Serotonin Depletion Attenuates AY-9944–Mediated Atypical Absence Seizures

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Summary: Purpose: To test the hypothesis that serotonin (5-HT) plays a role in the modulation of experimental atypical absence seizures.

Methods: Male Long-Evans hooded rats were treated from postnatal day (P) 2 to P20 with the cholesterol inhibitor AY-9944 (AY). Epidural electrodes were implanted for electrocorticography (ECoG) followed by serotonin depletion by using para-chlorophenylalanine (PCPA). High-performance liquid chromatography (HPLC) was used to measure the levels of serotonin and its metabolite (5-HIAA) in various brain regions. Serotonin metabolism was computed by using the 5-HIAA/5-HT ratio and used to ascertain differences between groups.

Results: PCPA treatment was associated with a significant decrease in the total slow spike-and-wave discharge (SSWD) duration in AY-treated rats compared with controls (p < 0.01). HPLC data confirmed the PCPA depletion of 5-HT and 5-HIAA in cortex, thalamus, hippocampus, and brainstem compared with naïve rats. AY-treated rats showed higher levels of 5-HIAA and 5-HT in the same brain regions, with a concomitant decrease in rates of serotonin turnover.

Conclusions: The data indicate that serotonin depletion protects against experimental atypical absence seizures. The increased levels of 5-HIAA and 5-HT and altered rates of serotonin turnover suggest that the serotonergic neurotransmission may be perturbed in the AY rat.

Key Words: Atypical absence seizures—AY-9944—ECoG—5-HT—PCPA.

Atypical absence seizures (AASs) are clinically distinct from typical absence (petit mal) seizures in terms of cognitive outcome, ictal behavior, and electroencephalographic (EEG) manifestations (1–3). Unlike typical absence seizures, AASs often occur as a component of severe, medically refractory childhood epilepsy syndromes, such as the Lennox–Gastaut syndrome or other malignant epilepsy syndromes that are characterized by multiple seizure types (4). AASs rarely occur alone; they almost always coexist with tonic seizures during sleep, myoclonic jerks, and generalized tonic–clonic seizures. The recurrent, bilaterally synchronous 1.5- to 2.5-Hz slow spike-and-wave discharge (SSWD) that typifies AASs is highly predictive of a poor cognitive outcome (5), and AASs are almost always associated with cognitive deficits (6).

The clinical ictal events observed in patients with Lennox–Gastaut syndrome in both awake and sleep states are highly reproducible in the AY-9944 (AY) cholesterol inhibition model. The electrocorticographic (ECoG) signature of the AY model consists of spontaneously recurring, bilaterally synchronous generalized 5- to 7-Hz SSWDs that emanate from the cortex, the thalamus, and the hippocampus (7). AY-treated animals move about intermittently during the seizure, and, unlike that in animal models of typical absence seizures (8), the ictal behavior is not time-locked with the epileptiform discharge. Rather, a poor correlation exists between the timing of the onset and offset of the SSWD and the onset and offset of the behavioral ictal changes. In addition, unlike animal models of typical absence seizures, the AY model of AASs is characterized by cognitive impairment (9, 10).

The administration of AY during rat brain development leads to a prepubertal onset of SSWDs (11) that persists during the adult period, although the brain cholesterol returns to normal values (12). The SSWDs in the AY model are state dependent, going from intermittent bilaterally synchronous discharges during the awake state to a continuous SWD during slow-wave sleep, which is often interrupted by myoclonic jerks (13).

Administration of the precursor of serotonin, 5-hydroxytryptophan (5-HTP), is associated with the induction of myoclonic jerks in guinea pigs (14). These myoclonic jerking movements can be prevented by pre-treatment with para-chlorophenylalanine (PCPA) (15), a compound that depletes brain serotonin by selectively and...
irreversibly inhibiting tryptophan hydroxylase, the rate-determining step in serotonin synthesis (16). Recently it was shown that selective serotonin reuptake inhibitors (SSRIs) exacerbate typical absence seizures, presumably by the increase in serotonergic activity (17).

To date, inconsistent results have been seen regarding the effect of serotonin on absence seizures. For example, in the genetic absence epilepsy rat from Strasbourg (GAERS), decreasing serotonin via PCPA had no effect, nor did using 5-HTP or methysergide, a nonspecific mixed agonist/antagonist (18). With the WAG/Rij absence seizure model, Coenen and van Luijtenaar (19) reported that serotonin may indirectly modulate absence seizures via glutamate transmission. However, recent work with the WAG/Rij model has shown that SSRIs may exacerbate absence seizures, and this may be mediated via the 5-HT2C receptor subtype. Thus little work demonstrates a direct relation between the effect of specific serotonin-receptor subtypes and rodent absence seizures. To date, the effect of serotonin on the AASs in the AY model has not been elucidated. Because no receptor subtypes have been implicated in the AY model, our goal was to determine the outcome of global depletion of serotonin via PCPA. Serotonin depletion via administration of PCPA was chosen because this is widely used and very well characterized (16). Neurotoxic serotonin-depleting agents (5,7-dihydroxytryptamine, 5,7-DHT) are reliable but produce profound permanent axonal damage (20). Thus we chose to use PCPA because its effects are well described and reversible (16).

Because of the occurrence of state-dependent changes in the EEG and the occurrence of myoclonic jerks during sleep in AY-treated animals, we hypothesized that perturbation of the serotonergic system may play a role in the genesis of AASs in the AY model. Therefore the objective of this study was to determine the effect of PCPA-mediated serotonin depletion on SSWD duration in the AY model of AASs.

METHODS

Animals

Male Long-Evans hooded rats (250–300 g) and untimed pregnant Long-Evans hooded rats were obtained from Charles River (St. Constant, Quebec, Canada) and housed in the animal facility at the Hospital for Sick Children (Toronto, Ontario, Canada). The suckling rat pups were weaned at postnatal (P) day 21 and grouped in pairs. Animals were kept in a controlled environment at a 12-h light–dark cycle with lights on at 0600 h and ad lib access to food and water. All animal procedures were approved by the Animal Care Committee at the Hospital for Sick Children, which conforms to the rules and regulations of the Canadian Council on Animal Care and Animals for Research Act (Ottawa, Canada).

Drugs

AY-9944 [trans-1,4-bis(2-chloro-benzylaminomethyl) cyclohexane dihydrochloride] was a gift from Wyeth-Ayerst (Philadelphia, PA, U.S.A.). Para-chlorophenyl-lalanine methyl ester (PCPA) was purchased from Sigma (Oakville, Ontario, Canada). Drugs and chemicals used for biochemical assay were purchased from Sigma and were of the highest purity (HPLC grade). AY-9944 and PCPA were both dissolved in distilled water. All drugs were injected in a volume of 2 ml/kg of body weight, unless stated otherwise.

AY model

After birth, rat pups were randomly divided into two groups, receiving subcutaneous AY (7.5 mg/kg in 1 ml/kg of body weight) every 6 days from P2 to P20 (AY group, n = 12) or the equivalent volume of distilled water (control group = 8) as previously described (7). No female rats were used because of our previous finding that the female estrus cycle does modulate the SSWD duration in the AY model (11). All rats were randomly divided into two groups: the first group included the AY rats treated with either PCPA (n = 6) or vehicle (n = 6); and the second group of control (Ctrl) rats (n = 8) that were given the same PCPA treatment to ascertain any effect of serotonin depletion on AY-SSWD and on the spontaneous burst observed in controls (7).

Surgery and electrocorticography

On surgery day at P60, all animals received intraperitoneal (i.p.) atropine methyl bromide (0.5 mg/kg of body weight) as a preanesthetic agent to control secretions. Fifteen minutes later, animals were anesthetized with a single i.p. injection of Somnotol (sodium pentobarbital, 35 mg/kg), which provides anesthesia that lasts approximately 2 to 3 hours. Surgeries were done in the animal facility with sterile equipment, and the greatest care was taken to ensure minimal pain and discomfort. Rats were implanted with two monopolar epidural electrodes placed over the left and right frontal cortical regions and two placed over the left and right parietal regions. Four watchmaker screws were set in the lateral regions of the skull, and the preparation was secured with dental cement. After surgery, all animals were monitored daily by veterinary technicians for 5 days of recovery.

Electrocorticographic (ECoG) recordings were performed in unrestrained animals in individual Plexiglas chambers lined with bedding (Harvard Apparatus, Holliston, MA, U.S.A.). A 20-min adaptation period was given before every ECoG to minimize movement artifact. Paper ECoG recordings were made by using a Grass Polysonomnograph (78D; Grass Instruments, Quincy, MA, U.S.A.). Frontal and parietal differential recordings were performed on two distinct channels. ECoG recordings were made between 1000 and 1400 h to prevent circadian variations (21).
Experimental design

Each rat was monitored with a 1-h baseline ECoG recording 1 week after surgery day. On the following 3 consecutive days, PCPA (150 mg/kg in a volume of 2 ml/kg) or vehicle was administered between 0900 and 1100 h during the peak of serotonin levels (22). Then a test ECoG recording was obtained from each rat, 24 h after the last PCPA injection.

Brain serotonin analysis

After the test ECoG recording, all rats were anesthetized deeply with pentobarbital (65 mg/kg). The brains were removed and quickly dissected on ice. Thalamus, hippocampus, frontal–parietal cortex, and brainstem were individually stored in 1.5-ml microcentrifuge tubes and frozen at −80°C (23). Biochemical analysis of serotonin levels was performed as described by Fletcher et al. (24). Tissue was homogenized in 5 volumes of 0.2N perchloric acid (HClO4) containing 100 ng/ml of dihydroxybenzylamine (DHBA) as the internal standard. Homogenates were centrifuged at 20,800 g for 15 min at 4°C. The resulting supernatant aliquots (20 μl) were filtered by using 0.22-μm syringe filters and analyzed for serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) by using high-performance liquid chromatography with electrochemical detection (HPLC-ED) (ESA, Bedford, MA, U.S.A.). The analytic system consisted of a 100-μl sample loop (Rheodyne, Rhonert Park, CA, U.S.A.), a 3.2 × 150 mm, 3 μm C18 reverse phase column C18 reverse phase column (MD-150, ESA), and a pulse dampener. Electrochemical detectors consisted of ESA Coulochem II detector with 5020 guard cell set at +350 mV and 5014B analytical cells set at potential −150 mV (electrode 1) and +300 mV (electrode 2). Data were collected and integrated by using the ESA 500 Chromatography Data System. The mobile phase consisted of 75 mM sodium dihydrogen phosphate monohydrate, 1.7 mM octanesulfonic acid (sodium salt), 25 μM EDTA, and 8% acetonitrile adjusted to pH 3.00 with phosphoric acid. Stock solutions of 5-HT and 5-HIAA (HPLC grade, Sigma) were used to prepare external standard curves. Data were collected and integrated by using the ESA 500 Chromatography Data System.

Data analysis

Measure of SSWDs

The total duration of AY-SSWDs and the spontaneous bursting activity of controls were quantified over 1-h baseline and test ECoG recordings. AY-SSWDs were scored only if they appeared on both channels simultaneously at the frequency of 5–6 Hz with at least threefold higher amplitude compared with baseline. Total AY-SSWD duration was expressed as mean ± standard error of the mean (SEM). A two-way repeated analysis of variance (ANOVA) was used with treatment (PCPA or vehicle) as the between-subject and session (baseline or post) as the within-subject variables to test the effect of PCPA in AY-treated rats as compared with vehicle (interaction effect). Specific comparisons were made by using paired Student’s t test to test statistical significance within each group. All analyses were performed by using SigmaStat 2.0, and an alpha of p = 0.05 was considered to be significant.

HPLC analysis

Within the same chromatogram, sample peak areas were compared with an internal standard to yield the corrected amount of neurotransmitter and presented as picograms/milligram of wet brain tissue. In addition to measuring the levels of serotonin, we decided to study the functional activity of serotonin metabolism. Previous studies showed that the ratio of serotonin metabolite (5-HIAA) to serotonin (5-HT) can serve as an index of the serotonin turnover rate (25). Thus we used the 5-HIAA/5-HT ratio as an approximation of the serotonin turnover (26,27).

Statistical analysis

Statistical analyses of biochemical results were performed only within the same brain region and for the same neurotransmitter/turnover rate. One-way ANOVAs were used to analyze differences within each brain region. Comparisons were made a priori by using two-tailed unpaired Student’s t tests. Specific comparisons were made between naïve rats and AY-vehicle–treated rats and also between PCPA-treated rats and their respective controls. For all statistical analyses, an alpha of p = 0.05 was used as a measure of significance.

RESULTS

AY-induced atypical absence seizures

AY treatment resulted in spontaneous, recurrent, bilaterally synchronous 5- to 6-Hz SSWDs associated with AASs, as previously described (7). AY-induced AASs were characterized by staring, facial myoclonus, and whisker twitching, with a gradual onset and disappearance that usually began after the onset of the SSWDs and clearly outlasted them. The complete immobility or “frozen stare” that typifies pharmacologic and genetic rat models of typical absence seizures was not observed in the AY-treated rats. Instead, the AY-treated animals showed an ability to move intermittently during the seizures.

PCPA treatment–attenuated SSWDs in the AY model

As shown in Fig. 1, PCPA treatment significantly reduced the total SSWD duration, as demonstrated by an interaction effect (p < 0.001, two-way repeated ANOVA). Specifically, PCPA reduced total SSWDs from 484.42 ± 63.98 to 259.42 ± 13.48 s/h (p < 0.01, Student’s t test). No difference was observed when AY-treated rats were given vehicle. Similarly, no significant difference was observed when control naïve male rats were given a regimen of PCPA.
Brain serotonin levels

Levels of 5-HIAA differed significantly within each region examined (Fig. 2A). 5-HIAA levels were significantly elevated in AY rats treated with vehicle as compared with naïve rats in the brainstem (51% increase, p < 0.05, Student’s t test). PCPA treatment significantly reduced 5-HIAA levels by 92–95% in all brain regions of control-PCPA rats when compared with naïve rats and in AY-PCPA rats when compared with AY-vehicle.

Figure 2B shows the levels of 5-HT in the brain regions studied. With ANOVA, significant differences were obtained in all brain regions examined. Specific comparisons showed that AY-vehicle rats had significantly elevated 5-HT levels in both the thalamus (50% increase, p < 0.05, Student’s t test) and brainstem (61% increase, p < 0.05, Student’s t test). PCPA significantly depleted 5-HT levels by 92–95% in all brain regions studied when comparing control-PCPA with naïve rats, and AY-PCPA with AY-vehicle–treated rats.

As Fig. 3 shows, AY-treated rats given vehicle had significantly reduced turnover rates in the thalamus (21%, p < 0.05, Student’s t test) and cortex (19%, p < 0.05, Student’s t test) as compared with male naïve rats.

DISCUSSION

These data indicate that PCPA-mediated serotonin depletion protects against experimental AASs induced by AY in Long Evans hooded rats. Serotonin depletion with PCPA did not change the spontaneous and minimal spike-wave–like bursts duration seen in control rats. Biochemical data confirmed that PCPA caused a marked depletion of both 5-HT and 5-HIAA in all brain regions studied in both control and AY-treated rats. Furthermore, with the HPLC, we demonstrated that the AY developmental treatment produced an increase in 5-HT and 5-HIAA levels in some of the brain regions studied when compared with those in naïve rats. A concomitant significant decrease was noted in serotonin-turnover rates in both the thalamus and cortex.

When PCPA was administered to the GAERS model of chronic typical absence epilepsy, no effect was found in a variety of doses (18). The authors concluded that serotonin did not seem to have an effect in that model. PCPA has not been used in other chronic models of typical absence seizures. The effect PCPA on other types of experimental seizures appears to be model dependent. For instance, PCPA-mediated serotonin depletion prolonged the latency of audiogenic seizures in DBA/2J mice (28) and decreased the amplitude, but not frequency, of hippocampal seizures elicited by digitoxigenin (29). Short- and long-term PCPA treatment decreased the severity of seizures and delayed kindling in rats and rabbits (30, 31). Similarly, PCPA delayed amygdala kindling in rabbits but increased the severity of pentylenetetrazol-induced convulsions in rats (32,33). Alternatively, PCPA increased the severity of seizures induced by repetitive stimulations to the dorsal hippocampus in rat (34).

In our study, we confirmed that PCPA depleted the brain of 5-HIAA (94–99%) and 5-HT (92–95%). Such a profound reduction of serotonin in the PCPA-treated AY rat is important because it demonstrates that even with a near-complete depletion of brain serotonin, seizures were still apparent, albeit significantly reduced, in the AY model. Because thalamocortical hippocampal pathways are involved in the AY model (7,10), our data comport with the findings of Jakala et al. (35), who reported that thalamocortical oscillations (36) were still apparent after PCPA-mediated serotonin depletion. Similarly, PCPA had no effect on the GAERS rat model of typical absence seizures (18). These data suggest that serotonin depletion may not affect thalamocortical oscillatory activity. The ability of PCPA to reduce SSWDs in the AY model but not SWDs in the GAERS may lie in the inherent differences between these two models of absence seizures. Because the hippocampus displays seizure activity in the AY model (7) but not in the GAERS model (8,18), it could be hypothesized that the hippocampus may play a role in modulating seizures in the AY model (37). For example, Bertram and Zhang (38) showed strong and possibly monosynaptic connections between the thalamus and hippocampus. Thus it is conceivable that serotonin depletion may act on the hippocampus to block absence seizure generation or propagation.

The brain levels of 5-HT were significantly increased in the thalamus and brainstem, and the levels of 5-HIAA were significantly increased in the thalamus of AY rats treated with vehicle compared with naïve rats and less so in other brain regions. The lack of significant changes in the cortex may be attributed to a lack of sensitivity to small changes after AY treatment. The small effects are amplified when serotonin turnover is calculated and significance is reached. The inability of hippocampal transmitter levels...
FIG. 2. Monoamine measurements from four separate brain regions: levels of 5-hydroxyindoleacetic acid (5-HIAA) and serotonin (5-HT).

A: 5-HIAA levels are elevated in male AY-treated rats as compared with naive male rats. Treatment with para-chlorophenylalanine (PCPA) depleted 5-HIAA levels in AY and control rats as compared with their respective controls. 5-HT levels were markedly depleted in AY and control rats as compared with their respective controls. AY rats showed higher 5-HT levels in the thalamus and brainstem as compared with naive rats (*p < 0.05, two-tailed unpaired Student’s t test versus male naive; **p < 0.01, two-tailed unpaired Student’s t test versus male naive; #p < 0.01, two-tailed unpaired Student’s t test versus AY vehicle). NB: AY-PCPA cortex 5-HIAA value is correctly labeled but value is too small to produce noticeable bar.

The 5-HIAA/5-HT ratio has been used as a tool to approximate the serotonin-turnover ratio and metabolism (25,26) and can be altered in response to pentylenetetrazol (PTZ) (27) as well as audiogenic seizures in mice (32). In our study, we showed that AY rats had lower 5-HIAA/5-HT ratios when compared with naive rats, which suggests that the alterations in brain 5-HT and 5-HIAA levels could be due to reduced serotonergic metabolism in this model. In the PTZ seizure model, Szyndler et al. (28) demonstrated reduced serotonin-turnover rate in the hippocampus and prefrontal cortex. Concomitantly, in another study, Szyndler et al. (39) demonstrated that PTZ-induced seizures reduced the binding of the SSRI citalopram in the hippocampus. These data suggest that, at least in the PTZ model, reduced serotonin uptake can be associated with the reduced turnover rate. It could be hypothesized that these compensatory mechanisms occur to conserve serotonin. Whether similar mechanisms explain
FIG. 3. Serotonin turnover rates (5-HIAA/5-HT) in four distinct brain regions. AY-treated rats showed reduced serotonin-turnover rates as compared with naive rats (*p < 0.05, two-tailed unpaired Student's t test vs. male naive).

the reduced serotonin-turnover rate in the AY model remains to be elucidated.

We found that serotonin-turnover rates in the hippocampus or the brainstem were not significantly altered in AY-vehicle rats as compared with naive rats. In the hippocampus, these differences may be attributed to tissue variability, because both 5-HT and 5-HIAA were slightly elevated in AY-vehicle rats as compared with naive rats. This suggests that at least some degree of perturbation of the serotonergic system exists. In the brainstem, 5-HT and 5-HIAA were significantly elevated, but the overall ratio was unaltered. Because compensatory mechanisms are likely to occur at the extremities of serotonergic neurons, it is likely that these changes would not be observed in the brainstem where the serotonergic cell bodies are located.

It is conceivable that PCPA could attenuate AY-induced AASs by other mechanisms derived from serotonin depletion. PCPA-mediated serotonin depletion has been shown to induce insomnia in cats (40). It is possible that the rats in our study became more alert after serotonin depletion. Thus reduction of seizures could be attributed to an increase in wakefulness. However, studies investigating insomnia usually do so by using cats given single high doses of PCPA (500 mg/kg) (41). Rats seem to show more resistance to insomnia induced by PCPA, and they show a rapid rebound (42). Our dosing schedule would also minimize the amount of insomnia produced because the drug is delivered over a 3-day period. Nevertheless, changes in the sleep–wake cycle could in part contribute to the reduced seizure effects that we observed.

The actions of PCPA also can be explained by assessing long-term neurochemical changes in the rat brain after serotonin depletion. For example, PCPA-mediated serotonin depletion has been shown to be associated with changes in glutamate-receptor expression (43). Specifically, the authors reported that GluR2, which controls the influx of Ca2+ ions, was significantly increased, thus limiting neuronal Ca2+ influx. Alternatively, Bidzinski et al. (44) found that PCPA treatment was associated with a decrease in [3H]muscimol binding to the GABA_A receptor in the hippocampus (CA3 and dentate), entorhinal cortex, occipital cortex, and geniculate nucleus. These data raise the possibility that serotonin depletion also may modulate the GABAergic function within the thalamocortical circuitry with a resultant effect on SSWDs (35,45). Further studies are needed to investigate the possible effects of PCPA on the GABA and glutamate systems and their contribution to the attenuation of the developmentally induced AY-SSWDs. However, in the meantime, we will consider studying the effect of enhancing serotonergic tone via selective serotonin reuptake inhibitors (SSRIs) (17), in the AY-9944 model.

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