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Resistance to ethanol sensitization is associated with increased NMDA receptor binding in specific brain areas

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Abstract

Co-administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists is known to block the development of behavioral sensitization to ethanol and other psychostimulants. Since ethanol sensitization in mice does not occur uniformly in all treated animals, the present study examined the possibility that NMDA receptor binding would be selectively altered in mice susceptible to ethanol sensitization. Mice received 2.4 g/kg ethanol or saline i.p. daily for 21 days and were sacrificed 24 h later. No differences in [^{3}H]dizocilpine ([^{3}H](+)MK-801) binding were found between sensitized and vehicle-treated mice in any of the brain regions analyzed. However, ethanol-treated mice that did not develop sensitization showed significantly higher binding in the nucleus accumbens core (+32% and +40% compared to controls and ethanol-sensitized mice, respectively; $P<0.04$) and the prefrontal cortex (+15% and +22%; $P<0.02$). In a separate experiment, sensitization resistant mice challenged with 0.25 mg/kg (+)MK 801 showed significantly less motor activation than saline-treated or ethanol-sensitized mice. These results point to a clear association between elevated NMDA receptor binding in specific brain regions and resistance to ethanol sensitization.

Keywords: [^{3}H](+)MK-801; Glutamate; Nucleus accumbens; Cortex, prefrontal

1. Introduction

Behavioral sensitization, the potentiation of a drug's behavioural effects after repeated exposure, has been hypothesized to be an important factor in drug craving in general (Robinson and Berridge, 1993). In the case of ethanol, sensitization processes may contribute to the dependence and withdrawal syndromes (Eckardt et al., 1998; Lessov and Phillips, 1998), and individual propensity to develop sensitization to ethanol has been suggested as a screening tool for ethanol abuse liability (Hunt and Lands, 1992; Newlin and Thomson, 1991).

Ethanol sensitization does not occur in every mouse strain (Masur et al., 1986; Masur and Santos, 1988; Phillips et al., 1991). Further, within susceptible strains, there is considerable individual variability, such that some mice receiving chronic ethanol are likely to develop clear locomotor sensitization, whereas other similarly treated littermates will show little or no sensitization (Masur and Santos, 1988; Souza-Formigoni et al., 1999). This phenomenon provides a convenient paradigm to investigate neurochemical substrates of differential susceptibility to sensitization, without resorting to selective breeding or other manipulations. Using this paradigm, recent work from this laboratory has shown that mice showing high sensitization to ethanol have higher levels of dopamine D2 receptor binding in the ventral striatum in comparison to either non-sensitized ethanol-treated or vehicle-treated animals (Souza-Formigoni et al., 1999).

While dopaminergic systems remain the focus of much of the sensitization literature, increasing attention has been given to the role of glutamatergic neurotransmission in mechanisms of action of drugs of abuse, including ethanol (Kalivas, 1995; Koob et al., 1998; Wang and McGinty, 1999; Wolf, 1998). Glutamatergic systems may be involved in the behavioral and physiological effects of ethanol by

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mediating neuroplastic changes (Blitzer et al., 1990) and/or by interacting with dopaminergic systems mediating the reinforcing properties of ethanol (Hodge and Cox, 1998; Rassnick et al., 1992; Wang and McGinty, 1999).

Ethanol has strong inhibitory effects on *N*-methyl-D-aspartate (NMDA) receptor function (Wirkner et al., 1999), including NMDA receptor-mediated electrophysiological responses and ionic fluxes (Dildy and Leslie, 1989; Hoffman et al., 1989; Lovinger et al., 1989; Simson et al., 1993; Takadera et al., 1990). Chronic ethanol treatment increases NMDA receptor binding (Grant et al., 1990; Gulya et al., 1991; Hu and Ticku, 1995; but see also Tremwel et al., 1994; Ulrichsen et al., 1996), increases NMDA subunit expression (Kalluri et al., 1998; Trevisan et al., 1994) and potentiates a number of NMDA receptor-mediated responses including neurotoxic effects (Davidson et al., 1993; Tsai and Coyle, 1998). These effects are commonly regarded as compensatory responses to inhibition of NMDA receptors by ethanol. Accordingly, various ethanol withdrawal effects are antagonized by NMDA receptor blockers and are exacerbated by NMDA receptor agonists (Danysz et al., 1992; Grant et al., 1990; Morrisett et al., 1990).

NMDA receptor antagonists such as dizocilpine ((+)MK-801) acutely mimic some of the effects of ethanol, including a dose-dependent biphasic effect on locomotor activity (Danysz et al., 1992). (+)MK-801 fully substitutes for ethanol in a drug discrimination paradigm, when injected in the nucleus accumbens core or the CA1 region of the hippocampus (Hodge and Cox, 1998). On the other hand, (+)MK-801 blocks ethanol conditioned place preference (Biala and Kotlinska, 1999) and ethanol self-administration (Rassnick et al., 1992), suggesting that (+)MK-801 may antagonize ethanol's reinforcing properties. Likewise, prior administration of (+)MK-801 blocks ethanol's acute locomotor stimulatory effects as well as behavioral sensitization to the drug (Broadbent and Weitemier, 1999; Camarini et al., 2000). Indeed (+)MK-801 and other NMDA receptor blockers are able to prevent the development of behavioral sensitization to a wide variety of drugs. This prompted us to test the hypothesis that binding of [^{3}H](+)MK-801 to the channel site of the NMDA receptor–ionophore complex would be selectively altered in brains of mice showing an increased propensity to develop ethanol sensitization. We performed these analyses in brain material from the same animals where we recently identified selective changes in [^{3}H]raclopride binding to dopamine D2 receptors (Souza-Formigoni et al., 1999).

2. Methods

2.1. Animals

Male mice from the UNIFESP colony, originally derived from the Swiss Webster line, were housed in groups of 10 in plastic cages, with free access to food and water. They were kept in a temperature-controlled colony room (22 ± 1 °C), with lights on between 0700 and 1900 h. Mice were approximately 60 days old at the beginning of the experiment. All animal procedures were carried out in accordance with the National Institutes of Health (NIH) *Principles of Laboratory Animal Care* (1985).

2.2. Behavioral tests

Animals were individually tested in Opto-Varimex activity cages (Columbus Instruments, Columbus, OH) which detect locomotion by interruptions of horizontal photoelectric beams. Animals were initially subjected to a 15-min session, without any drug treatment. Two groups, equated in terms of basal activity scores, were then assigned to daily ethanol (2.4 g/kg, 15% w/v in 0.9% NaCl, given i.p., $n = 24$) or saline treatments ($n = 10$), which started 1 week after the basal test. Locomotor activity was measured for 15 min immediately after the injection on days 1, 7, 14 and 21. Daily injections of ethanol or saline were given in the colony room. On test days, animals were taken to a separate room and allowed at least 1 h before injections and activity testing. All procedures were carried out in the afternoon (between 1300 and 1700 h).

2.3. Classification

Animals treated with ethanol, as a group, showed a progressive increase in locomotor activity over the 21 days. As previously described, however, within-group variability was high (Souza-Formigoni et al., 1999). Ethanol-treated mice with activity scores in the upper 33% of the distribution on day 21 were classified as "Sensitized" ($n = 7$), whereas those in the lower 33% were classified as "Non-sensitized" ($n = 7$). Animals classified as sensitized or non-sensitized on day 21 were also found to have been in the upper or lower 30% of the distribution on days 7 and 14, with only two exceptions.

2.4. Autoradiography

Fourteen ethanol-treated and 8 randomly selected saline-treated mice were sacrificed 24 h after the last activity test. Brains were quickly removed, frozen over dry ice, and stored at − 80 °C. Serial 20-μm coronal sections were cut on a Hacker–Bright cryostat at − 20 °C and collected onto glass slides at 0.3-mm intervals, through the longitudinal extent of the brain. Slides were returned to − 80 °C storage until the day of the assays. [^{3}H](+)MK-801 autoradiographic assays followed the procedures of Sakurai et al. (1991) with minor modifications. Briefly, slices were brought to room temperature and then pre-incubated in 50 mM Tris-acetate buffer (pH = 7.4) for 30 min at 4 °C. Sections were then incubated for 2 h in buffer containing 5 nM [^{3}H](+)MK-801 (20.3 Ci/mmol, NEN Dupont), at 21

°C. An additional set of slides was incubated in the presence of 20 μM (+)MK-801, for determination of nonspecific binding. Sections were then washed in buffer for 60 min at 4 °C and allowed to dry at room temperature. Slides were exposed to [^{3}H]Hyperfilm (Amersham), in tungsten cassettes together with calibrated standards for 4 weeks. Films were developed and densitometric analyses performed using an M2 MCID system (Imaging Research, St. Catharines, Ontario). Anatomical regions were defined according to the Franklin and Paxinos (1997) atlas. For any subject the final binding value for any given brain region represented an average of multiple readings on 3–6 brain sections, performed by an investigator who was unaware of group membership of the samples. One brain from the non-sensitized group was lost during autoradiographic processing, resulting in a final $n=6$ for all behavioral and binding analyses in this group.

2.5. (+)MK-801-induced locomotion after chronic ethanol

In order to ascertain the possible functional significance of altered [^{3}H](+)MK-801 receptor binding, a second experiment was conducted where mice were again subjected to 3 weeks of daily ethanol ($n=36$) or saline ($n=16$) injections and weekly locomotor activity tests, exactly as described above. Twenty-four hours after the last activity test (day 21) all mice were challenged with 0.25 mg/kg (+)MK-801 given i.p. Thirty minutes after the injection, mice received a saline injection and were placed into the activity cages, where locomotor activity was recorded for 30 min. After the test, animals continued to receive daily ethanol or saline injections for another 4 days. On the fifth day, all mice received a saline injection and had their locomotor activity recorded for 15 min.

3. Results

3.1. Locomotor sensitization

Fig. 1 shows the locomotor activity during the course of the 21-day treatment for the three groups (Sensitized, Non-sensitized and Saline). A two-way analysis of variance (ANOVA) with repeated measures revealed a significant effect of Group ($F(2,18)=16.15$, $P<0.0001$), Time ($F(3,54)=23.74$, $P<0.0001$) and the Group × Time interaction ($F(6,54)=14.48$, $P<0.0001$). Separate one-way ANOVAs for each test day revealed significant group differences on days 7, 14, and 21 ($P<0.002$). Duncan's new multiple range tests indicated that ethanol-sensitized animals had higher activity levels than the other two groups on days 7 and 21 ($P<0.035$), while ethanol non-sensitized animals differed from saline controls only on day 14 ($P<0.003$). Within-group analyses confirmed that activity scores for sensitized animals on day 21 were significantly higher than their own scores on every preceding test day ($P<0.015$).

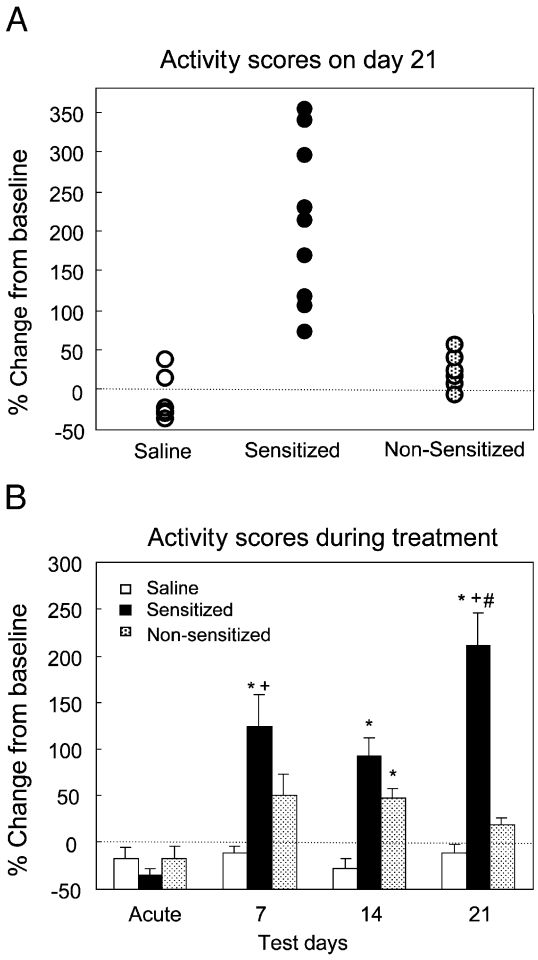

Fig. 1. **Panel A**: Locomotor activity scores at the end of treatment. Each point represents an individual animal. The Sensitized group ($n=7$) was defined as ethanol-treated mice that were in the upper 33% of the distribution of activity scores on day 21, whereas non-sensitized mice were in the lower 33% of the distribution on day 21. Saline controls ($n=8$) did not receive ethanol at any point. Baseline activity counts (mean ± S.E.M.) were as follows: saline group = 1319 ± 105; Sensitized = 1291 ± 128; Non-sensitized = 1172 ± 100. **Panel B**: Group changes in locomotor activity scores in the course of treatment. Bars represent percentage changes between each test day and baseline activity scores for each group (means ± S.E.M.) in 15-min sessions. Only data for animals included in the brain analyses are shown. *: Different from saline group on the same test day ($P<0.003$); +: Different from non-sensitized group ($P<0.035$) on the same test day. #: Different from the group's own score on all other test days ($P<0.015$).

Day 21 scores for the non-sensitized group did not differ from their previous scores on any other test day.

3.2. [^{3}H](+)MK-801 receptor binding

As shown in Table 1, separate ANOVAs indicated significant differences between the three groups in the nucleus accumbens core ($F(2,18)=4.14$, $P<0.04$) and in the prefrontal cortex ($F(2,18)=7.31$, $P<0.005$). Post-hoc comparisons using Duncan's new multiple range test indicated that [^{3}H](+)MK-801 binding levels in the sensitized group were

Table 1
[^{3}H]MK-801 binding after chronic ethanol treatment[a]

	Saline ($n=8$)	Ethanol	
		Sensitized ($n=7$)	Non-sensitized ($n=6$)
Prefrontal cortex	98.7 (3.8)	92.7 (3.3)	113.3 (4.1)[b]
Frontal cortex	112.7 (5.3)	106.4 (5.7)	120.4 (6.3)
Nucleus Accumbens—core	46.3 (3.2)	43.6 (5.2)	61.1 (4.9)[c]
Nucleus Accumbens—shell	52.2 (3.9)	49.2 (5.0)	63.2 (3.9)
Olfactory tubercle	67.1 (4.8)	62.1 (4.7)	75.2 (5.3)
Caudate–putamen			
anterior	45.1 (3.6)	45.1 (6.2)	58.1 (5.4)
dorsomedial	42.5 (3.7)	41.3 (4.0)	48.8 (5.9)
dorsolateral	46.9 (4.0)	45.3 (5.2)	51.8 (6.7)
ventrolateral	61.6 (4.4)	60.7 (4.7)	68.1 (8.1)
posterior	51.0 (4.5)	51.5 (6.9)	56.9 (9.1)

[a] Values are means (S.E.M. in parenthesis), in pmol/g tissue.
[b] $P<0.014$; [c]$P<0.031$ when compared to either saline-treated or sensitized groups.

not different from those in the saline control group. On the other hand, non-sensitized animals had significantly higher [^{3}H](+)MK-801 binding levels in the nucleus accumbens core (+40% compared to ethanol-sensitized animals, and +32% compared to saline controls, $P<0.031$) and in the prefrontal cortex (+22% compared to ethanol-sensitized animals, and +15% compared to saline controls, $P<0.014$). Binding in other brain regions examined (for example, amygdala, thalamus, hippocampus) did not differ among groups.

3.3. (+)MK-801-induced locomotion after chronic ethanol

Fig. 2 shows the locomotor activity effects of an acute 0.25 mg/kg (+)MK-801 challenge in sensitized ($n=10$), non-sensitized ($n=9$) and saline-treated ($n=16$) mice. A one-way ANOVA revealed a significant effect of Group ($F(2,32)=3.99$, $P<0.03$). While ethanol-sensitized and saline-treated mice showed similar locomotor activation after (+)MK-801, the non-sensitized group showed significantly lower activity levels than both sensitized and saline-treated mice ($P<0.03$, Duncan's test). During the saline challenge, there were no differences among the groups ($F(2,32)=3.14$, $P<0.06$), although there was a trend for higher locomotor activity levels in sensitized group. The inset in Fig. 2 shows the locomotor activity during the course of the 21-day treatment for the three groups. Duncan's new multiple range tests indicated that ethanol-sensitized animals had higher activity levels than the other two groups on days 7, 14 and 21 ($P<0.001$).

4. Discussion

The major finding in this study was that while [^{3}H](+)MK-801 binding was unaltered in ethanol-sensitized mice, it was significantly elevated in brains of ethanol-treated mice that failed to develop sensitization after 3 weeks of treatment. The increases were statistically significant in the core of the nucleus accumbens and the prefrontal cortex, both of which are part of neuroanatomical circuits commonly implicated in reward processes, psychomotor stimulation, behavioral sensitization and drug abuse (Pierce and Kalivas, 1997; Wang and McGinty, 1999; Wise and Bozarth, 1987). In a separate experiment sensitization-resistant mice were found to be less sensitive to the locomotor activating effects of systemic (+)MK-801, suggesting that altered receptor binding in brain may indeed

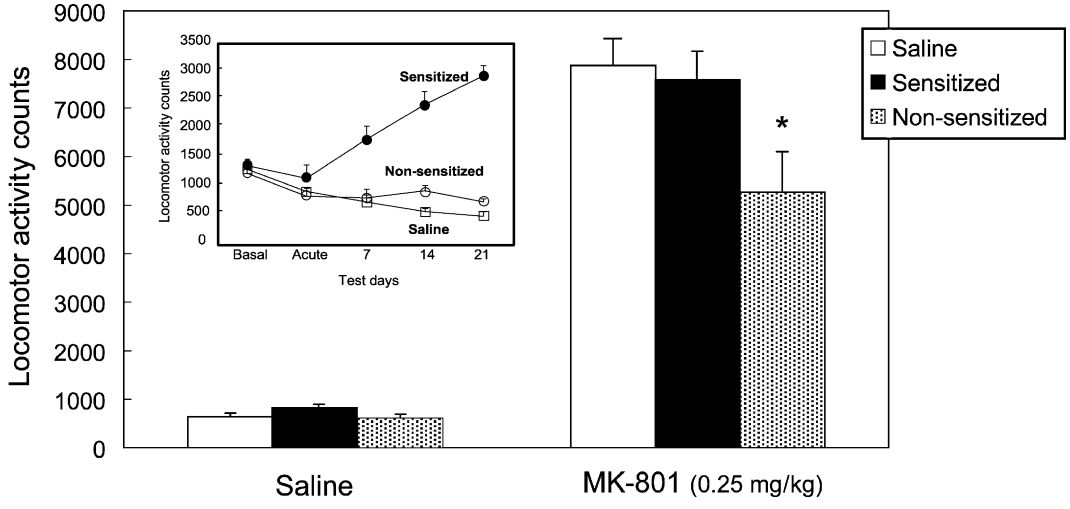

Fig. 2. Locomotor activity effects of an acute MK-801 challenge (0.25 mg/kg, i.p.) in sensitized ($n=10$), non-sensitized ($n=9$) and saline-treated ($n=16$) mice. Bars represent means ± S.E.M. *: The non-sensitized group showed significantly lower activity levels than both sensitized and saline-treated mice ($P<0.03$, Duncan's test). The inset shows locomotor activity during the course of the 21-day treatment for the three groups. Ethanol-sensitized animals had higher activity levels than the other two groups on days 7, 14 and 21 ($P<0.001$), while ethanol non-sensitized animals were not different from saline controls in any of the tests.

have functional correlates in these animals. It is conceivable that receptor supersensitivity in this group may have partly counteracted the behavioural effects of acute receptor blockade induced by (+)MK-801.

In agreement with previous studies in rats (Gulya et al., 1991), the present results indicate that increased NMDA receptor binding after chronic ethanol does not occur uniformly across brain regions. More importantly, the present data demonstrate that such binding increases do not occur uniformly among ethanol-treated animals but are largely restricted to animals that do not develop behavioral sensitization. This type of within-group variability may be a factor in explaining previous discrepancies in the literature concerning NMDA receptor changes after chronic ethanol (Tremwel et al., 1994; Ulrichsen et al., 1996).

The present design does not allow definitive conclusions to be drawn as to whether the observed group differences in [^{3}H](+)MK-801 binding existed prior to ethanol treatment or were induced by the treatment. It may be noted that in both experiments animals defined as sensitized and non-sensitized on day 21 had similar locomotor activity levels when first exposed to ethanol, which could suggest that the behavioral and binding differences between these groups would have developed as a result of chronic ethanol treatment. However, different mechanisms may underlie ethanol's acute stimulant effect and behavioral sensitization, since mice which are insensitive to ethanol's acute stimulant effect are able to develop behavioral sensitization to chronic treatment (Phillips et al., 1997). Thus, it would not be appropriate to rely on activity levels after acute administration to conclude that NMDA receptors were at the same state in all groups prior to ethanol treatment. On the other hand, the well-documented ability of ethanol to increase [^{3}H](+)MK-801 binding (Grant et al., 1990; Gulya et al., 1991; Hu and Ticku, 1995) does suggest that the binding increases observed here were to a large extent induced by the treatment.

The fact that ethanol-sensitized animals did not present higher [^{3}H](+)MK-801 binding is in agreement with data on [^{3}H](+)MK-801 binding in animals sensitized to other drugs of abuse. Behavioral sensitization to nicotine (Shoaib et al., 1997) has also been reported not to involve increased [^{3}H](+)MK-801 binding in brain. Likewise, cocaine sensitization in rats does not appear to be associated with consistent changes in [^{3}H](+)MK-801 binding (Bhargava and Kumar, 1999).

In the present study, increased [^{3}H](+)MK-801 binding was observed only in the ethanol-treated group that did not develop sensitization. One possible speculation is that ethanol may have had a stronger inhibitory effect on NMDA receptors in this sensitization-resistant group. This in turn would impair sensitization development, similarly to what is observed after lesions of glutamatergic pathways (Li et al., 1999; Pierce et al., 1998; Wolf, 1998) or when NMDA receptor antagonists are co-administered with ethanol or other psychostimulants. In this context, it is conceivable that co-administration of NMDA antagonists might upregulate NMDA receptors, and that this could be the general mechanism underlying the effectiveness of co-administration of NMDA antagonists in blocking sensitization.

Regardless of mechanisms involved, the observed differential effects of ethanol in the non-sensitized group are not likely to have been due to ethanol pharmacokinetic differences between groups. A parallel study showed that blood ethanol levels after acute ethanol injection did not correlate with locomotor activity levels in these mice (Pearson's $r = -0.5449$). Furthermore, other experiments in this laboratory using the same protocol in mice from the same colony have consistently failed to identify differences in blood ethanol concentrations between sensitized and non-sensitized mice at any point in the course of treatment (unpublished observations).

While NMDA receptor changes do not seem necessary for sensitization to develop (our data suggest the opposite), the literature consistently points to increased glutamatergic transmission as being essential for the development of behavioral sensitization to psychostimulants (for reviews see Pierce and Kalivas, 1997; Trujillo and Akil, 1995; Wang and McGinty, 1999). For example, Pierce et al. (1996), working with rats classified as sensitized or non-sensitized to cocaine, showed that glutamatergic neurotransmission in the nucleus accumbens was increased only in rats that developed behavioral sensitization to cocaine. If the same were true in the case of our ethanol-treated mice, one might expect the sensitization-resistant group not to show increased glutamate levels in the accumbens. Interestingly, it has been recently shown in cell cultures that chronic ethanol upregulates specific NMDA receptor subunits only in conditions associated with low glutamate levels (Chandler et al., 1999). One could speculate that increased [^{3}H](+)MK-801 binding in the sensitization-resistant group may be associated with low synaptic levels of glutamate, a hypothesis that is amenable to experimental testing.

In summary, the present study has confirmed that after chronic ethanol mice fall into clearly distinct subgroups in terms of ability to develop behavioral sensitization. Mice that did not develop sensitization were found to have elevated [^{3}H](+)MK-801 binding levels in nucleus accumbens and prefrontal cortex and, in a separate experiment, to be less sensitive to the behavioural effects of NMDA receptor blockade. This effect may be associated with an increased blockade of NMDA receptors by ethanol and/or decreased glutamate levels in the sensitization-resistant subgroup. It will be of interest to determine whether similar effects occur in animals showing decreased capacity for sensitization to other psychoactive drugs.

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