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ITF mapping after drugs of abuse: pharmacological *versus* perceptional effects

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Abstract. Analysis of inducible transcription factors (ITFs) expression is often applied to map drug-induced changes of neuronal activity in brain. Administration of cocaine and alcohol induces ITFs in a large number of brain structures. However, induction of ITFs in a brain region does not necessarily indicate a pharmacological effect of the drug in this brain region. Many of the brain regions could be activated by secondary effects. Perception of stimulus properties of the drug or locomotor effects of the drug are possible secondary effects. Anesthesia can block induction of ITFs by cocaine and alcohol suggesting that ITF expression in a majority of brain regions is more sensitive to secondary effects than to pharmacological effects of these drugs. In agreement with this hypothesis is our finding that the majority of brain regions responding with ITF expression to alcohol administration do not respond to voluntary alcohol self-administration in animals. Only a few brain regions show similar ITF induction after both administration and self-administration of this drug. Presumably these brain regions could be responding to pharmacological effects of alcohol. Given the low resolution of invasive techniques, ITF mapping experiments will continually contribute to our understanding of mechanisms of drug addiction and alcoholism.

Key words: c-Fos, FosB, Egr1, Zif268, psychostimulants, ethanol

ITF EXPRESSION AFTER ADMINISTRATION OF DRUGS OF ABUSE

Drug addiction and alcoholism are well known problems in human society, and are of long standing interest to neuroscientists. The rate of progress in drug abuse research has been uneven. Whereas the molecular targets for cocaine and morphine (i.e., dopamine and serotonin transporters and opioid receptors, respectively) have been identified, the molecular mechanisms of alcohol are less understood and probably involve several neurotransmitter systems (Koob and Bloom 1988, Nestler and Aghajanian 1997). Despite the success of molecular studies with cocaine and morphine, it is still unclear how binding of target molecules by these drugs will lead to such behavioral effects as sensitization, craving, withdrawal and addiction. Mapping of ITFs in the brain has been applied to identify brain regions possibly involved in development of these behaviors. This approach to studies of drugs of abuse was pioneered by S.L. Chang et al. and H.A. Robertson et al. in the late eighties (Chang et al. 1988, Robertson et al. 1989), and followed up in many laboratories around the world.

Psychostimulants are potent inducers of ITFs (Robertson et al. 1989, Graybiel et al. 1990, Young et al. 1991, Cole et al. 1992). Strong induction of c-Fos in the striatum became a hallmark of ITF induction after administration of cocaine (Graybiel et al. 1990, Young et al. 1991, Hope et al. 1992, Torres and Rivier 1993). Cocaine-induced ITF induction is not specific to c-Fos, and has been shown to be accompanied by induction of a number of other ITFs (Young et al. 1991, Moratalla et al. 1992, 1996, Hope et al. 1994, Nestler and Aghajanian 1997).

Chronic administration of this drug leads to a desensitization of ITF response, such that only a subset of ITFs, including the *fosB*-encoded Chronic Fras, become elevated (Young et al. 1991, Hope et al. 1994, Rosen et al. 1994, Chen et al. 1997, Hiroi et al. 1997). Striatum has long been implicated in regulation of reinforcing effects of drugs of abuse (Wise 1996, Koob and Moal 1997), and finding ITF induction in this area after administration of cocaine seems now not surprising. However, researchers in the field also know that cocaine increases c-Fos expression not only in the striatum, but also in a number of subregions of amygdala, thalamus, hypothalamus, septum and neocortex (Brown et al. 1992, Merlo-Pich et al. 1997, Ryabinin et al. 200.

Patterns of c-Fos expression have also been studied after administration of alcohol (Chang et al. 1995, Ryabinin et al. 1995, Thiele et al. 1996, Hitzemann and Hitzemann 1997, Ryabinin et al. 1997). Surprisingly, these patterns are significantly different from the ones

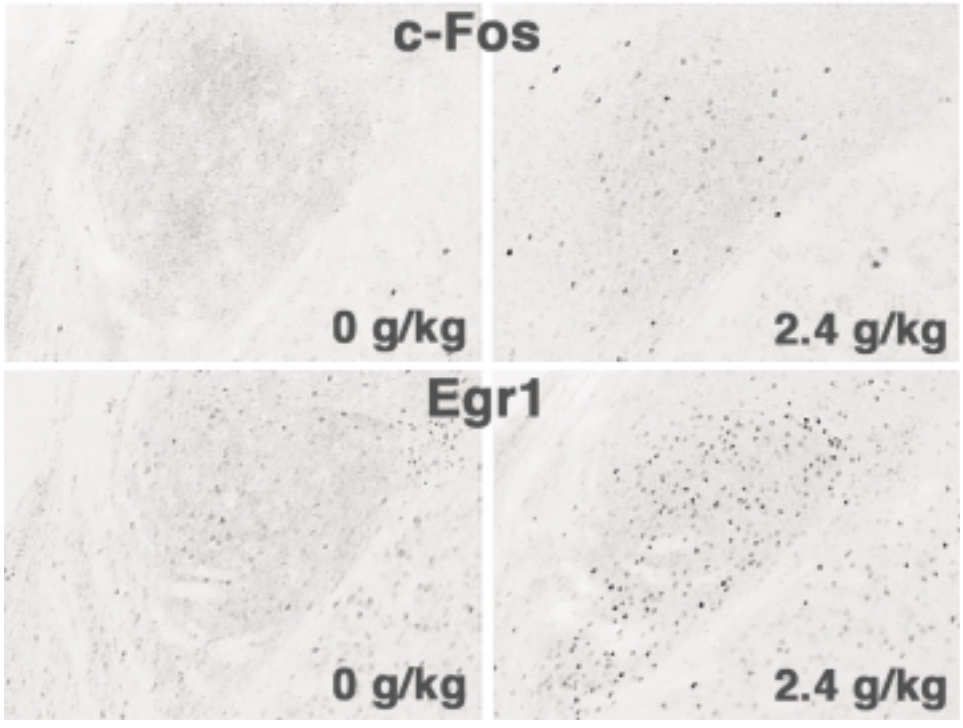

Fig. 1. Expression of c-Fos and Egr1 in the central nucleus of amygdala of C57BL/6J mice after injection of saline or alcohol (2.4 g/kg). Note low number of c-Fos-positive cells (top right panel) versus large number Egr1-positive cells (bottom left panel) after alcohol administration.

produced by cocaine. The hallmark of alcohol-induced ITF expression is the c-Fos expression is the lateral portion of central nucleus of amygdala. Besides this nucleus, c-Fos is also strongly induced in the paraventricular nuclei of thalamus and hypothalamus, the bed nucleus of stria terminalis, the Edinger-Westphal nucleus and nucleus of the solitary tract (Chang et al. 1995, Thiele et al. 1996, Hitzemann and Hitzemann 1997, Ryabinin et al. 1997, Ogilvie et al. 1998). In contrast, c-Fos expression in nucleus accumbens is not strong, and its elevation is often not significant statistically (Hitzemann and Hitzemann 1997, Ryabinin et al. 1997).

Alcohol also leads to induction of ITFs FosB and Egr1 in many of the areas showing induction of c-Fos (Ogilvie et al. 1998, Ryabinin and Wang 1998). Similarly to cocaine, repeated alcohol administration can lead to desensitization of the acute response, but can leave the expression of FosB-related proteins (presumably the Chronic Fras) elevated (Ryabinin and Wang 1998). Analysis of several ITFs instead of just one is an important concern in ITF mapping studies. For example, C57BL6/J mice have a very low level of c-Fos induction in the central nucleus of amygdala, compared to rats or DBA2/J mice (Hitzemann and Hitzemann 1997). However, this apparent insensitivity to alcohol is misleading, as C57BL6/J show a very strong induction of Egr1 after alcohol administration (Fig. 1).

Interestingly, we have shown that alcohol not only can induce c-Fos in several areas of the brain, but also suppresses c-Fos expression in the hippocampus. Since basal levels of c-Fos in the hippocampus are low (Kaczmarek et al. 1988), this effect is noticable when c-Fos expression in this structure has been elevated by other factors, for example by restraint stress, fear conditioning or placing the animals in a novel environment (Ryabinin et al. 1995, 1997, Melia et al. 1996). The suppressive effects of alcohol on such experience-induced c-Fos expression does not undergo desensitization with repeated treatment (Ryabinin et al. 1997). This suppression of c-Fos has been shown to have a similar dose response as alcohol's effect on hippocampal learning, and is proposed to be involved in alcohol's amnestic effects (Ryabinin 1998). However, the low number of c-Fos--positive cells detected by immunohistochemistry in the hippocampus even after behavioral stimulation casts doubt on this possibility. Recently, alcohol's suppressive effects on c-Fos expression have been shown to be accompanied by parallel changes in the expression of ITF Egr1 (Ryabinin and Wang 1999, Ueyama et al. 1999). In contrast to c-Fos, Egr1 is expressed at a very high level in the CA1 region of the hippocampus, and it is possible that alcohol-mediated changes in hippocampal Egr1 expression could have a functional significance for learning.

The complex pattern of changes in ITF expression after drug administration probably reflects the plethora of effects produced by administration of these drugs. Among such effects are changes in locomotor activity, changes in body temperature, stress of involuntary drug administration, and stress of the first unpredicted intoxication. Therefore, ITF expression after administration of these drugs may not only reflect changes in neuronal activity mediating these effects, but may also be secondary to them, for example due to perception of these effects.

DRUGS OF ABUSE-INDUCED ITF EXPRESSION IN ANESTHETIZED ANIMALS

Anesthesia completely blocks many effects of drugs of abuse, especially the perceptional and motor effects, whereas many pharmacological effects remain (e.g., cocaine still blocks dopamine uptake in the striatum) (Torres et al. 1994). Hence, one could expect anesthesia to leave intact the drug-induced c-Fos expression in the zones of its pharmacological activity, and to block c-Fos expression in the brain regions regulated by locomotor activity or perception. Presumably such analysis could restrict the large number of brain regions activated by administration of drugs of abuse to only structures in which the activity is mediated by pharmacological effects.

We have attempted such analysis in recent experiments, in which Sprague-Dawley rats were injected with cocaine or alcohol in the presence or absence of pentobarbital anesthesia (Ryabinin et al. 2000). Since c-Fos expression is highly sensitive to environmental novelty, we first habituated animals to repeated anesthesia sessions or saline injections. On the day of experiment animals were first injected with pentobarbital (50 mg/kg, i.v.) or equal volume of saline, and then injected with saline, cocaine (15 mg/kg, i.v.), or ethanol (2 g/kg, i.p.). To our surprise, pentobarbital anesthesia completely blocked both cocaine- and alcohol-induced c-Fos expression in all brain regions that showed induced c-Fos in awake (saline-treated) animals, including striatum, amygdala, hypothalmus and neocortex (Fig. 2). Pentobarbital showed induction of c-Fos expression in the lateral habenula, which is consistent with previous

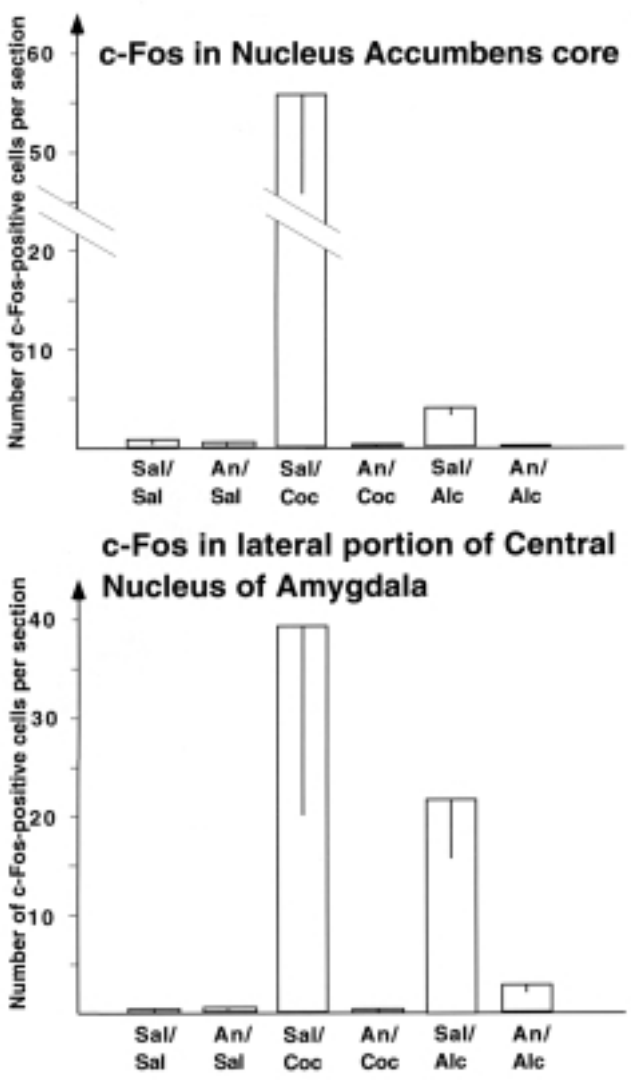

Fig. 2. Effects of pentobarbital anesthesia on alcohol- and cocaine-induced c-Fos expression in the nucleus accumbens and lateral portion of central nucleus of amygdala of Sprague-Dawley rats. Note strong elevation of c-Fos expression in nucleus accumbens and central nucleus of amygdala of cocaine-injected rats (Sal/Coc group), and in central nucleus of amygdala of alcohol-injected rats (Sal/Alc) group, and absence of this elevation in anesthetized animals (An/Coc and An/Alc groups). Graph based on (Ryabinin et al. unpublished).

studies (Takayama et al. 1994), but this induction did not differ between the cocaine-, alcohol-, or saline-injected animals. We have also analyzed expression of ITFs FosB and Egr1 in the striatum of these animals, and found a complete blockade of cocaine-induced ITF expression by pentobarbital anesthesia.

This result has two potential explanations. One explanation is that the extensive pattern of drug-induced c-Fos expression in the brain is all due to secondary (perceptional and motor) effects of cocaine and alcohol, and ITF expression is less sensitive to pharmacological effects of these drugs. An alternative explanation is that pentobarbital interferes with the pharmacological effects of cocaine and alcohol, thereby preventing c-Fos expression. We are in favor of the first explanation based on the earlier finding by G. Torres and C. Rivier which showed that a ketamine mixture anesthetic blocked cocaine-induced c-Fos in the striatum (Torres and Rivier 1993). Since ketamine acts through NMDA receptors, and not through GABA receptors, like pentobarbital, it seems that the suppressive effects of ketamine and pentobarbital on c-Fos induction are due to anesthetic properties of these drugs, and not due to their interference with pharmacological effects of cocaine. Interestingly, the same study also showed two other NMDA antagonists, decreased c-Fos expression in awake animals to a lesser extend than ketamine-induced anesthesia. It also seems unlikely that pentobarbital-mediated suppression of cocaine- and alcohol-mediated suppression occurs in diverse brain structures receiving non-overlapping projections, i.e. striatum, neocortex and amygdala. Taken together, our studies and those of Torres and Rivier suggest that ITF induction after administration is mostly due to secondary, rather than pharmacological effects. These secondary effects are not without importance, however, as both ketamine and pentobarbital anesthesia have been shown to interfere with sensitization to repeated cocaine, while ketamine did not affect dopamine uptake in the striatum (Torres et al. 1994). This explanation contradicts studies that observed *c-fos* mRNA expression in dissociated striatal cultures (Konradi et al. 1996). One can not exclude, however, that sensitivity of striatal neurons to pharmacological agents increases during preparation of dissociated cultures. Clearly, more studies have to be performed to compare the roles of perception of drug-associated cues and pharmacological effects in induction of ITFs.

EFFECTS OF "VOLUNTARY" ALCOHOL ADMINISTRATION ON ITF EXPRESSION

Another approach to distinguish between pharmacological and secondary effects of the drugs on ITF expression, is to examine ITF expression after administration

of drugs of abuse *via* different routes. By definition, if the effects of the drugs are pharmacological, they should be observed after any route of administration. Several research groups have used this approach in alcohol research. Thus, we have not only administered alcohol to the animals *via* intraperitoneal injections, but also *via* alcohol vapor inhalation. Although this route of administration allows less control on the dosage, we have observed very similar patterns of c-Fos expression after both injection and inhalation of alcohol in rats (Ryabinin et al. 1997). Another group described c-Fos and Egr1 expression in the paraventricular nucleus of hypothalamus, one of the alcohol-sensitive brain regions, after both intraperitoneal injections and intragastric intubation with alcohol (Ogilvie et al. 1998). However, these studies only partially overcome the concern of analyzing ITF expression due to secondary effects. Thus, in all of these cases alcohol was given to the animals involuntarily, by an experimenter. Involuntary alcohol administration is known to be aversive to rodents. For example, it has been shown to lead to place aversion and taste aversion (Cunningham 1981, Cunningham et al. 1993, Risinger and Cunningham 1995).

In our recent studies, we have been looking at the effects of voluntary alcohol drinking on ITF expression in brain. This approach is obviously important because this is the main route administration in humans. Rodents, however, do not readily self-administer large quantities of this drug, and the challenge of this approach was to train animals to self-administer alcohol in a short drinking session. We took advantage of the tendency of C57BL/6J to consume larger quantities of alcohol than other mouse strains, and trained them to self-administer 10% alcohol supplemented 10% sucrose solution during a thirty minute drinking session in a procedure developed by H. Samson and K. Grant (Grant and Samson 1985, Samson 1986). Animals had access to water for 20 h per day in this procedure, and thus were not water-deprived. Control animals consumed a 10% sucrose solution. In our first experiments animals consumed approximately 1.5 g/kg of alcohol during a single drinking session. After the last drinking session half of the animals remained in their home cage, and half of the animals were exposed to a 30-minute restraint stress session, and then placed back to their home cage. As expected restraint stress produced a significant increase in c-Fos expression in many areas of the forebrain. In contrast, consumption of the alcohol/sucrose solution did not change c-Fos expression in any forebrain structures of the unstressed mice compared to unstressed sucrose-consuming animals. However, animals that consumed the alcohol/sucrose solution and were exposed to restraint stress had a significantly higher c-Fos expression in the core of nucleus accumbens and lower levels of c-Fos in the CA3 region of hippocampus than sucrose-drinking restraint-stressed mice (Fig. 3) (Ryabinin et al. 1999). Similarly, although alcohol consumption did not show any expression of Egr1 in unstressed animals, it significantly suppressed restraint--induced Egr1 expression compared to sucrose-drinking

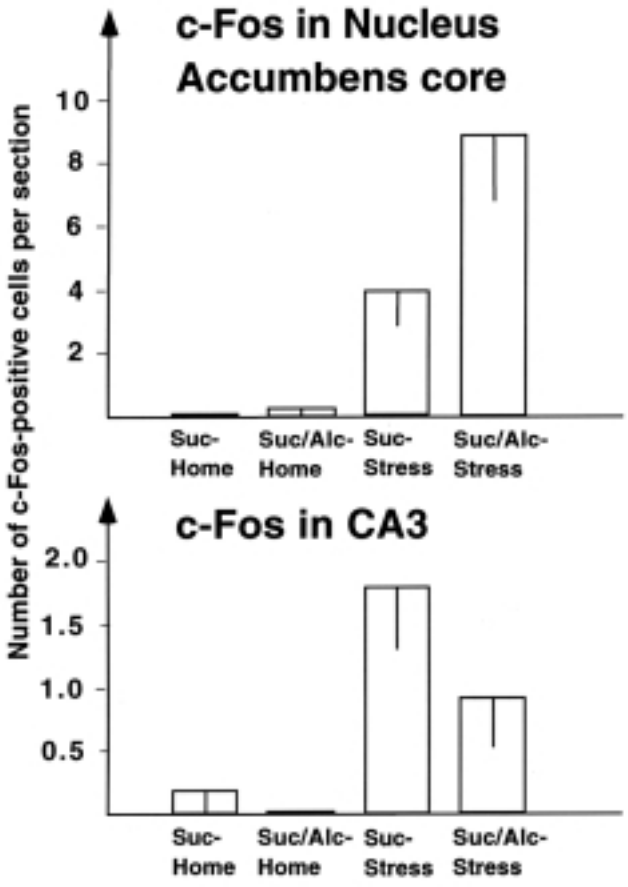

Fig. 3. Expression of c-Fos in nucleus accumbens and CA3 regions of hippocampus in sucrose/alcohol or sucrose self-administering C57BL/6J mice exposed to restraint stress. Note higher expression of c-Fos in nucleus accumbens of sucrose/alcohol-drinking mice exposed to restraint stress (Suc/Alc-Stress group) than in other groups, and decrease in c-Fos expression in CA3 in sucrose/alcohol-drinking restraint-stressed (Suc/Alc-Stress group) *versus* sucrose-drinking restraint-stressed animals (Suc-Stress group). Graph based on reference (Ryabinin et al. 1999).

restraint-stressed animals. The changes in FosB expression did not overlap with changes in Egr1 and c-Fos, and alcohol drinking animals had lower levels of FosB in lateral and basolateral amygdala and lateral hypothalamus than sucrose-drinking mice.

These data confirmed that the suppressive effects of alcohol on experience-induced hippocampal c-Fos and Egr1 expression are independent of the route of administration, and could be considered pharmacological. They also showed that c-Fos expression in nucleus accumbens, an area important for reinforcing effects of drugs of abuse, could be dependent on the presence of stress, providing a potential substrate for the long-suggested but difficult to prove role of stress in alcoholism (Masserman and Yum 1946, Conger 1956, Pohorecky 1990). However, these data were difficult to interpret since alcohol-drinking mice consumed lower doses of the drug than used in previous injection studies. Therefore, in our next experiments we trained our C57BL/6J mice to consume higher doses of alcohol.

In our next studies animals were trained to self-administer the alcohol/sucrose solution in a similar procedure, but due to minor modifications they consumed about 2.5 g/kg of ethanol per drinking session (Bachtell et al. 1999). Two groups of control animals were used: animals consuming sucrose and animals consuming water. These two controls are important because sucrose-consuming animals drank more solution during the drinking session than animals exposed to the sucrose/ethanol solution, while water-consuming animals drank less than the sucrose/ethanol group. Therefore, if we would find differences in ITF expression in the sucrose/ethanol group *versus* both the sucrose and water groups, these differences would not be due to differences in consumption, but to the actions of ethanol. The effects of interactions of alcohol and restraint stress were not assessed in these experiments due to complexity of experimental design. However, these studies identified ethanol-induced changes in ITF expression even in the absence of restraint stress. Thus, ethanol strongly induced c-Fos expression in the Edinger-Westphal nucleus, and to a lesser extend in the core of nucleus accumbens and the medial portion of central nucleus of amygdala, and decreased c-Fos expression in the dentate gyrus (Fig. 4) (Bachtell et al. 1999). No changes in Egr1 expression were identified in these studies, while FosB expression was elevated in the Edinger-Westphal nucleus and the medial portion of central nucleus of amygdala (the induction in the latter, however, was not specific for ethanol, and was also present in the sucrose-consuming animals).

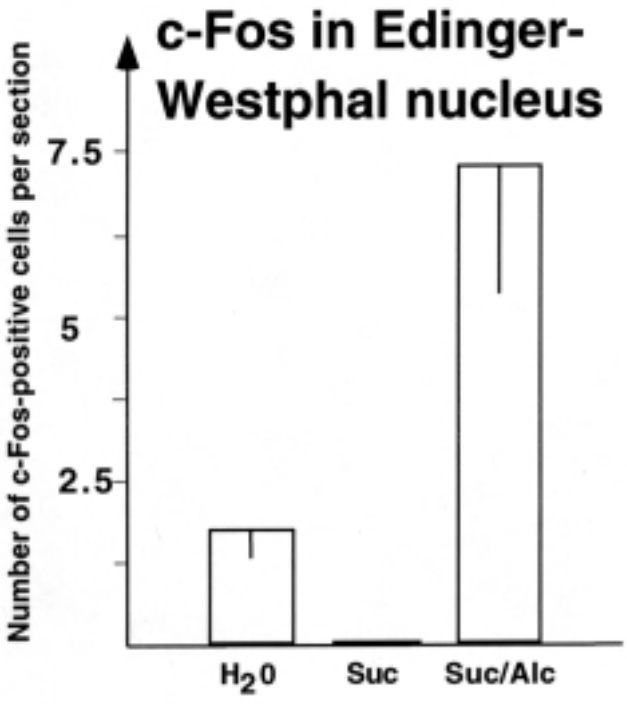

Fig. 4. Expression of c-Fos in the Edinger-Westphal nucleus of C57BL/6J mice self-administering water, sucrose, or sucrose/alcohol. Note higher levels of c-Fos expression in sucrose/alcohol-drinking mice (Suc/Alc group). Graph based on reference (Bachtell et al. 1999).

Taken together with previous alcohol-related studies, these findings allow us to classify brain ethanol-responsive regions in following four categories:

1. Brain regions strongly responsive to ethanol administration *via* any route, and hence presumably responsive to pharmacological effects of this drug. The Edinger-Westphal nucleus is the only brain region showing such response. This is in agreement with a recent study showing that Edinger-Westphal is the only nucleus showing increased c-Fos expression after self-administration of alcoholic beer in rats (Topple et al. 1998). This brain region is traditionally regarded as an oculomotor nucleus (Trimarchi 1992), and finding it in this category is surprising. However, recent discoveries showing that this nucleus is the primary site of expression of urocortin, the alternative CRF-receptor ligand, suggest that this nucleus could play an undefined role in stress responses (Vaughan et al. 1995, Weninger et al. 1999).

2. Brain regions showing less robust changes in ITF expression, which could be dependent on stress (or environmental novelty). The core of nucleus accumbens (in which stress can increase ethanol-induced c-Fos expression) and hippocampal areas (in which alcohol can suppress experience-mediated induction of c-Fos and Egr1) are among these regions.

3. Brain regions showing ITF expression only after alcohol self-administration, and not after administration of ethanol *via* other routes. The medial portion of the central nucleus of amygdala, is one such structure. Finding FosB expression in both alcohol/sucrose- and sucrose-drinking animals complicates interpretation of the role of this structure in response to alcohol, and suggests that this structure could be involved in consumatory responses.

4. A large number of brain regions responding to alcohol injections, but not to alcohol self-administration. It seems quite likely that these brain regions are responding to the stress of "involuntary" administration of this drug. Interestingly, these brain regions include the lateral portion of the central nucleus of amygdala, previously regarded as the "hallmark" of alcohol-responsive areas.

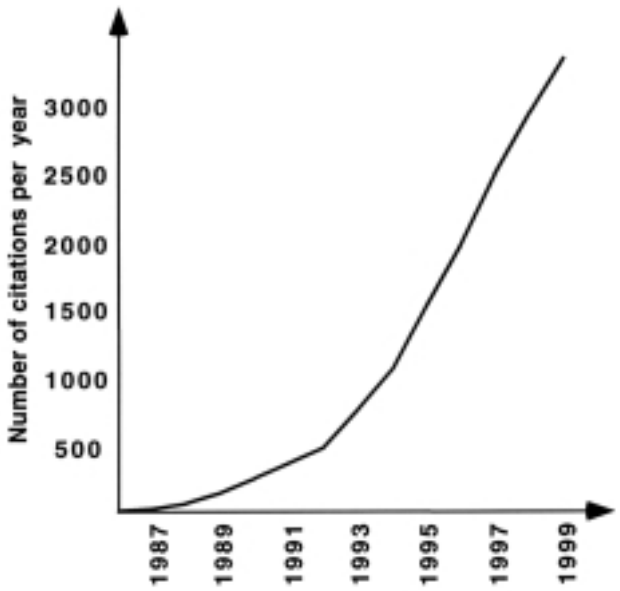

Fig. 5. Growth rate of publications containing words "fos" and "brain" in their title or abstract (based on National Center for Biotechnology Information PubMed database search performed in February, 22, 2000).

CONCLUDING REMARKS

The number of publications using ITF mapping methodology enjoys a steady growth (Fig. 5). According to the National Center for Biotechnology Information PubMed database beginning with 1995 one and a half percent of all new publications mentioning the word "brain" in the abstract, also mention the word "fos". As we grow accustomed to ITF mapping as a conventional methodology in neuroscience, the following considerations have to be kept in mind in analyses of drug-induced expression.

First, individual ITFs can have different profiles of induction. It is beneficial to simultaneously study changes in expression of several ITFs to not miss changes in neuronal activity of a brain structure. This notion has been stressed in ITF literature numerous times previously (cf. Herdegen and Leah 1998).

Second, ITF expression may be insensitive to the pharmacological effects of a drug. It is possible that ITF expression after drug administration is more sensitive to secondary (perceptional and locomotor) effects of a drug than to their pharmacological effects, which may lead to inaccurate assessment of drug-induced changes.

Third, when secondary effects of the drug are eliminated, the relevant changes in ITF expression after drug administration can be occurring in very small neuronal structures, perhaps just in several specific neurons. The typical continuation of ITF mapping study is to manipulate the identified brain region with invasive techniques (by lesions or microinjections) to reveal causal relation of its activity to the function of interest. However, if the identified region is small, the manipulation without perturbing adjacent brain areas is challenging. It seems that ITF mapping studies with appropriate controls will have a long future until the invasive techniques sufficiently increase their resolution.

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