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7-Chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine *S,S*-dioxide (IDRA 21), a congener of aniracetam, potently abates pharmacologically induced cognitive impairments in patas monkeys

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ABSTRACT **We report here on the ability of IDRA 21 and aniracetam, two negative allosteric modulators of glutamate-induced DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor desensitization, to attenuate alprazolam-induced learning deficit in patas monkeys working in a complex behavioral task. In one component of a multiple schedule (repeated acquisition or "learning"), patas monkeys acquired a different four-response chain each session by responding sequentially on three keys in the presence of four discriminative stimuli (geometric forms or numerals). In the other component (performance) the four-response chain was the same each session. The response chain in each component was maintained by food presentation under a fixed-ratio schedule. When alprazolam (0.1 or 0.32 mg/kg p.o.) was administered alone, this full allosteric modulator of γ-aminobutyric acid type A ($GABA_A$) receptors produced large decreases in the response rate and accuracy in the learning component of the task. IDRA 21 (3 or 5.6 mg/kg p.o.) and aniracetam (30 mg/kg p.o.) administered 60 min before alprazolam, having no effect when given alone, antagonized the large disruptive effects of alprazolam on learning. From dose–response studies, it can be estimated that IDRA 21 is ≈10-fold more potent than aniracetam in antagonizing alprazolam-induced learning deficit. We conclude that IDRA 21, a chemically unrelated pharmacological congener of aniracetam, improves learning deficit induced in patas monkeys by the increase of GABAergic tone elicited by alprazolam. Very likely IDRA 21 exerts its behavioral effects by antagonizing AMPA receptor desensitization.**

In the central nervous system, γ-aminobutyric acid (GABA) is the most important and abundant inhibitory neurotransmitter, acting at $GABA_A$ and $GABA_B$ receptors (1, 2). Glutamate is the most potent and abundant excitatory neurotransmitter, acting at ionotropic DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate, *N*-methyl-D-aspartate receptors, and on metabotropic receptors (3). Together these two neurotransmitters maintain a fragile balance between neuronal excitation and inhibition. Regulation of such balance establishes a time-related association of functional neuronal assemblies and changes the strength of neuronal circuits (i.e., long-term potentiation) underlying various forms of learning and memory processes (4, 5), thereby establishing functional neuronal maps in the neocortex and the limbic system attending cognitive and sensory-motor function expression (6–8). Drugs that up-regulate GABAergic or down-regulate glutamatergic transmission in neocortical and limbic brain areas produce dramatic sensory-motor and learning impairments (9–13). For example, a facilitation of GABAergic transmission elicited at specific GABA receptors by benzodiazepines acting as full or selective positive allosteric modulators (i.e., alprazolam, triazolam, or diazepam) or an inhibition of glutamatergic transmission elicited by competitive and noncompetitive inhibitors of glutamate action at *N*-methyl-D-aspartate receptors (phencyclidine, dizocilpine, etc.) reduces acquisition and retention in rodents and in human and nonhuman primates (9–13). These considerations are central to the working model that neuropharmacologists have recently adopted in order to develop animal models to evaluate potency and efficacy of cognition-enhancing drugs acting through neocortical or limbic glutamatergic or GABAergic neurons (5, 9, 10, 13).

In the past several years in our and others' laboratories, attention has been focused on allosteric modulatory sites located on GABAergic and glutamatergic receptors that might be targets for the action of drugs (i.e., partial allosteric modulators) improving neurological or neuropsychiatric disorders, including learning and memory abnormalities, without or with minimal adverse side effects (1, 5, 9, 10–12, 14).

A role for the negative allosteric modulation of glutamate-induced AMPA receptor desensitization in long-term potentiation and cognition has been recently proposed (5, 10) following the discovery that a pyrrolidinone derivative, aniracetam, enhances cognition in rodents (15) and primates (16) by attenuating glutamate-induced AMPA receptor rapid desensitization (17). This modification is induced via the aniracetam ability to bind to a putative allosteric site located in the extracellular loop between the TM_3 and TM_4 regions of various molecular forms of AMPA receptors (18, 19). It has been reported that aniracetam administered to rodents (20) and monkeys (16) antagonizes the cognition deficit induced by scopolamine, an acetylcholine muscarinic antagonist. Since aniracetam neither binds to nicotinic or muscarinic receptors nor inhibits acetylcholinesterases or high-affinity choline uptake, it has been considered an indirect cholinomimetic (21). However, in light of aniracetam's action on AMPA receptors, its reduction of scopolamine-induced amnesia could be attributed to the strengthening of the excitatory synaptic function linked to regulation of the cholinergic neurons that innervate neocortical and limbic structures (10). Although aniracetam and its congeners did not achieve a strong clinical endorsement because their action is weak and short lasting (22), these pyrrolidinone derivatives might nevertheless be viewed as prototypes of cognition enhancers that allosterically facilitate glutamatergic transmission without producing excitotoxicity or other adverse effects (5, 23). These considerations prompted us to view this action as a departing point for the discovery of

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Abbreviations: GABA, γ-aminobutyric acid; AMPA, DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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more potent and efficacious cognition-enhancing drugs acting as negative modulators of glutamate-elicited AMPA receptor desensitization and devoid of unwanted side effects (10, 24). The present studies came to fruition when it was shown that in addition to pyrrolidinone derivatives, the benzothiadiazide derivatives cyclothiazide, diazoxide, and the newly discovered 1,2,4-benzothiadiazine IDRA 21 (7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine *S,S*-dioxide) bind to the AMPA receptor at a site presumably isosteric to that of aniracetam (19, 20, 24), thereby allosterically facilitating AMPA receptor activity by inhibiting desensitization of glutamate-mediated synaptic transmission in hippocampus; the potency of IDRA 21 was found to be severalfold higher than that of aniracetam (24).

Among various 1,2,4-benzothiadiazine derivatives that allosterically modulate AMPA cationic current in primary hippocampal neuronal cultures or in hippocampal slices (24, 25), IDRA 21 given orally is the only known compound enhancing cognition in normal rats and potently antagonizing the cognition deficit elicited by the AMPA receptor antagonist 2,3-dihydro-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) (10). Moreover, in rats, the alprazolam-induced cognition deficit mediated by its positive modulation of GABA action at various $GABA_A$ receptor subtypes was inhibited by IDRA 21 with a potency 20 times greater than that of aniracetam (10).

In spite of IDRA 21 potency and efficacy in antagonizing the cognitive consequences elicited by AMPA/kainate receptor blockers *in vivo*, our preliminary evidence suggests that this drug in doses 10 times greater than those maximally active on rodent cognitive tests fails to potentiate kainic acid convulsant activity in rodents or to elicit neurotoxicity in primary cultures of neonatal rat cerebella. Thus, IDRA 21 is an appealing drug to use as a model compound in development of more efficacious and safer therapeutic agents directed at alleviating cognition disorders related to an operational deficit of glutamatergic transmission in humans.

To explore the validity of such a pharmacological strategy further, we have administered IDRA 21 and aniracetam orally to patas monkeys and demonstrated that IDRA 21 is at least 10-fold more active than aniracetam in abating alprazolam-induced cognition deficit.

MATERIALS AND METHODS

Subjects. Two male and two female patas monkeys, ≈12 years old, served as subjects. All subjects had extensive experience with the behavioral procedure used and had been exposed to a variety of drugs in the past but were drug-free for at least 2 weeks prior to the present study. The subjects were maintained at ≈90% of their free-feeding weights (5.1–10.9 kg) on a diet consisting of Noyes banana-flavored food pellets, Purina Monkey Chow, fruit, and vitamins. Water was continuously available.

Apparatus and Behavioral Procedure. The subjects were individually housed in standard primate cages, which were equipped with response panels (26, 27). A multiple schedule with acquisition and performance components served as the behavioral baseline. During the acquisition component for the female subjects, one of four geometric forms was projected onto a red background on three response keys (press plates). The subject's task was to learn a four-response chain by pressing the correct key in the presence of each form—e.g., horizontal line, left correct; triangle, right correct; vertical line, center correct; circle, right correct. When the chain was completed, the key lights turned off and a pilot lamp near the pellet dispenser was illuminated. A press on the pilot lamp, mounted on a switch, then reset the chain. The four-response chain was maintained by food presentation under a fixed-ratio (FR5) schedule; i.e., every fifth completion of the chain produced a food pellet (500 mg) when the pilot lamp was pressed. When the subject pressed an incorrect key, the error was followed by a 5-sec time out, during which the keys were dark and responses were ineffective. An error did not reset the chain; i.e., the stimuli after the time out were the same as before the time out. The four-response chain in the acquisition component was changed from session to session. The chains were carefully selected to be equivalent in several ways and there were restrictions on their ordering across sessions (28). During the performance component, the four geometric forms were projected onto a green background and the four-response chain remained the same (left–center–left–right) from session to session. In all other aspects (e.g., FR5 schedule), the performance component was identical to the acquisition component. For the male subjects, the multiple schedule was similar except that levers were used and the discriminative stimuli were white numerals (i.e., 1, 2, 3, 4) on a black (acquisition) or green (performance) background.

Sessions were conducted daily for all subjects. Each session began in the acquisition component, which then alternated with the performance component after 10 reinforcements (food pellets) or 15 min, whichever occurred first. Each session was terminated after a fixed number of reinforcements (60 for females, 100 for males) or 2 hr, whichever occurred first.

Drug Testing. Alprazolam alone was tested first. The drug was administered orally 60 min presession twice a week in doses varying from 0.01 to 0.32 mg/kg (0.03–1.04 μmol/kg). Oral administration was done by suspending alprazolam (with a drop of dimethyl sulfoxide) in a 5% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma) and then mixing it (0.5 ml/kg) with 15 ml of fruit punch, which the subject drank. Alprazolam was tested on Tuesdays and Fridays, with control sessions (vehicle) occurring on Thursdays.

The monkeys were then pretreated with various doses of racemic IDRA 21 (0.3–5.6 mg/kg), synthesized according to the procedure described by Bertolino et al. (24). IDRA 21 was suspended in a few drops of Tween 80 and given orally in the same way as alprazolam. IDRA 21 was administered 60 min before alprazolam (0.1 or 0.32 mg/kg), which was administered 60 min presession. Next, each drug was tested alone at the same presession time used with the drug combinations (IDRA 21, 120 min; alprazolam, 60 min). Finally, to provide a direct comparison with IDRA 21, aniracetam (1–30 mg/kg, suspended in Tween 80) was administered orally alone and 60 min before alprazolam.

RESULTS

Fig. 1 documents individual differences in the control response rates for the four monkeys in both acquisition and performance (V in Fig. 1). Independently from such individual variability, when high doses of alprazolam were administered alone (0.32 mg/kg for the female monkeys, Alice and Gail; 0.1 mg/kg for the males, Murray and Fred) the overall response rates in both schedule components were decreased (AL in Fig. 1). Note that the rate-decreasing effects of alprazolam were greater in the acquisition component. Pretreatment with IDRA 21 abated the rate-decreasing effects of alprazolam in a dose-dependent fashion in both acquisition and performance. When given alone, IDRA 21 at the highest dose tested in each monkey had no effect on rate in either schedule component (I in Fig. 1). The lowest dose of IDRA 21 that reduced alprazolam's rate-decreasing effects was either 0.3 or 1 mg/kg depending on the monkey. At the highest dose of IDRA 21 (3 or 5.6 mg/kg), the antagonism was complete in some monkeys (e.g., Alice and Murray) but not in other monkeys (e.g., Fred), where noticeable rate-decreasing effects of alprazolam can be seen in relation to control (V in Fig. 1).

Fig. 2 shows that alprazolam (AL) alone increased percentage errors in both acquisition and performance in all subjects, although the error-increasing effects were much greater in

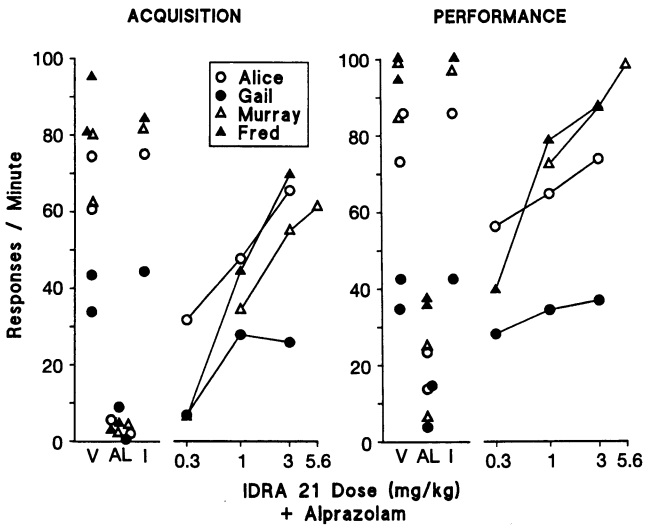

FIG. 1. Effects of varying doses of IDRA 21 in combination with alprazolam (0.32 mg/kg for Alice and Gail, 0.1 mg/kg for Murray and Fred) on the overall response rate (total responses per min excluding time outs) in acquisition and performance for four monkeys. Data at V show the control range for each monkey based on 10–12 vehicle sessions. Data at AL show the range of values for alprazolam alone (at the doses indicated above), which was tested three times in each monkey (before, during, and after the combinations were tested). Data at I are for IDRA 21 alone at the highest dose tested in each monkey. A drug (or drug combination) was considered to have an effect on rate to the extent that the drug data fell outside of the control range.

acquisition. Pretreatment with IDRA 21, at a dose having no effect on percentage errors when given alone (I in Fig. 2), blocked the error-increasing effects of alprazolam in both schedule components. As shown, the antagonism was both dose and component dependent, with complete antagonism generally occurring (except in Gail) at 3 mg/kg in acquisition and at 1 mg/kg in performance. Interestingly, at these doses of IDRA 21, the antagonism of the rate-decreasing effects of alprazolam was incomplete in most cases (Fig. 1).

Figs. 3 and 4 show that aniracetam (AN), at a dose having no effect on rate or accuracy when given alone, was similar to IDRA 21 (Figs. 1 and 2) in producing a dose-dependent attenuation of alprazolam's rate-decreasing and error-increasing effects. Note, however, that aniracetam was ≈10-fold less potent than IDRA 21 in this regard; e.g., the attenuation seen with aniracetam at 30 mg/kg (137 μmol/kg) was generally similar to that seen with IDRA 21 at 3 mg/kg (13 μmol/kg).

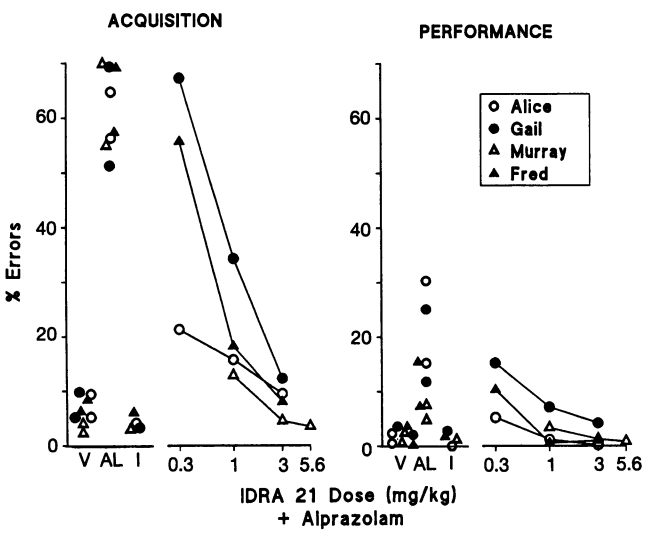

FIG. 2. Effects of various doses of IDRA 21 in combination with alprazolam on the overall accuracy as measured by percentage errors (errors/total responses × 100) in acquisition and performance. Other details are the same as in Fig. 1.

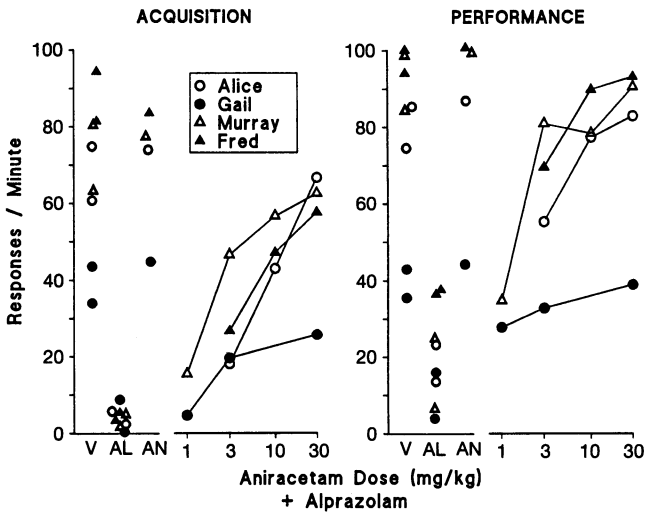

FIG. 3. Effects of various doses of aniracetam in combination with alprazolam on the overall response rate in acquisition and performance. Data at AN are for aniracetam alone (30 mg/kg). Other details are the same as in Fig. 1.

DISCUSSION

With the present understanding of the role of GABAergic and glutamatergic transmission in initiation, maintenance, and expression of the rhythmic electrical oscillations that underpin the functional properties of the thalamocortical neuronal projections and of the hippocampal neuronal circuits (6–8, 29), it is beginning to be appreciated that a cooperative interaction of neuronal assemblies that utilize either glutamate or GABA as transmitters represents the basic organization of brain operation attending not only visual function but also working memory (30).

The findings reported here demonstrate that administration of alprazolam results in a profound learning deficit in monkeys. This effect of alprazolam on cognition is presumably due to the

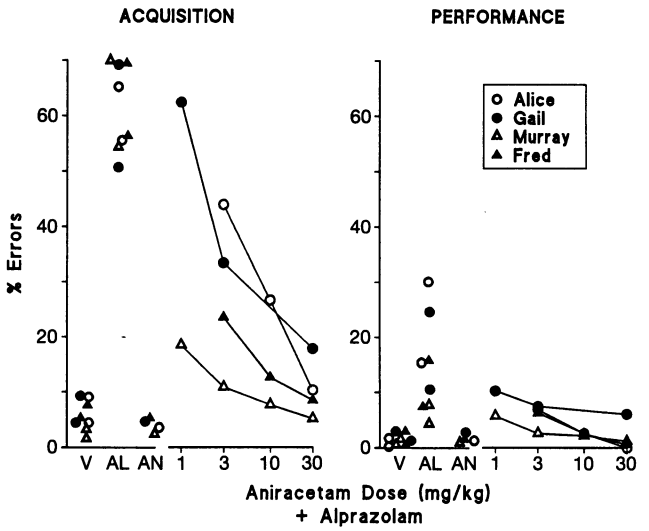

FIG. 4. Effects of various doses of aniracetam in combination with alprazolam on percentage errors in acquisition and performance. Data at AN are for aniracetam alone (30 mg/kg). Other details are the same as in Fig. 1.

allosteric modulatory action of this drug at the $GABA_A$ receptors because it occurs with drug doses that produce brain concentrations sufficient to occupy a significant percentage of $GABA_A$ receptors (31) and its behavioral effects are reverted or attenuated by either flumazenil, a competitive allosteric modulator antagonist, or imidazenil, a partial allosteric modulator at the benzodiazepine binding site of the $GABA_A$ receptor (11, 12).

The findings reported here also show that IDRA 21 and aniracetam, which are devoid of action at $GABA_A$ receptors (10, 21) but increase excitatory synaptic strength by attenuating the rapid desensitization of AMPA-selective glutamate receptors (24), administered systemically to primates (patas monkeys) blocked the acquisition deficit elicited by an increase of GABAergic tone induced by alprazolam. Moreover, aniracetam, which is $\approx$1 order of magnitude weaker than IDRA 21 in preventing AMPA receptor desensitization in hippocampal neurons *in vitro* (24), is 1 order of magnitude weaker than IDRA 21 in preventing alprazolam-induced cognition deficit in monkeys.

Although pharmacokinetics and time course studies with IDRA 21 and aniracetam have not been performed in patas monkeys, a similar difference in potency between IDRA 21 and aniracetam was observed in rats (10) when their effects were measured at the peak of their respective responses. Thus, from the experiments in monkeys and previous studies in rats (10) a correlation can be proposed between potency of IDRA 21 and aniracetam derivatives to attenuate AMPA receptor desensitization and to reverse alprazolam-induced cognition deficits. This correlation, however, does not hold for the benzothiadiazine derivative cyclothiazide, which is significantly more potent than IDRA 21 and aniracetam in preventing the desensitization of AMPA receptors *in vitro*, but is virtually ineffective when tested in our monkey model of cognition deficit (data not shown). This discrepancy between *in vitro* and *in vivo* data is presumably due to the poor brain penetration of cyclothiazide because of the presence of a $SONH_2$ group in the cyclothiazide ring.

When given in the absence of alprazolam, IDRA 21 and aniracetam fail to produce significant changes in the behavioral parameters we measured, indicating that the pharmacological action on cognition does not result from a central nervous system stimulatory action similar, for instance, to that of amphetamine or caffeine, drugs known to increase attention in animals and humans by increasing dopamine release or inhibiting adenosine receptors, respectively (32, 33). It has been reported that attention-improving drugs can increase motor performance and antagonize benzodiazepine-induced sedation or anxiolytic action but cannot consistently enhance acquisition (34, 35) or reverse benzodiazepine-induced impairment in recall (36, 37). Thus, the dose-related action of IDRA 21 and aniracetam on alprazolam-induced cognition deficit in the absence of an action on performance in normal animals suggests that IDRA 21 and aniracetam must have a primary effect on learning and memory processes rather than on attention processes. Drugs that increase glutamatergic transmission may produce behavioral adverse effects even at pharmacologically active doses; for example, exaggerated increase in sensory motor responses and seizures. In this respect, IDRA 21 and aniracetam, presumably because they are allosteric modulators with a relatively low intrinsic activity at AMPA-sensitive glutamate receptors, do not disrupt the physiological oscillation of glutamatergic transmission. They also differ from direct AMPA receptor agonists such as kainate in that they fail to produce changes in the complex behavioral processes or any other gross behavioral abnormalities triggered by a persistent receptor stimulation achieved by administering kainate.

The findings reported here also demonstrate that IDRA 21 and aniracetam at doses that antagonize the acquisition deficit induced by alprazolam fail to affect acquisition in normal monkeys. We have obtained preliminary data showing that the lack of effect of cognition-enhancing drugs in improving acquisition in normal animals is related to the difficulty level of the behavioral task. In the present research with normal monkeys, the acquisition component required that the monkey learn a four-response chain by pressing in sequence three response keys labeled by four geometric forms. Even though the four-response chain was different in each session, the monkeys made relatively few errors in learning each chain. To make the task more sensitive to drugs that may decrease the number of errors in learning, we removed the geometric forms as discriminative stimuli; thus, the monkeys were required to learn "tandem" response sequences (based only on serial position) instead of response "chains" (38). After $\approx$3 months of training, the behavior in this difficult learning task stabilized, and aniracetam, at doses similar to those that antagonize the alprazolam-induced cognition deficit, had a clear error-decreasing effect. Experiments with IDRA 21 in this behavioral model remain to be done. IDRA 21, however, has been shown to enhance learning in normal young rats subjected to a difficult water maze task (10).

In previous experiments, we have also demonstrated that in rats IDRA 21 is a potent antagonist of alprazolam-induced cognition deficit by a mechanism that does not include a direct action on $GABA_A$ receptors (10). Electrophysiological and biochemical experiments have also shown that IDRA 21 does not affect *N*-methyl-D-aspartate, nicotine, glutamate metabotropic, or muscarinic receptor function (10). Thus, to explain how blockade of the spontaneous AMPA receptor desensitization by IDRA 21 reverts the cognition deficit elicited by an increase of $GABA_A$ receptor function induced by administration of alprazolam in monkeys, we must assume that the effect of IDRA 21 on behavior results from an indirect functional down-regulation of GABAergic transmission that occurs as a consequence of the increase in the AMPA receptor-mediated excitatory synaptic strength elicited by IDRA 21. Recent observations have demonstrated that a dynamic relationship in the tone of neuronal excitatory and inhibitory interneurons in monkey prefrontal cortex and hippocampus correlates with changes in behavioral events (29).

Whittington *et al.* (7) using cortical or hippocampal slices have identified that glutamatergic metabotropic receptor stimulation elicits a 40-Hz oscillation of inhibitory GABAergic interneurons and suggest that such oscillations are a crucial component in determining time-based associations of neurons, which appear to have functional significance. It is our working hypothesis that an alteration of the GABAergic tone elicited by alprazolam, a full allosteric modulator of GABA action at the majority of benzodiazepine-sensitive cortical $GABA_A$ receptor subtypes (39), perturbs the GABA-dependent oscillatory activity that delineates the time-based recruiting of hippocampal or neocortical neuronal assemblies thereby altering cognition, attention, and sensory-motor functional responses in rats and monkeys.

IDRA 21 and presumably aniracetam fail to act on $GABA_A$ receptors directly, but very likely they act indirectly by reinforcing the strength of glutamatergic transmission. This may reinstate the appropriate time-based drive for recruiting pyramidal cortical or hippocampal neuronal populations to discharge at the required frequency, which may be optimal to express neuronal firing putatively related to their computational activity and thereby reestablish the functional interaction that was disrupted by alprazolam. In conclusion, our observations provide further evidence in support of the theory that, in primates, cognitive function may depend on a coordinated participation of hyperpolarizing and depolarizing synaptic events mediated by $GABA_A$ and AMPA receptors and further suggest that any cognition disorders due to an imbalance between GABAergic and glutamatergic transmission may benefit from the treatment with drugs, which, like aniracetam

or IDRA 21, positively modulate AMPA-gated currents and facilitate glutamatergic transmission without occupying the recognition site of glutamate.

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