxylase activity in deep cerebellar nuclei of the rat. *Brain Res* 347:154–158.
- Oltmans GA, Beales M, Lorden JF (1986) Glutamic acid decarboxylase activity in micropunches of the deep cerebellar nuclei in the genetically dystonic rat. *Brain Res* 385:148–151.
- Ordway GA, O'Donnell JM, Frazer A (1987) Effects of clenbuterol on central beta-1 and beta-2 adrenergic receptors of the rat. *J Pharmacol Exp Ther* 241:187–195.
- Paxinos G, Watson C (1982) The rat brain in stereotaxic coordinates. New York: Academic.
- Porter T, Martin D (1988) Non-steady-state kinetics of brain glutamate decarboxylase resulting from interconversion of the apo- and holoenzyme. *Biochim Biophys Acta* 874:235–244.
- Schultz JE, Siggins GR, Schocher FW, Turck M, Bloom FE (1981) Effects of prolonged treatment with lithium and tricyclic antidepressants on discharge frequency, norepinephrine responses and beta-receptor

- binding in rat cerebellum: electrophysiological and biochemical comparison. *J Pharmacol Exp Ther* 216:28–38.
- Segovia J, Tillakaratne NJK, Tobin AJ (1991) cAMP regulates GAD₆₇ gene expression in C6 cells. *Soc Neurosci Abstr* 17:159.
- Sessler FM, Mouradain RD, Cheng J-T, Yeh HH, Liu W, Waterhouse BD (1989) Noradrenergic potentiation of cerebellar Purkinje cell responses to GABA: evidence for mediation through the β -adrenoceptor-coupled cyclic AMP system. *Brain Res* 499:27–38.
- Siggins GR, Hoffer BJ, Oliver A, Bloom F (1971) Cyclic adenosine monophosphate and norepinephrine: effects on transmembrane properties of cerebellar Purkinje cells. *Science* 171:192–194.
- Stratton SE, Lorden JF, Mays LE, Oltmans GA (1988) Spontaneous and harmaline-stimulated Purkinje cell activity in rats with a genetic movement disorder. *J Neurosci* 8:3327–3336.
- Sukin D, Skedros DG, Beales M, Stratton SE, Lorden JF, Oltmans GA (1987) Temporal sequence of motor disturbances and increased cerebellar glutamic acid decarboxylase activity following 3-acetylpyridine lesions in adult rats. *Brain Res* 426:82–92.
- Sutin J, Minneman KP (1985) Adrenergic beta receptors are not uniformly distributed in the cerebellar cortex. *J Comp Neurol* 236:547–554.
- Tan HS, Collewyn H (1992) Cholinergic and noradrenergic stimulation in rabbit flocculus have synergistic facilitatory effects on optokinetic responses. *Brain Res* 586:130–134.
- Tondo L, Conway PG, Brunswick DJ (1985) Labeling *in vivo* of beta-adrenergic receptors in the central nervous system of the rat after administration of [¹²⁵I]-iodopindolol. *J Pharmacol Exp Ther* 235:1–9.
- Vos R, Davenport PA, Artymyshyn RP, Frazer A, Wolfe B (1987) Selective regulation of beta-2 adrenergic receptors by the chronic administration of the lipophilic beta adrenergic receptor agonist clenbuterol: an autoradiographic study. *J Pharmacol Exp Ther* 242:707–712.
- Wood PL (1991) Pharmacology of the second messenger, cyclic guanosine 3',5'-monophosphate, in the cerebellum. *Pharmacol Rev* 43:1–25.
- Wuensche CW, Fishcr RS, Kaufmann DL, Tobin AJ (1986) *In situ* hybridization to localize mRNA encoding the neurotransmitter synthetic enzyme glutamate decarboxylase in mouse cerebellum. *Proc Natl Acad Sci USA* 83:6193–6197.