ELSEVIER

European Journal of Pharmacology 287 (1995) 35–41

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Brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 maintain functional tolerance to ethanol

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Received 20 July 1995; accepted 28 July 1995

Abstract

Neurotrophins and growth factors not only affect neuronal development, but also maintain neuronal survival and influence neuronal function in the adult brain, and affect various cognitive processes related to learning and memory. Functional tolerance to ethanol represents an adaptive change in the central nervous system that has been hypothesized to have mechanisms in common with those underlying learning or memory. In the present work, the effects of neurotrophins on ethanol tolerance were compared to the effect of the neuropeptide, arginine vasopressin, which maintains (reduces the rate of dissipation of) both ethanol tolerance and memory. Functional tolerance to ethanol was induced in C57BL/6J mice by feeding them an ethanol-containing liquid diet, and the effect of neurotrophins on the rate of dissipation of tolerance to the hypnotic effect of ethanol was assessed. Human recombinant brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5, injected intracerebroventricularly once daily following ethanol withdrawal, maintained ethanol tolerance, while tolerance dissipated in ethanol-fed mice injected with vehicle (artificial cerebrospinal fluid) or with basic fibroblast growth factor. The results demonstrate that some neurotrophins can modulate neuroadaptation to ethanol, supporting the hypothesis that these factors can influence the function of postmitotic neurons in the adult brain.

Keywords: Ethanol tolerance; BDNF (brain-derived neurotrophic factor); Neurotrophin-3; Neurotrophin-4/5

1. Introduction

A number of soluble proteins influence the developmental growth, differentiation and survival of neuronal populations. The best characterized of these neurotrophic factors (neurotrophins) is nerve growth factor (NGF), which affects peripheral sympathetic and sensory neurons as well as central cholinergic neurons (Thoenen et al., 1987). A family of related neurotrophins has been cloned which includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (also known as hippocampal-derived neurotrophic factor) and neurotrophin-4/5 (Leibrock et al., 1989; Maisonpierre et al., 1990; Hallböök et al., 1991; Berkemeier et al., 1991). Neuronal survival and growth can also be influenced by factors that are thought to primarily target non-neuronal cells. These include fibroblast growth factors (FGF) and epidermal growth factor (EGF) (Cheng and Mattson, 1991; Patterson and Nawa, 1993).

While the role of neurotrophins and growth factors in the development and differentiation of embryonic or post-embryonic neurons has been well studied (e.g., see Patterson and Nawa, 1993; Klein, 1994), there is increasing evidence that these factors can also influence the function (e.g., neurotransmitter turnover) (Martin-Iverson et al., 1994) and enhance the survival (Chadi et al., 1993) of neurons in the adult brain. These actions, as well as the ability of the factors to influence gene expression in neurons (Nawa et al., 1993), and the widespread expression of brain-derived neurotrophic factor and the other neurotrophins, and their receptors, in the adult brain (Maisonpierre et al., 1990; Hofer et al., 1990; Ip et al., 1992; Merlio et al., 1992), suggest a potential role for the neurotrophins and

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SSDI 0014-2999(95)00466-1

growth factors in modulating cognitive functions. In fact, there is evidence that human nerve growth factor can improve the performance of aged rats in a test of recent memory (delayed alternation in a T maze) (Markowska et al., 1994), that nerve growth factor can ameliorate deficits in retention of memory in lesioned rats (Garofalo and Cuello, 1994) and that administration of antibodies to nerve growth factor can interfere with cognitive processes (Nabeshima et al., 1991). These results support the possibility that neurotrophins, perhaps by maintaining or enhancing neuronal integrity, can influence processes related to learning or memory in the adult brain.

We and others have argued that functional tolerance to ethanol and other drugs (i.e., the acquired resistance of the central nervous system (CNS) to the effects of the drug) may have underlying mechanisms in common with learning or memory, since both phenomena represent adaptations of the CNS to external stimuli (LeBlanc and Cappell, 1977; Hoffman et al., 1978). It has previously been shown that the neuropeptide, arginine vasopressin, has effects on tolerance that are similar to its effects on memory processes. That is, vasopressin, administered to animals that have acquired functional ethanol tolerance, will maintain that tolerance (reduce the rate of tolerance dissipation) (Hoffman et al., 1978; Szabó et al., 1988). Similarly, vasopressin inhibits the extinction of an active avoidance response in rats (maintains the learned avoidance behavior), which has been suggested to represent an effect on the consolidation of memory (De Wied and Bohus, 1966). Based on the actions of the neurotrophins to maintain the survival and affect the functional capacities of adult neurons, and also to influence cognitive processes in adult animals, in the present study we evaluated the ability of these compounds to maintain functional tolerance to the hypnotic effect of ethanol in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (22–25 g) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and housed ten per cage under controlled environmental and lighting conditions (12-h light/dark circle) in an AAALAC-accredited facility for at least 1 week, with food and water available ad libitum, before being used in experiments.

2.2. Reagents

Human recombinant brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 were provided by Genentech (South San Francisco, CA, USA). Basic fibroblast growth factor (basic FGF) was obtained from Boeringher-Mannheim, Mannheim, Germany and arginine vasopressin (vasopressin) was from Bachem, Torrance, CA, USA.

2.3. Determination of the effects of vasopressin and neurotrophins on ethanol tolerance

Measurements of the effect of vasopressin and the neurotrophins on the rate of loss of ethanol tolerance were performed as previously described (Szabó et al., 1988). Mice were implanted with a cannula in the lateral cerebral ventricle under pentobarbital anesthesia (75 mg/kg) and then housed individually. After a 3-day recovery period, the animals were fed a liquid diet for 5 days. Experimental animals received liquid diet composed of chocolate-flavored Carnation Slender (Carnation Corporation, Los Angeles, CA, USA), vitamin supplement (3 g/l diet; ICN, Cleveland, OH, USA) and 7% (v/v) ethanol (Ritzmann and Tabakoff, 1976). Control animals were pair-fed a liquid diet in which sucrose equicalorically replaced the ethanol (Ritzmann and Tabakoff, 1976). At the end of the 5-day ethanol ingestion period (i.e., on the morning of the sixth day), all animals were given the control liquid diet (withdrawal). This 5-day ethanol treatment regimen produces mild ethanol withdrawal signs in the ethanol-fed mice upon cessation of ethanol intake, indicating that physical dependence on ethanol has not yet developed maximally (Ritzmann and Tabakoff, 1976). At 24 h after withdrawal, when ethanol had been eliminated, mice were tested for tolerance to the hypnotic effect of ethanol by determination of the duration of loss of righting reflex after intraperitoneal (i.p.) injection of a challenge dose of 3.2 g/kg of ethanol (21% solution, 20 ml/kg). Tolerance testing was carried out between 9:00 a.m. and noon. After the initial tolerance test, groups of control and ethanol-fed mice were subdivided into groups that received intracerebroventricular (i.c.v.) injections of protein or vehicle, which was artificial cerebrospinal fluid (CSF) (Szabó et al., 1988). Daily injections, in a volume of 2 μl, were given between 4:00 and 5:00 p.m., until the fifth day after withdrawal. The dose of vasopressin was 1 ng (Szabó et al., 1988), and the dose of neurotrophins or basic FGF was 100 ng. Tolerance was again tested on the mornings of days 3, 6 and 10 after withdrawal (Szabó et al., 1988). This procedure allowed us to test the effects of neurotrophins and basic FGF not only on the rate of dissipation of ethanol tolerance, but also on the acute response to ethanol, as measured in control mice that had not ingested ethanol chronically. These latter data were used to determine if the neurotrophin treatments acutely interfered with the measurements of tolerance, for example, by altering the metabolism of the challenge dose of ethanol.

After all studies, the placement of cannulas was determined by injection of 10 μl of methylene blue and examination of the brain. Data from animals with improper cannula placement were excluded from the analysis.

2.4. Statistical analysis

Statistical analysis was carried out with the SAS program (SAS, 1986). One-way analysis of variance (ANOVA) was used to determine the differences among treatment groups on a given day after withdrawal. Post-hoc comparisons among the groups were made using Tukey's test. Values of $P < 0.05$ were considered to be significant.

3. Results

The response of control mice (mice fed control liquid diet) to a challenge dose of ethanol was not affected by prior treatment with any of the proteins tested (Fig. 1). Furthermore, the responses of mice fed the control liquid diet and given daily injections of neurotrophins, basic FGF or vasopressin did not differ significantly on any day after withdrawal from the responses of the control mice that received daily injections of artificial CSF vehicle (ANOVA, $P < 0.1$). Therefore, responses of ethanol-fed mice were compared to those of control mice that received vehicle injections (see Figs. 2 and 3). The response to the challenge dose of ethanol of the mice fed control liquid diet also did not change significantly over the 4 test days (days 1, 3, 6 and 10 after withdrawal) (Figs. 2 and 3; ANOVA, $P < 0.08$).

As previously reported (Szabó et al., 1988; Ritzmann and Tabakoff, 1976), mice developed maximal tolerance to the hypnotic effect of ethanol after 5 days of ingestion of the ethanol-containing diet (Figs. 2 and 3). In ethanol-tolerant mice treated daily after withdrawal with i.c.v. injections of vehicle, tolerance dissipated by 6 days after withdrawal (i.e., the response of the ethanol-fed mice that were treated with vehicle was no longer significantly different from the response of the control group on day 6 after withdrawal). Treatment with vasopressin, which was used as a positive control in these experiments, maintained tolerance to ethanol: as shown in Figs. 2 and 3, the response of the ethanol-fed mice treated with vasopressin was significantly lower than the response of the control group (and also significantly lower than the response of the ethanol-vehicle group) on days 3 and 6 after withdrawal (i.e., the vasopressin-treated mice were still tolerant on the sixth day after withdrawal). Five days after termination of vasopressin treatment (10 days after withdrawal), tolerance had completely dissipated

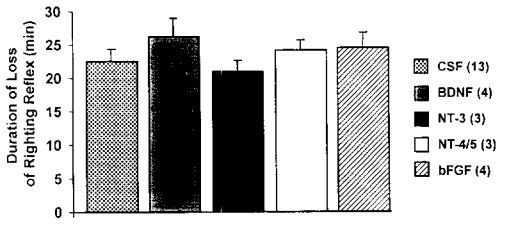

Fig. 1. Effect of neurotrophins and basic fibroblast growth factor (bFGF) on the acute response to a challenge dose of ethanol. Control C57BL/6J mice that had ingested liquid diet containing sucrose for 5 days were injected i.c.v. (through previously implanted cannulas; see Materials and methods) with vehicle (artificial cerebrospinal fluid (CSF)) or 100 ng of the indicated protein (brain-derived neurotrophic factor, BDNF; neurotrophin-3, NT-3; neurotrophin-4/5, NT-4/5) between 4:00 and 5:00 p.m. on days 1 and 2 after withdrawal. The next morning (9:00 a.m.–noon) the mice were injected i.p. with 3.2 g/kg ethanol, and duration of loss of righting reflex was measured as described in the text. Values represent mean ± S.E.M. from the number of mice in parentheses. There was no significant difference among the responses (ANOVA, $P < 0.1$).

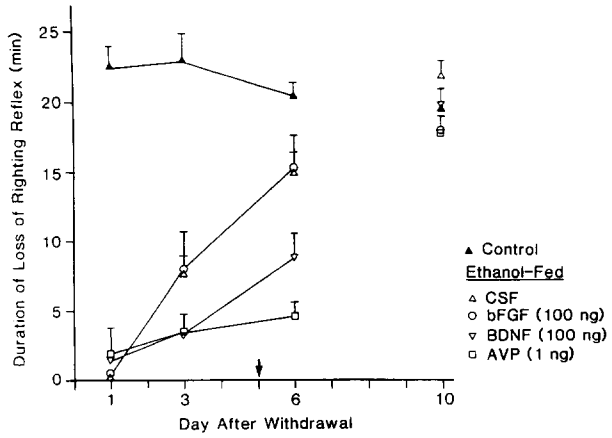

Fig. 2. Effects of brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) on the rate of dissipation of ethanol tolerance. Male C57BL/6J mice were fed ethanol in a liquid diet (open symbols) or were pair-fed control liquid diet (closed symbols) for 5 days. At 24 h after ethanol withdrawal, control and ethanol-fed mice were tested for tolerance by measuring the duration of loss of righting reflex after a challenge dose of 3.2 g/kg of ethanol. Control and ethanol-fed mice were then subdivided into groups that received once-daily i.c.v. injections of vehicle (CSF), 100 ng of basic fibroblast growth factor (bFGF) or brain-derived neurotrophic factor (BDNF), or 1 ng of arginine vasopressin (AVP) for 5 days. The arrow (↓) denotes termination of vasopressin or neurotrophin treatments. Tolerance was tested again on days 3, 6 and 10 after withdrawal. Values represent mean ± S.E.M. from two separate experiments, and from 8–11 mice in each group. Protein treatments did not significantly affect the response of control mice to ethanol, and values shown are for control mice treated with vehicle (CSF) (▲). Responses of the ethanol-fed mice in all groups are different from that of controls on days 1 and 3 after withdrawal; responses of ethanol-fed mice treated with brain-derived neurotrophic factor (BDNF) (△) or vasopressin (AVP) (□), but not with vehicle (CSF) (▽) or basic fibroblast growth factor (bFGF) (○), were different from that of controls on day 6 after withdrawal ($P < 0.05$, ANOVA and Tukey test).

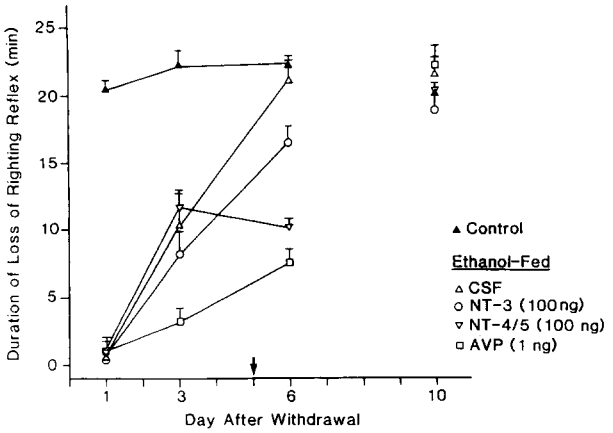

Fig. 3. Effects of neurotrophin-3 and neutrophin-4/5 on the rate of dissipation of ethanol tolerance. Male C57BL/6J mice were fed ethanol in a liquid diet, or control liquid diet, for 5 days, treated with 100 ng of the indicated neurotrophins (neurotrophin-3, NT-3; neurotrophin-4/5, NT-4/5) or 1 ng of vasopressin (AVP) i.c.v. for 5 days after ethanol withdrawal, and tested for tolerance to the hypnotic effect of ethanol as described in the text and legend to Fig. 1. The arrow (↓) denotes termination of treatments with vasopressin or the neurotrophins. Values represent mean ± S.E.M. from two separate experiments, with a total of 4–14 mice in each group. Neurotrophin treatment did not affect the response of control mice to ethanol, and values for control mice treated with vehicle (CSF) (▲) were used for comparisons. On the first and third days after ethanol withdrawal, the response of all ethanol-fed groups was significantly different from that of the control group; on the sixth day after withdrawal, the responses of the ethanol-fed mice treated with neurotrophin-3 (NT-3) (○), neurotrophin-4/5 (NT-4/5) (▽) or vasopressin (□) were still significantly different from that of the control group ($P < 0.05$, ANOVA and Tukey test), while the response of the ethanol-fed mice treated with vehicle (CSF) (△) was not.

in the ethanol-fed, vasopressin-treated mice (no difference between control, ethanol-vehicle and ethanol-vasopressin groups), in agreement with previous studies (Szabó et al., 1988).

The results depicted in Fig. 2 demonstrate that treatment of ethanol-fed mice with basic FGF did not maintain tolerance. The response of these mice was not different from that of ethanol-fed, vehicle-treated mice at either 3 or 6 days after withdrawal, and was no longer different from that of controls at 6 days after withdrawal. However, treatment with brain-derived neurotrophic factor (BDNF), similar to treatment with vasopressin, reduced the rate of tolerance dissipation (maintained functional ethanol tolerance). The response of the ethanol-fed, BDNF-treated mice remained significantly different from that of control mice on day 6 after withdrawal, i.e., the ethanol-fed, BDNF-treated mice were still tolerant to ethanol at a time when ethanol-fed, vehicle-treated mice had lost tolerance. In preliminary studies, we found that daily treatment of mice with 10 ng of brain-derived neurotrophic factor did not maintain tolerance (data not shown), suggesting a steep or an 'all-or-none' dose-response relationship for brain-derived neurotrophic factor. The data presented in Fig. 3 illustrate that both neurotrophin-3 and neurotrophin-4/5 also maintained ethanol tolerance: on the sixth day after withdrawal, the response of the groups of mice treated with these neurotrophins was still significantly lower than the response of control mice on the sixth day after withdrawal. However, neurotrophin-3 appeared to be less effective than neurotrophin-4/5 in maintaining tolerance: on the sixth day after withdrawal, the response of the ethanol-fed mice treated with neurotrophin-3, in contrast to the responses of ethanol-fed mice treated with neurotrophin-4/5 or vasopressin, was not significantly different from the response of the ethanol-fed mice treated with vehicle. The pattern of tolerance dissipation was somewhat different in the ethanol-fed, neurotrophin-4/5-treated mice than in the other groups: in two separate experiments, the responses of these animals did not change between days 3 and 6 after withdrawal, while the responses of the mice in the other groups increased toward control levels over this time (Fig. 3).

4. Discussion

Tolerance to ethanol is most simply defined as an acquired resistance to the effects of the drug. However, there are several forms of tolerance, which may have differing underlying mechanisms (for review, see Tabakoff et al., 1982). A basic distinction is that between pharmacokinetic (metabolic, dispositional) (Cederbaum et al., 1977) and pharmacodynamic (functional) ethanol tolerance (Tabakoff et al., 1986). The former results from a change in the metabolism or distribution of ethanol, such that the organism is exposed to a lower level of the drug after administration of a given dose, while the latter reflects an alteration at the cellular level in the central nervous system that renders the animal resistant to the effects of ethanol. The tolerance produced by the liquid diet paradigm used in the present study is functional (Ritzmann and Tabakoff, 1976).

In addition to the drug itself, both environmental and behavioral variables can influence ethanol tolerance. Under certain experimental conditions, for example, ethanol tolerance can be demonstrated only in the presence of cues associated with drug administration (Lê et al., 1979; Melchior and Tabakoff, 1981). This type of tolerance has been called 'environment-dependent' or associative tolerance (Melchior and Tabakoff, 1981; Poulos and Cappell, 1991), and it can be considered a form of Pavlovian conditioning (Poulos and Cappell, 1991). Under other conditions, which include high doses of drug and short inter-drug intervals, the tolerance that occurs is 'environment-independent' or non-associative (Melchior and Tabakoff, 1981; Poulos

and Cappell, 1991), and can be demonstrated regardless of the environments in which drug administration and testing take place. The liquid diet method used in the present study produces non-associative tolerance, one of the hallmarks of which is rapid dissipation after cessation of drug administration (Poulos and Cappell, 1991). In the homeostatic theory proposed by Poulos and Cappell (1991), all tolerance is contingent on the detection of a drug-induced disturbance, which is required for the generation of adaptive, homeostatic responses. The loss or dissipation of both associative and non-associative forms of tolerance requires a counteradaptation, i.e., the original adaptive response, in the absence of continued drug administration, presents a new functional disturbance to the organism which requires an adaptation to return to the initial state.

These behavioral considerations provide a framework for proposing CNS mechanisms which may underlie the development or dissipation of functional ethanol tolerance. Although non-associative tolerance is not tied to cues, and is not governed by learning as is associative tolerance (Poulos and Cappell, 1991), it is still determined by an adaptation of the CNS which, like learning, may be hypothesized to depend on changes in synaptic efficacy. Numerous biochemical manipulations have been shown to have similar effects on learning and on functional tolerance to ethanol or other drugs (see LeBlanc and Cappell, 1977), including the administration of the neuropeptide, arginine vasopressin. This peptide not only inhibits the extinction of an active avoidance response (De Wied and Bohus, 1966), but also inhibits the dissipation of functional ethanol tolerance in mice (Hoffman et al., 1978; Szabó et al., 1988) and rats (Lê et al., 1982). If these actions are placed within the framework described above, vasopressin can be viewed either as maintaining the adaptation that is produced in response to chronic ethanol ingestion, or preventing the counteradaptation that normally occurs when ethanol administration is stopped. In either case, influences on synaptic plasticity may be involved.

As described in the Introduction, the rationale for investigating the effects of neurotrophins on the maintenance of ethanol tolerance derives from their well-described effects on neuronal survival and function in the adult, as well as the developing brain, and their widespread localization in adult brain. The regulation of neurotrophin expression by neurotransmitters (Wetmore et al., 1994) also supports the possibility of an autocrine function for these proteins. This proposed action in adult brain is consistent with the present findings that brain-derived neurotrophic factor and the structurally related neurotrophin-3 and neurotrophin-4/5 can maintain ethanol tolerance in mice. Thus, chronic ethanol exposure, in conjunction with neuronal activity (Tabakoff and Ritzmann, 1977), produces an altered synaptic efficacy that is expressed as functional tolerance. One may speculate that the neurotrophins, by maintaining the integrity of these changes, can reduce the rate of loss of tolerance.

As mentioned above, the tolerance generated in this study is functional. It is possible that administration of the effective neurotrophins could acutely alter ethanol metabolism, and therefore appear to prolong functional tolerance by changing the response to the challenge dose of ethanol during tolerance testing. However, if that were the case, there would also be changes in the response to the challenge dose of ethanol in control animals (those that did not ingest ethanol chronically) that were treated with the neurotrophins. Since the ethanol response of these animals was not affected, it can be concluded that the neurotrophins (under the conditions of this study) do not influence ethanol metabolism.

The neurotrophins that are effective in maintaining tolerance are structurally related members of a family of which nerve growth factor is the prototype (see Klein, 1994). We had previously found, however, that nerve growth factor, at the same dose as that used in the present study, had only a marginal effect on the maintenance of tolerance (Szabó et al., 1991). Basic FGF, a member of the family of heparin-binding growth factors that is present in brain (Pettmann et al., 1986), and that has neurotrophic influences on a wide variety of neurons (Westermann et al., 1990), also did not maintain tolerance. Among the factors that could contribute to the differential ability of the various neurotrophins to affect ethanol tolerance is their localization in the brain. The mRNA for brain-derived neurotrophic factor, for example, is widespread in the adult rodent brain (Hofer et al., 1990), while that for basic FGF is primarily found in the hippocampus (Emoto et al., 1989). There is less information available regarding regional distribution of neurotrophin-3 and neurotrophin-4/5 in the adult brain. Other possible contributors to the differential effects of neurotrophins on tolerance are their receptor specificity and the localization of the receptors. Fibroblast growth factors use a dual receptor system to activate signal transduction pathways. The primary receptor component of this system is a protein tyrosine kinase, while the second component consists of heparin-related molecules that are required for activation of the receptor (Ornitz et al., 1995). The mRNA for the fibroblast growth factor receptor is widely expressed in the CNS (Wanaka et al., 1990). The receptors for the members of the nerve growth factor family of neurotrophins are more closely related to one another. An essential component of the high-affinity nerve growth factor receptor is the protein tyrosine kinase product of the proto-oncogene *trk*, while the products of two other structurally related genes, *trkB* and *trkC*, are essential components of the

high-affinity receptors for brain-derived neurotrophic factor and neurotrophin-3, respectively (see Merlio et al., 1992). Neurotrophin-3 also binds to the TrkB and Trk receptors and neurotrophin-4/5 can elicit phosphorylation of both Trk and TrkB (Berkemeier et al., 1991), while nerve growth factor does not appear to interact with either TrkB or TrkC (Klein et al., 1991; Lamballe et al., 1991). Thus, a common element among the neurotrophins that can affect ethanol tolerance is their ability to activate the TrkB receptor. This receptor, like TrkC, but unlike Trk, has a widespread distribution in rat brain (Merlio et al., 1992), and its presence in the adult brain is consistent with mediation of the observed effects of brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 on tolerance. The finding that neurotrophin-3 appeared to be somewhat less effective than brain-derived neurotrophic factor or neurotrophin-4/5 at maintaining tolerance could reflect the relative affinities or efficacies of these compounds at the TrkB receptor. However, more complete dose-response studies are needed to evaluate the relative potencies and efficacies of the various neurotrophins to maintain tolerance.

The similarity in the actions of vasopressin and neurotrophins in maintaining ethanol tolerance suggests the possibility of sequential or parallel actions of these compounds on the maintenance of tolerance. In particular, we have shown that a vasopressin-induced increase in expression of c-*fos* in the mouse septum may be related to the ability of vasopressin to maintain tolerance (Giri et al., 1990; Szabó et al., 1991). There is also evidence that the neurotrophins can increase c-*fos* expression in various cells (Ip et al., 1993; Giri et al., 1990). Depending on the localization of this effect in brain, such an increase could be directly involved in effects on ethanol tolerance. It is of interest that the dose of vasopressin needed to maintain ethanol tolerance was lower than the dose of neurotrophins (approximately 20-fold lower, on a molar basis). This difference could result from factors such as pharmacokinetic parameters, receptor affinities, or signal transduction pathways that are involved in tolerance to the hypnotic effect of ethanol.

In summary, these studies are the first to demonstrate that neurotrophins can influence an adaptive response to ethanol in the adult brain. The results support the view that neurotrophins can act not only as differentiation or growth factors, but also can modulate the function of postmitotic neurons, possibly by altering gene expression, and produce significant behavioral consequences.

Acknowledgements

This work was supported in part by the National Institute on Alcohol Abuse and Alcoholism, NIH (AA 9086). Recombinant human brain-derived neurotrophic factor, neurotrophin-3 and NT-4/5 were supplied by Genentech, Inc., South San Francisco, CA, USA.

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