Pentobarbital induces nocifensive hyperreflexia, not hyperalgesia in rats

David P. Archer MD,*
Naaznin Samanani BSc,*
Sheldon H. Roth PhD*†

Purpose: To seek behavioural, reflexive and histochemical evidence of long-lasting changes in nociceptive stimulus transmission induced by exposure to doses of pentobarbital that induce nocifensive hyperreflexia.

Methods: Nocifensive hyperreflexia was induced in 12 rats with 30 $mg \cdot kg^{-1}$ pentobarbital *ip*. Reflex latency times for withdrawal of the hind paw from noxious radiant heat were measured with an automated electronic timer. Subjective responses to noxious stimulation (licking or biting of the stimulated hindpaw) and the level of sedation were recorded. Histological sections of lumbar spinal cord were stained for immunoreactivity of the immediate-early-gene (IEG), *c-fos,* in three rats that received repeated threshold noxious radiant heat stimulation during the period of nocifensive hyperreflexia induced by 30 $mg \cdot kg^{-1}$ pentobarbital *ip*.

Results: Reflex withdrawal latency decreased by 32 ± 8% of control values ($P < 0.001$) following pentobarbital injection and returned to control values 120 min after drug injection. Once fully alert, pentobarbital-treated animals did not show any increase in nociceptive behaviour relative to saline-injected controls ($P = 0.41$). Sustained noxious stimulation to the hindpaw in halothane-anesthetized animals was associated with an increase in *c-fos* immunoreactivity in the dorsal horn of the lumbar spinal cord ipsilateral to the stimulation ($P < 0.001$). Threshold stimulation in the pentobarbital-treated animals was not associated with any increase in *c-fos* expression.

Conclusions: During pentobarbital-induced hyperreflexia, rats did not show any reflexive, behavioural, or histochemical evidence of long-lasting enhancement of nocifensive signal transmission. The results are consistent with previous observations that, in the absence of tissue injury, nocifensive hyperreflexia induced by barbiturates is a short-lived pharmacological effect.

Objectif : Découvrir les manifestations comportementales, réflexes et histochimiques de modifications persistantes de la transmission d'un stimulus nociceptif induit par l'exposition à des doses de pentobarbital qui provoquent une surréflectivité défensive.

Méthode : La surréflectivité défensive a été induite chez 12 rats avec 30 $mg \cdot kg^{-1}$ de pentobarbital *ip*. Les temps de latence réflexe nécessaire au retrait de la patte arrière d'une source de chaleur radiante ont été mesurés avec un chronomètre électronique automatisé. Les réponses subjectives à la stimulation désagréable (lécher ou mordre la patte stimulée) et le niveau de sédation ont été enregistrés. Des sections histologiques de la moelle épinière lombaire ont été colorées pour vérifier l'immunoréactivité du gène précoce immédiat (GPI), *c-fos,* chez trois rats qui ont reçu une stimulation liminale nocive répétée de chaleur radiante pendant la période de surréflectivité défensive induite par les 30 $mg \cdot kg^{-1}$ de pentobarbital *ip*.

Résultats : Le temps de latence réflexe a baissé de 32 ± 8 % par rapport aux valeurs témoins ($P < 0{,}001$) après l'injection de pentobarbital et est revenu aux valeurs témoins 120 min après l'injection du médicament. Une fois complètement réveillés, les animaux traités au pentobarbital n'ont pas affiché de comportement nociceptif accru comparés aux animaux témoins à qui on a injecté une solution salée ($P = 0{,}41$). La stimulation nocive à la patte arrière, subie par les animaux anesthésiés à l'halothane, a été associée avec un accroissement de l'immunoréactivité au gène *c-fos* dans la corne supérieure de la moelle épinière lombaire homolatérale à la stimulation ($P < 0{,}001$). La stimulation liminale chez les animaux traités au pentobarbital n'était pas accompagnée d'une augmentation de l'expression de *c-fos* .

Conclusion : Pendant la surréflectivité liée au pentobarbital, les rats n'ont pas donné de signe réflexe, comportemental ou histochimique d'une augmentation persistante de la transmission du signal défensif. Ces résultats confirment des observations antérieures qui montraient qu'en l'absence de lésion aux tissus la surréflectivité défensive induite par les barbituriques présente un effet pharmacologique de courte durée.

From the Departments of Anesthesiology,* and Pharmacology and Therapeutics,† Faculty of Medicine, The University of Calgary, Calgary, Canada.

Address correspondence to: Dr. David P. Archer, Department of Anesthesiology, Foothills Medical Center, 1403 29th St. N.W., Calgary, Alberta, Canada T2N 2T9. Phone: 403-670-1991; Fax: 403-670-2425; E-mail: archerd@cadvision.com

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IN subhypnotic doses, many anesthetics facilitate flexor withdrawal (nocifensive) reflexes.[1–4] In addition, pentobarbital has been shown to produce long-lasting changes in synaptic transmission in the hippocampus.[5,6] The latter effect shares many characteristics with conventional hippocampal synaptic plasticity. It is not clear whether anesthetic-induced hyperreflexia is accompanied by long-lasting changes in transmission in pain pathways, similar to what has been observed during stimulation of hippocampal pathways.[6] If this were the case, then anesthetics could potentially facilitate an enhanced pain response to innocuous stimulation (hyperalgesia). To test this hypothesis we examined rats treated with pentobarbital for evidence of increased nociceptive behaviour and persistence of enhanced nocifensive reflexes during emergence from anesthesia. Finally, since nocifensive reflex enhancement by pentobarbital is nitric oxide- (NO) dependent,[7] we examined the lumbar spinal cords of stimulated animals for evidence of induction of the immediate-early-gene (IEG), *c-fos* in the spinal cord, which is proposed to be a marker for N-methyl-D-aspartate (NMDA) receptor-mediated, NO-dependent spinal cord plasticity.[8–10] Here we show that despite repeated stimulation during hyperreflexia induced by pentobarbital 30 $mg \cdot kg^{-1}$, rats showed no behavioural, reflexive, or histochemical signs of long-lasting changes in nociceptive transmission.

Methods and materials

The study protocols were approved by the Faculty of Medicine Animal Care Committee and complied with guidelines of the Canadian Council for Animal Care and the International Association for the Study of Pain. Thirty-six male Sprague-Dawley rats (250-350 g) were housed in the medical vivarium (lights on: 0700-1800) for at least one week before study. Studies were performed between 10:00 and 15:00 hr.

Protocol 1 - Nociceptive behaviour evaluation

In this protocol, 24 rats were studied, 12 treated with 30 $mg \cdot kg^{-1}$ pentobarbital *i*) and 12 saline-injected control animals. Hind paw withdrawal (HPW) latency and response to vibrissal (whisker) stimulation were determined as previously described,[5,11,12] before and every ten minutes after the pentobarbital injection for a total of two hours. During the measurement of the HPW latency, licking or biting of the stimulated hind paw was noted. At each measurement time the degree of sedation was evaluated by the response to whisker stimulation: 0-awake, no signs of sedation; 1-responds quickly to light whisker stimulation; 2-responds only to vigorous whisker stimulation; 3-no response to whisker stimulation. Since sedation may interfere with nociceptive behaviour, for analysis of the latter variable, we compared pentobarbital-treated animals to saline controls for the period 80-120 min after injection.

Protocol 2 - Induction of c-fos immunoreactivity in spinal cord neurons

In this protocol we examined the influence of noxious stimulation to the hind paw on expression of *c-fos* immunoreactivity in the lumbar spinal cord in 12 rats. To minimize *c-fos* protein induction by non-specific stimulation, experiments were performed in a darkened room between 11:00 and 15:00 hr. The experimental chamber[1,2] was fitted with a cover to reduce auditory stimulation and air was provided to the chamber at a rate of 3 $L \cdot min^{-1}$.

Four experimental conditions were used. The first group (n=3) served as stimulated controls. These rats were injected with intraperitoneal saline (1 ml).

One hind paw received radiant heat stimulation[1,2] once every five minutes for two hours. The duration of the stimulus was determined by movement of the hind paw, terminating the stimulus after the normal HPW latency of 9-12 sec. These animals were assumed to be representative of *c-fos* activity in awake, stimulated animals.

The second group (n=3) consisted of the animals with pentobarbital-induced facilitation of nocifensive reflexes. These animals were injected with 30 $mg \cdot kg^{-1}$ pentobarbital and then subjected to transient radiant heat stimulation as described above.

The third group (n=3) was used to prove that, using our immunohistochemical techniques, we could detect the presence of *c-fos* immunoreactivity in an injury model previously reported by others.[8] Since the choice of anesthetic was not important for this part of the experiment, we used halothane[8] for the ease of maintaining an adequate depth of anesthesia for the duration of the experiment. Animals were anesthetized with halothane in oxygen (1.5-2%) provided by face mask. We set the depth of anesthesia by adjusting the vaporizer output so that each animal was unconscious and immobile but retained withdrawal reflexes. In this group, the hindpaw was submerged in 54C water for 20 sec every five minutes for two hours.[8] To ensure that halothane was not inducing *c-fos* immunoreactivity, the fourth group of animals (n=3) was anesthetized with halothane but not subjected to any thermal injury. These animals were lightly anesthetized with halothane for two hours. Animals were placed in the prone position on a soft towel. Inspired halothane concentrations were adjusted to preserve the response to an air puff directed at the cornea.

After the two-hour treatment period, the rats were injected with 50 mg·100g^{-1} sodium pentobarbital, *ip*, and perfused through the heart with 100 ml physiologic saline, followed by 0.5 L formaldehyde 4%. The spinal cord was dissected free and immersed in formaldehyde 4% for one hour and then 30% sucrose solution overnight. The spinal cord was cut into 40 µ thick sections using a cryostat (-70°C). Sections were collected into phosphate buffered saline (PBS)(pH 7.4) and then stained for *c-fos* immunoreactivity as described below.

Immunohistochemistry

Spinal cord sections were incubated in normal goat serum for one hour, primary antibody for 48 hr at 4°C (anti- *fos* antiserum, raised in rabbit, titer 1:1000, Oncogene Science, Inc. Scarborough, Canada), and secondary antibody for 30 min (biotinylated anti-rabbit IgG, Vector Laboratories, Mississauga, Canada). Tissue sections were washed in PBS for 10 min before and after each incubation. Sections were stained using the horseradish peroxidase-diaminobenzidine (DAB) method. The sections were incubated with Avidin DH - biotinylated horseradish peroxidase complex (Vectastain ABC reagent, Dimension Laboratories, Mississauga, Canada) for 30 min, washed in PBS and then incubated in peroxidase substrate solution (DAB Kit, Dimension Laboratories) until reaction completion. The stained sections were again washed in PBS (three times) mounted onto slides, air-dried for 24 hr and then dehydrated for two minutes each in ethanol and xylene 50%, 70%, 95%, 100%.

Tissue sections were examined at 100 times magnification with lightfield microscopy. Mean cell counts of immunoreactive nuclei were determined in lumbar spinal cord sections L3-5 (4 sections per animal) and expressed as total cell count per animal on each side of the spinal cord.

Sample size and data analysis

Sample size for the nocifensive reflex and behavioural studies was estimated from previous studies in which pentobarbital reduced the withdrawal threshold by approximately 30 ± 10% of control values.[13] Power analysis predicted that such differences could reliably be detected with groups of 3-5 animals. We were concerned that behavioural measures would show a greater variability and therefore increased the group size to 12 in anticipation of this potential problem.

All values are reported as mean values ± standard deviation. The HPW latencies were expressed as the percentage of the pre-injection HPW latency (% of control). Mean values of these normalized HPW values were then calculated for each measurement time for each experimental group. Mean HPW latency values for the two groups (pentobarbital-treated and saline controls) over the two-hour experimental period were then compared by one-way analysis of variance (ANOVA). The frequency of licking behaviour pooled for the period 80-120 min after injection was compared between the two groups with the Mann-Whitney rank sum test.

Mean cell counts per spinal cord per animal were compared between groups and between sides by two way ANOVA, using treatment as one factor and stimulation (ipsilateral *vs* contralateral) as the second factor. For all analyses, $P < 0.05$ was selected to imply statistical significance.

Results

Nocifensive reflex latency

The HPW latency was reduced in pentobarbital-injected rats (Figure 1) to a minimum of 68 ± 8% (mean value ± SD) of the control values ($P < 0.001$). As the effects of the pentobarbital injection wore off, the HPW latency returned towards the values recorded in the saline-injected animals (Figure 1). Saline treated animals showed no change in HPW latency.

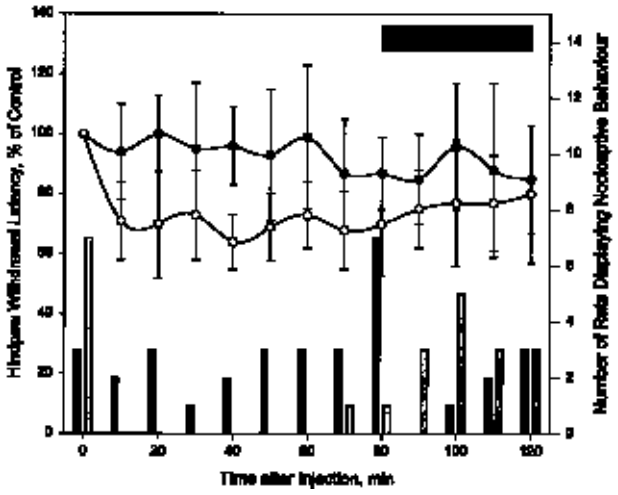

FIGURE 1 Normalized values for the nocifensive withdrawal threshold (left axis, circles). Pentobarbital injection results in a decrease in hindpaw withdrawal (HPW) latency (empty circles – mean values ± SD from 12 rats) when evaluated over the entire 120 treatment period, compared to saline-injected controls (filled circles, n=12)($P < 0.001$). Vertical bars (right axis) represent the number of animals manifesting nociceptive behaviour. Once the pentobarbital-injected animals (empty bars) had regained full responsiveness at 80 min after injection, they did not manifest more nociceptive behaviour (foot licking or biting) than the control group (solid bars ($P = 0.41$)). The black horizontal rectangle represents the period of time during which nociceptive behaviours were compared.

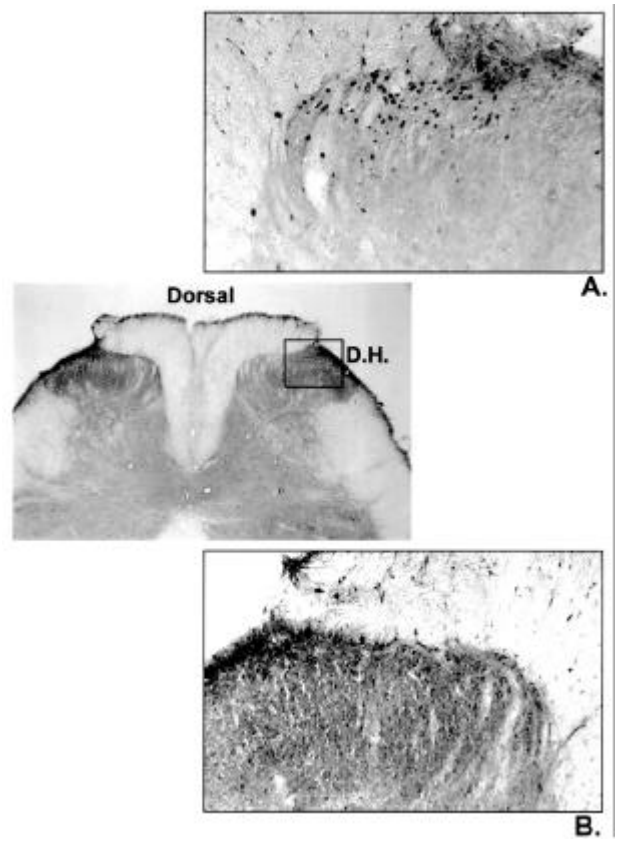

FIGURE 2 *c-fos* immunoreactivity in the dorsal horn (D.H.) of the lumbar spinal cord was increased by tissue injury but not by threshold noxious stimulation during pentobarbital-induced hyperreflexia. The lower magnification (x25) of the cross-section of lumbar spinal cord shows the location (black rectangle) of the representative high-power (x50) magnifications. A. Dorsal horn ipsilateral to repeated stimulation of 20 sec duration by immersion of the left hind paw of halothane-anesthetized animals in 54°C water. Dark staining in neuronal nuclei indicates the presence of c-fos immunoreactivity. B. A representative section in an animal that received repeated threshold noxious stimulation to the ipsilateral hind paw during sedation with pentobarbital.

Nociceptive behaviour

Rats regained responsiveness to vibrissal stimulation 40 ± 10 min after pentobarbital injection, but appeared sedated for a further 40 min. Rats appeared fully awake 80 ± 10 min after pentobarbital injection. After the pentobarbital injection, no licking behaviour was noted until the animals were fully alert and, once fully alert, pentobarbital-treated rats were not more likely to demonstrate licking behaviour than the control animals ($P = 0.41$, Mann-Whitney rank sum test).

c-fos Immunoreactivity in the spinal cord

The results are summarized in the Table and Figure 2. Radiant heat stimulation did not result in increased expression of *c-fos* immunoreactivity in either the control animals or in the pentobarbital-treated group. Peripheral tissue injury by immersion in hot water did result in increased *c-fos* expression ipsilateral to the injury in Rexed laminae I and II (Figure 2) despite halothane anesthesia ($P < 0.001$).

Discussion

In the present study, pentobarbital decreased nocifensive reflex thresholds in a manner similar to our previous report.[13] As the sedative effects of the pentobarbital wore off, nocifensive reflex thresholds returned to normal and, once awake, the animals did not show any increase in subjective responses to noxious stimulation. Stimulation resulting in tissue injury in the halothane anesthetized animals induced the expression of *c-fos* in the dorsal horn of the spinal cord, but threshold stimulation in the pentobarbital-anesthetized group failed to do so. We interpret these findings to mean that, although pentobarbital may enhance nociceptive transmission, repeated stimulation under these conditions is not sufficient to provoke long-lasting changes in nociception. The results of the present study extend findings previously reported using the rat formalin model.[14] Goto *et al.*[14] showed that pentobarbital enhanced flinching behaviour in the five minutes after formalin injection (phase 1) suggesting that pentobarbital enhanced nociception during the acute activation of C-fibre afferents by formalin. In contrast, the pentobarbital-treated animals showed less flinching behaviour during the second phase of formalin-induced hyperalgesia. The authors interpreted these findings to be consistent with pre-emptive analgesia by pentobarbital inhibiting central sensitization and thereby blunting the hyperalgesia in phase 2. Taken together with our present findings, these results suggest that, although pentobarbital may enhance nociception during acute painful stimuli, this phenomenon does not lead to activity-related spinal cord neuroplasticity.[15]

The lack of *c-fos* expression in the pentobarbital-treated animals must be treated with some caution. Although increases in the expression of *c-fos* immunoreactivity commonly increases with noxious stimulation and nociceptive behaviour can be blocked by intrathecal anti-sense antibodies to *c-fos*.[8–10] However, the absence of *c-fos* expression is not always correlated with an absence of nociceptive behaviour,[16] making *c-fos* an imperfect marker for hyperalgesia.

Since we did not know the relationship between nocifensive reflex thresholds and nociceptive behav-

TABLE *c-fos* Immunoreactivity in lumbar spinal cord

Experimental Group	*Number of rats*	*Mean Immunoreactive Cell Count Per Spinal Cord, ± SD*	
		Ipsilateral to Stimulation	*Contralateral to Stimulation*
Group 1 – Stimulated Controls	3	14 ± 7	14 ± 6
Group 2 – Halothane Controls	3	1 ± 1*	3 ± 2*
Group 3 – Halothane Injured	3	227 ± 56†	12 ± 10
Group 4 – Pentobarbital Stimulated	3	13 ± 5	13 ± 8

* signifies a lower cell count than that seen contralateral to stimulation in all other groups ($P < 0.05$).
† signifies a higher cell count than that seen ipsilateral to stimulation in all other groups.

iour under these experimental conditions, it was difficult to estimate the increase in frequency of nociceptive behaviour that we could expect to be associated with a decrease in nocifensive threshold of approximately 30%. The results suggest that the present study, with twelve animals in each group, is likely (power > 0.8) to detect a doubling in the frequency of nociceptive behaviour in the treatment group.

The results of the present study may help to explain why some investigators have failed to find any decrease in pain threshold[17] (which may reflect pain perception) after subhypnotic doses of thiopental and propofol that reliably decrease nocifensive reflex thresholds in the rat.[13] Our findings do not explain the conflicting results obtained in human studies of the effects of subhypnotic effects of thiopental on the response to painful stimulation.[2,17]

The present results differ from our findings concerning the facilitation of synaptic transmission in the hippocampus by pentobarbital.[6] There we reported that during stimulation that was not sufficient to induce synaptic plasticity, the addition of pentobarbital enhanced of synaptic transmission for at least three hours following removal of the drug. Our findings suggested that the facilitation of hippocampal synaptic plasticity by pentobarbital was dependent on -aminobutyric acid ($GABA_A$) and NMDA receptors, and was dependent upon bicarbonate ion.[6] In contrast, in the present study, a threshold stimulus repeated during a period of hyperreflexia did not produce any evidence of long-lasting changes, even though similar molecular mechanisms may be operative in the enhancement of signal transmission.[7] We speculate that additional influences resulting from tissue injury may be necessary for the induction of hyperalgesia.

In summary, although pentobarbital facilitates nocifensive reflexes, stimulation during the period of hyperreflexia does not produce evidence of enhanced nociceptive behaviour or increased *c-fos* immunoreactivity in the spinal cord. These findings suggest that, in the absence of tissue injury, pentobarbital does not promote the development of hyperalgesia or nociception-induced neuroplasticity.[18]

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