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Terfenadine induces toxicity in cultured cerebellar neurons: a role for glutamate receptors

R. Díaz-Trelles[1], A. Solana-López[3], J. R. Fernández-González[3], A. Novelli[1,2], and M. T. Fernández-Sánchez[1]

[1] Department of Biochemistry and Molecular Biology and [2] Department of Psychology, University of Oviedo, Oviedo, Spain
[3] Astur-Pharma S.A., Silvota, Asturias, Spain

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Summary. Exposure of cultured cerebellar neurons to the histamine H1 receptor antagonist terfenadine resulted in neuronal degeneration and death. Terfenadine neurotoxicity was dependent upon concentration and time of exposure. After 2 h exposure, 20 μM terfenadine reduced the number of surviving neurons by 75%, and as low as 10 nM terfenadine induced significant neurotoxicity after 5 days of exposure. Neuronal sensitivity to terfenadine changed with age in culture, and at 25 days in culture neurons appeared to be much less sensitive than at 5 or 9–17 days in culture. Neurotoxicity by terfenadine could not be prevented by high concentrations of histamine (5 mM), but it was significantly delayed by blocking NMDA or non-NMDA glutamate receptors with MK-801 or CNQX respectively, suggesting the involvement of excitatory transmission mediated by glutamate in the neurotoxicity induced by terfenadine in these neurons. We also found that the presence of terfenadine (5 μM) unveiled the potential excitotoxicity of the non-NMDA receptor agonist AMPA (100 μM), and reduced the concentration of glutamate necessary to induce excitotoxicity, compared to untreated cultures. These results suggest a role for terfenadine in the modulation of the excitotoxic response mediated in cerebellar neurons through ionotropic glutamate receptors.

Keywords: Amino acids – Cerebellar granule cells – Neurotoxicity – Terfenadine – Excitatory amino acids – Histamine

Introduction

Histamine has been established to be a neuromodulator in the mammalian central nervous system (Prell and Green, 1986; Pollard and Schwartz, 1987; Schwartz et al., 1991). Histamine is considered to act at three pharmacologically distinct receptor subtypes, termed H_1, H_2 and H_3, which

belong to the superfamily of receptors with seven transmembrane domains, and are coupled to guanylnucleotide-sensitive G proteins (Schwartz et al., 1995).

Histaminergic neurons are mainly located in the hypothalamus, from where they send diffuse projections to most brain areas including the molecular and the granule cell layer of the cerebellum (Panula et al., 1993). Several lines of evidence indicate that the role of histaminergic fibers could be the modulation of the neurotransmission mediated by glutamate. Thus, histamine can depress synaptic transmission in the dentate gyrus through a H3-mediated mechanism (Brown and Reymann, 1996), and an enhancement by histamine of NMDA-mediated responses has been reported in hippocampal neurons (Bekkers, 1993; Vorobjev et al., 1993). Histamine has been also shown to increase glutamate release from hippocampal synaptosomes (Rodríguez et al., 1997), further suggesting that histamine release may eventually contribute to glutamate-mediated excitotoxicity. Indeed, a role for histamine in glutamate N-methyl-D-aspartate (NMDA)-mediated excitotoxic neuronal death in a rat model of Wernicke's encephalopathy has been reported (Langlais et al., 1994).

The H1-receptor antagonist terfenadine (TEF) is widely used as a prototype of non-sedating antihistamines for the relief of symptoms in patients with allergic rhinitis, allergic dermatological conditions and other histamine-mediated disorders. TEF is thought to penetrate poorly the blood brain barrier. However, reported adverse effects including sedation, drowsiness, fatigue, and weakness (McTavish et al., 1990) indicate that therapeutic concentrations of the drug may also act at the CNS. Although very little is known about the effect of antihistamine compounds on neuronal survival and function, neurotoxicity has been observed in animals treated with the H1-receptor antagonist mepyramine (AU Sharma, personal communication). In this paper we have investigated the effects of TEF on rat cerebellar neurons in primary culture. These cells are known to express histamine H1 receptors (Hösli and Hösli, 1984; Xu and Chuang, 1987; Dillon-Carter and Chuang, 1989). We present evidence indicating that TEF may induce neurodegeneration through an H1-receptor independent mechanism involving excitatory amino acid receptor-mediated neurotransmission. We have also found that exposure of neurons to subtoxic concentrations of TEF resulted in significant excitotoxity by either the non-NMDA receptor agonist AMPA, or subtoxic concentrations of glutamate, suggesting a role for TEF in the modulation of the excitotoxic response mediated in cerebellar neurons through ionotropic glutamate receptors.

Materials and methods

Cell culture

Primary cultures of rat cerebellar neurons were prepared as described (Novelli et al., 1988). Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated first

enzymatically, by adding trypsin (0.25 mg/ml), followed by mechanical dissociation with a fire-polished Pasteur pipette. Cells were then suspended in basal Eagle's medium (GIBCO-BRL, Life Technologies) supplemented with 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamycin and 10% fetal calf serum, seeded in poly-L-lysine coated (5 μg/ml) dishes (NUNC) at 2.5×10^5 cells/cm^2 and incubated at 37°C in a 5% CO_2, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added after 20–24 h of culture to prevent proliferation of non-neuronal cells. After 8 days in culture (DIC), morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture (Nicoletti et al., 1986) Cerebellar granule neurons were kept alive for more than 40 DIC, by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation.

Neuronal treatment and survival

Neurons were used between 6 and 34 DIC. Drugs were added in the growth medium for the indicated times. Then, growth medium was removed and cultures were incubated for 5 min. with 1 ml incubation buffer containing 154 mM NaCl, 5.6 mM glucose, 8.6 mM HEPES, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, pH 7.4, to which the vital stain fluorescein diacetate (5 μg/ml) was added. The staining mixture was then aspirated, replaced with incubation buffer, and cultures were examined for neurotoxicity. Under fluorescent light, live neurons showed a bright green color in the cell body and neurites, while dead neurons did not retain any fluorescein diacetate, and their nuclei could be stained in red by 1 min. exposure to 50 μg/ml ethidium bromide. Photographs of three randomly selected culture fields were taken, and live and dead neurons were counted. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the picture (~3,000).

Materials

Terfenadine was provided by Dr. J. R. Fernández (ASTUR-PHARMA, Llanera, Asturias, Spain). (6-(2-(4-Imidazolyl) ethylamino)- N-(trifluoromethylphenyl) heptane carboxamide (HTMT dimaleate), (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from Tocris Cookson, Bristol, United Kingdom). All other chemicals were from Sigma.

Data presentation and analysis

The mean ± SD of the data is reported. For statistical analysis the one-way or the two-way analysis of variance (ANOVA) was used to identify overall treatment effects. The unpaired two-tailed Studen's *t*-test was used for selective comparison of individual data groups. Only significances relevant for the discussion of the data are indicated in each figure.

Results

Terfenadine neurotoxicity

Cerebellar neurons at 10 DIC were exposed to terfenadine (TEF) at concentrations from 10 nM to 20 μM in the growth medium for up to 120 hours. Signs of neurotoxicity, including neurite degeneration, swelling of cell bodies, and cell death, were timed as they developed. Neurotoxicity was time and concen-

Table 1. Development of terfenadine-induced neurotoxicity

Terfenadine (μM)	Time of exposure (hours)						
	2	12	24	48	72	96	120
0	−	−	−	−	−	−	−
0.01	−	−	−	−	−	−	±
0.1	−	−	−	−	−	±	±
0.5	−	−	−	−	±	+	
1	−	−	−	±	+		
5	−	−	±	+			
10	−	+					
20	+						

Neurons were exposed to terfenadine in the growth medium for the indicated times. (+) indicates >75% cell mortality; (±) indicates degeneration of neurites; (−) indicates not affected cells.

tration dependent. As summarized in Table 1, 2h exposure to 20μM TEF reduced the number of live neurons by more than 75%, while 5μM TEF required about 48h exposure. Neurotoxicity was also visible in cultures exposed to lower concentrations of the drug for longer periods of time. Thus, neurites from neurons exposed to 10nM TEF for 5 days appeared weaker as compared to those of untreated cultures (see Table 1).

We next investigated whether neuronal sensitivity to TEF depended upon differentiation of neurons in culture. For this purpose, we compared the neurotoxic effect of 12h exposure to 10μM TEF on neurons maintained in culture for 5, 9, 11, 13, 15, 17 and 25 days respectively. Neurotoxicity changed with the age of the culture (Fig. 1), although the neurotoxicity pattern described above, i.e. neuronal network disruption and cell body swelling, did not. Thus, TEF significantly decreased neuronal survival by approximately 60% at 5 DIC, while in neurons between 9 and 17 DIC neuronal survival decreased by approximately 80%. However, older neurons appeared to be much less sensitive to the drug and at 25 DIC neuronal survival was similar in TEF-treated and untreated cultures (Fig. 1).

In order to establish whether TEF-induced neurotoxicity was due only to the blockade of histamine receptors, we tried to antagonize its effects by adding, prior to TEF, high concentrations of histamine to the growth medium. However, the presence of up to 5mM histamine did not significantly protect from the reduction in neuronal survival induced by 5μM TEF (Fig. 2), suggesting that other mechanisms must be involved in the neurotoxic process. A possible contribution of histamine H2-receptors in TEF toxicity is also unlikely, since exposure of neurons to the selective histamine H2-receptor antagonist famotidine at concentrations up to 500mM for 24h had no effect on neuronal survival (data not shown).

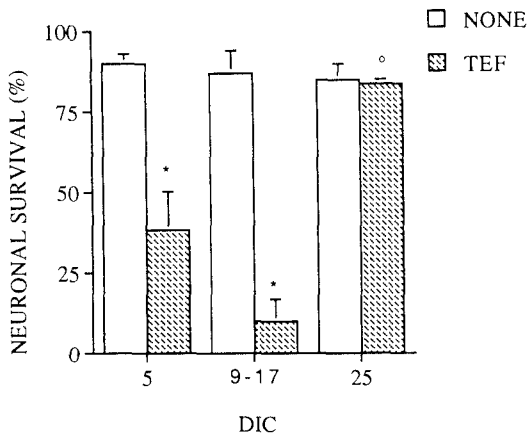

Fig. 1. Neurotoxicity by terfenadine is dependent upon neuronal age in culture. Neurons at the indicated days in culture (*DIC*) were exposed for 12 h to 10 μM terfenadine (*TEF*) in the growth medium, and the percentage of surviving neurons was determined and compared to that of untreated neurons of the same age (*NONE*). Values represent the mean ± SD (n = 6 − 8). *P < 0.01 vs NONE at the same age; °P < 0.01 vs TEF at 5 and 9–17 DIC

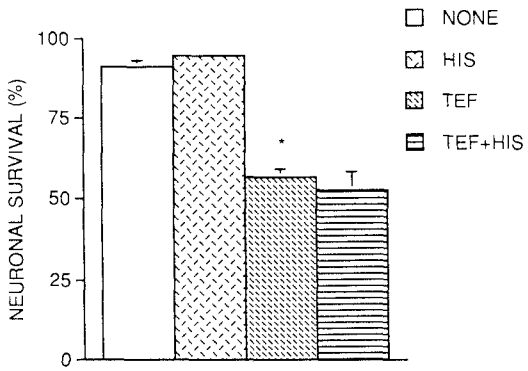

Fig. 2. Terfenadine-induced neurotoxicity is not prevented by histamine. Neurons at 13–14 DIC were exposed for 24 h to 5 μM terfenadine (*TEF*) in the absence or in the presence of 5 mM histamine (*HIS*). Survival of untreated neurons (*NONE*) is also reported for comparison. HIS was added 15 min prior to TEF. Values represent the mean ± SD of two independent experiments. *P < 0.01 vs NONE

To examine whether the toxicity process initiated by TEF could be reversed upon removal of the drug, we exposed neurons at 10 DIC to 10 μM TEF for different periods of time, after which the TEF-containing growth medium was substituted by TEF-free medium from sister cultures. Cultures were then examined for morphological signs of neurotoxicity after 1, 2, 3, 4 and 5 days, and the results obtained are summarized in Table 2. Exposures

Table 2. Reversibility of terfenadine-induced neurotoxicity

Time of exposure (min)	Time of observation (days)				
	1	2	3	4	5
0	−	−	−	−	−
1	−	−	−	−	±
5	−	−	−	−	±
15	−	−	−	±	+
30	−	−	−	+	+
60	−	−	±	+	+
90	−	±	+	+	+
120	±	+	+	+	+
180	+	+	+	+	+

Neurons were exposed to 10μM terfenadine in the growth medium for the indicated times before changing the medium by terfenadine-free medium from sister cultures. (+) indicates >75% cell mortality; (±) indicates degeneration of neurites; (−) indicates not affected cells.

ranging from 15 min to 3 h were able to extensively reduce neuronal survival, and the time required for neurotoxicity was proportionally longer as the time of exposure decreased. Thus, the presence of TEF for 3 h in the culture medium decreased after 24 h the number of live neurons by more than 75%, while neurons exposed to TEF for 15 min. required 5 days to show a similar reduction in neuronal survival (Table 2). It is worth noting that neurons exposed to TEF for only 1 min. showed after 5 days the characteristic signs of TEF toxicity including swelling of cell bodies and degeneration of neuritis.

Terfenadine and glutamate receptors

Most of cerebellar neurons in culture are glutamatergic granule cells which may release excitatory amino acids such as glutamate (Thomas et al. 1989). Moreover, glutamate has been demonstrated to achieve a steady-state concentration of approximately 6μM in the growth medium of cultured cerebellar granule cells (Didier et al., 1996; Novelli et al., unpublished results). Thus, we have explored the possibility that the neurotoxic effects induced by TEF could be at least partially due to an overactivation of glutamate receptors by endogenous excitatory amino acids. For that purpose, neurons were exposed to 10μM TEF in the presence of either MK-801 (2μM) or CNQX (15μM), specific antagonists for NMDA and non-NMDA ionotropic glutamate receptors respectively (Hollmann and Heinemann, 1994). Both MK-801 and CNQX were able to delay TEF neurotoxicity (see Fig. 3). After 8 h exposure to TEF, neuronal survival in the presence of MK-801 or CNQX was 63% and 86% respectively, while only 30% of the neurons treated with TEF alone survived at that time. Observation of cultures 2 h later revealed a significant

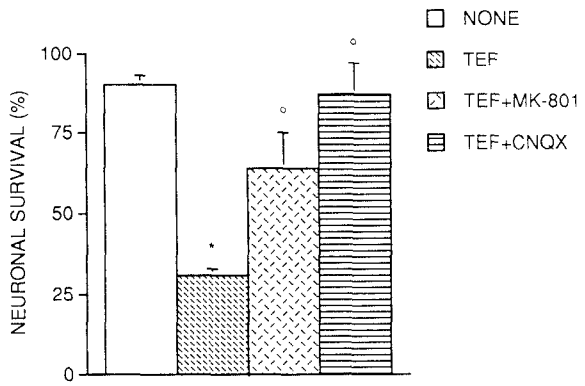

Fig. 3. Neurotoxicity by terfenadine is attenuated by NMDA and non-NMDA glutamate receptor antagonists. Neurons at 13–15 DIC were exposed to 10μM terfenadine (*TEF*) alone or in the presence of 2μM MK-801 or 15μM CNQX, and neuronal survival was determined 8 h later. Values represent the mean ± SD of three independent experiments. *NONE*, untreated neurons. *P < 0.01 vs NONE; °P < 0.01 vs TEF

decrease in neuronal survival in neurons treated with MK-801 + TEF (15% live neurons) or CNQX + TEF (20% live neurons), while only 5% of the neurons survived in cultures treated with TEF alone. Taken together these results suggest a role for ionotropic glutamate receptors activation on the toxic effect induced by TEF.

We considered the possibility that TEF could actually facilitate the occurrence of neurotoxicity via ionotropic excitatory amino acid receptors. To test this hypothesis we used AMPA, a specific agonist for some non-NMDA receptors (Hollmann and Heinemann, 1994). Overstimulation of non-NMDA receptors by AMPA has been shown not to induce excitotoxicity in cerebellar granule neurons unless its rapid desensitization is prevented (Hack et al., 1995). Indeed, no reduction in neuronal survival was observed in neurons exposed to 100μM AMPA for 24 h (Fig. 4). However, exposure to 100μM AMPA in the presence of a low concentration of TEF (5μM), significantly reduced the number of live neurons by approximately 80% after 12 h, while for neurons exposed to TEF alone neuronal survival at that time and up to 24 h, was similar to that of untreated cultures (Fig. 4). Stimulation of AMPA receptors was crucial for the potentiation of neurotoxicity observed in TEF-treated neurons, for it could be fully antagonized by the non-NMDA receptor antagonist CNQX (Fig. 4). The effect of TEF on glutamate receptor agonists-induced excitotoxicity was further confirmed by using subtoxic concentrations of glutamate. The presence of TEF (5μM) dramatically decreased the number of surviving neurons exposed to subtoxic concentrations of glutamate, and this potentiation was completely abolished by the NMDA receptor antagonist MK-801 (data not shown).

The results above clearly indicated a role for TEF in glutamate receptors stimulation. Since exposure of neurons to TEF alone eventually results in

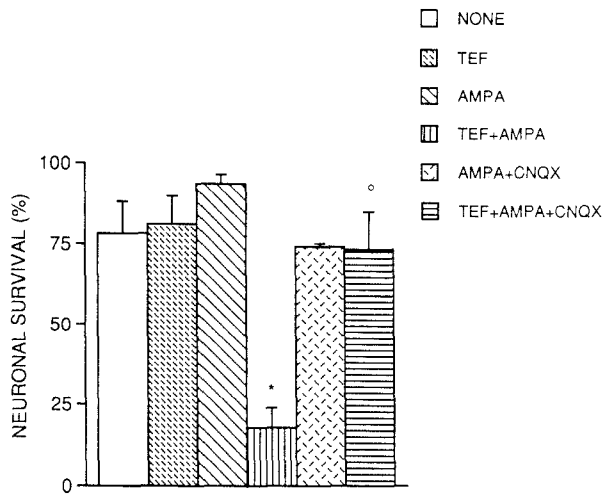

Fig. 4. Terfenadine potentiates excitotoxicity mediated by the non-NMDA glutamate receptor agonist AMPA. Neurons at 14–15 DIC were exposed to the indicated drugs in the growth medium. Terfenadine (*TEF*) was added between 1 and 2 h before AMPA depending on the experiment, with similar results. CNQX was added 2 min before AMPA. Neuronal survival was determined between 7 h and 10 h (depending on the experiment) after the addition of AMPA. Concentrations were *TEF*, 5 μM; *AMPA*, 100 μM; *CNQX*, 15 μM. *NONE*, untreated cultures. Values represent the mean ± SD of three independent experiments. *P $<$ 0.01 vs AMPA and vs TEF; °P $<$ 0.01 vs TEF + AMPA

neuronal death (see Table 1), we asked whether TEF potentiated the excitotoxic response induced by either AMPA or subtoxic concentrations of glutamate, or by contrast, these agonists were somehow facilitating the neurotoxic process induced by TEF in granule neurons. To distinguish between these two possibilities we examined the morphological signs of neurotoxicity in neurons exposed to TEF and AMPA or TEF and subtoxic concentrations of GLU and found that the morphological characterization of neurotoxicity was rather consistent with the former possibility. Indeed, neurons showed the neurotoxic effects characteristic of excitatory amino acids, i.e. rapid swelling and darkening of cell bodies already visible after 15 min. exposure, followed by complete degeneration of the neurites (Novelli et al., 1988). In contrast to this fast neurotoxic process, TEF (5 μM) produced a slow pattern of neurotoxicity characterized first by the disintegration of the neurites and later by swelling of cell bodies and cellular death. No dark inclusions could be observed in neuronal cell bodies in TEF-treated cultures in the absence of excitatory amino acids. The presence of CNQX or MK-801 completely blocked fast toxicity in TEF + AMPA and TEF + glutamate treated neurons respectively, while slow degeneration and death did proceed as in cultures exposed to TEF alone (see Table 3).

Table 3. Terfenadine potentiated neurotoxicity by excitatory amino acids

	NONE		AMPA		AMPA + CNQX		GLU		GLU + MK-801	
	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
NONE	−	−	−	−	−	−	−	−	−	−
TEF	−	+	++	−	−	+	++	−	−	+

Terfenadine (*TEF*) alone or in combination with the indicated drugs was added to neurons at 15 DIC, and the neurotoxic effects were evaluated from 15 min to 24 h later. Fast toxicity was characterized by rapid darkening and swelling of cell bodies visible after 15 min exposure to the drugs followed by degeneration of the neurites. Slow toxicity defines the propressive disintegration of the neurites visible after 24 h, followed by swelling of cell bodies and cellular death. (+) and (++) indicate the different degrees of toxicity; (−) indicates the absence of signs of toxicity. Concentrations were *TEF*, 5 μM; *AMPA*, 100 μM; glutamate (*GLU*), 20 μM; *CNQX*, 15 μM; *MK-801*, 2 μM. Similar results were obtained in three independent experiments.

Discussion

We show in this paper for the first time that the specific non-sedating histamine H1-receptor competitive antagonist terfenadine (TEF) may induce neuronal degeneration and death of cultured neurons, and that excitatory amino acid receptors may play a role in TEF-induced neurotoxicity. TEF toxicity was characterized by the progressive disintegration of the neurites followed by swelling of cell bodies and cellular death, and was time and concentration dependent.

Although further studies using cells lacking H1-receptors are necessary to evaluate the actual contribution of these receptors to the toxic effects of TEF, the observation that TEF-induced neurotoxicity cannot be antagonized by high concentrations of histamine in the culture medium (Fig. 2), or by the specific H1-receptor agonist HTMT dimaleate (data not shown), suggests that TEF toxicity occurs independently from H1-receptor activation. Among possibilities, TEF could associate to receptors other than H1, accumulate inside neurons and act intracellularly, or associate to membranes. Both the extent of toxicity and the time required for its induction after TEF withdrawal, strongly depended upon how long cultures were exposed to TEF. Thus, as long as five days were necessary to cause neuronal death in cultures exposed to 10 μM TEF for 15 min., while only 24 h were necessary to induce a similar degree of toxicity in neurons exposed to the same concentration of TEF for 180 min. (see Table 2). This observation would be consistent with a progressive accumulation of TEF in neurons. Indeed, preliminary data from HPLC analysis of TEF in TEF-treated neurons suggest that TEF associates to neurons and that the amount of neuronal associated-TEF is maximum after approximately 30 min. exposure to the drug (data not shown). Characterization of the specific mechanism by which TEF interacts with neurons and leads to neurotoxicity will deserve further studies.

Neuronal sensitivity to TEF changed with time in culture. Thus, at 25 DIC (Fig. 1) and up (data not shown), neurons appeared to be much less sensitive to TEF, although this resistance was overwhelmed by longer exposures to the toxin (not shown). Such changes in sensitivity may reflect developmentally acquired mechanisms that slow down the pathways triggered by TEF and leading to toxicity. Thus, location and/or affinity of the target site for TEF could be different in old neurons, or it is also possible that the amount of target sites may be decreased in old neurons. Alternatively, there could be developmentally related changes in the modulation of biochemical pathways, resulting in a delay of the neurotoxicity process.

The observation that TEF-induced neurotoxicity could be significantly delayed by blocking NMDA or non-NMDA receptors with MK-801 or CNQX respectively (Fig. 3), strongly suggests a role for TEF in the modulation of excitatory transmission mediated by glutamate through ionotropic glutamate receptors in cerebellar granule neurons. This role was further confirmed by TEF unveiling of AMPA excitotoxicity and potentiation of glutamate toxicity (Fig. 4 and data not shown). However, our results also indicate that this modulation contributes only partially to the toxicity induced by TEF, and therefore mechanisms independent of excitatory amino acid receptors stimulation must be also involved in TEF-induced neuronal death. Studies are currently in progress to investigate these mechanisms.

To our knowledge, this is the first study showing that anti-histamine drugs may modulate the response following glutamate receptors activation. Although further experiments are necessary to elucidate the specific mechanisms underlying the potentiation of excitatory amino acid toxicity we show here, preliminary results indicate that it can be antagonized, at least partially, by high concentrations of histamine (5 mM) in the growth medium, thus suggesting that such potentiation is probably related to the anti-histamine H1-receptor activity of TEF. Several lines of evidence indicated a role for histamine in the modulation of glutamatergic transmission. Thus, histamine increased the release of glutamate evoked by depolarization (Rodríguez et al., 1997), and it potentiated NMDA responses in hippocampal neurons (Bekkers, 1993; Vorobjev et al., 1993). Interestingly, the later effect could not be ascribed to the activation of any of the known histamine receptors, and an interaction of histamine with the polyamine site on the NMDA receptor complex was suggested (Vorobjev et al., 1993). It is possible to speculate that a direct interaction of TEF with excitatory amino acid receptors could be also responsible for the potentiation of toxicity by AMPA or glutamate we observed here. Alternatively, TEF may interfere at the intracellular level with biochemical pathways triggered by excitatory amino acids and leading to toxicity. To test the possibility that the enhanced excitotoxicity observed in the presence of TEF could be due to inhibition of the desensitization of the receptors further studies are warranted.

Although the specific functions of histamine and histamine receptors in the CNS remain unclear, the variety of collateral effects produced by the use of antihistaminergic drugs (McTavish et al., 1990), as well as the wide distribution of histamine receptors in almost all areas of the brain (Palacios et al.,

1981), including the cerebellum (Hösli and Hösli, 1984), reflect the great importance of histamine receptors in a variety of physiological functions, and suggest the existence of complex interactions between neurotransmitters (Schwartz et al., 1991). Consistent with this idea, locomotor activity and exploratory behavior have been reported to be impaired in mutant mice lacking histamine H1-receptors (Inoue et al., 1996), and H1 receptor antagonists significantly increased heat stress-induced changes in blood-brain barrier permeability, cerebral blood flow, brain edema, and serotonin levels, in conscious rats (Sharma et al., 1992). According to our data, at least part of the effects exerted by anti-histamine drugs may not be directly related to their anti-histamine activity, and may involve the interaction with other neurotransmitter receptors and/or pathways.

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Authors' address: María Teresa Fernández-Sánchez or Antonello Novelli, Departamento Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, E-33071 Oviedo, Spain, Fax (+34) 98-5103157, Email: Neurolab@sauron.quimica.uniovi.es

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