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Ketogenic diet decreases circulating concentrations of neuroactive steroids of female rats

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Abstract

Ketogenic diet (KD) is used to manage intractable epilepsy; however, the mechanisms underlying its therapeutic effects are not known. Steroid hormones, such as progesterone and testosterone, are derived from cholesterol, and are readily 5 α -reduced to dihydroprogesterone and dihydrotestosterone, which are subsequently converted to 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP) and 3 α -androstenediol, neuroactive steroids that can influence seizures. The present study examined the effects of the KD on circulating concentrations of these neuroactive steroids, and their precursors, in intact female rats. Thirty-six, 22-day-old female Sprague–Dawley rats (weaned at 21 days) were fasted for 8 hours prior to placement on one of three dietary regimens for 6 weeks: ad libitum chow, calorie-restricted chow, or KD. After 6 weeks of the diet, when six rats in each dietary condition were in diestrus and six were in behavioral estrus, all rats were administered pentylenetetrazole (PTZ, 70 mg/kg, IP). The latency and incidence of seizures were recorded by an observer who was uninformed of the estrous cycle and dietary treatment conditions of the rats. Immediately after each test, trunk blood was obtained for later measurement of pregnane (progesterone, dihydroprogesterone, 3 α ,5 α -THP) and androstane (testosterone, dihydrotestosterone, 3 α -androstenediol) neuroactive steroid concentrations in plasma by radioimmunoassay. KD tended to lengthen the latency to, and significantly reduced the number of, PTZ-induced barrel roll seizures. KD also significantly reduced plasma levels of the pregnane (dihydroprogesterone, 3 α ,5 α -THP) and androstane (dihydrotestosterone, 3 α -androstenediol) 5 α -reduced metabolites. These data suggest that levels of pregnane and androstane neuroactive steroids, or their precursors, may underlie some of the antiseizure effects of KD.

Keywords

Progestins; Androgens; Nongenomic; Seizures; Pentylenetetrazole; Ketogenic diet; Epilepsy

1. Introduction

Epilepsy, a complex brain disorder involving excessive, synchronous, abnormal electrical firing patterns of neurons, is one of the most common and challenging neurologic disorders [1]. Management of seizure disorder with the available antiepileptic drugs (AEDs) is often successful [2]. However, there are serious AED-associated side effects, including weight gain, risk of cardiovascular disease, bone density loss, and neurobehavioral sequelae, such as deficits in activity, attention, and/or cognitive functioning [3,4]. Moreover, up to 30% of those with seizure disorders are resistant to AEDs [5]. Other treatment modalities, such as

surgery, vagus nerve stimulation, and the ketogenic diet (KD), are alternatives for those with seizure disorders that are refractory to AEDs [6–9]. Of these options, the KD has relatively few serious complications [10], is least invasive, has the lowest risk of morbidity, and is a cost-effective alternative to other non-AED treatments [11,12]. Furthermore use of the KD as mono- or adjunctive therapy can enable AED use to be minimized and thereby improve behavior [13–17].

The KD, a high-fat, low-carbohydrate, low-protein diet developed in the 1920s and used for more than eight decades for the treatment of refractory epilepsy, is one of the most effective methods of non-AED epilepsy therapy [18]. The KD has been proven to be a well-tolerated and effective treatment for children [19], adolescents [20], and adults [21] who are refractory to standard AEDs. The KD is remarkably effective, with two-thirds of individuals showing significant reduction in seizure frequency and one-third becoming nearly seizure-free [22]. The KD, originally introduced in the 1920s, has been undergoing a recent resurgence as an adjunctive treatment for refractory epilepsy [23,24]. Despite this long history, the mechanisms by which the KD exerts its antiseizure actions are not fully understood.

Steroids have had a role in the treatment of epilepsy for some time [25,26]. Adrenocorticotrophic hormone (ACTH) is used to therapeutically manage infantile spasms and other developmental epilepsies. Although the mechanisms underlying ACTH's clinical benefits are not understood, it has been proposed that ACTH has stimulatory effects on the release of adrenocortico-steroids and neuroactive steroids. In support, agents that are allosteric modulators of GABA_A receptors, such as vigabatrin, benzodiazepines, ganaxolone (a synthetic neuroactive steroid), and progesterone (a natural neuroactive steroid), are at least as effective as ACTH in acutely controlling infantile spasms [27]. Indeed, neuroactive steroids (and adrenocorticosteroids) are emerging as effective treatment approaches for children with epilepsy [27]. Progesterone has also demonstrated clinical benefits in women with catamenial epilepsy [28,29].

One putative mechanism that may underlie some of the antiseizure effects of the KD are alterations in formation of neuroactive steroids. First, production of neuroactive steroids is increased in response to stress [30], and elevated cortisol levels have been observed in children maintained on the KD for 3–4 weeks [31]. Second, dietary cholesterol is the essential precursor for production of neuroactive steroids, which are potent modulators of GABA_A receptor function [32–36]. Cholesterol is converted by the P450 side-chain cleavage enzyme to pregnenolone, from which progesterone and testosterone are derived [37]. Progesterone is then metabolized by 5 α -reductase to dihydroprogesterone, which is then converted to 5 α -pregnan-3 α -ol-20-one (3 α ,5 α -THP) by 3 α -hydroxysteroid dehydrogenase. Testosterone is similarly converted to dihydrotestosterone and 3 α -androstenediol. Notably, endogenous production, or administration, of these pregnane and androstane neuroactive steroids can have anticonvulsant effects [38–40]. As well, the efficacy of the KD has long been known to be mitigated by the amount of fat in the diet [41,42] and efficacy in modulating GABA_A receptors [43]. Third, formation of neurosteroids varies across developmental and hormonal states [44–48]. As well, efficacy of the KD may be influenced by developmental and/or hormonal status [49–51]. Fourth, both the KD and neuroactive steroids have neuroprotective effects to inhibit cell death of hippocampal neurons [52,53]. Together, these findings suggest that the KD may influence formation of neuroactive steroids [54].

The present study examined seizure incidence and levels of pregnane and androstane neuroactive steroids of female rats on the KD, calorie-restricted, or ad libitum diet. We hypothesized that if the KD has antiseizure effects that involve synthesis of neuroactive

steroids, then circulating levels of neuroactive steroids and/or their precursors should be altered in rats on the KD, compared with those in rats on a calorie-restricted or ad libitum diet. Revealing the effects of the KD on seizures and neuroactive steroid levels may help to elucidate the mechanisms that underlie the antiseizure effects of the KD.

2. Methods

2.1. Animals

Thirty-six 22-day-old (weaned at 21 days) female Sprague–Dawley rats (Charles River Laboratories, Boston, MA, USA) were housed individually in cages at an ambient room temperature of 22 °C on an alternating 14/10-hour light/dark cycle with lights on at 2100 hours. All animals had free access to water at all times. Animals were housed and tested at the University of Hartford.

2.2. Dietary treatments

Animals were fasted for 8 hours upon arrival, weighed, and divided randomly into three dietary treatment groups ($n = 12/\text{group}$, weight = 45–54 g). As per Harney et al. [55], rats were fed either: (1) rodent chow ad libitum diet, (2) rodent chow calorie-restricted diet (70% of ad libitum consumption), or (3) KD, for 6 weeks. Previous research suggests that calorie restriction alone can be anticonvulsant [56]. As such, ad libitum food consumption was calculated every 3 days to adjust the calorie-restricted diet. The KD (Bio-Serv product, Frenchtown, NJ, USA; No. F3666) contains more than 93% of its calories from fat. The rodent chow ad libitum and calorie-restricted groups were fed rodent chow (Purina 5001). Fresh diets were administered 2 hours prior to lights off and were left in the cages at all times.

2.3. Vaginal opening and estrous cyclicity

Animals were weighed every other day and were checked daily for vaginal opening. Although rats treated with the KD were active and seemed healthy, consistent with previous reports, their weight gain was substantially lower than that of rats that received regular rat diet. After vaginal opening occurred, female rats were cycled daily by vaginal lavage to determine the phase of the estrous cycle [57]. Given that cycle phase can influence seizures [58], this variable was controlled to prevent confounds. That is, all rats were tested on a single day when 6 of the rats in each dietary group were in diestrus (a phase of the cycle associated with low hormone levels) and the remaining 6 rats in each dietary group were tested as described below when in proestrus (a phase of the cycle associated with higher hormone concentrations).

2.4. Seizure induction with pentylenetetrazole

After 6 weeks of their assigned diet regimen (rats' approximate age = day 65), all rats in each group were administered pentylenetetrazole (70 mg/kg; i.p.) and latency to, and incidence of, seizures were examined according to previously published methods [59] by an observer who was uninformed of the estrous cycle and dietary condition of each rat.

2.5. Tissue collection

Rats were killed by rapid decapitation immediately after seizure testing. Trunk blood was collected on ice, centrifuged, and transported on dry ice to the University at Albany, where it was stored at -70°C until used for radioimmunoassay.

2.6. Neuroactive steroid measurement

Plasma levels of progesterone, dihydroprogesterone, 3 α ,5 α -THP, testosterone, dihydrotestosterone, and 3 α -androstenediol were measured by radioimmunoassay (RIA) according to previously published methods [46].

2.7. Radioactive probes

Tritiated progesterone (NET-208: specific activity = 47.5 Ci/mmol), 3 α ,5 α -THP (used for dihydroprogesterone and 3 α ,5 α -THP; NET-1047: specific activity = 65.0 Ci/mmol), testosterone (NET-387: specific activity = 51.0 Ci/mmol), dihydrotestosterone (NET-302: specific activity = 43.5 Ci/mmol), and 3 α -androstenediol (NET-806: specific activity = 41.00 Ci/mmol), used for RIAs, were purchased from New England Nuclear (Boston, MA, USA).

2.8. Extraction of steroids from plasma

Steroids were extracted from plasma with ether following incubation with water and 800 cpm of tritiated steroid. After snap-freezing twice, test tubes containing steroid and ether were evaporated to dryness in a Savant. Dried down tubes were reconstituted with phosphate assay buffer to the original plasma volume.

2.9. Setup and incubation of radioimmunoassays

Standard curves were prepared in duplicate. For progestins and androgens, the ranges of the standard curves were 5 to 4000 and 50 to 2000 pg, respectively. The standards were added to BSA assay buffer, followed by addition of the appropriate antibody (see below) and ³H-labeled steroid. The total assay volume for 3 α ,5 α -THP and androgens was 1200 μ l. For progesterone and dihydroprogesterone, the total assay volumes were 800 and 950 μ l. All assays were incubated overnight at 4 °C, except that for 3 α -androstenediol, which was incubated at room temperature.

2.10. Antibodies

The progesterone antibody (No. 337) was obtained from Dr. G.D. Niswender (Colorado State University) and used at a concentration of 1:30,000. Dihydroprogesterone (X-947) and 3 α ,5 α -THP (921412-5) antibodies were purchased from Dr. Robert Purdy (Veteran's Medical Center, La Jolla, CA, USA); the concentration of these antibodies used was 1:5000. The testosterone (T3-125) and dihydrotestosterone (DT3-351) antibodies were purchased from Endocrine Science (Calabasas Hills, CA, USA) and used at concentrations of 1:20,000 and 1:10,000, respectively. 3 α -Androstenediol antibody (X-144) was purchased from Dr. P.N. Rao (Southwest Foundation for Biomedical Research, San Antonio, TX, USA); a 1:20,000 concentration of this antibody was used.

2.11. Termination of binding

Separation of bound and free steroid was accomplished by the rapid addition of dextran-coated charcoal. Following incubation with charcoal, samples were centrifuged at 1200g and the supernatant was pipetted into a glass scintillation vial with scintillation cocktail. Sample tube concentrations were calculated using the logit-log method of Rodbard and Hutt [60], interpolation of the standards, and correction for recovery. Intraassay and interassay coefficients of variance for each assay were (progesterone) 9 and 10%; (dihydroprogesterone) 12 and 14%; (3 α ,5 α -THP) 14 and 14%; (testosterone) 7 and 6%; (dihydrotestosterone) 6 and 15%; and (3 α -androstenediol) 10 and 11%.

2.12. Statistical analyses

Initial analyses revealed that there were no estrous cycle differences (which may have been due to insufficient power or a “washing out” of group effects); thus this variable was collapsed so that the only treatment variable was diet. One-way analyses of variance (ANOVAs) were used to determine effects of diet on seizures. Repeated one-way ANOVAs were used to examine effects of diet on steroid levels in plasma to account for the fact that hormone levels are not entirely independent of one another. Where appropriate, ANOVAs were followed by Fisher’s least-squares difference post hoc tests. The α level for significance was considered $P = 0.05$; trends were considered when the α level for was $P = 0.10$.

3. Results

3.1. Seizures

As expected, the KD was associated with longer latencies to, and fewer numbers of, seizures. Notably, although there were modest apparent effects for less severe aspects of seizures, the KD produced statistically significant reductions only in the most severe parameters of pentylenetetrazole-induced seizures. For example, there was no effect of dietary condition on the latency to or number of myoclonic (latency: $F(2, 33) = 1.13$, $P = 0.33$); (No.: $F(2, 33) = 0.433$, $P = 0.65$) or forelimb (latency: $F(2, 33) = 1.50$, $P = 0.23$); (No.: $F(2, 33) = 1.13$, $P = 0.33$) seizures (Table 1). There were nonsignificant increases in the latency to ($F(2, 33) = 1.46$, $P = 0.24$), and reductions in the number of ($F(2, 33) = 1.00$, $P = 0.37$) tonic seizures by rats on the KD as compared with rats on other dietary regimens (Table 1). The latency tended to be longer ($F(2, 33) = 2.46$, $P = 0.10$), and there were nonsignificant reductions in the incidence of hindlimb extensions ($F(2, 33) = 1.73$, $P = 0.19$) for rats on the KD (Table 1). The latency to the most severe aspect of pentylenetetrazole-induced seizures, barrel rolls, tended to be longer ($F(2, 33) = 2.64$, $P = 0.08$) (Fig. 1, top). There were significantly fewer barrel rolls exhibited by rats on the KD compared with rats on other dietary regimens ($F(2, 33) = 5.306$, $P = 0.01$) (Fig. 1, bottom).

3.2. Pregnane neuroactive steroids

There was an overall significant effect for diet type to alter levels of circulating pregnane neuroactive steroids ($F(2, 33) = 7.83$, $P = 0.01$). Post hoc tests revealed that progesterone levels were not significantly altered by the KD ($P = 0.33$) (Fig. 2, top). However, the KD did significantly decrease plasma concentrations of dihydroprogesterone ($P = 0.001$) (Fig. 2, middle) and $3\alpha,5\alpha$ -THP ($P = 0.004$) (Fig. 2, bottom) compared with levels of rats on the ad libitum diet.

3.3. Androstane neuroactive steroids

There was also an overall effect for diet to alter androstane neurosteroids ($F(2, 33) = 1.69$, $P = 0.01$). Post hoc analyses revealed that plasma levels of dihydrotestosterone ($P = 0.004$) and 3α -androstenediol ($P = 0.01$), but not testosterone ($P = 0.23$) (Fig. 3, top), were altered by diet condition. Rats on the KD had lower circulating concentrations of testosterone (Fig. 3, top), dihydrotestosterone (Fig. 3, middle), and 3α -androstenediol (Fig. 3, bottom) than did rats on ad libitum feeding.

4. Discussion

The present results support our hypothesis that the KD decreases seizures and alters circulating levels of neurosteroids. Rats on the KD had significantly fewer of the most severe aspects of pentylenetetrazole-induced seizures than did rats that were fed ad libitum. There were apparent but nonsignificant reductions in levels of the precursors for the 5α -

reduced metabolites, progesterone and testosterone, that were produced by the KD compared with the ad libitum standard diet. The KD had robust and statistically significant effects in reducing plasma concentrations of pregnane and androstane 5 α -reduced metabolites. Circulating levels of both dihydroprogesterone and 3 α ,5 α -THP were significantly reduced by the KD compared with the ad libitum diet. Similarly, both dihydrotestosterone and 3 α -androstenediol in plasma were significantly reduced by the KD as compared with the ad libitum diet. Together the present results demonstrate that a KD regimen that increases thresholds to the most severe aspects of pentylenetetrazole-induced seizures can also decrease levels of 5 α -reduced neuroactive steroids in plasma.

While the present findings that circulating levels of neuroactive steroids were actually lowered by the KD may seem somewhat contradictory, they are congruous in several ways with previous results. Although animals are typically protected when there are acute endogenous changes in neuroactive steroids or when physiological levels of neuroactive steroids are administered, there is some evidence that the most effective anticonvulsant hormonal milieu occurs when levels of neuroactive steroids are stable [61]. Indeed, abrupt variations in levels of progestins or androgens can be proconvulsant [62,63]. In support, precipitous withdrawal from 3 α ,5 α -THP is associated with lowered seizure thresholds in women and in animal models [64,65]. Clinically, it is well known that even inhaled steroids can have profound effects in producing dyscontrol of seizures [66]. As such, the beneficial effects of the KD observed here on pentylenetetrazole-induced seizures may have been in part due to the stable levels of neuroactive steroids. Another consideration is that although the absolute levels of neuroactive steroids found in the KD group were lower than those in the ad libitum group, perhaps this reflects more turnover of the neuroactive steroids in the KD group. We have previously demonstrated that activity of the 5 α -reductase enzyme, which converts progesterone and testosterone to their neuroactive metabolites, is increased by seizures [67]. Given that the rats on the KD had more dietary cholesterol from which to form neuroactive steroids, it is possible that the lower neuroactive steroid levels observed in this group reflect a greater capacity for their metabolism. Although these findings indicate that the KD lowered circulating levels of neuroactive metabolites, it will also be essential in the future to examine central levels of neuroactive steroids that are produced by the KD and also to examine measures of activity at GABA_A receptors, a putative target for the effects of neuroactive steroids and KD.

These are intriguing data that suggest that KD may produce antiseizure effects by altering pregnane and androstane neurosteroids. However, several factors need to be addressed in future investigations of the mechanisms of KD on seizure susceptibility. First, levels of neuroactive steroids produced by the KD in the absence of pentylenetetrazole-induced seizures were not examined. This is an important question that will allow us to more definitively determine the extent to which the KD alters seizures through actions of neurosteroids. Second, the fact that decreased seizure incidence was associated with lower steroid levels suggests that acute fluctuations in neurosteroid levels may be more important than absolute levels. In the present study, steroid levels were measured at only one time point, which limits our interpretation of the data. Ideally, steroid levels would be measured prior to, during, and following seizures to reveal whether abrupt changes in or absolute levels of neuroactive steroids underlie antiseizure effects of the KD. However, collection of blood at each of these time points would require restraint, which is a stressor. It is well known that stress can alter neurosteroid levels and seizures [30]. Thus, to avoid this confound we collected blood only after testing and measured levels at one time point. Third, demonstrating altered endogenous steroid levels that occur concomitant with changes in seizure incidence suggests that neuroactive steroids may be implicated in the antiseizure effects of the KD. However, this does not rule out other factors that might be involved in antiseizure effects of the KD. Despite these limitations, the present study supports the notion

that alterations in neuroactive steroids may underlie some of the antiseizure effects of the KD.

Other possible mechanisms that may underlie the benefits of the KD and/or neuroactive steroids are alterations in energy metabolism. For example, increasing the ketogenic ratio, but not the levels of β -hydroxybutyrate, may be directly involved in the anticonvulsant mechanism of the KD [68]. Although elevated levels of β -hydroxybutyrate were observed in children on the KD (as compared with prediet), levels of β -hydroxybutyrate did not correlate with their seizures. Instead, ketosis induced by a very high fat “ketogenic” diet or injection or infusion of free (nonesterified) polyunsaturates, such as arachidonate and docosahexaenoate, both increase ketosis and reduce seizure susceptibility [69]. However, the level of ketosis is also not predictive of seizure protection by the KD [70,71]. Indeed, it has been proposed that beneficial effects of the KD may not be due to higher, but rather steady, ketosis [72,73]. Given that steroids are antiketogenic and will lower ketosis, only the latter notion could potentially account for any of the observed effects in our study.

Another possibility is that the KD and/or neuroactive steroids lead to alterations in the metabolism of brain amino acids. The KD may have effects in multiple ways to increase how much GABA is available. KD feeding may increase how much glutamate is available to glutamate decarboxylase, which increases brain GABA [74]. Ketosis can increase levels of acetate in the brain, which glia convert to glutamine, and then GABAergic neurons use as a precursor to GABA [74]. Enzymes that metabolize brain amino acids may also be altered by the KD. For example, mRNA for glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis, is increased in several brain regions by calorie restriction and the KD [75]. These findings from animal models are also supported by results in clinical studies. Two (of three) patients on the KD showed low initial GABA levels that increased over time [76]. It is important to note that neuroactive steroids also enhance levels of GABA [77]. Interestingly, neuroactive steroids are potent modulators of GABA_A receptors [33]. Thus, antiseizure effects of the KD may involve an interaction between alterations in neuroactive steroids and their putative substrates.

Although the majority of research on anticonvulsant mechanisms of neuroactive steroids has focused on actions through GABA and its receptors, neuroactive steroids may also have other actions that influence seizure processes. It has been proposed that steroids may regulate the synthesis and activity of transporters for GABA, dopamine, and/or serotonin, which may be substrates for regulation by protein kinase C and protein kinase A signaling [78]. Indeed, cDNA microarray analyses have demonstrated that the KD downregulates the expression of protein kinase C (PKC) in the hippocampus [79]. Our laboratory has also found that inhibiting protein kinase C and protein kinase A signaling attenuates many behavioral effects of neuroactive steroids [80]. However, we have not yet examined their role in seizure regulation by neuroactive steroids.

The KD can be very beneficial in the management of epilepsy, and the present data suggest that alterations in neuroactive steroids may underlie some of these effects. There are also other beneficial effects produced by the KD. For example, the KD may act as a mood stabilizer. Rats on the KD demonstrate less depressive behavior, and rats in which formation of neuroactive steroids was blocked show more depressive behavior [80]. The KD also decreases activity levels in an animal model of attention deficit/hyperactivity disorder [81]. Notably, both of these processes may involve actions of neurosteroids. However, not all of the effects of the KD are favorable. Rats that were treated with the KD had significantly impaired brain growth and visual-spatial memory. Although the KD has impressive effects in mitigating seizure thresholds, to fully harness the therapeutic potential of this diet for seizure disorders and other conditions in the future, it is essential to further elucidate the

processes that underlie both the beneficial effects of the therapy and the negative side effects.

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Fig. 1.

Mean ($\pm$ SEM) latencies to (top) and incidence of (bottom) pentylenetetrazole-induced seizures of rats on ad libitum (left, white), calorie-restricted (middle, black), and ketogenic (right, striped) diets. [#]Tendency ($P = 0.08$) for latencies to be longer for rats on the KD. ^{*}Number of seizures was significantly lower ($P = 0.01$) for rats on the KD.

Fig. 2.

Mean ($\pm$ SEM) plasma progesterone (top), dihydroprogesterone (middle), and 3 α ,5 α -THP (bottom) levels for rats on an ad libitum (left, white), calorie-restricted (middle, black), and ketogenic (right, striped) diets that were administered pentylenetetrazole. #Tendency for 3 α , 5 α -THP levels to be lower in rats on the KD compared with calorie-restricted diet. *Dihydroprogesterone and 3 α ,5 α -THP levels were significantly lower in rats on the KD than in rats on an ad libitum diet.

Fig. 3.

Mean ($\pm$ SEM) plasma testosterone (top), dihydrotestosterone (middle), and 3 α -androstanediol (bottom) levels for rats maintained for 21 days on ad libitum (left, white), calorie-restricted (middle, black), and ketogenic (right, striped) diets that were administered pentylentetrazole. [#]Tendency for dihydrotestosterone or 3 α -androstanediol to be lower in rats on the KD compared with the calorie-restricted diet. ^{*}Dihydrotestosterone and 3 α -androstanediol levels were significantly lower in rats on the KD than in rats on an ad libitum diet.

Table 1

Effects of diet on the mean latency to and incidence of myoclonus, forelimb clonus, tonic, and hindlimb extension seizures induced by administration of pentylenetetrazole (70 mg/kg, IP)

Seizure type	Measure	Dietary regimen		
		Ad libitum	Calorie-restricted	Ketogenic diet
Myoclonus	Latency (s)	93 ± 46 ^a	41 ± 2	46 ± 3
	Incidence (No.)	2.8 ± 0.3	2.9 ± 0.2	2.5 ± 0.1
Forelimb clonus	Latency (s)	116 ± 46	56 ± 6	60 ± 6
	Incidence (No.)	3.8 ± 1.1	4.0 ± 0.7	6.4 ± 1.8
Tonic	Latency (s)	165 ± 59	207 ± 68	327 ± 80
	Incidence (No.)	0.8 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
Hindlimb extensions	Latency (s)	177 ± 57	218 ± 66	378 ± 76
	Incidence (No.)	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1

^aValues are means ± SEM.