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## *c-fos* and ornithine decarboxylase gene expression in brain as early markers of neurotoxicity

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An increase of proto-oncogene *c-fos* expression in cerebral cortex of rats treated with subconvulsant doses of the pesticide organochlorine lindane ( $\gamma$ -hexachlorocyclohexane) has been detected using Northern blots. Immunohistochemical studies show that Fos protein was already increased in neuronal nuclei 3 h after treatment. The administration of the benzodiazepine diazepam prior to lindane totally blocked the activation of this proto-oncogene expression. Parallel to this increased expression of *c-fos* an activation of the ornithine decarboxylase (ODC) gene and enzyme was also observed. High levels of ODC mRNA and increased enzyme activity in cortex were found in rats following lindane treatment. These changes were attenuated by prior treatment of animals with diazepam. The co-induction of *c-fos* and ODC suggests a potential link between the ODC/polyamine system and the short-acting proto-oncogenes in stimulus–transcription coupling events.

### INTRODUCTION

Lindane ( $\gamma$ -hexachlorocyclohexane) is a potent and widely used organochlorine pesticide employed in veterinary and human medicine as an ectoparasiticide<sup>25</sup>. The compound is known to be able to cause hyperexcitability, tremors and convulsions in humans and other mammals<sup>10,27,30,32</sup>. Although it is uncertain by what mechanism lindane effects excitation, enhanced synaptic release of neurotransmitters<sup>22</sup> may be involved. It has been proposed that an increased level of intracellular calcium underlies this effect<sup>32,33</sup>. Low level exposures ( $10^{-6}$ – $10^{-7}$  M) to lindane increased <sup>45</sup>Ca uptake into isolated rat brain synaptosomes<sup>9,20</sup>. Direct measurement of free intrasynaptosomal Ca<sup>2+</sup> revealed that lindane increases [Ca<sup>2+</sup>]<sub>i</sub> in a dose-dependent manner<sup>3</sup>. In other studies, lindane and the  $\alpha$  and  $\beta$  isomers of hexachlorocyclohexane have been reported to produce both time- and dose-related increases in free intracellular calcium in a neurohybridoma cell line<sup>11</sup>. Lindane may thus exert its neurotoxic effects in part by affecting calcium homeostasis in neurons<sup>10,33</sup>.

Increases in levels of intracellular free calcium can also bring about rapid and profound changes in numerous cellular processes like protein phosphorylation and gene transcription. One of the earliest transcriptionally activated genes is the *fos* proto-oncogene<sup>16</sup>. *c-fos*, together

with other genes, is rapidly induced after cell activation, and is thought to take part in the early cellular response to a wide range of stimuli. Such genes may function as third messengers within an intracellular cascade linking extracellular stimuli to long-term adaptive processes<sup>6</sup>. Some of these so-called early genes are components of known transcription regulation complexes<sup>23</sup> and thus may be directly responsible for alterations in the expression of other target genes. A model for such a cascade has been proposed by Morgan and Curran<sup>17</sup>.

Ornithine decarboxylase (ODC) catalyses the first and rate-limiting step in the synthesis of polyamines in animal cells. Polyamines are believed to be essential for DNA replication and cell proliferation. ODC is rapidly induced in many tissues by a variety of stimuli<sup>12</sup>. The stimulation of neural tissue can induce this enzyme<sup>4,21</sup>. The protein has an unusually short half-life (10–30 min) in higher organisms and its activity is subject to multiple regulatory controls. It can undergo drastic and transient elevation in activity. It is known that a common feature of lesions of the nervous system is the rapid induction of ODC mRNA and enzyme activity<sup>31</sup>. Furthermore, ODC activity has also been detected at various times following the initiation of seizures<sup>2</sup>. The ODC/polyamine system and the nuclear proto-oncogenes thus share some similar properties.

In a previous study electroconvulsive shock (ECS) was

shown to result in the induction of ODC mRNA and enzyme activity as well as *c-fos* mRNA<sup>34</sup>. In this communication we report that lindane treatment of rats results in induction of cortical *c-fos* mRNA followed by accumulation of Fos protein, and also in the induction of ODC mRNA and enzyme activity. The rapid induction of *c-fos* and ODC following neural insult precedes major morphological changes and thus they may be considered as early markers of response to neurotoxic damage.

## MATERIALS AND METHODS

### *Animals and treatment*

Male Wistar rats weighing 250–270 g were used for all experiments. Animals were kept under standardized conditions with a 12-h dark/12-h light schedule. They were given free access to food and water.

Four different treatment groups were utilized: (1) animals orally gavaged with lindane at 30 mg/kg (subconvulsant dose) dissolved in pure olive oil in a dose volume of 1 ml/kg; (2) animals that were orally gavaged with olive oil alone (control animals); (3) animals injected with diazepam (i.p., 10 mg/kg, in dimethyl sulfoxide (DMSO)) 5 min prior to receiving the same treatment as group 1; (4) animals that were injected with diazepam 5 min prior to the same treatment as group 2. For analysis of mRNA expression, animals were decapitated 1 h and 5 h after treatments for *c-fos* mRNA and ODC mRNA, respectively. Cortices were immediately removed, frozen in dry ice, pooled and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Two additional groups of rats were treated with either pentylene tetrazol (PTZ) 60 mg/kg b.w.t. (convulsant dose) or also with diazepam (10 mg/kg b.w.t. in DMSO) 5 min prior to the administration of PTZ. These animals were used as positive controls for our treatment groups.

For immunohistochemical studies of Fos proteins, rats of each group were perfused 3 h after toxicant administration (details described later).

The animals were sacrificed 5 h after treatments for ODC assay.

### *Northern blot analysis*

All solutions contained 0.1% diethylpyrocarbonate to inhibit RNA degradation by ribonucleases during the extraction procedure. Frozen tissues were homogenized in a 3 M LiCl, 6 M urea plus 0.2% SDS solution. The homogenates were kept on ice for 4 h and RNA was then extracted by a modification of the LiCl and urea procedure<sup>1,8</sup>. RNA samples (7  $\mu\text{g}$ ) were size fractionated by electrophoresis through 1.2% agarose/2% formaldehyde gels stained with 1  $\mu\text{g}/\text{ml}$  ethidium bromide. After electrophoresis, the gels were photographed to ensure that equal amounts of intact RNA had been applied to each lane of the gel. RNAs were transferred overnight to a nylon membrane (Hybond-N, Amersham) by capillary blotting in  $20 \times \text{SSC}$  (3 M NaCl, 0.3 M  $\text{Na}_3$  Citrate) according to standard procedures<sup>13</sup>. The membranes were then rinsed in  $2 \times \text{SSC}$  and exposed to a UV-light source for 5 min to cross-link the RNA. They were afterwards prehybridized in a solution containing 50% formamide,  $5 \times \text{SSC}$ , 5  $\times$  Denhardt's solution (1  $\times$  contains 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), 20 mM Na phosphate, 1% SDS and 50  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA at  $42^{\circ}\text{C}$  overnight. The hybridization with the  $^{32}\text{P}$ -labeled nick-translated *c-fos* and ODC probes ( $1\text{--}2 \times 10^6$  cpm/ml) was carried out at  $42^{\circ}\text{C}$  overnight in the solution described for prehybridization. Preliminary washings of the blots were performed in  $2 \times \text{SSC}$ , 0.1% SDS at room temperature for 40 min. Stringent washings were in  $0.1 \times \text{SSC}$ , 0.1% SDS at  $45^{\circ}\text{C}$  for 30 min. The filters were exposed to AGFA CURIX RP 2 film with an intensifying screen at  $-80^{\circ}\text{C}$ .

### *ODC assay*

Animals were treated in the same manner as those used for mRNA isolation. ODC activity was determined by the measurement of evolved  $^{14}\text{CO}_2$  from carboxy- $^{14}\text{C}$ ornithine (50 mCi/mmol). Tissue was homogenized in 19 vols. of 0.04 M Tris-HCl. After centrifugation (26,000 g, 20 min), 0.9 ml of various tissue supernatants were added to 50  $\mu\text{l}$  pyridoxal phosphate solution (1 mM) and 50  $\mu\text{l}$   $^{14}\text{C}$ ornithine, in the presence of 0.045 M dithiothreitol. The final ornithine concentration was 2.5  $\mu\text{M}$ . Incubations were carried out at  $37^{\circ}\text{C}$  for 45 min in a sealed tube and terminated by injection of 1 ml of 2 M acetic acid into the reaction mixture<sup>24</sup>. Evolved  $\text{CO}_2$  was trapped on a paper wick containing hyamine suspended above the reaction mixture. The decarboxylation process is linear for up to 1.5 h under these conditions. Decarboxylation not attributable to ODC was determined by running a parallel incubation in the presence of the specific ODC inhibitor 5 mM difluoromethylornithine<sup>15</sup>.

### *Immunohistochemistry of Fos proteins*

Three hours after administration of lindane, animals from different treatment groups were anesthetized with sodium pentobarbital (6 mg/100 g b.w.t. i.p.) and perfused intracardially with 100 ml saline to largely remove blood from the brain. This was followed by perfusion with 300 ml of 3% fresh paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains were removed, postfixed in the latter solution for 1 h and washed in phosphate buffer for 1 h (3 washes 20 min each) before being dehydrated and embedded in paraffin. Five-micron coronal brain slices were cut and after rehydration were washed in phosphate buffered saline (PBS) (0.1 M, pH 7.2). Sections were then incubated in a buffer containing 10 mM Tris-HCl, 5 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 7.4 to reduce background staining. The immunological reactions were carried out using the peroxidase-antiperoxidase (PAP) method<sup>28</sup>.

Brains from animals of each treatment group previously fixed were frozen in isopentane after fixation and cooled with liquid nitrogen. Sections were cut in a cryostat, mounted on clear glass slides, air dried for 1 h and washed with PBS. Immunological analysis was carried out with the PAP method.

Both paraffin and frozen sections were incubated with: (1) anti-Fos antibody diluted 1:20 in PBS containing 2% defatted milk powder; incubation was at  $4^{\circ}\text{C}$  overnight; (2) rabbit anti-mouse immunoglobulin serum (1:100) in 2% milk powder PBS, 1 h at room temperature. The reaction was developed with 0.025% diaminobenzidine in PBS pH 7.6 containing 0.03% hydrogen peroxide. Sections were washed, dehydrated in alcohol, cleared in xylene and mounted in DPX mountant. As a negative control, sections were stained as described above but with omission of the primary antibody or with pre-absorption of the primary antibody with an excess of *c-fos* synthetic peptide.

### *Materials*

*c-fos* probe was kindly donated by Dr. Tom Curran and ODC probe was a generous gift from Dr. Phillip Coffino. Monoclonal antibody against Fos proteins and Fos peptide were from Microbiological Associates, Maryland. 5'-( $\alpha$ - $^{32}\text{P}$ )-ATP (3000 Ci/mmol) and  $^{14}\text{C}$ ornithine (50 mCi/mmol) were obtained from Amersham International (U.K.) and New England Nuclear (Boston, MA), respectively. Secondary antibodies, rabbit anti-mouse and PAP complex (mouse) were from Dakopatts (Denmark) and diaminobenzidine from Sigma Chemical Company (St. Louis, MO). Diazepam was kindly given by Prodesfarma, Barcelona. All other reagents were of the highest purity grade commercially available.

## RESULTS

In an earlier study, we reported increased *c-fos* expression after ECS-induced seizures<sup>34</sup>. In this work, we have used lindane as a paradigm for *c-fos* induction.

Subconvulsant doses of lindane (30 mg/kg) significantly increased *c-fos* mRNA levels 1 h after treatment when compared to control values (Fig. 1A). Ethidium bromide staining confirmed that all lanes were equally loaded with mRNA (Fig. 1B). While administration of lindane resulted in increased expression of *c-fos* mRNA, there were no apparent differences between the oil-treated group (control) and those treated with diazepam (10 mg/kg, i.p.) 5 min prior to lindane administration. No induction was seen when diazepam was given before gavaging with the oil vehicle. The induction elicited by PTZ was of the same magnitude as that of lindane. Its effect was also blocked by diazepam (Fig. 1A).

ODC mRNA expression in the different treatment groups is presented in Fig. 2. In a previous study we had determined time courses for ODC and *c-fos* mRNA following ECS<sup>34</sup>. ODC mRNA increased until 5 h post-stimulus where it reached a plateau. In the present study we have chosen this time to study ODC mRNA levels, which were also elevated after treatment with lindane (Fig. 2). Basal levels of ODC mRNA were found in those animals pretreated with diazepam. Variations in ODC mRNA levels correlated with changes in ODC

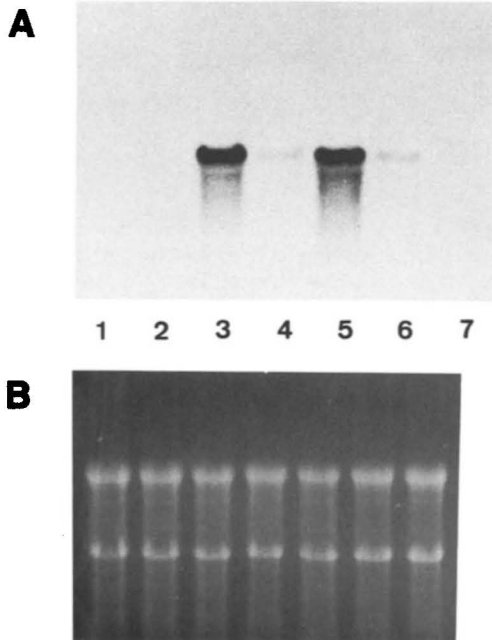


Fig. 1. A: induction of *c-fos* mRNA 1 h after treatment with lindane (30 mg/kg b.w.t. in oil) and PTZ (60 mg/kg b.w.t. in saline). Pretreatment with diazepam (10 mg/kg b.w.t., in DMSO) was 5 min prior to the administration of lindane or PTZ. Lane 1, untreated control animals; lane 2, oil vehicle-treated animals; lane 3, PTZ-treated animals; lane 4, diazepam preceding PTZ-treated animals; lane 5, lindane-treated animals; lane 6, diazepam preceding lindane-treated animals; lane 7, diazepam preceding oil-treated animals. Each lane is the result obtained from 4 pooled animals. B: ethidium bromide-stained agarose gel showing equally loaded lanes.

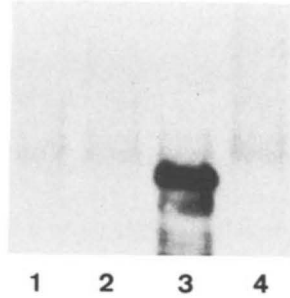


Fig. 2. Induction of ODC mRNA 5 h after treatment with lindane (30 mg/kg b.w.t. in oil). Pretreatment with diazepam (10 mg/kg b.w.t. in DMSO) was 5 min prior to the administration of the toxic agent. Lane 1, oil-treated animals; lane 2, diazepam preceding oil-treated animals; lane 3, lindane-treated animals; lane 4, diazepam preceding lindane-treated animals.

enzyme activity (Fig. 3). Levels of ODC activity increased 2.8-fold 5 h after lindane treatment. However this increase was only 1.6-fold in rats treated with diazepam before lindane (Fig. 3).

Immunohistochemical studies were carried out to evaluate any changes in Fos protein levels and distribution in the cortex. Although Fos-positive neurons were observed throughout the brain 1 h after treatment with lindane (data not shown), we preferred to sacrifice the animals 3 h postadministration because by that time essentially all neurons in the cortex were labeled. Fig. 4 shows a sequence of images corresponding to the cingulate cortex of brains from the different treatment groups. The immunostaining was highly specific, being abolished when the antibody was either omitted or pre-absorbed with the Fos peptide. There was intense staining of cell nuclei in those animals treated with lindane, with far weaker staining in the cytoplasm (Fig. 4a-c). The nucleolus did not appear to be stained. This pattern was also true for other brain areas such as different cortical

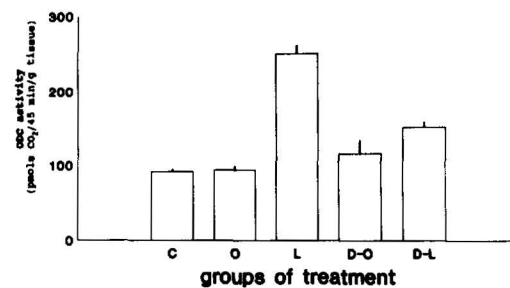


Fig. 3. Effect of lindane on ODC enzyme activity. ODC was determined 5 h after lindane administration by the measurement of evolved <sup>14</sup>CO<sub>2</sub> from [<sup>14</sup>C]ornithine. The mean ODC enzyme activity (± S.E.M.) of 3-4 individual determinations is shown. Groups L and D-L significantly differ from each other and from all other groups (Duncan's test with *P* < 0.001 taken as significant). C, control; O, oil; L, lindane; D-O, diazepam-oil; D-L, diazepam-lindane.

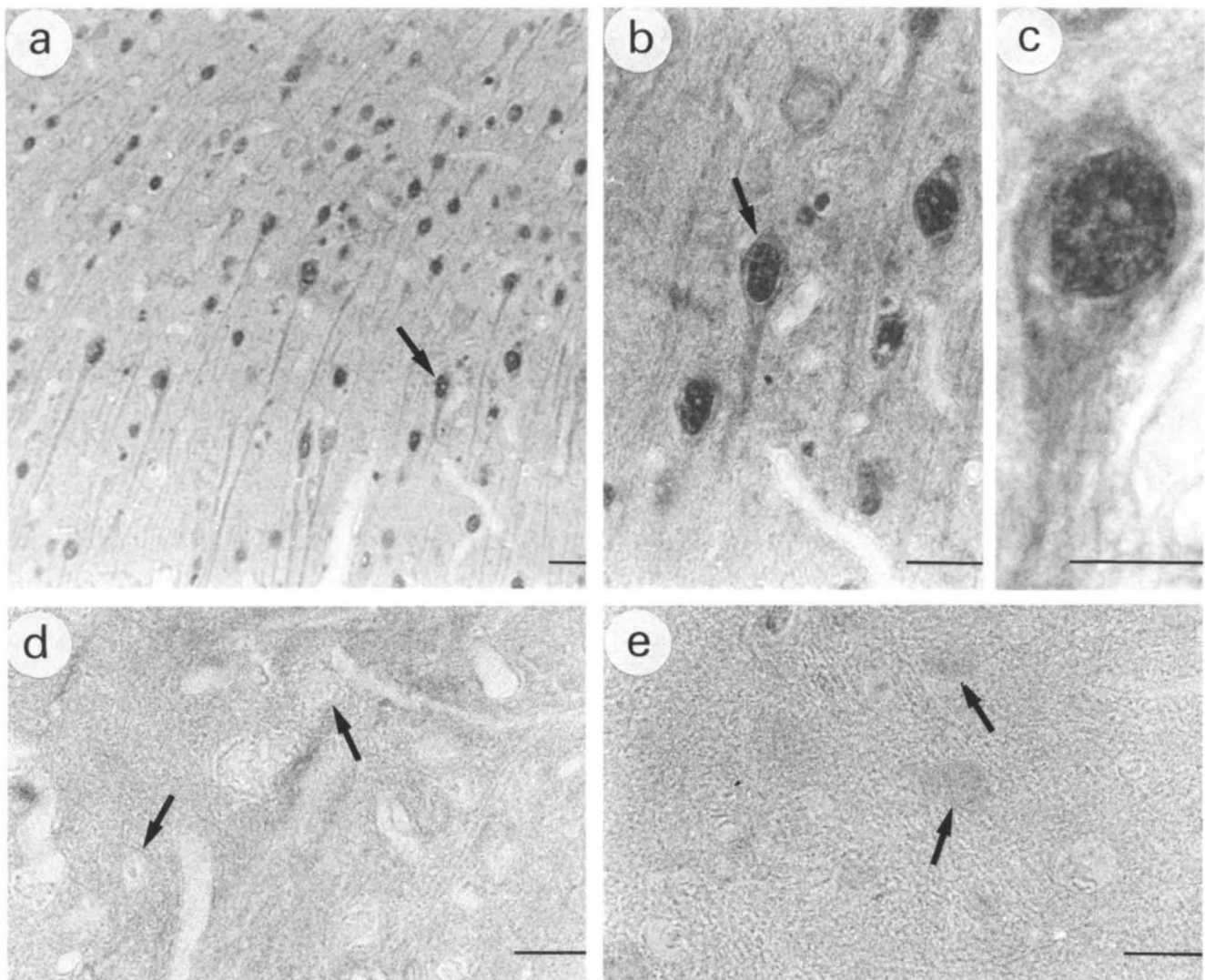


Fig. 4. Immunohistochemistry with Fos monoclonal antibody diluted 1:20. Photomicrographs of rat cingulate cortex 3 h postadministration. a–c show the nuclear staining at different magnifications in lindane-treated animals. Note the lack of staining in the nucleolus (c). d shows the absence of immunoreactivity in the cortex of an animal pretreated with diazepam 5 min before administration of lindane. e is from an oil-treated animal. Arrows point at nuclei. Bar = 20  $\mu\text{m}$  in a,b,d and e; and 10  $\mu\text{m}$  in c.

areas, the hippocampus and the amygdala (data not shown). Animals treated with the oil vehicle showed no nuclear staining (Fig. 4e). This result was consistent with the lack of induction of *c-fos* mRNA seen in the Northern blots. The administration of diazepam before treatment with lindane decreased protein levels to basal values (Fig. 4d).

#### DISCUSSION

Lindane treatment has been shown to elevate the expression of proto-oncogene *c-fos*. This effect has been described for other seizure-eliciting agents like PTZ or ECS<sup>18,34</sup>. Under the conditions described here this induction occurred in the absence of convulsions. Nevertheless, we do not exclude that undetectable seizures

might be taking place. Diazepam completely blocked the induction elicited by lindane although the administration of diazepam alone did not modify the level of expression of *c-fos*. Administration of lindane solvent (olive oil) alone also did not induce *c-fos*. Thus, we infer that the increased levels of *c-fos* mRNA that we find are attributable to the action of lindane.

Fos protein displayed a similar induction pattern to that already observed for the mRNA. There was an intense nuclear staining involving only neural cells. In all Fos-positive cells, the nucleoli appeared as immunonegative translucent vacuoles. There was also no staining in the perinucleolar heterochromatin. Fos-like immunoreactivity has been reported to be exclusively associated with dispersed chromatin (euchromatin), where all actively transcribing DNA sequences are thought to

reside<sup>19</sup>. This supports the concept of a role for Fos protein in regulating gene transcription.

Fos induction an hour after administration of a convulsant has been reported to occur selectively in brain and lungs<sup>18</sup>. This indicates that such events are not attributable to a generalized induction of transcription as a response to stress, possibly mediated by glucocorticoids<sup>34</sup>. Fos staining in the brain follows a characteristic distribution both regionally and temporally<sup>18</sup>. Three hours after the administration of lindane high levels of Fos can be found in the hippocampus, several nuclei in the thalamus and the amygdala and the pyriform, anterior cingulate and temporal cortex. This pattern of expression is parallel to that previously reported with other convulsants<sup>6</sup>.

We have already reported an induction in ODC mRNA expression after ECS<sup>34</sup>. The present data show that this can also be accomplished by subconvulsant doses of lindane. The pattern of ODC expression paralleled that of *c-fos* with respect to the inhibition exerted by diazepam on mRNA expression and enzyme activity induced by lindane. A parallel activation of both genes in PTZ-treated rats was also found (data not shown).

While the mechanism underlying lindane toxicity is not clear, recent findings suggest that it interacts with the  $\gamma$ -aminobutyric acid (GABA) receptor–benzodiazepine receptor–chloride ionophore complex<sup>32</sup>. Lindane seems to act on the picrotoxin component of this receptor which may subsequently interfere with the inhibitory neurophysiological action of GABA in opening the chloride channel<sup>14</sup>. Since GABA is the principal inhibitory neurotransmitter in the mammalian brain, lindane might exert its convulsant effects through ‘inhibition of inhibition’, leading to increased activation of neurons. A similar pattern of action has been proposed for another convulsant, PTZ, widely used as experimental paradigm to study the activation of proto-oncogene *c-fos* in the CNS<sup>18,19,26</sup>. The effects of both lindane and PTZ are abolished by the administration of diazepam. The benzodiazepines are neuropharmacological agents that have anti-convulsant effects via their agonistic interaction with the GABA receptor–chloride ionophore complex. The fact that diazepam counteracts the convulsant effects of

lindane and PTZ may indicate that the effects of these agents all center upon the GABA receptor complex.

It is still unclear how these convulsants bring about changes in early gene expression. Several studies with PTZ have reported the coincidence of the distributions of Fos-like immunostaining and the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor in the CNS<sup>6,17,26</sup>. ODC expression after ECS is also partially inhibited by MK-801, an NMDA receptor antagonist<sup>34</sup>. A partial inhibition by MK-801 has also been described for *c-fos* induction after PTZ<sup>26</sup>. Although the NMDA receptor may only constitute a portion of the pathway of early gene activation, the remarkable resemblance that its distribution in the brain bears with that of Fos suggests its participation in triggering the nuclear event<sup>18</sup>. This type of glutamate receptor can gate calcium entry into the cell. Thus, calcium could play the role of the common intracellular mediator of transcriptional activation<sup>6,7</sup>. Studies conducted with calcium channel agonists and antagonists demonstrate the importance of calcium in the onset of early gene transcription<sup>16,17</sup>. As already mentioned, lindane increases calcium uptake into neurons, their synaptosomes and possibly other cells<sup>3,11</sup>. Furthermore, influx of calcium through cell membrane channels has been linked to glutamate neurotoxicity<sup>5</sup>.

A neurotoxic agent has here been shown to activate both the proto-oncogene *c-fos* and the ODC/polyamine system. Although such activation is not temporally coincident, this suggests that ODC may be regulated by a gene comprising part of the early response of the cell to an external stimulus. The polyamines may modulate the expression of proto-oncogene target genes as well as having their own target sites. Both systems may be part of the initially activated cellular machinery implicated in the coupled stimulus-transcription cascade that is ultimately responsible for long-term adaptive changes.

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