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Enhanced locomotor stimulation by NMDA receptor antagonists in alcohol-sensitive ANT rats

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Abstract

The ability of the antagonists for the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor to modulate locomotor activity were compared in alcohol-sensitive (or alcohol-nontolerant, ANT) and alcohol-insensitive (or alcohol-tolerant, AT) rat lines. Both rat lines showed altered locomotor activity after acute injections of a competitive antagonist (LY235959), a glycine-site antagonist (L-701,324), or noncompetitive antagonists [MK-801, phencyclidine (PCP), and ketamine] of the NMDA receptor. MK-801 at 0.5 mg/kg caused a strong increase in horizontal activity in both rat lines, the effect being significantly greater in the ANT rats. There was a subpopulation among AT rats that was almost completely unresponsive to MK-801. This insensitivity to MK-801 correlated with the lack of c-*fos* induction in the retrosplenial and cingulate cortices. Fos immunoreactive cells in these brain regions after MK-801 treatment were more numerous in ANT than AT rats, although c-*fos* induction in the inferior olivary nucleus was similar in all animals after MK-801. The ANT rats showed greater locomotor stimulation also after ketamine and LY235959, while stimulation induced by PCP and depression induced by L-701,324 did not differ between the rat lines. The data suggest that altered NMDA receptor-mediated processes may correlate with differences in innate alcohol sensitivity in the ANT/AT rat model.

Keywords: NMDA receptor antagonists; Locomotion; Innate alcohol sensitivity; Selected rat lines; Fos immunoreactivity

It has been suggested that many of the behavioral effects of ethanol might be caused by enhancement of the A type of inhibitory γ-aminobutyric acid ($GABA_A$) receptors and blockade of the *N*-methyl-D-aspartate (NMDA) type of excitatory glutamate receptors [14,26,37]. Although the precise mechanisms for the action of ethanol on some receptors are known at the molecular level [28], still their roles in behaving animals remain to be clarified.

The study of ethanol-induced behaviors often has been done by using selectively bred animal lines. The alcohol-insensitive (or alcohol-tolerant, AT) and alcohol-sensitive (or alcohol-nontolerant, ANT) rat lines are examples of such lines. These rat lines were developed by selection on the basis of differential sensitivity to acute motor-impairing effects of a moderate dose (2 g/kg, ip) of ethanol. AT rats are rather similar to nonselected heterogeneous rats and serve as alcohol-insensitive controls for the ANT rats [10,11]. In addition to higher sensitivity to ethanol, ANT rats also show higher sensitivity to benzodiazepine agonists and sodium barbital, which suggests for a role for $GABA_A$ receptors in ethanol sensitivity [15,42]. In the search for the basis for the differential alcohol sensitivity of these two rat lines, a point mutation in the cerebellar $GABA_A$ receptor α6 subunit was discovered [20]. This point mutation alone cannot, however, explain the enhanced ethanol sensitivity of the ANT rats [21], and therefore, studies were recently undertaken to determine the possible role of NMDA recep-

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tors in the effects of ethanol on ANT and AT rats. No differences between the lines were found in the binding of a noncompetitive NMDA receptor antagonist, [^{3}H]MK-801, in various brain regions [30], in the ethanol-induced decrease in cerebellar and hippocampal cGMP levels [39], or in the NMDA receptor subunit mRNA expression [39]. At the behavioral level, MK-801 impaired motor function in a tilting plane test more in ANT than AT rats, and this effect was potentiated by low doses of ethanol in the ANT rats [39]. It was concluded that neither dopaminergic nor GABAergic mechanisms were involved in the motor impairment induced by MK-801 and ethanol [39].

The divergence of molecular and behavioral results observed by Toropainen et al. [39] prompted us to conduct a more detailed analysis of NMDA receptor functions in the AT and ANT rats at the behavioral level. For this purpose we used, in addition to MK-801, two other noncompetitive NMDA antagonists: phencyclidine (PCP), which has been reported to be less specific than MK-801, binding not only to NMDA receptors but also sigma receptors [6], and ketamine, which more closely resembles MK-801 but has a low affinity to a number of other neurotransmitter receptors as well [18]. On the NMDA receptor, these drugs have the same binding site [41], and one of their well-known behavioral effects is an increase in spontaneous locomotor activity [24]. Since it has been shown that competitive and noncompetitive NMDA antagonists not only differ by having distinct binding sites on the NMDA receptor [22], but also in their electrophysiological, neurochemical, and behavioral features [12], we also employed a competitive NMDA antagonist, LY235959, and a glycine-site antagonist, L-701,324 [36]. Antagonists binding to the latter site have been shown to suppress spontaneous activity in rats [3].

1. Methods

1.1. Animals

The adult male rats [111 ANT and 112 AT [10,11], weighing 250–320 g] of generation F_{50} and F_{54} were housed in polypropylene cages with stainless steel-mesh lids (three to four rats per cage) on a 12/12-h light/dark cycle (lights on at 7:00 a.m.) under standard conditions at a temperature of 21 ± 1°C. The animals had tap water and food ad libitum. All animals were naive to ethanol and drugs before experiments. Drugs and vehicles were injected intraperitoneally (ip), the injections being delivered in a volume of 1 ml/kg. Experiments were carried out between 9:00 a.m. and 1:00 p.m.

All experimental protocols were approved by the appropriate Institutional Animal Use and Care Committees.

1.2. Apparatus

The horizontal activity was measured by placing the animals in transparent $25 \times 42 \times 15$ cm^3 plastic cages equipped with computer-controlled photocells (placed 11 cm from the bottom of the cages) to automatically monitor the movements (PAS system, San Diego Instruments, San Diego, CA). The data were calculated for each 10 min of the test. A similar manner of measurement was used during all consecutive experiments. In some experiments, vertical activity (representing mostly lifting of the head and, less frequently, actual rearing) was also monitored with another set of photocells positioned at 14 cm from the bottom.

1.3. Design and procedures

A preliminary study and three independent experiments were performed. In the preliminary experiment, ANT and AT rats were given MK-801 (Research Biochemicals, Natick, MA) at 0.5 mg/kg and their horizontal activity monitored using self-constructed, computer-controlled photocells surrounding Macrolon III cages as described in detail in Ref. [17].

Experiment 1 also tested 0.5 mg/kg MK-801 but used the locomotor activity monitoring as described in the Apparatus section above. On 2 days preceding the first experimental day, the animals were pre-adapted to the measuring cages for 60–120 min. On the experimental days, after a 40-min habituation period, all animals were given MK-801. Immediately after the injection, the animals were placed into the activity cages, and the locomotor activities registered for 4 h.

In Experiment 2, horizontal and vertical activities were measured in ANT and AT rats after acute injections of noncompetitive NMDA antagonists MK-801 (1.0 mg/kg), PCP (6.5, 12.5, and 25 mg/kg; Research Biochemicals), and ketamine (12.5, 25, and 50 mg/kg; Parke-Davis, Barcelona, Spain). Control animals from both lines received saline, and their activity was registered in parallel.

The concluding experiment (Experiment 3) consisted of the measurement of the horizontal and vertical activities of ANT and AT rats after acute injection of L-701,324 (10 mg/kg; Tocris-Cookson, Bristol, UK) and LY235959 (1 and 3 mg/kg; Tocris-Cookson).

1.4. Immunostaining for Fos protein

In parallel with Experiment 1, a separate group of naive ANT and AT rats was habituated to handling and individual cages to prevent experimenter-induced c-*fos* gene induction. The adapted animals were injected with MK-801 (0.5 mg/kg) and allowed to stay undisturbed in their home cages for 2 h. Then the animals were anesthetized with a lethal dose of sodium pentobarbital (125 mg/kg) and perfused through the ascending aorta with 200 ml of physiological saline followed by 400 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The perfused brains were immersed for 12 h in the fixative and 50-μm sections were cut using a Leitz cryostat. The immunohistochemistry was conducted as described in Ref. [30] using the mono-

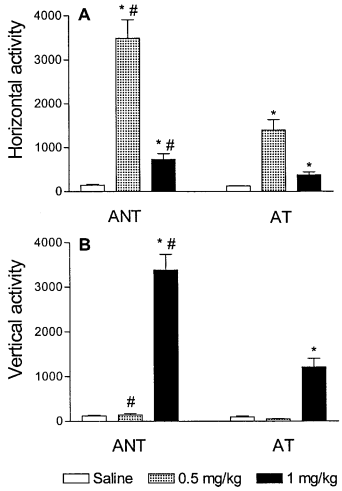

Fig. 1. The effect of various doses of MK-801 on horizontal (A) and vertical (B) activities of ANT and AT rats. The pooled activity scores are as means ± S.E.M. for 12–14 animals per group. * Significance of the difference in comparison with the saline group. # Significance of the difference between rat lines according to Student–Newman–Keuls test ($P < .05$).

clonal antibody (LA041) raised to a synthetic peptide representing residues 4–17 from the N-terminus of Fos protein (Microbiological Associates, Bethesda, MD) as a primary antibody. The number of Fos immunoreactive cells in several brain regions was estimated using MCID M4 image analysis program (Imaging Research, St. Catharines, Canada) using Olympus BH2 microscope and spatial areal calibration with a reticule for automatic detection of positive cells by target size and optical density adjustment. Two to three consecutive sections were analyzed for each brain region of each animal. The results are expressed as immunoreactive cells/mm^2.

1.5. Statistical analyses

All data are presented as means ± S.E.M.

Analyses of locomotor activities for the whole measuring period (pooled activity scores) were performed with two-way ANOVA, the two factors being rat lines and drug doses. A one-way ANOVA test was used for Experiment 1, where the factor was rat lines only. ANOVAs were performed using SAS-STAT software (version 6.12, SAS Institute, Cary, NC, USA). Individual comparisons were performed using post-hoc Student–Newman–Keuls test (only when ANOVA revealed significant effects).

An alternative criterion was also used to assess differences within the rat lines: the number of AT or ANT rats that had a level of locomotor activity less than the mean locomotor activity of the corresponding ANT rats minus two standard deviations was calculated (representing weakly responding animals). The ANT rat line was used as the baseline population, since their responses to drugs were more homogeneous.

2. Results

In the preliminary experiment, MK-801 was given at 0.5 mg/kg to AT and ANT rats and horizontal activity was recorded. A one-way ANOVA revealed significant differences for the 10-min periods between the 80th and 120th min of the test [$F(1,16) = 7.74$, $P < .05$; $F(1,16) = 5.06$, $P < .05$; $F(1,16) = 5.06$, $P < .05$, respectively], with the ANT rats showing higher stimulation of horizontal activity. A more detailed analysis showed that while the ANT rats behaved homogeneously, the AT rats could be divided into two groups with respect to horizontal activity. Four rats out of eight showed high stimulation by MK-801, whereas another four animals responded only weakly and did not exceed the level of alternative criterion.

In Experiment 1, both horizontal and vertical activities of AT and ANT rats were characterized after MK-801 (0.5 and 1.0 mg/kg) injections to a larger number of animals ($n = 12–14$). At 0.5 mg/kg, vertical activity was not altered in ANT rats, but in AT rats a significant decrease was observed [$F(1,54) = 5.08$, $P < .05$; Fig. 1B]. Pooled activity showed that the increase in horizontal activity was significantly higher in ANT rats than AT rats [$F(1,54) = 10.31$, $P < .01$; Fig. 1A]. As in the preliminary experiment, some AT rats were almost completely unresponsive to the stimulatory effect of MK-801. Alternative criterion analysis of pooled activity scores showed that 3 out of 12 AT rats responded poorly to MK-801 treatment, but since the range of the scores for all the ATs (488–3281, median 1376) was clearly set at a lower level than that of the ANTs (1425–6055, median 3381), the line difference was not only due to a small number of weak responders. When MK-801 was given at 1.0 mg/kg, the analysis of pooled activity scores indicated that ANT rats were more sensitive to MK-801 than AT rats [$F(1,53) = 6.37$, $P < .05$; Fig. 1A and B]. The alternative criterion showed that 5 out of 13 AT rats were not responding to the effect of MK-801.

Table 1
Quantification of Fos immunoreactivity in selected brain regions of the ANT and AT rats 2 h after the administration of MK-801 (0.5 mg/kg, ip)

Treatment	Rat line	Posterior cingulate cortex	Retrosplenial cortex	Inferior olive, subnucleus β
Saline	ANT	4, 4	0, 7	16, 0
	AT	13, 0	0, 2	3, 0
MK-801	ANT	1187 ± 278 (111–2008)	1315 ± 319 (105–2392)	560 ± 102 (325–965)
	AT	308 ± 193 (3–1014)	409 ± 283 (0–1686)	586 ± 125 (59–929)

The data (number of immunoreactive cells/mm^2) are for two saline-treated and six MK-801-treated animals/rat line, and are given as means ± S.E.M. (the smallest and largest values) for the MK-801-treated samples and as individual values for the saline-treated samples. Two-way ANOVA on MK-801-treated samples: $F(1,30) = 9.60$, $P < .01$ for the rat line effect; $F(2,30) = 0.79$, $P = .5$ for the brain region effect; and $F(2,30) = 2.62$, $P = .09$ for the interaction between rat line and brain region.

In order to investigate more closely the subpopulations of AT rats discovered above, six ANT and six AT rats were treated with MK-801 (0.5 mg/kg), and the brains examined for neurotoxicity 2 h later. Visual monitoring of these animals revealed that MK-801 strongly increased locomotor activity in five ANT rats, whereas only two AT rats responded in a similar fashion. All animals did show frequent head-weaving, starting at about 10 min after the drug administration. Fos immunoreactivity was stronger in the retrosplenial and cingulate cortices of ANT rats than AT rats [two-way ANOVA: $F(1,30)=9.60$, $P<.01$ for the rat line effect; Table 1; Fig. 2]. The intensity of Fos immunoreactivity correlated with MK-801-induced locomotor activity so that the number of Fos immunoreactive cells was always high in the animals that responded to the drug with strongly increased locomotion. The weakly responding four AT rats had a very low level of Fos immunoreactivity, and the one ANT rat that had a reduced locomotor response also had diminished Fos immunoreactivity. All animals, irrespective of their locomotor responses, had a similar number of Fos immunoreactive cells in the subnucleus β of the inferior olive (Table 1).

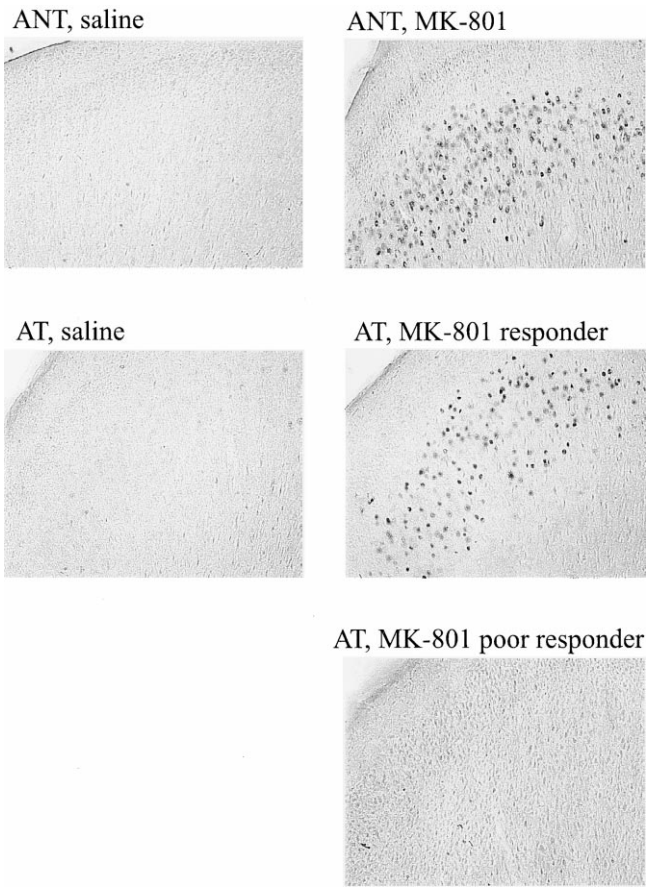

Fig. 2. Representative micrographs of sections on retrosplenial cortex processed for Fos immunoreactivity in the ANT and AT rats after treatment with saline (control) and MK-801 (0.5 mg/kg, ip). The images illustrate the extensive induction of c-*fos* gene expression at 2 h after MK-801 treatment in an ANT rat and in an AT responder, but not in a poorly responding AT rat or in the corresponding saline controls. Sections represent retrosplenial cortex just caudal from the corpus callosum.

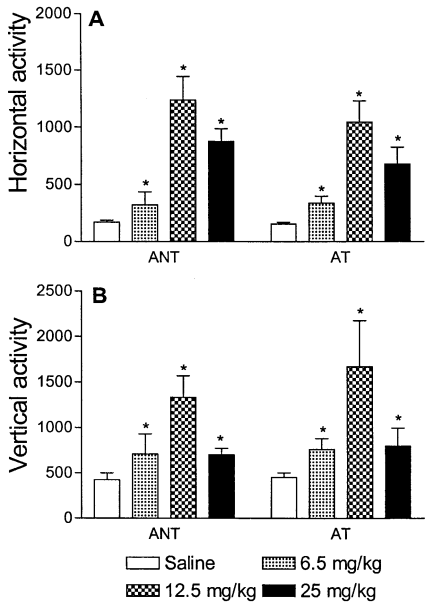

Fig. 3. The effect of various doses of PCP on horizontal (A) and vertical (B) activity in ANT and AT rats. The data are presented as pooled activity scores (means ± S.E.M. for eight to nine animals per group). * Significance of the difference in comparison with the saline group according to Student–Newman–Keuls test ($P<.05$).

In Experiment 2, PCP at all three doses induced a significant stimulation of horizontal and vertical activities in both rat lines [$F(3,100)=13.72$, $P<.001$; $F(3,100)=35.54$, $P<.001$, respectively, for pooled activity scores], but no

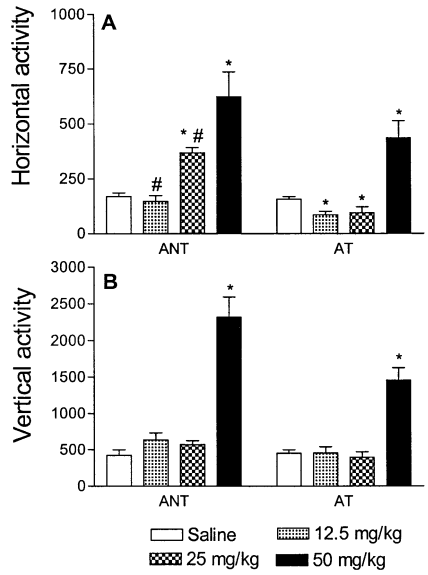

Fig. 4. The effect of various doses of ketamine on horizontal (A) and vertical (B) activity in ANT and AT rats. The data are presented as pooled activity scores (means ± S.E.M. for eight to nine animals per group). * Significance of the difference in comparison with the saline group. #Significance of the difference between rat lines according to Student–Newman–Keuls test ($P<.05$).

significant difference was observed between the rat lines (Fig. 3A and B). Ketamine at doses of 12.5 and 25 mg/kg induced a differential response in horizontal activity between ANT and AT rats [$F(1,101)=25.55$, $P<.05$ for pooled activity scores]. In AT rats these doses of ketamine decreased horizontal activity, but in ANT rats an increase was observed at 25 mg/kg [$F(3,101)=22.89$, $P<.05$ for the factor of dose; Fig. 4A]. According to the alternative criterion, seven out of eight AT rats were insensitive to the effect of ketamine. In both lines, 50 mg/kg ketamine stimulated horizontal and vertical activities to the same extent [$F(3,101)=22.34$, $P<.01$ for the factor of dose; Fig. 4A and B], and no significant difference was obtained between the lines.

In Experiment 3, the competitive NMDA receptor antagonist LY235959 increased horizontal activity at 3 mg/kg in ANT rats only. In AT rats, the only effect of LY235959 was a small, but significant decrease in horizontal activity at 1 mg/kg [$F(2,58)=8.05$, $P<.001$; Fig. 5A]. Vertical activity was decreased in both rat lines at both concentrations of the compound (Fig. 5B). At 3 mg/kg a rat line difference was observed, the locomotor activity-decreasing effect of LY235959 being more pronounced in AT than ANT rats (Fig. 5B).

The glycine-site antagonist L-701,324 (10 mg/kg) caused no significant changes in horizontal activity, the pooled activity scores being: after the saline treatment, 149 ± 19 and 124 ± 14 (means ± S.E.M., $n=14–16$), and after the drug treatment, 124 ± 14 and 78 ± 21 ($n=6$) for the ANT and AT rats, respectively. In contrast, vertical activity scores (saline: 122 ± 14 and 103 ± 15 and L-701,324: 36 ± 12 and 50 ± 20 for the ANT and AT rats, respectively) were significantly decreased similarly in both rat lines [$F(1,42)=21.89$, $P<.001$]. The doses below 10 mg/kg failed to alter the activity (not shown).

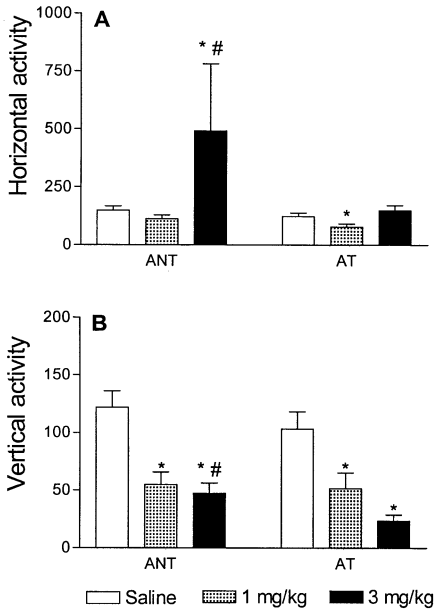

Fig. 5. The effect of various doses of LY235959 on horizontal (A) and vertical (B) activity in ANT and AT rats. The data are presented as pooled activity scores (means ± S.E.M. for 7 animals per group and 14–16 animals in saline group). * Significance of the difference in comparison with the saline group. # Significance of the difference between rat lines according to Student–Newman–Keuls test ($P<.05$).

3. Discussion

The results from the present study show that ANT rats are more sensitive to the locomotor activity-stimulating effect of the noncompetitive NMDA receptor antagonist MK-801 than AT rats. This difference in sensitivity was not dependent upon the dose of MK-801 used, since at both 0.5 and 1.0 mg/kg, the ANT rats showed higher horizontal activity. Furthermore, in our previous report [39], MK-801 at 0.2 mg/kg tended to induce higher locomotor activity in the ANT than AT rats. In that study, ANT rats were also more sensitive to the ataxic effects of MK-801. Of the noncompetitive antagonists tested, ketamine also induced greater horizontal activity in ANT rats, although it was less efficient than MK-801 or PCP in inducing horizontal activity, in keeping with Danysz et al. [7]. PCP, on the other hand, was equally effective in stimulating locomotor activity in both rat lines. The failure of PCP to reproduce the differential effects obtained with MK-801 and ketamine might be due to the fact that it is less specific than the other two. PCP has been shown to have higher affinity for the σ and σ-like receptor than MK-801 and ketamine [13], and to be a more potent inhibitor of serotonin uptake than MK-801 [16]. In addition, PCP affects several other signaling systems differently from MK-801, e.g., its effect on dopaminergic mechanisms is distinct from that of MK-801 [27]. Thus, non-NMDA effects of PCP might mask or compensate for a possible difference in locomotor activity induced by this agent. This conclusion is strengthened by the fact that the competitive NMDA receptor antagonist LY235959 [1,2] also stimulated horizontal activity in ANT rats, whereas no stimulation was observed in AT rats. On the other hand, the glycine-site antagonist L-701,324 failed to stimulate locomotor activity in both rat lines (doses higher than 10 mg/kg, however, were not tested). Our result is in accordance with a previous report showing that the compound fails to stimulate locomotor activity in rodents [4]. Taken together, the present results indicate that pure NMDA receptor antagonists produce stronger effects in ANT than AT rats (Table 2).

In previous studies, no apparent difference has been detected in the NMDA receptor functions in ANT and AT rats. Binding of [^{3}H]-MK-801 to various brain regions of the two rat lines failed to reveal any differences either in binding constants or distribution. Neither were there any differences in the expression of the NMDA receptor subunit NR1 and NR2A-D mRNAs [39]. Although levels of cGMP tended to be higher in ANT rats, no significant line differences were observed upon MK-801 treatment [39]; it

Table 2
Summary of drug effects on horizontal locomotor activity of the ANT and AT rats

Drug	Dose (mg/kg)	Horizontal activity		% Nonresponders	
		ANT rats	AT rats	ANT rats	AT rats
MK-801	0.5	↑*	↑	0	25
	1	↑*	↑	0	38
PCP	6.5	↑	↑	22	11
	12.5	↑	↑	13	14
	25	↑	↑	25	38
Ketamine	12.5	= *	↓	38	50
	25	↑*	↓	38	100
	50	↑	↑	38	63
LY235959	1	=	↓	29	43
	3	↑*	=	14	43
L-701,324	10	=	=	17	50

↑↓: significantly different from the saline effect; =: no significant effect. The % nonresponders was calculated by alternative criterion as described in Methods.

* Significantly different from the AT rat line according to Student–Newman–Keuls test ($P < .05$).

should be noted, however, that 0.2 mg/kg (which produced ataxia in the ANT rats) was the highest dose used. It would thus appear that NMDA receptor functions do not differ between ANT and AT lines, but we cannot, however, exclude a possible difference in the subcellular locations of the receptors and their intracellular signals, and in expression of various functionally significant subunit splice variants. The differential behavioral responses observed in the present study resemble those reported in a recent study on the effects of noncompetitive and competitive NMDA receptor antagonists in three different inbred strains of mice [24]. Significant differences in locomotor activity were observed between the mouse strains, and these were concluded to be genetically determined. Interestingly, ethanol-induced impairment was also greater in the CBA mouse line, which was the most sensitive to MK-801, than in the C57 and NMRI lines [23]. Since the motor coordination was impaired in the ANT rats after combined administration of low doses of ethanol and MK-801, the role of the NMDA receptor as the genetic correlative factor of enhanced alcohol sensitivity appears likely. However, the exact molecular mechanism remains still unknown in the cases of ANT/AT rat lines and CBA/C57/NMRI mouse strains.

It has been suggested that the effect of MK-801 on locomotor stimulation is mediated indirectly via enhanced dopamine release in the nucleus accumbens [5,31,40]. Since ANT rats have been reported to have higher levels of dopamine in the limbic brain regions than AT rats, it would appear reasonable to hypothesize that the enhanced sensitivity to NMDA antagonists is due to differential activation of dopaminergic systems in ANT and AT rats. The role of dopamine in the action of MK-801 has been disputed, however [9]. Depletion of dopamine or 6-hydroxydopamine lesions in the nucleus accumbens have failed to block the locomotor stimulating effect of MK-801 [25]. Thalamic injection of MK-801 produces neurotoxic damage in the retrosplenial cortex similar to that from systemic MK-801 [38], indicating an indirect mechanism of action. Therefore, the reason for the lower general sensitivity of the AT than ANT rats to MK-801 (and the heterogeneous response within the AT line, see below) can reside in neuronal mechanisms other than NMDA receptors.

NMDA receptor antagonists are usually neuroprotective, but they produce a paradoxical excitation and neurotoxicity [33,34], which can be visualized by the induction of c-*fos* gene in selected limbic brain regions such as the retrosplenial and cingulate cortices [8]. A surprising finding in the present study was that MK-801 at 0.5 mg/kg reproducibly induced a heterogeneous behavioral response within the AT line but less so in the ANT rat line. This suggests that the AT line is not fully inbred and that certain selection pressure keeps the line inhomogeneous, as is the case with the ANT rat line and the cerebellar $GABA_A$ receptor $\alpha 6$ subunit mutation [20]. The low locomotor stimulation by MK-801 correlated with the low expression of Fos protein in the retrosplenial and cingulate cortices. An obvious explanation for the presence of low-responding AT rats would be either disturbances in the absorption of MK-801 or genetic differences in the pharmacokinetics of the drug. These explanations are unlikely because Fos expression in the subnucleus β of the inferior olive, the only inferior olivary nucleus sensitive to MK-801 in nonselected rats [29], was equally stimulated in both responders and nonresponders, indicating that MK-801 was acting in the brain even in the "weak responders." This bimodal effect was most striking with MK-801, since the other two noncompetitive NMDA receptor antagonists, PCP and ketamine, affected locomotor activity in AT rats without showing any more heterogeneity within the line than ANT rats showed (Table 2). Since the excitatory effects of the NMDA receptor antagonists have been suggested to be used as an animal model of psychosis [19,32,35], further study of the mechanisms of action of MK-801 in the two AT line subgroups seems warranted.

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