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Ligand Binding to CNS Muscarinic Receptor Is Transiently Modified by Convulsant 3-Mercaptopropionic Acid Administration*

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The administration of convulsant drugs has proven a powerful tool to study experimental epilepsy. We have already reported that the administration of convulsant 3-mercaptopropionic acid (mp) at 150 mg/kg enhances binding affinity of muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to certain rat CNS membranes during seizure and postseizure without affecting site number. Results obtained with a 100-mg/kg dose of mp have shown reversible increases in [^{3}H]QNB binding to cerebellum and hippocampus, whereas a delayed response has been found in striatum. Neither a subconvulsant dose nor in vitro addition modifies binding. In order to evaluate preseizure, seizure as well as early (30 min) and late (24 h) postseizure stages, we employed a 50 mg/kg dose and tested [^{3}H]QNB binding to CNS membranes. Changes in binding were as follows (in %): in cerebellum, +37, +86, and +40 at preseizure, seizure and early postseizure stages, respectively, but there was a decrease at late postseizure; in hippocampus, +27 at pre- and seizure stages, but a decrease at early and late postseizure. No changes were found in striatum or cerebral cortex membranes at any stage studied. Saturation curves analysed by Scatchard plots indicated that changes in [^{3}H]QNB binding to cerebellar membranes are attributable to an increase in ligand affinity at seizure, followed by a decrease in binding site number at postseizure. A similar profile was observed for hippocampus except that the decrease in binding site number, though lower than at postseizure, was already evident at seizure stage.

Results confirm a region-specific response to the convulsant and transient changes provide an example of neuronal plasticity.

KEY WORDS: Muscarinic receptor; experimental epilepsy; [^{3}H]QNB binding; convulsant 3-mercaptopropionic acid; seizures; neuronal plasticity.

INTRODUCTION

Considerable progress has been made by experimental studies in epilepsy models in which seizures are initiated in response to the administration of convulsant drugs.

Cholinergic neurotransmission relates to epilepsy in an essential and complex manner. The role of the

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cholinergic system in the generation and propagation of electrical discharges in epilepsy has been disclosed by several studies (see Ref. 1 for a review). Experimental generalized epileptic seizures can be induced by direct stimulation of the central cholinergic system by agents such as acetylcholine enhancers or analogues and acetylcholinesterase inhibitors, and may be abolished by certain cholinergic antagonists (2).

The role of acetylcholine in seizure maintenance as occurs in *status epilepticus* has been advanced; in fact, acetylcholine appears increased in cerebral cortex and hippocampus, at variance with in vitro experiments showing that excessive stimulation leads to decreased acetylcholine levels (3,4). Acetylcholine itself is able to produce epileptiform discharges when applied to the cerebral cortex (5) and induced epileptic foci are known to correlate with decreased muscarinic receptor activity (6).

The administration of convulsant drugs such as the GAD inhibitor 3-mercaptopropionic acid (MP) also leads to generalized tonic-clonic seizures. Previous work from this laboratory has shown that the administration of a 150 mg/kg dose of MP increases binding of muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to cerebellar and striatal membranes though not to cortical and hippocampal membranes, an effect owing to an increase in affinity without changes in binding site number (7). Further work has shown that a 100 mg/kg dose of MP is also able to enhance binding not only to cerebellar and striatal membranes but also to hippocampal ones. Changes in cerebellum and hippocampus become evident after a short delay with a trend to reversibility, whereas those in striatum appear later and seem more persistent (8).

In order to evaluate a longer time course, given MP effect reversibility with a 100 mg/kg dose, we employed a lower dose (50 mg/kg) and tested [^{3}H]QNB binding to rat CNS membranes at preseizure and late postseizure stages as well.

EXPERIMENTAL PROCEDURE

Materials. 3-mercaptopropionic acid, 2,5-diphenyloxazole, and atropine sulfate were purchased from Sigma, St. Louis, MO, USA. L-[^{3}H]-quinuclidinyl benzilate (specific activity, 43.6 Ci/mmol) was puchased from Du Pont, New England Nuclear, Boston, MA, USA. All other chemicals were of the highest purity commercialy available.

Animals and Drug Treatment. Male Wistar rats weighing 100–150 g were used. Animals were maintained at least 1 week in a 12:12 h light-dark cycle (from 9.00 a.m. to 9.00 p.m.) with free access to food and water. Rats were injected i.p. between 9.30 and 11.30 a.m. with MP solutions to reach a 50 mg/kg dose. Fresh MP solutions containing 0.15 M NaCl and neutralized with Tris base were used.

After 6–10 min, the given dose induced generalized tonic-clonic seizures of moderate intensity which lasted 1 to 2 min (seizure period). Animals recovered the normal state 25–30 min after injection.

After MP administration, rats were decapitated at the following stages: before seizure (4 min), during seizure (6–10 min), and at early (25–30 min) and late (24 h) postseizure. For each experimental condition, groups of three animals were used.

Since saline administration produces no change in any of the areas studied (5), uninjected rats were used as controls. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Membrane Preparations. For each experimental condition the cerebellum, hippocampus, cerebral cortex and striatum were harvested and separately pooled. Tissues were rapidly homogenized at 10% w/v, except for cerebral cortex at 4% w/v, in 0.32 M sucrose neutralized with Tris base solution (0.4 mM Tris final concentration) in a Teflon glass Potter-Elvehjem homogenizer.

Homogenates were centrifuged at 900 *g* for 10 min and pellets discarded; resulting supernatants were diluted with 0.16 M sucrose to achieve a final concentration of 0.25 M sucrose, centrifuged at 100,000 *g* for 30 min and membrane pellets stored at −70°C until use.

[^{3}H]QNB Binding Assay. [^{3}H]QNB binding was determined according to the method described by Yamamura and Snyder (9) with slight modifications. Membrane pellets were resuspended and later diluted in 50 mM sodium-potassium phosphate buffer (pH 7.4) to reach 0.1 mg protein per ml concentration. Triplicate membrane aliquots were incubated (2 ml final volume) at 30°C for 60 min in the presence of 0.5 nM of L-[^{3}H]QNB. Non-specific binding was defined as tracer binding in the presence of 5 μM atropine sulfate. After incubation, 3 ml of ice-cold sodium-potassium phosphate buffer were added and samples vacuum-filtered through Whatman GF/B glass disks. Filters were washed twice with 3 ml of ice-cold buffer, placed in plastic vials and dried overnight at 70°C. To each vial 3 ml of 0.4% 2,5-diphenyloxazole in toluene were added and radioactivity quantified in a liquid scintillation counter.

Specific binding was calculated as the difference between the binding in the absence and presence of atropine, and represented *circa* 95% of total binding.

For saturation studies, membranes were incubated in the presence of [^{3}H]QNB concentrations ranging from 0.125 to 2.00 nM and processed as described above.

Protein was assayed according to Lowry et al. (10) using bovine serum albumin as standard.

Data Analysis. A one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls Multiple Comparisons Test was used for statistical examination of differences between control animals and MP-treated groups *inter se.* ANOVA *F* values are reported in the text and *post-hoc* test results are listed in figure captions.

Scatchard analysis was performed using the EBDA computer program (G. A. Mc Pherson 1983 V 2.0).

RESULTS

Intraperitoneal rat injection of 50 mg/kg MP resulted in the development of generalized tonic-clonic seizures. After drug administration, [^{3}H]QNB binding to rat CNS membranes was examined for changes in muscarinic receptors at preseizure, seizure as well as at early and late postseizure stages (4, 6–10, 25–30 min and 24 h after MP injection, respectively).

Table I. Effect of Convulsant MP on [^{3}H]QNB Binding to Rat Cerebellar and Hippocampal Membranes

	Cerebellum		Hippocampus	
Stage	[^{3}H]QNB binding (pmoles/mg protein)	Δ (%)	[^{3}H]QNB binding (pmoles/mg protein)	Δ (%)
Control	0.41 ± 0.02		1.40 ± 0.17	
Preseizure	0.57 ± 0.06^{a}	+ 37	1.78 ± 0.11^{aa}	+ 27
Seizure	0.77 ± 0.06^{aa,b}	+ 86	1.78 ± 0.10^{a}	+ 27
Early postseizure	0.58 ± 0.06^{a,c}	+ 40	1.18 ± 0.06^{b,c}	– 16
Late postseizure	0.31 ± 0.02^{b,cc,d}	– 24	1.12 ± 0.08^{b,c}	– 29

Animals were decapitated during preseizure (4 min), seizure (6–10 min), early (25–30 min) and late (24 h) postseizure. Results are expressed as pmoles [^{3}H]QNB bound per mg membrane protein and are mean values (± SEM) from 5–6 separate experiments. A one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls Multiple Comparisons Test was used for statistical examination of differences between control animals and MP-treated groups *inter se.* ANOVA *F* values are reported in the text.

Cerebellum

[a] $P < 0.05$ *vs* control; [aa] $P < 0.001$ *vs* control
[b] $P < 0.01$ *vs* preseizure
[c] $P < 0.01$ *vs* seizure; [cc] $P < 0.001$ *vs* seizure
[d] $P < 0.01$ vs early postseizure

Hippocampus

[a] $P < 0.05$ *vs* control, [aa] $P < 0.01$ *vs* control
[b] $P < 0.001$ *vs* preseizure
[c] $P < 0.001$ *vs* seizure

Cerebellum. At preseizure stage, [^{3}H]QNB binding to cerebellar membranes exhibited a significant 37% increase, since ligand binding in pmol per mg protein was 0.57 versus 0.41 in control group. At seizure stage, [^{3}H]QNB binding peaked at +86%, with a value of 0.77 pmoles per mg protein.

In order to examine changes in [^{3}H]QNB binding for a longer time course, two postseizure conditions were studied. Although 40% higher than control at early postseizure, binding was significantly lower than at seizure stage (0.58 pmol per mg protein), with a further drop at late postseizure (0.31 pmol per mg protein) which attained statistical significance versus two other treated groups, though not versus control group (see Table 1).

A one-way ANOVA disclosed significant differences across MP groups for [^{3}H]QNB binding ($F_{4,23}$ = 16.09, $P < 0.001$).

Hippocampus. At both preseizure and seizure stages, [^{3}H]QNB binding to hippocampal membranes was significantly increased (+27%) as compared to the control group: 1.78, 1.78 and 1.40 pmol per mg protein, respectively. At early and late postseizure values were 1.18 and 1.12 pmol per mg protein, respectively, providing a statistically significant difference versus both preseizure and seizure stages, though not versus control (Table 1).

A one-way ANOVA disclosed statistically significant differences across MP groups for [^{3}H]QNB binding ($F_{4,22}$ = 12.78, $P < 0.001$).

Striatum. No changes were found in [^{3}H]QNB binding to striatal membranes. Mean values (S.E.M.) in pmol per mg protein were 1.51 ± 0.09 for control, 1.47 ± 0.07 at preseizure, 1.51 ± 0.17 at seizure, 1.56 ± 0.13 at early postseizure and 1.49 ± 0.15 at late postseizure stages.

One-way ANOVA confirmed the absence of significant differences between treated groups *inter se* or versus control group ($F_{4,23}$ = 0.06, $P < 0.99$) at any stage studied.

Cerebral Cortex. No changes were found in [^{3}H]QNB binding to cortical membranes. Mean values in pmol per mg protein were: 1.28 ± 0.06 for control, 1.26 ± 0.11 at preseizure, 1.43 ± 0.14 at seizure, 1.36 ± 0.13 at early postseizure and 1.47 ± 0.15 at late postseizure stages.

Again, one-way ANOVA showed no significant differences between treated and control groups ($F_{4,20}$ = 0.64, $P < 0.64$).

Time Course. In order to examine changes in [^{3}H]QNB binding to rat CNS membranes for a longer time course, preseizure, seizure and two postseizure (early and late) stages were studied. Among the areas analyzed, hippocampus and cerebellum responded promptly to convulsant MP administration with a significant increase in binding. However time elapsed to achieve control values differed, because recovery occured at 30 min (early postseizure stage) and 24 h (late postseizure stage) in hippocampus and cerebellum, respectively (Fig. 3).

Saturation Studies. In order to determine whether binding changes observed during seizure and late postseizure in cerebellum and hippocampus were due to modifications in affinity and/or site number, [^{3}H]QNB binding was studied at variable ligand concentration. It was observed that binding to membranes of both areas reached saturation values with a maximum at 0.75–1.00 nM for all stages studied (Fig. 1 and 2).

In seizure period, cerebellum K_d value decreased 36%, with no significant change in B_{max}. At late postseizure, K_d was partially recovered, while B_{max} dropped significantly (30%). N_H was close to unity and remained unaltered at both experimental stages (Table II). In seizure period, hippocampus K_d value decreased 37%, with a concomitant 15% reduction in B_{max}. At late postseizure, Kd recovered control value but B_{max} dropped twice as much as at seizure. Again, N_H was close to unity and remained unaltered at both experimental stages (Table III).

DISCUSSION

We have previously shown that 150 mg/kg of convulsant MP significantly increases [^{3}H]QNB binding affinity to striatal and cerebellar membranes (7), as well as decreasing acetylcholinesterase activity in striatum and cerebellum and enhancing enzyme activity in hippocampus (11). More recently, we have observed that a 100 mg/kg MP dose is able to enhance [^{3}H]QNB binding to CNS membranes following a similar pattern for cerebellum and hippocampus, which featured a rapid increase in binding with a trend to reversibility. At variance, changes in striatum appear later and seem more persistent, supporting that MP effect is area-specific (8).

Herein we assayed [^{3}H]QNB binding to CNS membranes after the administration of a non-lethal, though convulsant, MP dose, 50 mg/kg, low enough to allow the study of a longer time course. In cerebellum, [^{3}H]QNB binding increased at preseizure, seizure and early postseizure, with maximal changes at seizure stage; a statistically significant decrease below control was recorded at late postseizure stage. A similar pattern of changes was found in hippocampus, though delta values were lower and binding dropped below control values not only at late postseizure but at early postseizure as well. Since the treatment was unable to induce changes in [^{3}H]QNB binding to striatum, the participation of striatal muscarinic receptor in seizure onset is hardly tenable.

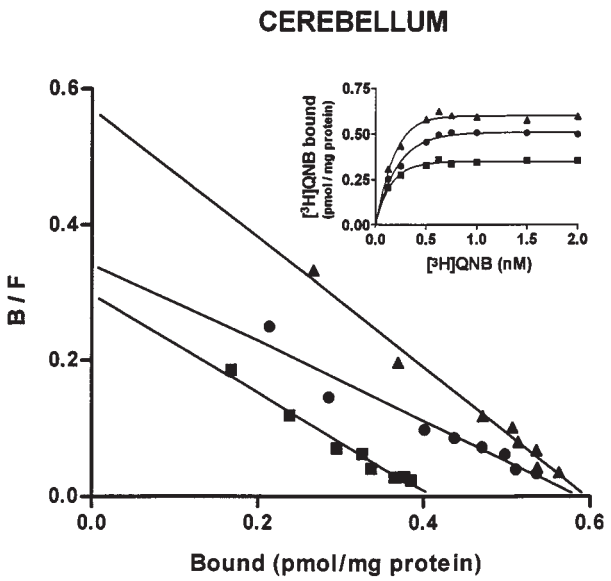

Fig. 1. Scatchard plots for L-[^{3}H]QNB binding to rat cerebellum membranes after administration of convulsant MP (50 mg/kg). Results are from a single experiment representative of a set of three, each performed in triplicate. Inset, saturation curves. (●) Control; (▲) MP seizure; (■) MP late postseizure.

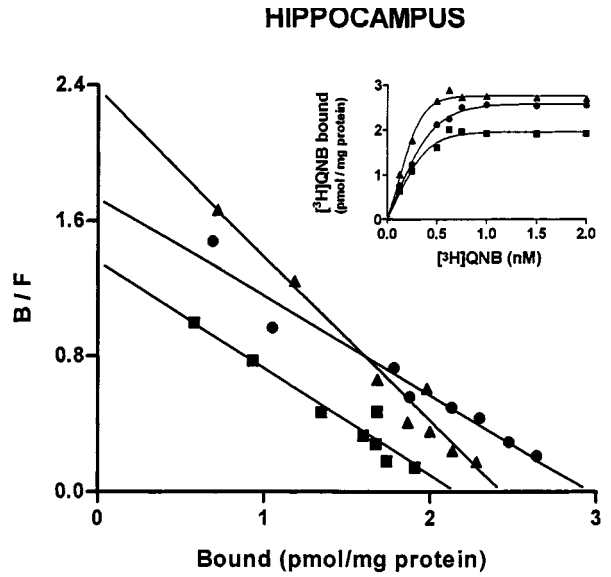

Fig. 2. Scatchard plots for L-[^{3}H]QNB binding to rat hippocampus membranes after administration of convulsant MP (50 mg/kg). Results are from a single experiment representative of a set of three, each performed in triplicate. Inset, saturation curves. (●) Control; (▲) MP seizure; (■) MP late postseizure.

Scatchard plots indicated that changes in binding to cerebellar membranes are attributable to an increase in ligand affinity at seizure, followed by a decrease in

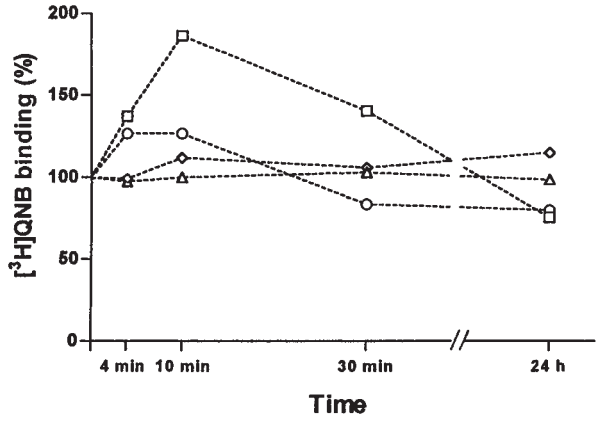

Fig. 3. Time course of [^{3}H]QNB binding to rat CNS membranes after administration of convulsant MP (50 mg/kg) at preseizure, seizure, early and late postseizure. Findings summarized in Results section are expressed as a percentage of binding measured in membranes from untreated animals.
(□) Cerebellum; (Δ) Striatum; (o) Hippocampus and (◇) Cerebral cortex.

Table II. [^{3}H]QNB Binding Constants in Rat Cerebellum Membranes after Administration of Convulsant MP

Condition	K_d (pM)	B_{max} (pmol. mg prot^{-1})	N_H
Control	148.5 ± 9.3	0.56 ± 0.01	1.08 ± 0.03
Seizure	94.6 ± 4.4*	0.58 ± 0.02	1.09 ± 0.05
Late postseizure	126.3 ± 3.2	0.39 ± 0.01**	1.03 ± 0.04

Rats were injected (i. p.) with 50 mg/kg MP and decapitated at two stages: seizure and late postseizure. Cerebella from four rats were pooled, membranes separated and [^{3}H]QNB binding performed. Results were analyzed by Scatchard plots. Data from three experiments were processed to calculate kinetic constants. Results presented are mean values (± SEM).
* $P < 0.01$; ** $P < 0.001$.

binding site number at postseizure. A similar binding profile was recorded for hippocampal membranes except that the decrease in binding site number, though lower than at postseizure, was already evident at seizure stage. Here again, there is a different area-response perhaps due to variability in the combination of functional versus non-functional populations of receptor sites.

Since Hill number was close to unity and Scatchard plots rendered linear profiles, it is suggested that [^{3}H]QNB binds to a homogeneous population.

Binding to cerebral cortex or striatum failed to change at any stage studied, though the latter responds to higher MP dose (7).

Table III. [^{3}H]QNB Binding Constants in Rat Hippocampus Membranes after Administration of Convulsant MP

Condition	K_d (pM)	B_{max} (pmol. mg prot^{-1})	N_H
Control	169.0 ± 2.9	3.05 ± 0.08	0.99 ± 0.03
Seizure	107.6 ± 4.1**	2.57 ± 0.07*	1.03 ± 0.03
Late postseizure	143.1 ± 16.1	2.09 ± 0.06**	0.94 ± 0.02

Rats were injected (i. p.) with 50 mg/kg MP and decapitated at two stages: seizure and late postseizure. Hippocampus from four rats were pooled, membranes separated and [^{3}H]QNB binding performed. Results were analyzed by Scatchard plots. Data from three experiments were processed to calculate kinetic constants. Results presented are mean values (± SEM).
* $P < 0.02$; ** $P < 0.001$.

Hippocampus is an epileptogenic area, where structural, functional and persistent changes have been observed after short convulsive episodes (12). In support, experimental epilepsy models have been shown to induce long-lasting cellular and structural alterations in hippocampus, suggesting the participation of this area in generation of abnormal excitability and development of epilepsy (13,14).

Our findings of decreased binding in hippocampal membranes at early MP postseizure is compatible with that observed after administration of kainic acid, suggesting that in this area the cholinergic neuromodulatory system is altered through activity-dependent mechanisms at early times following seizures (15). Interestingly, reduced binding in anterior hippocampus ipsilateral to the lesion has been detected in epileptic patients, attributable in part to transient postictal muscarinic receptor decrease (16).

Participation of cerebellum in motor activity is well known, a function modified during seizures. On the whole, results of [^{3}H]QNB binding to CNS membranes after MP administration lead us to conclude that cerebellum was the most affected area since changes were found at all experimental stages, and binding increases were greatest though this area presents the lowest density of muscarinic receptor sites (7). On the other hand, changes appeared promptly after MP administration and were preferentially persistent at all experimental points (Fig. 3).

Muscarinic receptors are associated with the glutamatergic system and are involved in inhibiting the release of excitatory transmitters, which in turn may well modulate neurotransmission through presynaptic muscarinic receptors located at glutamatergic nerve endings (17). Concomitant changes in muscarinic and

glutamatergic receptors have been observed. It has been reported that repeated administration of tacrine, a potent acetylcholinesterase inhibitor, decreases binding to M_2 muscarinic receptor sites in several cortical regions, while reduction in M_1 muscarinic receptor binding was restricted to the cingulate and entorhinal cortex as well as to caudate putamen. Tacrine administration also induces a decrease in AMPA receptor, while NMDA and $GABA_A$ receptor binding remain unchanged (18). On the other hand, ibotenic acid lesion produces a slight increase in M_2 muscarinic receptor binding in parietal cortex, while the M_1 subtype remains unchanged one week after lesion; reduced acetylcholinesterase activity has been observed in cortical regions where NMDA receptor is markedly diminished in comparison to the unlesioned brain side, whereas AMPA, and kainate, as well as $GABA_A$ receptor binding are significantly increased (19). Lastly, there is evidence that $GABA_B$-receptor blockade facilitates muscarinic agonist-induced epileptiform activity (20).

GABA is recognized as the major inhibitory neurotransmitter present in cerebellum. We have previously shown that convulsant MP modifies GABA system with significant decreases in glutamate decarboxylase activity and GABA levels (21,22), as well as remarkably alters morphology of cerebellar Purkinje cells (22). Cholinergic-GABA systems' interaction in striatum and hippocampus has been described (23–27) and GABAergic inhibitory neurons possess muscarinic receptors, whose activation increases excitability (28), and GABA release from cortical inhibitory neurons is modulated by presynaptic M_1 muscarinic receptors (29).

It may be speculated that increased muscarinic receptor binding by MP is due to a compensatory mechanism diminishing the participation of excitatory influences during seizure processes. In all likelihood, changes recorded herein are attributable not only to region-specific subtype distribution (30), but also to cortical and cerebellar reorganization to compensate reduced GABA synthesis, but await experimental confirmation.

Present findings showed increased [^{3}H]QNB binding to CNS membranes preceding seizure onset and indicate the ability of cerebellum and hippocampus to respond promptly to convulsant MP, so that a direct effect of the drug on these areas may be postulated and changes cannot be attributed to seizures but rather seem to indicate a major role in seizure triggering. In support, we found significant changes in [^{3}H]QNB binding to hippocampal membranes after MP doses of 100 mg/kg (8) or 50 mg/kg (present findings). However, no plausible explanation is available for the lack of effect of a higher MP dose (150 mg/kg) on binding to hippocampal membranes (7).

In hippocampal formation after amygdala kindling, electroshock, entorhinal kindling and entorhinal lesion, down regulation of muscarinic receptor has been shown, which seems an endogenous inhibitory response of neurons intensely and repeatedly depolarized during seizures (31). In agreement, we observed a tendency to lower binding at postseizure stages.

Neuronal plasticity manifests the response of a circuit, a neurotransmitter or a receptor modified as a result of diverse factors or altered processes. Changes in plasticity exhibit a wide duration range, from transient, lasting seconds to hours, to more permanent, lasting days or even the whole lifespan (32). Plasticity is associated with normal neurophysiological functions (adaptation, inhibition and facilitation, among others) participating in compensatory processes by which the CNS adapts to pathological conditions, exposure to drugs and neuronal damage or loss. Thus, permanent or transient alteration of neuronal areas is a feature of several disorders, including epilepsy, suggesting that similar mechanisms may underlie the genesis or maintenance of these dysfunctions. Present results showing that convulsant MP induces transient stimulation followed by inhibition of ligand binding to muscarinic receptor may provide an example of neuronal plasticity.

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