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Aflatrem: A Tremorgenic Mycotoxin with Acute Neurotoxic Effects

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Tremorgenic mycotoxins induce neurologic symptoms ranging from mental confusion to tremors, seizures and death, and are apparently the only class of mycotoxins with significant central nervous system activity. Tremorgens have been implicated in a number of neurologic diseases of cattle collectively known as staggers syndromes, and pose significant agricultural and health problems for both cattle and humans. Although the effects of tremorgens are thought to result from transient perturbations of amino acid neurotransmitter release mechanisms, there is reason to believe that acute exposures to toxins with such synaptic effects may result in degeneration of neuronal fiber processes. To test this hypothesis, rats were given a single tremorgenic (3 mg/kg, IP) dose of aflatrem, and kinetics of amino acid neurotransmitter uptake was assessed in isolated hippocampal nerve terminals at 1 day, 1 week, and 2 weeks after injection. Results indicate a decrease in the capacity of the GABA and glutamate uptake systems, which was interpreted as a loss of nerve terminals. The affinity constants suggest a decrease in release of these transmitters as well. In addition to its transient influence on transmitter release, a single low dose of aflatrem is able to induce degeneration of neuronal processes in hippocampal neurotransmitter systems and therefore represents a long-term health threat.

Introduction

A group of fungal metabolites collectively known as tremorgenic mycotoxins have been identified as the causative agents of a neurological disease of cattle known as "staggers syndrome"(*1*). This disease is characterized by muscle tremors and hyperexcitability, and similar responses have been observed in laboratory animals treated with the mycotoxins (*2,3*). The fungi which produce these compounds have been isolated from corn, silage, and various forages (*2*), and so pose a health threat for both livestock and humans who consume these commodities.

The mechanism of action of these tremorgens is unknown, but indirect evidence suggests a neurochemical (*4*), rather than a cytotoxic, basis for the symptoms. Two lines of evidence seem to support this conclusion. First, tremorgenic mycotoxins do not appear to produce any gross histopathological lesions (*5,6*). Second, glutamate and γ-aminobutyric acid (GABA), which are, respectively, generally excitatory and inhibitory amino acid neurotransmitter candidates, have been reported to respond to exposure to tremorgens. Rats treated with tremorgenic doses of verruculogen, the most potent compound in this group, evince increased glutamate, and decreased GABA, levels in brain tissue (*4,7,8*). These effects apparently occur in subcortical tissue and are completely reversible (*4*), leading to the inference that the tremorgens perturb biochemical processes involved with neurotransmitter release. This conclusion, however, is based on indirect evidence.

Excitotoxic (*9*) compounds are known to induce cell death possibly as a result of sustained depolarization, and some neurotoxins with presynaptic activity such as black widow spider venom (*10*) and Bungarus snake venoms (*11*) produce nerve terminal degeneration. Analogously, it follows that the biochemical alteration triggered by tremorgenic mycotoxins could induce morphological changes in the nerve terminal which would be manifest some time after the exposure, and would not appear as gross lesions.

Neurotransmitter uptake is a specific and sensitive marker for the presence or absence of nerve terminals utilizing a particular neurotransmitter (*12*). Kinetic analysis of such data yields an estimate of alterations in the number (V_{max}) or affinity (K_m) of these terminals, from which inferences about nerve terminal degeneration can be made. Both glutamate and GABA are important neurotransmitters in the hippocampus, and the low seizure threshold of this structure (*13*) makes it a

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likely target for tremorgenic mycotoxins. Accordingly, the effects of the classic tremorgen aflatrem (*14*) on amino acid neurotransmitter uptake were assessed in rat hippocampal synaptosomes isolated at various times after injection of one tremorgenic dose.

Materials and Methods

Subjects

Male albino [AMRI(SD × WI) BR; $N=12$] rats weighing 220–330 g were housed singly in metal cages and allowed *ad libitum* access to laboratory chow (Purina) and tap water. They were housed under conditions of controlled temperature (24 ± 2°C) and humidity (30–70%) on a 12 hr light/dark schedule and allowed to acclimate to their surroundings for 1 week prior to the experiments.

Radioisotopes

Tritiated γ-aminobutyric acid (^{3}H-GABA: 0.33 Ci/mM) and glutamic acid (^{3}H-Glu: 44.1 Ci/mM) were obtained from New England Nuclear. The following stock concentrations of ^{3}H-GABA (0.33 Ci/mM) and ^{3}H-glutamate (1.1 Ci/mM) were used in the experiment: 1.0×10^{-5} M, 7.5×10^{-6} M, 5.0×10^{-6} M, 2.5×10^{-6} M, 1.0×10^{-6} M, 7.5×10^{-7} M. Final incubation concentrations represented a 100× dilution.

Toxins

Purified aflatrem crystals were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 3 mg/cm^3. Toxin was prepared by fermentation from *Aspergillus flavus* and purified to greater than 98% using its UV extinction coefficient.

Reagents and Buffers

Modified Krebs–Henseleit buffer was made up of 1.2 mM $MgSO_4$, 1.5 mM CaCl, 10 mM glucose, 2.5 mM HEPES, 145 mM NaCl, 5 mM KCl, 10 μM aminooxyacetic acid (pH 7.4).

HEPES–Sucrose homogenization medium consisted of 0.32 M sucrose and 2.5 mM HEPES (pH 7.4).

Procedures

Preliminary Experiments. The effects of 20μL DMSO on uptake was assessed in a pilot study. Experimental procedures were as described in Uptake Section. Since DMSO at this concentration did not alter transport, it was used in subsequent studies due to its superior ability to dissolve the mycotoxins.

Uptake: In Vivo Toxin Exposure. Rats (N = 12) were given intraperitoneal injections of either DMSO or a tremorgenic dose of aflatrem (3 mg/kg IP), and decapitated at 24 hr, 1 week, and 2 weeks after the injection. Day of injection was staggered so that any given experiment contained tissue from rats killed at each time point post-injection. The brains were removed to an ice cold dissecting plate; hippocampus (HIP) was rapidly dissected, weighed, and homogenized in 20 volumes sucrose–HEPES medium with a Teflon pestle and matched smooth glass homogenizer (Wheaton, 10 strokes on ice, setting 3). The volumes were adjusted to 20 mL, and the homogenates were centrifuged at 1000*g* (JA-20 rotor) for 10 min at 6°C.

The pellets were discarded and the supernatants were centrifuged at 17000*g* (JA-20 rotor) for 20 min at 6°C. Pellets were resuspended in 5 mL modified Krebs–Henseleit buffer by hand homogenization (5 strokes). The following were combined in duplicate test tubes: 1880 μL Krebs-Henseleit; 20 μL either ^{3}H-GABA (six concentrations prepared as in Radioisotope section) or ^{3}H-glutamate (six concentrations prepared as in Radioisotope section); 100 μL HIP tissue suspension. The tubes were incubated at either 38°C or 0°C for 10 min, uptake being linear in this preparation over this time period, and the contents were aspirated onto filter strips (Whatman GF-B) with a Brandel tissue harvester and washed three times with cold physiological saline (0.9%). The filter disks were placed in Hang-in vials (Packard) to which 5 mL Formula 947 (New England Nuclear) was added, and counted in a Packard 300-c scintillation spectrometer (63% efficiency).

Data Treatment

The data were expressed as moles of transmitter per microgram of synaptosomal protein per minute (mole/μg P/min) of incubation. The 0°C incubation was used as control for nonspecific uptake and subtracted from that obtained at 38°C to yield temperature-sensitive uptake. The data for each neurotransmitter were combined across experiments and graphed as a Lineweaver-Burk plot (*15*) for visual representation only, with kinetic constants K_m and V_{max}, representing the affinity and capacity, respectively, of the uptake system, determined by computer using a program based on a weighted least-squares analysis derived from the Wilkinson equations (*16*). Each data point in each of the Lineweaver-Burk plots shown in Figures 3 and 4 below represents the mean of the uptake values of three rats at that transmitter concentration. The kinetic constants reported in Results were computed using these values. Additionally, kinetic constants K_m and V_{max} were computed from the data from each rat at each time point for both transmitters. These values were then subjected to statistical analyses using one-way analysis of variance and Newman-Keuls test for subsequent comparisons among time points.

Results

Uptake of both ^{3}H-GABA (Fig. 1) and ^{3}H-glutamate (Fig. 2) was linear over the range of neurotransmitter

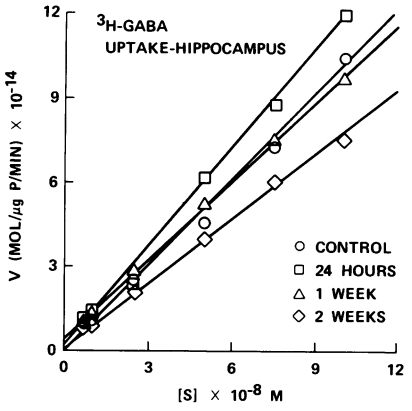

FIGURE 1. ^{3}H-GABA uptake velocity (mole/μg P/min) as a function of transmitter concentration.

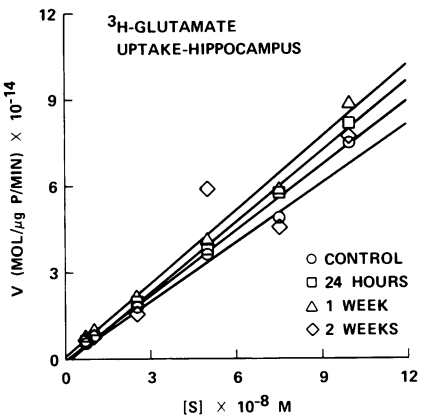

FIGURE 2. ^{3}H-Glutamate uptake velocity (mole/μg P/min) as a function of transmitter concentration.

concentrations and for all time points assessed. Kinetic analyses indicated a similar pattern of alterations for both neurotransmitters. The data for ^{3}H-GABA uptake are presented as a double reciprocal plot in Figure 3. At 24 hr post-injection, ^{3}H-GABA uptake was characterized by a 106% increase in K_m (K_m = 20.2 × 10^{-7}M) and a 125% increase in V_{max} (25.3 × 10^{-13} mole/μg P/min) relative to control values (K_m = 9.8 × 10^{-7} M, V_{max} = 11.2 × 10^{-13} mole/μg P/min). At one week post-injection, K_m (K_m = 4.8 × 10^{-7}M) and V_{max} (V_{max} = 5.6 × 10^{-13} mole/μg P/min) were decreased by 51% and 50%, respectively, compared to control values. This trend was stable at two weeks post-injection, with K_m (K_m = 4.8 × 10^{-7}M) and V_{max} (V_{max} = 4.4 × 10^{-13} mole/μg P/min) being reduced 51% and 60%, respectively, relative to control values. Statistical analysis of these data using one-way ANOVA indicates a significant alteration of ^{3}H-GABA K_m over time after aflatrem injection (F = 72, df = 3,8, p<0.001). Subsequent comparisons using the Newman-Keuls test showed a statistically significant increase of K_m at 24 hr (p<0.01), and decreased K_m at one week (p<0.01) and two weeks (p<0.01) relative to control. When the V_{max} data were subjected to ANOVA, a similar pattern of significant alterations occurred (F = 91, df = 3, 8, p<0.001). V_{max} increased significantly at 24 hr (p<0.01) relative to control. The data for ^{3}H-glutamate uptake are presented as a double reciprocal plot in Figure 4. At 24 hr post-injection, ^{3}H-glutamate uptake was characterized by a 113% increase in K_m (K_m = 4.7 × 10^{-7}M) and a 150% increase in V_{max} (V_{max} = 5.0 × 10^{-13} mole/μg P/min) relative to control values (K_m = 2.2 × 10^{-7}M; V_{max} = 2.0 × 10^{-13} mole/μg P/min). These values returned to approximate control levels by 1 week post-injection (K_m = 2.6 × 10^{-7}M; V_{max} = 2.5 × 10^{-13} mole/μg P/min), and then decreased by 77% (K_m = 0.5 × 10^{-7}M) and 45% V_{max} = 1.1 × 10^{-13} mole/μg P/min) by 2 weeks. Statistical analysis of these data

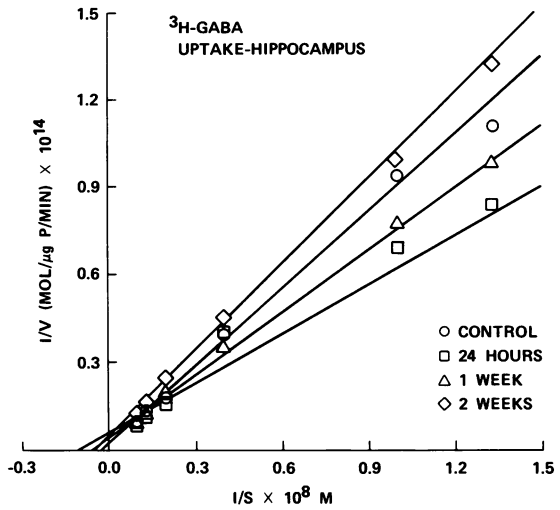

FIGURE 3. Lineweaver-Burk plot of ^{3}H-GABA uptake.

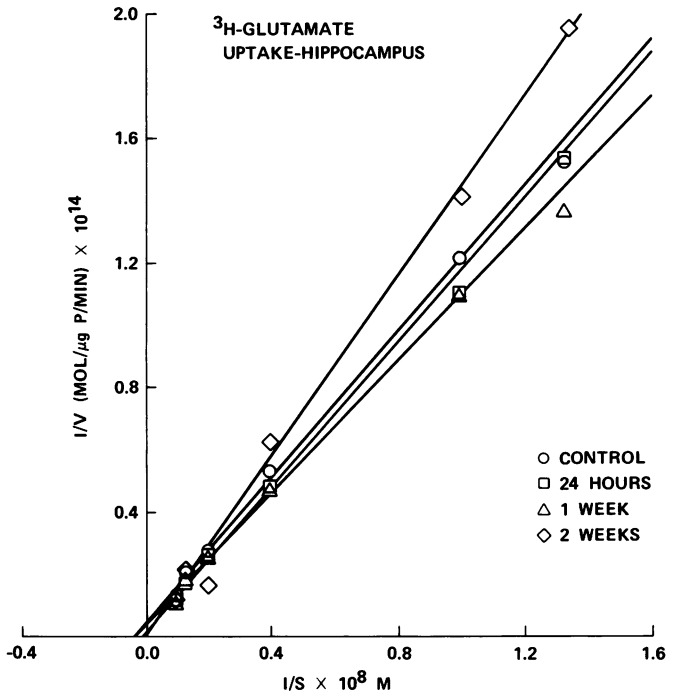

FIGURE 4. Lineweaver-Burk plot of ^{3}H-glutamate uptake.

using one-way ANOVA indicates a significant alteration of ^{3}H-glutamate K_m over time after aflatrem injection ($F = 155$, df = 3, 8, $p<0.001$). Subsequent comparisons using the Newman-Keuls test showed a statistically significant increase of K_m at 24 hr ($p<0.01$), a smaller, but statistically significant increase at 1 week ($p<0.05$), and a statistically significant decrease at 2 weeks ($p<0.01$). Analysis of the V_{max} data via one-way ANOVA revealed significant alterations of this kinetic constant over time after injection ($F = 45$, df = 3, 8, $p<0.001$). Subsequent comparisons indicated an increase of V_{max} at 24 hr ($p<0.01$) and a gradual decrease over time, reaching values significantly lower than control at two weeks ($p<0.01$). In summary, both neurotransmitter uptake systems initially respond to the aflatrem exposure with decreased affinity and increased capacity, and then reverse this trend within two weeks.

Discussion

Neurotransmitter uptake is a dynamic process which is primarily responsible for inactivating released transmitter and providing material for the depleted stores. The rate and amount of uptake are regulated by demand; increased release or decreased synthesis induces increased uptake. Therefore, an interpretation of the biphasic nature of these uptake data must account for the potential acute effects of aflatrem on transmitter release. Specifically, if aflatrem modulates transmitter release as has been hypothesized for verruculogen (*4,7,8*), uptake measurements made during this acute phase of toxicity would be confounded by the compensatory response of the uptake system to the altered demand.

Kinetic analyses of the data at 24 hr post-injection indicates large increases in uptake capacity (V_{max}) and decreases in affinity (i.e., increased K_m) for both transmitter uptake systems. Two interpretations are immediately apparent. First, if aflatrem has, as an acute effect, the enhanced release of transmitters from the nerve terminals, the uptake systems would be required to step up activity in order to replenish transmitter stores to meet demand. Second, the heightened levels of endogenous transmitters in the synaptic cleft which are consequences of increased release creates a situation in which the endogenous and exogenous (i.e., tritiated) transmitters compete for the available uptake sites. These two interpretations are complementary, the first accounting for the increased V_{max} and the second for the increased K_m.

This sequence of events would probably be confined to the initial acute stages of toxicity and would not be involved as an explanation for later changes. By two weeks post-injection, both systems show decreased capacity and increased affinity for their respective transmitters. Since high affinity uptake is a reliable marker for the presence of nerve terminals utilizing a particular transmitter (*12*), the decreased V_{max} values indicate either a loss of both GABAergic and glutaminergic nerve terminals, or an impairment of the storage mechanism (e.g., failure to repackage vehicles in these treated animals). A definitive resolution of this issue awaits histopathological studies using stains specific for neuronal degeneration.

The increased affinity at the two week point is compatible with an explanation based on perturbation of release mechanisms. Specifically, decreased release from the intoxicated nerve terminals would result in lower levels of transmitter in the synaptic cleft, hence less competition for the available uptake sites. The result would be an apparent increase in affinity. Alternately, and less likely, the toxin may differentially damage some subset of nerve terminals with particular (i.e., lower affinity) uptake characteristics, thus favoring the survival of a population of nerve terminals with higher affinities for the transmitters.

It is apparent that an explanation of aflatrem toxicity must address the possibility that a single tremorgenic dose induces neuronal pathology which is expressed over a period of weeks. This pathology is most likely nerve terminal degeneration as indicated by the decreased synaptic uptake of amino acid neurotransmitters. Other possibilities, such as interference with transmitter synthesis or storage, cannot be definitively ruled out, however. Several questions remain: it is an open question as to how the hypothesized degeneration is induced, whether it is specific to amino acid transmitter systems or the result of some general metabolic mechanism common to all types of neurons, and whether this neurotoxicity is a characteristic of all tremorgenic mycotoxins.

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