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The Influence of Cryogenic Brain Injury on the Pharmacodynamics of Pentobarbital

Evidence for a Serotonergic Mechanism

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To study of the influence of brain injury on the pharmacodynamics of pentobarbital, the authors examined the effect of a focal cortical freezing lesion in rats on the brain concentration of pentobarbital associated with lack of response to tail clamp. The freezing lesion was made with a probe (−50 °C) applied through a craniotomy to the intact dura over the left parietal cortex. Three days after injury the rats were anesthetized with a continuous intravenous infusion of pentobarbital until they first did not respond to tail clamp stimulation. The brains were then removed for determination of pentobarbital by high-performance liquid chromatography. The brain pentobarbital concentration required to prevent response to tail clamp (EC_{50}) was reduced from 209 ± 39 nmol/g (mean ± standard deviation) in rats without brain injury to 149 ± 28 nmol/g in the injured animals ($P = 0.005$). The cortical serotonin (5-HT) concentration was increased from 1904 ± 358 pmol/g in uninjured rats to 2513 ± 598 pmol/g ($P < 0.01$) in injured animals ipsilateral to the lesion. Pretreatment of the rats with p-chlorophenylalanine (PCPA, 200 mg/kg by intraperitoneal injection) to inhibit 5-HT synthesis abolished both the increase in 5-HT concentration associated with the injury (left cortex, 708 ± 389 pmol/g; right cortex, 911 ± 979 pmol/g) and the effect of the lesion on EC_{50} (uninjured, EC_{50} = 186 ± 24 nmol/g; injured, EC_{50} = 179 ± 47 nmol/g). Prevention of the decrease in EC_{50} by inhibition of 5-HT synthesis provides support for a functional role for 5-HT in the influence of cold injury on the pharmacodynamics of pentobarbital. (Key words: Pharmacodynamics: depth of anesthesia. Anesthetics, intravenous: pentobarbital. Hypnotics, barbiturates: pentobarbital. Brain: injury. Brain, neurotransmitters: serotonin. Measurement techniques: high-pressure liquid chromatography.)

BRAIN IMAGING of head-injured patients has revealed regions of the brain in which no structural abnormality was seen by magnetic resonance or computed tomography imaging but in which glucose metabolism was depressed.[1] Since there is a close relationship in the brain between functional activity and glucose metabolism,[2] these results suggest that regional alteration of neurologic function may occur in humans after trauma without evidence of associated tissue damage. In this study we selected an animal model of focal brain injury to determine whether a focal cortical freezing lesion would alter the anesthetic requirements for pentobarbital.

Pappius and colleagues[3–6] used a model of cryogenic brain injury in the rat to investigate the effects of focal cerebral injury on brain function and monoamine content. Glucose utilization (cerebral metabolic rate of glucose utilization [CMR_{glu}]) was used to characterize the functional state of the brain. These studies showed that the depression of CMR_{glu}, widespread throughout the cortex ipsilateral to the injury, was associated with changes in indolamine content in the brain.[5,7] Treatment before brain injury with p-chlorophenylalanine (PCPA) to inhibit serotonin (5-hydroxytryptamine [5-HT]) synthesis[8,9] prevented the metabolic depression after the freezing lesion. The depression of CMR_{glu} was not evenly distributed; CMR_{glu} in cortical regions decreased more than in subcortical or brainstem structures. The depression of CMR_{glu} also varied with time; the nadir occurred 3 days after the lesion.[1,3] There was evidence that these effects were not caused by cerebral edema[1] or by changes in cerebral blood flow.[1]

In this study we proposed that the cortical freezing lesion would alter the pharmacodynamics of pentobarbital. Specifically, we speculated that during a continuous intravenous infusion of pentobarbital the brain pentobarbital concentration associated with lack of response to noxious stimulus (tail clamp) would be less in injured than in normal animals. If a reduction of anesthetic requirements was confirmed to occur in injured animals, then, we hypothesized, this effect would be abolished by inhibition of 5-HT synthesis with PCPA, supporting a role for 5-HT in the functional effects of the injury.

Materials and Methods

These studies were approved by the University of Calgary Animal Care Committee. Male Sprague-Dawley rats, 250–400 g, were used in this investigation.

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Determination of Anesthetic Requirements

Untreated Animals

On the initial day of the protocol, brain injury was produced in eight animals with a standardized cold lesion as previously described.[3] Briefly, while the animals were under general anesthesia with halothane (2–3 vol%) in oxygen, a 4-mm square craniectomy was made with a dental drill, 2 mm to the left of the midline and immediately behind the coronal suture. A circular brass probe, 3 mm in diameter, cooled to −50 °C with a slurry of dry ice and acetone, was applied to the intact dura mater over the left parietal cortex for 5 s. The wound was closed with silk sutures and the animal returned to the cage. Animals not operated upon served as controls.

On the 3rd day of the protocol, while the animals were under general anesthesia (halothane in oxygen as above), catheters were inserted into the femoral artery and vein, and a partial restraint consisting of a plaster cast from the ankles to the mid-chest was applied.[3] The animals were allowed 4 h in a warm environment to recover from the anesthetic, and satisfactory physiologic status was confirmed with measurements of blood pressure, hematocrit, rectal temperature, and arterial blood gas tensions. Measurements of mean arterial pressure and arterial blood gas tensions were made before (initial) and at the completion of (final) the pentobarbital infusion.

Normal and injured animals then received a continuous intravenous infusion of sodium pentobarbital (4 nmol · kg^{-1} · min^{-1} = 1 mg · kg^{-1} · min^{-1}). Anesthetic depth was initially assessed with the eyelash and corneal reflexes repeated three or four times. Once these reflexes were abolished, response to noxious stimulus was tested with a dedicated hemostatic clamp applied to the distal 2 cm of the tail and closed to the first ratchet.[10] This stimulus was repeated, more proximally with each trial, for three to six trials per animal. When there was no movement or vocalization response to this stimulus, the animal was decapitated and the brain rapidly removed and frozen to −20° C. Blood samples (40 μl, at 10-min intervals) were obtained for plasma pentobarbital determination. With this protocol, the time to decapitation varied from animal to animal.

PCPA-treated Animals

Eighteen animals received one intraperitoneal injection of PCPA (sodium salt, Sigma, St. Louis, MO) in a dose of 200 mg/kg. Twenty-four hours later, eight animals received a brain injury as described above; ten animals not operated upon served as PCPA controls. Anesthetic requirements in all animals were determined 4 days after PCPA injection in the same manner as described above for the untreated animals, except that final values for physiologic measurements were not obtained in this group.

Determination of Brain Indolamine Concentrations

Twenty-seven animals were studied. Eight animals received an intraperitoneal injection of PCPA, 200 mg/kg 24 h before brain injury; 10 animals received brain injury alone; and 9 untreated animals without brain injury served as controls. All injured animals were studied on the 3rd day after the injury. All animals were given an intraperitoneal injection of pentobarbital (120 nmol/kg = 30 mg/kg). When the animal did not respond to tail clamp, it was decapitated and the brain rapidly removed. Samples of cortex from the frontoparietal region were dissected at room temperature and then placed in weighed vials cooled to between −30 and −50° C in a slurry of dry ice and acetone and stored at −20° C until they were analyzed for 5-HT and 5-hydroxyindoleacetic acid concentration.

Analytic Methods for Pentobarbital and Indolamines

Measurement of Pentobarbital

Pentobarbital was measured in serum and brain samples using *n*-butyl chloride extraction and high-performance liquid chromatography (HPLC).[11] For brain samples, a portion of the right frontal lobe (approximately 0.25 g) was combined with 1.5 ml of water and homogenized with a ground-glass bit in homogenizer tubes. The serum samples and brain homogenates, with secobarbital (40 nmol/ml) added as the internal standard, were extracted into 4 ml of n-butyl chloride to which 0.5 ml of 0.1 M phosphate buffer (*p*H 7.0) had been added. The organic phase was separated from the aqueous phase by centrifugation at 5,000 *g* for 25 min. The organic phase was evaporated to dryness with nitrogen at 40° C and reconstituted with 100 μl acetonitrile 50% in deionized water, 20 μl of which was injected onto the HPLC column.

Chromatographic analysis was performed with a Perkin-Elmer Series 4 HPLC device equipped with a spectrophotometric detector (model LC 85) (both from Perkin-Elmer, Norwalk, CT) set at 215 nm and a loop-type injector (20-μl volume, Rheodyne, Cotati, CA). The column used was a Whatman RAC II, composed of octadecyl sulfate, 5-μm particle size, and 4.6 mm in diameter and 10 cm in length (C_{18}, Whatman Laboratory Products, Clifton, NJ). The mobile phase was acetonitrile 50% in deionized water infused at a rate of 1 ml/min. Chromatograph results were printed with an electronic integrator (Varian Vista 402 Data Console, Varian, Sunnyvale, CA), at attenuation 4 for serum samples and at attenuation 16 for tissue samples.

For the preparation of the standard curves, pentobarbital and secobarbital were obtained as the sodium salts in pure crystalline form (Sigma, St. Louis, MO). The standard curve was constructed as follows. Linear regression analysis was performed, comparing the pentobarbital standard concentration to the pentobarbital/secobarbital area ratio determined in plasma to which pentobarbital had been added in concentrations of 40, 60, and 100 nmol/ml. From the chromatograph from each unknown sample, the pentobarbital/secobarbital area ratio was calculated. The concentrations of pentobarbital in the brain and serum samples were then calculated using the line equation. In our laboratory this method has been determined to have a sensitivity of 4 nmol/ml and a coefficient of variation of 4% for pentobarbital values in the range in this study.

Measurement of Indolamines

The frozen brain samples were weighed and then homogenized as above in 0.5 ml of ice-cold 0.2 M perchloric acid. Two 200-μl aliquots were taken from the homogenate, and a standard containing a known amount of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) (both prepared from crystalline standards, Sigma, St. Louis, MO) was added to one of the aliquots. The samples were centrifuged cold (4° C) at 5,000 *g* for 10 min. The supernatants were removed and stored frozen until they were analyzed.

For analysis we used a HPLC device (Series 10, Perkin-Elmer) equipped with an amperometric detector (model LC-17, glassy carbon TL-5A, Bioanalytical Systems, West Lafayette, IN). The column used was a Nova-pak C_{18} (3.9 mm in diameter and 15 cm in length) stainless steel column (Waters, Milford, MA). The mobile phase consisted of 85/15 (volume/volume) 0.07 M KH_2PO_4 containing 0.6 mM hexane sulfonic acid adjusted to *p*H 4.1 with 0.1 M phosphoric acid buffer/methanol, at a flow rate of 1.2 ml/min. The detector potential was set at 0.85 V against a silver–silver chloride reference electrode. The concentrations of 5-HT and 5-HIAA were calculated from:

$$C = \frac{A_1 \times X}{(A_2 - A_1)}$$

where C is the concentration of pentobarbital in the sample, A_1 and A_2 are the areas of the samples without and with added internal standard, respectively, and X is the amount of internal standard added. In our laboratory this method has a sensitivity of 450 pmol/g and a coefficient of variation of 5% for both 5-HT and 5-HIAA.

All values are expressed as means ± standard deviations. For the four groups (untreated, normal and injured; PCPA-treated, normal and injured) the values obtained for the physiologic variables were analyzed by one-way analysis of variance. Individual differences were isolated by unpaired *t* test. The brain pentobarbital concentrations for the four groups were analyzed by two-way analysis of variance to identify differences related to 1) the freezing lesion, 2) PCPA treatment, and 3) the interaction between the effect of the freezing lesion and the effect of the PCPA treatment.

For the serum pentobarbital concentration *t* tests were used to compare the injured and uninjured animals at individual sample times. A Bonferroni correction, with a denominator of 4, was used to adjust for the four comparisons done.

Brain concentrations of 5-HT and 5-HIAA in the untreated injured and PCPA-treated injured animals were compared to their respective values in the untreated normal group using one-way analysis of variance followed by Bonferroni *t* tests to isolate individual differences. For all tests statistical significance was inferred when $P < 0.05$.

Results

Physiologic State of the Animals

The average values of the physiologic measurements made during each experiment are presented in table 1. No significant differences were found for weight, hematocrit and arterial oxygen tension. In the untreated group, normal animals had an average mean arterial pressure less than that of the injured rats both initially and at completion of infusion. In the comparison of the physiologic variables before and after the infusion, mean arterial pressure decreased in both the normal and injured animals. The normal animals demonstrated a significant decrease in *p*H during the infusion. In the PCPA-treated group, the only difference identified was that the mean temperature of the normal animals was less than that of the injured animals ($P < 0.05$).

Pharmacokinetics of Pentobarbital

Figure 1 shows the serum pentobarbital concentrations during the first 40 min of the pentobarbital infusion. Because some animals were decapitated before 40 min, the numbers of subjects contributing to the means changes with time. The only time in either group at which the injured animals show a difference in serum pentobarbital concentration from the normal animals is at 20 min after the start of the infusion in the PCPA-treated animals ($P = 0.016$).

Pharmacodynamics of Pentobarbital

Figure 2 shows the the brain pentobarbital concentrations in normal and injured animals in the untreated and PCPA-treated groups. Analysis of variance demonstrated

TABLE 1. Physiologic State of the Animals

	Untreated		PCPA-treated	
	Normal	Injured	Normal	Injured
Numbers of animals	13	8	10	9
Weight (g)	341 ± 65	305 ± 24	269 ± 32	292 ± 25
Temperature (°C)	36.8 ± 0.9	36.2 ± 0.3	35.2 ± 1.4*	36.9 ± 0.4
Hematocrit (%)	50 ± 6	47 ± 4	49 ± 4	48 ± 3
MAP (mmHg)				
Initial	100 ± 5	137 ± 8*	125 ± 15	123 ± 10
Final	78 ± 3†	113 ± 10*†		
*p*H (arterial)				
Initial	7.39 ± 0.03	7.33 ± 0.03	7.41 ± 0.03	7.43 ± 0.16
Final	7.32 ± 0.03†	7.30 ± 0.02		
Pa_{CO_2} (mmHg)				
Initial	35 ± 2	36 ± 4	35 ± 2	33 ± 2
Final	40 ± 6	36 ± 14		
Pa_{O_2} (mmHg)				
Initial	80 ± 3	83 ± 12	84 ± 5	78 ± 8
Final	78 ± 12	78 ± 11		

Values are means ± SD.
PCPA = p-chlorophenylalanine; MAP = mean arterial pressure; Pa_{CO_2} = arterial carbon dioxide tension; Pa_{O_2} = arterial oxygen tension.

* $P < 0.05$ untreated injured *versus* normal.
† $P < 0.05$ untreated animals before and after pentobarbital infusion, normal and injured compared respectively.

a significant effect of the freezing lesion ($F = 8.808$, $P = 0.005$), a significant difference in the effect of the lesion between the untreated and PCPA-treated animals ($F = 4.562$, $P = 0.04$), and no significant effect of PCPA treatment ($F = 0.013$, $P = 0.910$). In the untreated animals, brain injury was associated with a 29% reduction in the brain pentobarbital concentration required to prevent response to tail clamp (EC_{50}, 149 ± 28 nmol/g compared to 209 ± 39 nmol/g in the normal animals; $P < 0.05$). No such difference was observed in the PCPA-treated animals (normal, EC_{50} = 186 ± 24 nmol/g; injured, EC_{50} = 179 ± 47 nmol/g).

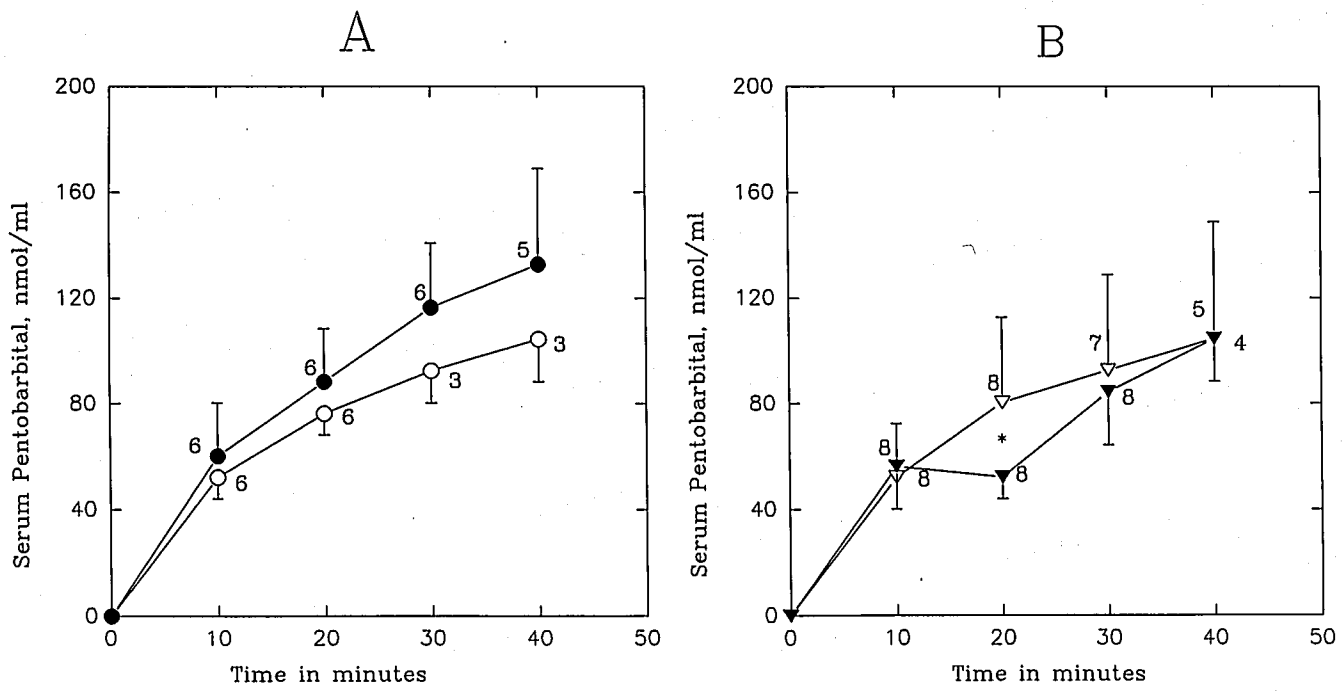

FIG. 1. Serum pentobarbital concentrations during continuous pentobarbital infusion in untreated animals (*A*) and animals pretreated with *p*-chlorophenylalanine (PCPA) (*B*). Open circles represent animals with lesions, and filled circles represent uninjured animals. The numbers of animals at each time is shown adjacent to the mean value. *$P < 0.05$ *versus* uninjured value at 20 min.

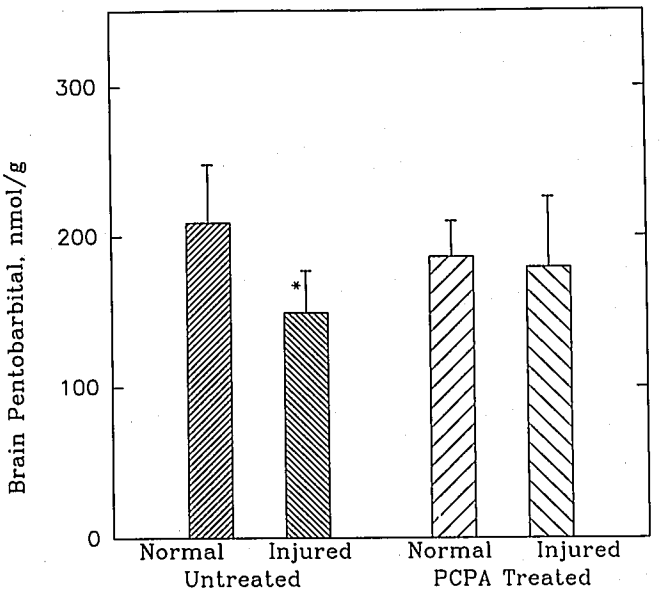

FIG. 2. Concentrations of pentobarbital (mean values ± SD) in brain samples taken at the first failure to respond to tail clamp. *P = 0.005 *versus* normal untreated animals.

CORTICAL INDOLEAMINE CONCENTRATIONS

Figure 3 shows the mean values for 5-HT and 5-HIAA in frontoparietal cortex. On the left side, ipsilateral to the injury, the cortices from ten injured, untreated animals had higher concentrations of both 5-HT and 5-HIAA than did nine normal animals ([5-HT] = 1904 ± 358 pmol/g and [5-HIAA] = 1522 ± 255 pmol/g; $P < 0.05$). On the right side, contralateral to the injury, only the 5-HIAA concentrations were greater than in the normal animals ([5-HIAA] of injured animals = 3125 ± 1744 pmol/g, $P < 0.05$).

PCPA treatment reduced the cortical 5-HT and 5-HIAA concentrations of nine PCPA-treated injured animals to the limits of sensitivity of the technique: in chromatographs for many animals, no peaks for either moiety could be found.

Discussion

This study shows that the freezing lesion injury altered the pharmacodynamics of pentobarbital. The EC_{50} of pentobarbital in brain at the first lack of response to tail clamp was reduced 29% in the injured animals. This reduction of EC_{50} associated with the injury was not observed in animals pretreated with PCPA to block synthesis of 5-HT. These results show a pattern similar to a previous study[5] of CMR_{glu} in this experimental model: although PCPA pretreatment had little effect on CMR_{glu}[5] or anesthetic requirements in normal animals, PCPA pretreatment prevented the depression of CMR_{glu}[5] and the decrease in anesthetic requirements associated with the freezing lesion. Despite the suggestion by these results that 5-HT may play a role in the reduction of the EC_{50} after the freezing lesion, other factors are probably involved, since near-complete depletion of cortical 5-HT and 5-HIAA did not alter the EC_{50}.

Previous studies of the effect of manipulation of the serotonergic pathways in the central nervous system on anesthetic requirements have given divergent results: halothane MAC was unchanged in dogs treated with PCPA (350 mg/kg),[12] whereas MAC was decreased after stereotactic ablation of the dorsal raphe nucleus in rats.[13] The effects of PCPA treatment on measures of anesthetic and analgesic potency are complex, because the effect of PCPA on 5-HT metabolism is not uniform throughout the central nervous system[14]; thus, some responses to noxious stimulation may be profoundly altered by PCPA administration whereas others are not.[14]

Criticisms of the study design include 1) that the observers were not blinded and 2) that the PCPA-treated and the untreated animals were studied not concurrently but sequentially.

The results of this study do not establish a relationship between the alteration in EC_{50} after cryogenic injury, and the depression of cortical function or the change in cortical indolamine content previously demonstrated in this model. Serotonergic input to the cortex[15] is derived

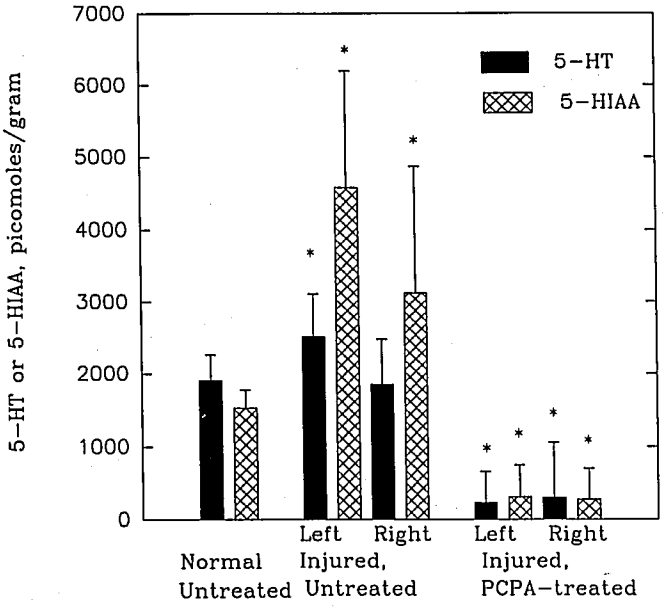

FIG. 3. Concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) (mean values ± SD) in cortical samples from normal (n = 9); untreated, injured (n = 10); and *p*-chlorophenylalanine (PCPA)-treated, injured (n = 8) rats. Brain injury is associated with an increase in 5-HT and 5-HIAA on the side of the lesion, whereas on the side contralateral to the lesion, only 5-HIAA is increased. PCPA pretreatment leads to profound bilateral reduction of both 5-HT and 5-HIAA. *$P < 0.05$ *versus* normal animals.

mainly from cell bodies in the raphe nuclei,[16] nuclei that also project to the spinal cord.[17] One hypothesis concerning the mechanism of the reduction of CMR_{glu} after the freezing lesion is that the increase in 5-HT and 5-HIAA observed in the cortex after injury represents an increased release of 5-HT.[5] If the bulbospinal serotonergic pathways[17] are activated in a similar fashion, then the resultant inhibition of the response of dorsal horn neurons to noxious stimulation[18] may explain the reduction in EC_{50} observed in this study.

In studies of anesthetic requirements using serum measurements it is conventional to calculate the appropriate concentration by interpolating the values at the last response and at the first lack of response. We were able to determine the brain concentration only once, at the first lack of response. By use of a continuous infusion, the end-point was approached gradually (fig. 1). With this design, intubation and controlled ventilation were impractical. Judging from a previous study,[6] we believed that the changes in physiologic variables caused by the anesthetic depth chosen would not be marked, and this was confirmed (table 1).

First lack of response to tail clamp was chosen to define anesthetic depth because from our previous study[6] we knew that with pentobarbital anesthesia, CMR_{glu} was the same in normal and injured animals at this end-point. Thus, at this depth of anesthesia, the normal and injured animals were expected to be similar in both the behavioral and metabolic measurements of the functional state of the brain. Comparison of the EC_{50} in normal animals in the present study (209 ± 39 nmol/g) with the EC_{50} for abolishing the righting reflex (58 ± 16 nmol/g)[19] confirms that animals that do not respond to tail clamp are profoundly anesthetized, consistent with the reduction observed previously of cortical CMR_{glu} to 35% of normal.[6]

In this study the cortical concentrations of 5-HT and 5-HIAA were greater those previously reported in this experimental model.[5] This difference may be due in part to decreased decapitation-induced changes in 5-HT related to the pentobarbital anesthesia, similar to the effect seen with high-energy compounds of the brain.[20] Preliminary studies of cortical 5-HT and 5-HIAA in unanesthetized rats in our laboratory showed lesser concentrations of 5-HT and greater concentrations of 5-HIAA than those reported in this study (unpublished observations). Additional factors that may have affected the 5-HT and 5-HIAA determinations are the brain-sampling techniques and the rate of freezing the brain samples.

In summary, this study shows that unilateral experimental cold injury, previously shown to be associated with decreased cortical CMR_{glu}[3-6] and increased cortical indolamine content,[6] also alters the pharmacodynamics of pentobarbital, reducing the EC_{50} by 29%. The observation that reduction of EC_{50} after the freezing lesion did not occur in PCPA-pretreated animals suggests that 5-HT may play a role in the reduction in anesthetic requirements after the freezing lesion.

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