

## Selective Effects of Pentobarbital and Halothane on *c-fos* and *jun-B* Gene Expression in Rat Brain

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The effects of pentobarbital and halothane anesthesia on the expression in brain of the immediate-early genes *c-fos* and *jun-B* were investigated. Pentobarbital-anesthetized rats ( $n = 10$ ) received a single intraperitoneal injection of pentobarbital 65 mg/kg in a vehicle of 40% propylene glycol and 10% ethanol and then were placed in a plexiglass box flushed continuously with 100% oxygen at 5 l/min. Halothane-anesthetized rats ( $n = 10$ ) received an intraperitoneal injection of vehicle only and were transferred to a box flushed continuously with oxygen plus 1.5% halothane. Unanesthetized control rats ( $n = 10$ ) received an intraperitoneal injection of vehicle and were placed in a box flushed continuously with 100% oxygen. Four additional rats received no intraperitoneal injection but were handled and otherwise treated identically to the control group, and six others had a femoral arterial catheter inserted for blood pressure and blood gas measurements. Five animals from the control and both anesthetized groups were killed at 30 and 120 min postinjection and their brains rapidly removed and frozen. The messenger ribonucleic acid transcription products of the genes *c-fos*, *jun-B*, and  $\beta$ -actin from whole cerebral hemispheres were analyzed autoradiographically after Northern blot hybridization with  $^{32}\text{P}$ -labeled deoxyribonucleic acid probes. Relative levels of *c-fos* and *jun-B* messenger ribonucleic acid were determined from optical density measurements of the autoradiographic bands, with  $\beta$ -actin measurements being used to correct for sample-to-sample variation. Rats became immobile within minutes of drug administration and remained anesthetized until they were killed. The anesthetics had little effect on mean arterial blood pressure, rectal temperature, and  $\text{PaO}_2$ ; however, pentobarbital-anesthetized rats were hypercarbic at both 30 and 120 min. Because halothane also produced hypercarbia at 120 min, there were no physiologic differences between anesthetics at this later point. Handling of rats did not change *c-fos* or *jun-B* expression. Intraperitoneal injection produced a 2- to 3-fold increase in the whole brain levels of *c-fos* and *jun-B* messenger ribonucleic acid ( $P < 0.01$  for all comparisons) at 30 min versus 120 min. Neither anesthetic modified this increase in *c-fos* and *jun-B* expression at 30 min postinjection. At 120 min postinjection, selective anesthetic

effects were apparent. *Jun-B* expression was 25% greater than that of control in pentobarbital-anesthetized rats ( $P < 0.05$ ) and 38% lower in halothane-anesthetized rats ( $P < 0.01$ ), but neither anesthetic affected *c-fos* expression. Because immediate-early genes are thought to play a pivotal role in neural mechanisms of stress, memory, and pain/analgesia, anesthetic-induced changes in *jun-B* expression may pertain to modifications that occur in these biological processes during the anesthetic state. In a broader sense, these data indicate that general anesthetics selectively alter expression of genes in the central nervous system. (Key words: Anesthesia: general. Anesthetics, intravenous: pentobarbital. Anesthetics, volatile: halothane. Brain: metabolism. Cells: metabolism. Genetic factors: *c-fos*; *jun-B*; protooncogenes. Metabolism: deoxyribonucleic acid; ribonucleic acid.)

THE CENTRAL NERVOUS SYSTEM (CNS) is concerned almost exclusively with integrating, managing, storing, and responding to information contained in external cues. Although the precise details of these processes are unknown, they involve a cascade of events at and within the cell membrane, cytoplasm, and nucleus.<sup>1</sup> Inasmuch as the primary function of general anesthesia is to disrupt the normal process by which environmental stimuli are registered on the CNS, the molecular events occurring within each of these regions of the neuron are obvious potential sites of anesthetic action.<sup>2</sup> Interactions between general anesthesia and many of these potential cellular and molecular sites have been explored to some extent, the result being that general anesthesia can be described in terms of marked alterations in the physiology and biochemistry of neural tissue, cells, and even cell regions. General anesthesia profoundly changes the rates of neuronal discharge<sup>3</sup> and metabolism,<sup>4</sup> release and reuptake of neurotransmitters,<sup>2,5</sup> activity of excitable membrane-bound proteins,<sup>5</sup> and levels of key intracellular mediators, such as cyclic nucleotides.<sup>6</sup> However, one potential target of anesthetic action, gene expression within the cell nucleus, has been largely neglected.

Perhaps this is because until recently it seemed that changes in gene expression occurred slowly and therefore could not be important for a highly dynamic phenomenon such as neuronal information processing and the changes anesthesia produces in it. Abundant recent research, however, has altered this view of gene expression dramatically.<sup>7,8</sup> Genes capable of extremely rapid changes in transcription to messenger ribonucleic acid (mRNA) exist, and their protein products are important intracellular messengers or signal molecules. A particular family of protooncogenes called immediate-early genes, and two specific members of the family normally expressed in the

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CNS, *c-fos* and *jun-B*, are among the best characterized of these genes.<sup>8</sup> *c-Fos* and *jun-B* exhibit rapid and transient alterations in transcription after both stimulation and pharmacologic manipulation.<sup>7,8</sup> For example, the transcription products of *c-fos* (*i.e.*, mRNA) accumulate in appropriate regions of the CNS as early as 30 min after chemical, electrical, or intense mechanical stimulation of the periphery,<sup>9-14</sup> whereas morphine<sup>15,16</sup> and possibly other anesthetics<sup>14,16</sup> attenuate such increases. More important for neuronal function than transcriptional lability is the fact that the protein products of *c-fos* and *jun-B* act as intracellular signalling molecules,<sup>7,8</sup> modulating transcription of several target genes (including tyrosine hydroxylase<sup>17</sup> and the opiate peptide genes<sup>18,19</sup>) by binding at regulatory regions.<sup>20</sup> Therefore, expression of these genes seems to be crucial for converting transient membrane or cytoplasmic events into lasting changes in neurons. In this way, expression of immediate-early genes provides a molecular mechanism by which individual neurons "learn" from the environment. Examples of neural processes relevant to the anesthetic state that are closely associated with expression of immediate-early genes include stress,<sup>21</sup> pain,<sup>18,19</sup> and formation of memory.<sup>8,22</sup>

There has been no systematic investigation, however, of the effects of general anesthetics on immediate-early genes. Although many studies have measured expression of *fos* and *jun* genes in animals under general anesthesia,<sup>9,12,22</sup> other anecdotal reports<sup>14,16</sup> suggest that anesthetics modify expression of immediate-early genes. Because general anesthesia disrupts the normal process by which environmental and physiologic stimuli influence the CNS, a process in which immediate-early genes appear to play a pivotal role,<sup>7,8</sup> we hypothesized that anesthesia alters expression of these genes in brain. Accordingly, we focused on the immediate-early genes *c-fos* and *jun-B* in these initial experiments and, because anesthetics with different mechanisms of action might affect these genes differently, examined the effects of pentobarbital and halothane as representatives of two different classes of anesthetic agents.

### Materials and Methods

To determine the effects of pentobarbital and halothane anesthesia on *c-fos* and *jun-B* expression, mRNA derived from these genes was measured with quantitative Northern blot hybridization.

Experiments were performed with institutional Animal Care Committee approval on 40 male, 350–380-g Sprague-Dawley rats maintained in a 12-h light–dark cycle and allowed free access to food and water. Pentobarbital-anesthetized rats ( $n = 10$ ) received a single intraperitoneal

(ip) injection of pentobarbital 65 mg/kg in a vehicle of 40% propylene glycol and 10% ethanol (total injectate volume = 1 ml/kg) and then were placed in a Plexiglas box flushed continuously with 100% oxygen at 5 l/min. Halothane-anesthetized rats ( $n = 10$ ) received an ip injection of vehicle only and were transferred to a plexiglass box flushed continuously with 98.5% oxygen and 1.5% halothane (monitored with an anesthetic agent analyzer; Datex 222, Puritan Bennett). Unanesthetized control rats ( $n = 10$ ) received an ip injection of vehicle and were placed in a box flushed continuously with 100% oxygen. Four additional rats received no ip injection but were handled and otherwise treated identically to the control group. Once in the box, rats were left undisturbed. Temperature within the box was maintained with a warming lamp, and the temperature of the rats was measured rectally at the time they were killed. At either 30 or 120 min postinjection, five rats from the control and each treatment group were killed by decapitation and their brains rapidly removed, dissected into cerebral hemispheres, frozen in dry ice, and stored at  $-70^{\circ}\text{C}$  until later analysis. To determine the effect of anesthesia on mean arterial pressure and blood gases, a femoral artery catheter was inserted and tunneled out the back of the neck of six additional rats who had received approximately 10 min of halothane anesthesia. Rats recovered for at least 4 h, after which arterial blood gases, pH, and mean arterial pressure were measured. Rats then were anesthetized as described above with pentobarbital ( $n = 3$ ) or halothane ( $n = 3$ ), and arterial blood gases, pH, and mean arterial pressure were measured 30 and 120 min later.

Detailed methods for RNA extraction and analysis are contained in the Appendix. Briefly, RNA was extracted from whole cerebral hemispheres according to the method of Bradley *et al.*,<sup>23</sup> which results in an RNA sample containing predominantly mRNA. A single, frozen cerebral hemisphere from each rat was weighed and homogenized (Polytron, Brinkmann Instruments Inc., Westbury, NY) in the presence of 2% sodium dodecyl sulfate and proteinase K. The RNA was extracted by affinity binding to oligo-deoxythymidylic acid cellulose (oligo dT, New England Biolabs, Beverly, MA) and then concentrated by precipitation from ethanol. The relative quantities of mRNA transcripts for *c-fos*, *jun-B*, and  $\beta$ -actin were determined by Northern blot analysis.<sup>13</sup> From each rat, RNA was fractionated by size with electrophoresis through an agarose gel, transferred to nylon filters (nylon-66 membrane, 0.45  $\mu\text{m}$ , Schleicher & Schuell, Keene, NH) by capillary blotting, and immobilized by ultraviolet irradiation. Filters were hybridized serially with <sup>32</sup>P-labeled deoxyribonucleic (DNA) probes directed against *c-fos*, *jun-B*, and  $\beta$ -actin mRNA. Before each subsequent hybridization, nylon filters were washed in accordance with the manufacturer's directions to remove bound probe.

The DNA probe complementary to c-fos mRNA was prepared by digestion with restriction endonucleases EcoRI and BamHIII of plasmid pYN3158, which contains a 1,250-base-pair segment of the human c-fos gene.<sup>24</sup> The DNA probe complementary to jun-B mRNA was prepared by digestion with EcoRI of plasmid p465.20, which contains a 1,800-base-pair segment complementary to the entire coding region of the murine jun-B gene.<sup>25</sup> (Both plasmids were provided to Dr. Uhl's laboratory by Dr. Daniel Nathans.) The DNA probe complementary to  $\beta$ -actin was prepared by digestion with endonuclease PstI of a plasmid with an 819-base-pair insert containing a 403-base-pair 3' noncoding region and 416-base-pair coding region for amino acids 251–374 of the human  $\beta$ -actin gene (provided to Dr. Uhl's laboratory by Dr. E. Fuchs).<sup>26</sup> The DNA fragments used for generating the labeled complementary DNA probes were isolated by electrophoresis through low-melting-temperature agarose and then labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (specific-activity 3,000 Ci/mmol, Amersham, Arlington Heights, IL) by a random-primed DNA synthesis reaction with a commercially available kit (Boehringer Mannheim, Indianapolis, IN).<sup>27</sup>

After each hybridization, filters were washed and exposed to autoradiographic film (XAR, Kodak, Rochester, NY) at  $-70^{\circ}\text{C}$  with an intensifying screen (Cronex, DuPont, Wilmington, DE). Exposure and developing conditions of the autoradiographs were selected to avoid film saturation. Optical density measurements for autoradiographic bands in the regions of interest were made with a computerized image processing system (Dumas, Drexel University, Philadelphia, PA); the optical density of the film background was subtracted from all measurements. Because variation in the total quantity of mRNA applied to each lane of the gel results in variations in the amount of available hybridizable message independent of changes due to alteration in gene transcription, each filter also was hybridized with a DNA probe directed against  $\beta$ -actin mRNA. Probing with  $\beta$ -actin provides an internal standard to quantify precisely the amount of RNA applied to each lane of the gel because it is abundant in neural tissue, has a long half-life, and encodes a structural protein

that remains constant under physiologic conditions.<sup>13</sup> Thus, the optical densities of c-fos and jun-B autoradiographic bands were divided by the optical density of the  $\beta$ -actin band in the corresponding lane from the same filter. Data are expressed as the ratio of the c-fos or jun-B optical density to that of  $\beta$ -actin. To confirm results, each RNA sample was analyzed at least twice by Northern blot hybridization. Hybridization data from the unanesthetized group were compared to those of the pentobarbital and halothane-anesthetized rats at corresponding time points with analysis of variance and Dunnett's test for multiple comparisons. Comparisons within each treatment group were made with an unpaired Student's *t* test. Physiologic data from the anesthetized groups were compared with each other and with the control by the use of analysis of variance followed by Bonferroni correction.

## Results

General anesthesia, as assessed by loss of spontaneous movement, occurred in all rats within 3 min of ip injection of pentobarbital or inhalation of halothane. Anesthetized rats remained immobile in the box throughout the experiment and did not respond to handling or insertion of a rectal probe at the time they were killed. Control rats who received ip ethanol-containing vehicle were indistinguishable from normal uninjected rats: they were awake, walked without ataxia, groomed, and explored the cage normally. At the time they were killed, there was no difference in rectal temperature between either control ( $37.0 \pm 0.1^{\circ}\text{C}$  and  $37.1 \pm 0.1^{\circ}\text{C}$  at 30 min and 120 min, respectively) and anesthetized rats or between the anesthetized groups. After 30 min of anesthesia,  $\text{PaCO}_2$  was higher ( $P < 0.05$ ) in pentobarbital- versus halothane-anesthetized or control rats and arterial pH was lower than control pH in the pentobarbital-anesthetized group ( $P < 0.05$ ) (table 1). At 120 min of anesthesia, however, rats in both anesthetized groups were hypercarbic. Although there were statistically significant differences in  $\text{PaO}_2$  and mean arterial pressure between the anesthetized groups (table 1), the changes were small and both variables remained well within the normal physiologic range.

TABLE 1. Physiologic Variables

Arterial Blood Gases	Control (6)	Pentobarbital (3)		Halothane (3)	
		30 min	120 min	30 min	120 min
pH	$7.41 \pm 0.02$	$7.29 \pm 0.02^*$	$7.33 \pm 0.02$	$7.37 \pm 0.03$	$7.28 \pm 0.04^*$
$\text{PCO}_2$ (mmHg)	$43 \pm 1$	$62 \pm 4^*$	$64 \pm 3^{\dagger}$	$41 \pm 2^{\ddagger}$	$55 \pm 5^*$
$\text{PO}_2$ (mmHg)	$423 \pm 5$	$403 \pm 8$	$393 \pm 2^{\dagger}$	$425 \pm 19$	$477 \pm 7^{\dagger}\S$
Mean arterial blood pressure (mmHg)	$114 \pm 4$	$95 \pm 4^*$	$103 \pm 3$	$100 \pm 3$	$96 \pm 3^*$

Data are mean  $\pm$  SEM for the number of animals in parentheses.  
\*  $P < 0.05$ ,  $^{\dagger}P < 0.01$  for comparison with the control.

$^{\ddagger}P < 0.05$ ,  $\S P < 0.01$  for comparison with the corresponding value in pentobarbital-anesthetized animals.

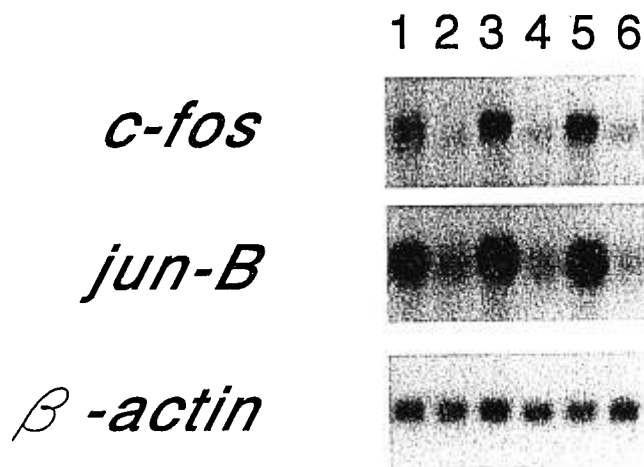


FIG. 1. Representative autoradiographs of portions of a single Northern blot. The filter was probed sequentially with  $^{32}\text{P}$ -labeled DNA probes complementary to *c-fos*, *jun-B*, and  $\beta$ -actin mRNA. Lanes 1, 3, and 5 are from animals killed at 30 min; lanes 2, 4, and 6 from animals killed 120 min after intraperitoneal injection. The samples in lanes 1 and 2 are from pentobarbital, lanes 3 and 4 from control (un-anesthetized), and lanes 5 and 6 from halothane-treated animals.

Sequential probing of a single Northern blot filter yielded detectable hybridization to *c-fos*, *jun-B*, and  $\beta$ -actin mRNA in each RNA sample (fig. 1), permitting determination of relative levels of all three mRNA species in each sample from the same filter. Brain *c-fos* and *jun-B* mRNA levels in rats that were simply handled did not change with time ( $0.48 \pm 0.09$  and  $0.69 \pm 0.13$  [ $n = 4$ ; mean  $\pm$  SEM], respectively). Control and anesthetized rats that received an ip injection, however, had much greater levels of *c-fos* and *jun-B* mRNA at 30 min than at 120 min after injection (table 2). In control rats, *c-fos* expression was 2.2 times greater ( $P < 0.001$ ) at 30 min than at 120 min, and there was 3.0 times more *jun-B* mRNA at 30 min after injection than there was at 120 min ( $P < 0.002$ ). Neither anesthetic modified this *c-fos* and *jun-B* response at 30 min postinjection (fig. 2). In pentobarbital- and halothane-anesthetized rats, *c-fos* expression was, respectively, 2.9 ( $P < 0.01$ ) and 2.5 times ( $P < 0.001$ ) greater than it was at 120 min, and *jun-B* expression was 2.6 ( $P < .001$ ) and 4.6 ( $P < 0.001$ ) times greater. At 120 min after injection, however, pentobarbital and halothane exhibited markedly different and opposite effects on *jun-B* but still had no effect on *c-fos* (fig. 2). Whereas *jun-B* expression was 25% greater than control ( $P < 0.05$ ) after 120 min of pentobarbital anesthesia, it was 38% lower in the halothane-anesthetized group ( $P < 0.01$ ) (fig. 2).

### Discussion

Immediate-early-response genes such as *c-fos* and *jun-B* normally are expressed throughout the CNS<sup>7,8</sup> but expression is enhanced rapidly and substantially in ap-

TABLE 2. Anesthetic Effects on *c-fos* and *jun-B* mRNA

	Control	Pentobarbital	Halothane
<i>c-Fos</i>			
30 min	$1.10 \pm 0.08$	$1.14 \pm 0.20$	$1.25 \pm 0.05$
120 min	$0.51 \pm 0.05$	$0.39 \pm 0.05$	$0.50 \pm 0.04$
<i>jun-B</i>			
30 min	$2.24 \pm 0.19$	$2.44 \pm 0.31$	$2.14 \pm 0.21$
120 min	$0.75 \pm 0.08$	$0.94 \pm 0.04^*$	$0.46 \pm 0.03^\dagger$

Data are mean  $\pm$  SEM of the optical density ratio of *c-fos* or *jun-B* to  $\beta$ -actin for five animals in each treatment group at each time point.

All differences between 30 and 120 min within groups are significant ( $P < 0.01$  for all comparisons by an unpaired *t* test).

\*  $P < 0.05$ ,  $^\dagger P < 0.01$  for comparison with the corresponding control value.

propriate regions by various stimuli.<sup>9-14</sup> This feature is well illustrated by the marked but transient increases in whole brain *c-fos* and *jun-B* mRNA observed in this study 30 min after ip injection. With respect to both the time course and magnitude of change, these changes agree with the results of a previous report<sup>21</sup> and were clearly a result of ip injection because the increase did not occur in un-anesthetized rats that were simply handled. Activation of somatic or visceral nociceptive afferents by electrical,

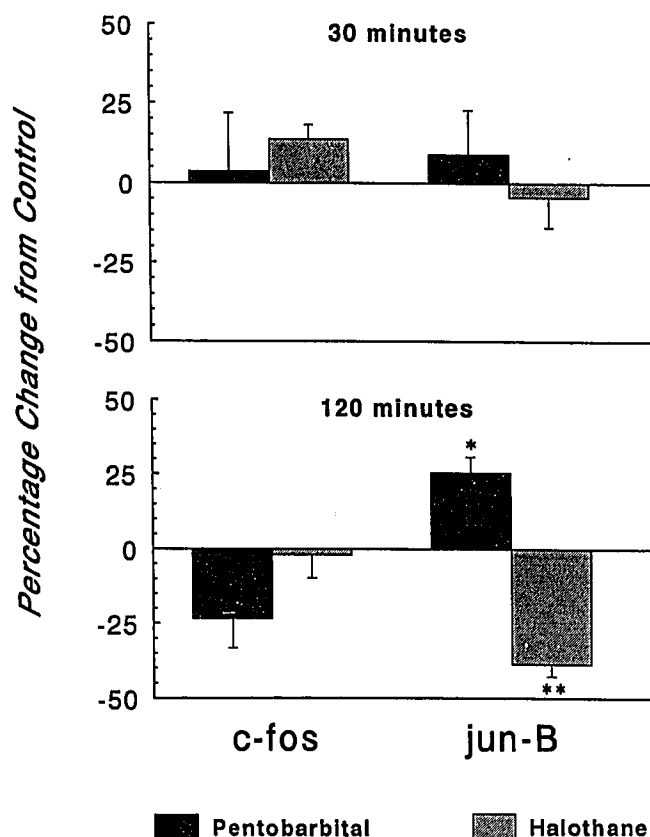


FIG. 2. *c-Fos* and *jun-B* data presented as percent change from control at each time point for pentobarbital and halothane anesthesia. Data represent mean  $\pm$  SEM. \* $P < 0.05$ . \*\* $P < 0.01$ .

thermal, chemical, or intense mechanical stimulation also produces induction within minutes to hours of several early-response genes, including *c-fos*,<sup>9,13</sup> *fos-B*,<sup>11,28</sup> *c-jun*,<sup>11,28</sup> *jun-D*,<sup>28</sup> and *jun-B*,<sup>28,29</sup> in subsets of neurons within pain-sensitive regions of the brain and spinal cord dorsal horn. Immediate-early genes, however, are differentially expressed in response to the same stimulus. For example, thermal or inflammatory stimulation induces expression of *jun-B*, but not *c-jun* or *jun-D*.<sup>29</sup>

The potential functional significance of such changes in early-response genes relates to the fact that they encode regulatory or signalling proteins that control expression of later response genes, which, in turn, serve more specific effector functions in the cell.<sup>7,8</sup> Thus, these genes are part of a cascade that couples cell surface receptors or ion channels to longer-term changes in the cellular phenotype and are the first activated in response to a chemical or physiologic stimulus.<sup>8</sup> The precise biochemical events that trigger this sequence are the subject of intense investigation but remain unclear. Transcriptional changes in immediate-early genes are initiated by neurotransmitters, membrane depolarization, and intracellular second messengers such as calcium, calmodulin, cyclic AMP, and phosphorylated regulatory proteins.<sup>7,8,30,31</sup> Once activated, the genes are transcribed in the nucleus to mRNA, which then is translated into protein in the cytoplasm.<sup>7,8</sup> These protein products of *fos* and *jun* then translocate back to the nucleus, where they form heterodimeric (*fos-jun*) or homodimeric (*jun-jun*) complexes that bind to regulatory regions of target genes.<sup>7,8</sup> Such a binding site, the so-called AP1 region,<sup>20</sup> is found in several genes, including tyrosine hydroxylase,<sup>28</sup> nerve growth factor,<sup>30</sup> and opiate peptide genes.<sup>18,19</sup> The promoter region of both *proenkephalin*<sup>18</sup> and *prodynorphin*,<sup>19</sup> for example, not only contains sequences that bind *fos-jun* protein complexes, but such binding is responsible for activation of transcription of these opiate genes. Different combinations of immediate-early genes can be induced by a particular extracellular stimulus or pharmacologic agent,<sup>11,19</sup> however, and certain dimers (*e.g.*, *fos-jun*) are more efficient than others (*e.g.*, *fos-fos*) at binding to DNA and affecting transcription.<sup>7,8,11,19</sup> Hence, although no simple relationship exists between induction of a single immediate-early gene and subsequent activation or repression of target genes, the variety of possible nuclear protein complexes provides a finely tuned mechanism for controlling transcription of target genes. Thus, ephemeral events at the cell membrane or within the cytoplasm leave an enduring trace within the neuron in the form of new nuclear proteins and altered gene expression.<sup>7,8</sup> In essence, the neuron is changed by its environment. These events, therefore, provide a molecular mechanism for converting short-term synaptic events into longer term functional changes in neurons<sup>7,8,32-35</sup> and are thought to be important for

forms of adaptational behavior that are influenced by anesthesia, including stress,<sup>21</sup> memory formation,<sup>8,22</sup> and pain and analgesia.<sup>18,19,33-35</sup>

There has been no other systematic investigation of the effects of general anesthesia on immediate-early gene expression. Inasmuch as expression of these genes is induced by stimuli that general anesthesia commonly is used to prevent, one might expect anesthesia also to prevent the gene response. This was not the case in our study. Neither halothane nor pentobarbital anesthesia blunted induction of *c-fos* expression, and *jun-B* was affected only after 120 min of anesthesia. The fact that neither anesthetic altered *c-fos* is especially surprising because expression of this gene in neurons is so activity-dependent and labile that it has been proposed as a marker of neuronal activity comparable to 2-deoxyglucose utilization.<sup>36,37</sup> Therefore, we anticipated that *c-fos* expression would decline because both anesthetics reduce neuronal activity and metabolic rate.<sup>4</sup>

Many experiments involving *c-fos* have been performed under general anesthesia with barbiturates,<sup>22</sup> halothane,<sup>15</sup> methoxyflurane,<sup>12</sup> ketamine,<sup>15</sup> or equithesin,<sup>9-11</sup> demonstrating that anesthesia does not entirely prevent stimulus-induced *fos* expression. There is some evidence, however, that certain anesthetic or analgesic agents modify expression of *c-fos*. An increase in the number of *c-fos* immunoreactive neurons in the olfactory cortex has been reported during methoxyflurane anesthesia,<sup>12</sup> but this probably reflects the odor of the drug rather than a selective effect on this brain region. Barbiturates interfere with *c-fos* induction in the hippocampus,<sup>22</sup> and anecdotally, halothane, ketamine, and the barbiturates<sup>14-16</sup> change the intensity and pattern of *c-fos* expression within spinal neurons. Morphine seems to have the greatest effect: at high doses, it completely blocks a stimulation-provoked increase in *c-fos*-like protein within the spinal cord and modifies it at lower dosages.<sup>16</sup> Our data do not resolve whether regional changes occur in *c-fos* mRNA related to anesthetic modulation of regionally specific neuronal events because the RNA was extracted from entire cerebral hemispheres. Nevertheless, it seems clear from this and other studies<sup>9-12,15,22</sup> that have measured *c-fos* expression during general anesthesia that *c-fos* cannot be a particularly sensitive marker of neuronal activity.

Although *c-fos* and *jun-B* expression were not initially affected by anesthesia, marked but selective changes were apparent after 120 min. Pentobarbital anesthesia did not change *c-fos* expression, whereas *jun-B* was expressed at a level 25% greater than that of the corresponding control group (fig. 2). Halothane reduced *jun-B* expression by 38% but also did not affect *c-fos* (fig. 2). These data therefore provide the first evidence that general anesthetics selectively alter gene transcription in the CNS. Moreover, it is unlikely that the results can be explained by nonspe-

cific effects of the drugs. The anesthetized groups were indistinguishable clinically, yet the anesthetics had opposite effects on jun-B and no effect on c-fos expression. A difference in the depth of anesthesia between the groups is not likely to be a factor either. Although a dose-response study would be necessary to exclude such a possibility, it is difficult to explain how rats still clinically anesthetized with pentobarbital could have greater jun-B expression than fully awake rats if level of consciousness or sensibility was important to the change in gene transcription. General anesthesia-induced cerebral metabolic depression probably does not explain the changes because it cannot account for selectively enhanced expression of jun-B during pentobarbital anesthesia, reduced expression during halothane, and no change in c-fos. The fact that changes in brain metabolism occur at induction of anesthesia,<sup>4</sup> yet no anesthetic-related differences in c-fos or jun-B expression were observed at 30 min, also argues against a simple metabolic mechanism. Abnormal physiology likewise fails to explain the differences between halothane and pentobarbital. When there were differences in  $P_{aCO_2}$  between the anesthetized at 30 min, there were no changes in gene expression. Conversely, when the physiology of the anesthetized groups was similar at 120 min, there were opposite anesthetic effects on jun-B. In fact, the mechanism by which these anesthetic agents selectively alter immediate-early gene products is not clear. Measurement of the level of a specific mRNA provides only a static index of the ratio of two dynamic processes: synthesis, which includes both transcription and posttranscriptional modification, and degradation. An alteration in the rate of either of these two processes changes the level of mRNA, and it is possible that pentobarbital and halothane affect c-fos and jun-B mRNA synthesis and degradation differently. Alternatively, it is possible that this selective effect on jun-B reflects actions of halothane and pentobarbital on intracellular regulators of jun-B expression, such as calcium or cyclic AMP.<sup>7,8,30,31</sup>

We cannot ascribe any particular behavioral characteristic of general anesthesia to changes in jun-B expression or the balance of c-fos and jun-B proteins within the brain. To do so requires analysis of immediate-early gene expression within specific brain regions and evidence that anesthetic-induced changes in jun-B actually are coupled to changes in expression of genes such as proenkephalin that are targets of jun-B. It is clear, however, that whole-brain changes in amount of jun-B mRNA play no role in producing or maintaining unconsciousness because anesthesia occurred immediately with both pentobarbital and halothane, but the result of jun-B expression (*i.e.*, jun-B mRNA) did not change until much later, and then did so in opposite ways. Because of the pivotal role these genes play in neural mechanisms of stress, formation of memory, and pain and analgesia,<sup>7,8,18,19,22,33-35</sup> it is conceivable that

anesthetic-induced changes in jun-B pertain to modifications that occur in these biological processes during and after anesthesia. There is a potentially broader, more important implication of this work, however. The fact that halothane and pentobarbital selectively change gene transcription in the CNS is good evidence that general anesthesia does not simply act on neurons: it changes them.

## Appendix: Detailed Experimental Methods

### RIBONUCLEIC ACID EXTRACTION AND ANALYSIS

Ribonucleic acid was extracted from whole cerebral hemispheres according to the method of Bradley *et al.*,<sup>23</sup> resulting in an RNA sample containing predominantly mRNA. In brief, a single, frozen cerebral hemisphere from each rat was weighed and homogenized in 10 ml/g 0.2 M NaCl, 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 1.5 mM  $MgCl_2$ , 2% sodium dodecyl sulfate, and 200  $\mu$ g/ml proteinase K. After incubation for 2 h at 45° C the NaCl concentration was adjusted to 0.5 M, and samples were centrifuged at 3,300 $\times$  g for 10 min. The supernatant was transferred to a clean tube containing 50 mg/g tissue prehydrated oligo dT in 1 ml binding buffer (0.5 M NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM ethylenediaminetetraacetic acid [EDTA]). After 1-h incubation at room temperature with gentle shaking, oligo dT was collected by centrifugation and the supernatant discarded. Oligo dT was washed four times by centrifugation after resuspension in binding buffer, transferred to a spin column, and washed until the eluant absorbance at 260 nm was less than 0.025. The RNA was eluted from the oligo dT with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA precipitated at -70° C in 0.3 M sodium acetate, and 70% ethanol and collected as a pellet by centrifugation. The sample was then resuspended in 1 mM EDTA (pH 8.0), and RNA content and yield were quantified by absorbance at 260 nm (1 optical density = 40  $\mu$ g RNA/ml).

The relative quantities of mRNA transcripts for c-fos, jun-B, and  $\beta$ -actin were determined by Northern blot analysis.<sup>13</sup> Approximately 6.5  $\mu$ g of RNA from each rat was fractionated by size with electrophoresis through an agarose gel containing 2% formaldehyde at 2-3 V/cm in 40 mM morpholinopropanesulfonic acid (pH 7.0) buffer containing 10 mM sodium acetate and 2 mM EDTA. The RNA was transferred from the gel to nylon filters (nylon-66 membrane, 0.45  $\mu$ m) by capillary blotting with 10 $\times$  saline-sodium citrate (SSC) (1 $\times$  SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and immobilized by ultraviolet irradiation. Filters were hybridized serially with <sup>32</sup>P-labeled DNA probes directed against c-fos, jun-B, and  $\beta$ -actin mRNA. After prehybridization for 12-24 h at 40° C in a solution of 50% formamide, 5 $\times$  SSC, 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, 1% sodium dodecyl sulfate, 2.5 $\times$  Denhardt's reagent (1 $\times$  Denhardt's reagent = 0.02% Ficoll, polyvinylpyrrolidone, and bovine serum albumen), and 10 mg/ml sheared, single-stranded salmon sperm DNA, filters were incubated for 24 h at 40° C in fresh solution containing 2-5  $\times$  10<sup>6</sup> decays per min (dpm)/ml DNA probe. Probe-specific activities were 1-1.8  $\times$  10<sup>9</sup> dpm/ $\mu$ g DNA.

After hybridization, filters were washed for 20 min at room temperature in 2× SSC, twice for 30 min at 55° C in 0.4× SSC and 0.5% sodium dodecyl sulfate, rinsed in 5× SSC, and exposed to XAR film at -70° C with an intensifying screen. Before each subsequent hybridization, bound probe was removed from filters by washing twice for 60 min at 68° C in 5 mM tromethamine-HCl (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1× Denhardt's reagent in accordance with the nylon manufacturer's protocol.

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