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16. Pregnant ferrets were injected with BrdU (5 mg per fetus, intrauterine) on E29. Fetal brains were removed at various times after injection and were dissociated (*10*). For the 0-hour time point, cells were labeled in vitro with BrdU (20 mM) during dissociation. Cells were fixed in ice-cold 70% ethanol, immunoreacted with a monoclonal antibody against BrdU (IU-4, Caltag, 1:150), then with fluorescein-conjugated antibody to a mouse IgG (Sigma), and stained with PI (10 μg/ml, Sigma) [C. A. Hoy, G. C. Rice, M. Kovacs, R. T. Schimke, *J. Biol. Chem.* **262**, 11927 (1987)]. Cells were analyzed by 488-nm laser excitation on an EPICS flow cytometer (Coulter), using a 514-nm band-pass filter for fluorescein and a 600-nm long-pass filter for PI fluorescence.
17 The total number of BrdU-labeled cells in each two-dimensional plot (Fig. 3) was determined with a Quadstat data analysis program. A horizontal line was positioned in the valley between the upper (BrdU-labeled) and lower (unlabeled) halves of each plot. A vertical line then delineated cells on the far right with G2/M DNA contents from those with intermediate (S-phase) DNA contents. Another vertical line was drawn to delineate G1 from S-phase cells, and the percentage of BrdU-labeled cells in each phase was calculated. The placement of vertical lines was based on the positions of unlabeled cells with G1 and G2/M DNA contents; although the exact placement of each line was somewhat arbitrary, identical positions were used for all suspensions in an experiment. Thus, any inaccuracies in this simple measurement of the fraction of cells in G1, S, and G2/M are consistent among all time points. Theoretically, 100% of labeled cells should be in S-phase at time 0 (Fig. 4A); however, our measurements underestimate this fraction because DNA contents of early S-phase cells overlap with G1, and those of late S-phase cells with G2/M. This underestimate should not affect the kinetics of cell movement between phases.
18. Times in Fig. 4 and text refer to the times at which cells were removed from donors; the dissociation procedure took an additional 1.5 to 2 hours. Thus, the real time intervals between labeling and transplantation were about 2 hours longer than those indicated in graphs. Because the total length of S-phase is about 8 hours (roughly a third of the cell cycle), even cells in early S-phase at the time of labeling would have had about 6 hours to reach late S-phase if removed from donors at 4 hours.
19. Lightly labeled cells were defined as cells labeled above background but not meeting the criterion for number of grains for a heavily labeled cell. Typical lightly labeled cells had 4 to 15 silver grains over their nuclei, compared to 20 or more for heavily labeled cells.
20. The normal destinations of lightly labeled cells after an E29 [^{3}H]thymidine injection are layers 5 and 6 (*2, 6*). The presence of lightly labeled cells exclusively in layer 2/3 suggests that the laminar distributions of heavily labeled cells do not result from the differential selection or survival of precommitted cells. According to a selection hypothesis, committed deep-layer cells would be incapable of migrating when transplanted in S-phase, whereas committed upper-layer cells would only be able to migrate when transplanted in S-phase. Because lightly labeled cells migrate to layer 2/3 at every time interval, the S-phase environment may determine laminar fate.
21 It is not known whether, as development proceeds, cortical progenitors retain the full developmental potential displayed by early precursors, or whether the competence of cells to respond to early environmental cues is lost over time.
22. A "feedback control" mechanism has been proposed to regulate neurogenesis in layered neural structures [T. A. Reh and T. Tully, *Dev. Biol.* **114**, 463 (1986); T. A. Reh and I. J. Kljavin, *J. Neurosci.* **9**, 4179 (1989); D. J. Anderson, *Neuron* **3**, 1 (1989); S. K. McConnell, *Annu. Rev. Neurosci.* **14**, 269 (1991)]. The production of layer 6 neurons could conceivably feed information back to progenitors to trigger the production of the next cortical layer. Interestingly, growth cones of recently generated cortical neurons extend near or along the upper surface of the ventricular zone (G. Y. Kim, C. J. Shatz, S. K. McConnell, *J. Neurobiol.*, in press), providing a possible source of communication between postmitotic neurons and progenitors.

23. We thank S. du Lac and N. A. O'Rourke for comments on the manuscript, and B. Holland, C. Hoy, C. Carswell, S. Schneider, and R. Schimke for help with flow cytometry. Supported by grants from NIH (EY08411), Pew Scholars, Searle Scholars/Chicago Community Trust, and a Clare Boothe Luce Professorship.

19 February 1991; accepted 12 July 1991

Long-Term High-Titer Neutralizing Activity Induced by Octameric Synthetic HIV-1 Antigen

C. Y. WANG,* D. J. LOONEY, M. L. LI, A. M. WALFIELD, J. YE, B. HOSEIN, J. P. TAM, F. WONG-STAAL

A titer for homologous viral neutralization activity (>1:19,683) was observed after a 3.5-year immunization period with an octameric, branching peptide representing the principal neutralizing determinant (PND) of the human immunodeficiency virus–1_{IIIB} envelope protein. Booster immunizations elicited persistent and potent antibodies in guinea pigs, exceeding responses produced by a conventional bovine serum albumin conjugate by 100-fold. Peptide length, central presentation of a conserved sequence, and inclusion of an upstream sequence contributed to immunogenicity. Titers (>1:1,000) of heterotypic neutralizing antibodies also developed. Octameric PND peptides are a promising approach for an acquired immunodeficiency syndrome (AIDS) vaccine.

THE THIRD HYPERVARIABLE REGION (V_3) of the human immunodeficiency virus–1 (HIV-1) external envelope glycoprotein (gp120) contains a cysteine loop that constitutes the PND (*1–6*). A relatively conserved GPGR (*7*) sequence lies at the tip of the loop, flanked by type-specific sequences (*1, 2*). The V_3 loop is also a target for cytotoxic T lymphocytes and for antibody-dependent, cell-mediated cytotoxicity; and the loop is involved in syncytium formation, replication, and cell tropism (*2, 8*). Neutralizing antibodies (NAs) to the V_3 loop, though low-titer, short-lived, and type-specific, can protect chimpanzees from viral challenge (*9, 10*) and correlate with a lower incidence of maternal transmission of HIV-1 in humans (*11, 12*). Investigators have shown that antibodies to a specific V_3 peptide neutralize a significant proportion of field HIV isolates (*6, 13*), suggesting that a limited repertoire of synthetic peptides based on varied PND epitopes may meet the requirements for a broadly protective vaccine (*14*).

In an effort to enhance the immunogenicity of PND peptides, we investigated the presentation of V_3-derived peptides as radial octamers (*5*) using the multiple-antigen peptide method (*15*). A heptalysyl core bearing eight reactive NH_2-termini served as a carrier onto which eight V_3 peptides were attached by solid-phase synthesis (*16*). This antigenic PND matrix, with an eightfold molar excess of peptide over the core, forms greater than 95% of the mass of the octamer, as opposed to 6 to 28% of the mass in a comparable V_3 peptide–bovine serum albumin (BSA) conjugate (range for molar ratio of peptide to carrier 1:1 to 6:1) and <3% of the mass of the envelope glycoprotein in the viral lysate.

The immunogenicity and neutralizing responses elicited by two forms of synthetic peptide (p127) [QSVEINCTRPNNNTRKSIRIQRGPGRAFVTIGK (residues 297 to 329 of HIV-1_{IIIB} gp120) (*7*)], which overlaps the PND-V_3 region, were evaluated. The immunogenicity of octameric p127 was compared to that of monomeric p127 (*16*) coupled to BSA by conventional glutaraldehyde cross-linking (p127-BSA) and to that of the whole, inactivated HIV. Both p127-BSA and octameric p127 induced specific and high-titer (>1:1000 to 1:10,000) antibodies to their respective peptides after one boost (3 weeks after initial immunization). Reactivity of both antisera to native HIV-1_{IIIB} gp120 protein was detected by protein immunoblot after 5 to 6 weeks

C. Y. Wang, M. L. Li, A. M. Walfield, J. Ye, B. Hosein, United Biomedical, Inc., Lake Success, NY 11042.
D. J. Looney, San Diego Veterans Administration Medical Center, San Diego, CA 92161, and Departments of Medicine and Biology, University of California, San Diego, La Jolla, CA 92093.
J. P. Tam, Rockefeller University, New York, NY 10021.
F. Wong-Staal, Departments of Medicine and Biology, University of California, San Diego, La Jolla, CA 92093.

*To whom correspondence should be addressed.

post-immunization (wpi) (Table 1). Titers against gp120 equaled p127 enzyme-linked immunosorbent assay (ELISA) titers by 14 to 16 wpi (Table 1). The reactivity to HIV-1_{IIIB} gp120 by antisera to octameric p127 improved over time until it was 30 to 50 times that of the p127-BSA antisera and 10^3 to 10^4 times that elicited in sera to inactivated HIV lysate (Table 1).

Both forms of p127 immunogen elicited antibodies effective in the neutralization of the homologous HIV-1_{IIIB} virus. The NA activities correlated with the ELISA and protein immunoblot titers to gp120 (Table 1 and Fig. 1). Although the p127-BSA immunogen elicited NAs slightly earlier than the octameric p127, animals immunized with octameric p127 developed higher titer NAs by week 37 (Table 1) and displayed stronger secondary immune responses to boosting thereafter. Once developed, these high amounts of HIV-1_{IIIB}–specific NAs were sustained over a 3.5-year period (Table 1 and Fig. 1).

Table 1. Activities of guinea pig immune sera to HIV-1 gp120 V_3 peptides. Guinea pigs (two per immunogen) were immunized and bled as in Fig. 1. Every value, except for the antibody to octameric p127 (anti–octamer p127) at 165 wpi, was for sera pooled from two animals. ELISA titers to p127 were determined as in Fig. 1 and expressed as the reciprocal logarithms of the end point serum dilutions to monomeric PND peptides or to HIV-1 lysate [for antisera to HIV (anti-HIV)]. Protein immunoblot titers to gp120 of isolate HIV-1_{IIIB} refer to reciprocal logarithms of the end point dilutions that gave consistent detectable gp120 signals on HIV-1 protein immunoblots (Biotech–Du Pont). Neutralization titers, determined as in Looney *et al.* (*18*) by immunofluorescence assays (in quadruplicate) for p24 of HIV clone HX10 (IIIB) or clone RFII, refer to the end point dilutions that caused complete inhibition of p24 expression at 2 weeks for ten times the median tissue culture infectious dose (10 × $TCID_{50}$). No end point was reached with anti–octameric p127 at 165 wpi with this procedure. The NA assay was repeated with a challenge dose of 100 × $TCID_{50}$ with higher dilutions (up to 1:19, 683 in a threefold series), but the end point was still not reached. HIV-1_{MN} NA titers were determined as in Fig. 2. Anti–p127-BSA, antisera to p127-BSA; anti–octamer p200, antisera to octamer p200; and anti–hexavalent octamer, antisera to the hexavalent immunogen [4 μg of each of the following octameric PND peptides: p127 (IIIB), p200 (MN), p280 (RFII), p281 (SC), p282 (WMJ2), and p283 (SF2); alum (2%) was substituted for the Freund's adjuvants used with this immunogen].

Antisera	ELISA	Protein immuno-blot	Neutralization		
			IIIB	RFII	MN
Preimmune	<1	<1	<4	<4	
Anti-HIV					
38 wpi	4*	<2	<4	<4	
Anti–p127-BSA (IIIB)					
5 wpi	4	2.5	16	<4	
16 wpi	3.5	3.5	256	<4	
38 wpi	3.0	3.0	128	<4	
45 wpi	2.8	3.0	64	<4	
75 wpi	2.8	2.8	64	<4	
Anti–octamer p127 (IIIB)					
6 wpi	4.5	2.8	<4	<4	
14 wpi	4.0	4.0	256	<4	
37 wpi	4.5	4.5	1,024	<4	
48 wpi	5.0	5.0	4,096	<4	
165 wpi†	5.0	5.5	>8,192 >19,683	64	512
Anti–octamer p200 (MN)					
54 wpi	5.0		128		8,912
Anti–hexavalent octamer					
21 wpi	4.0		1,024		4,096

*Anti-HIV was measured from pooled sera taken at 38 weeks after three immunizations of two guinea pigs with complete and incomplete Freund's adjuvants with inactivated HIV-1_{IIIB} (50 μg per dose). †Serum was obtained from the surviving animal, which was the lower responder of the pair.

The persistent serological response to the octameric antigen was best illustrated at 163 wpi, when one surviving guinea pig had maintained a titer of 4.5 (titers are expressed as the reciprocal logarithms) to p127, 97 weeks after the most recent boost (Fig. 1). After additional boosting, the p127 ELISA titer rose to 4.9 at 165 wpi (Fig. 1), and the gp120-specific antibody titer rose to 5.5 (Table 1). The 165 wpi serum exhibited high NAs (>1:19,683 and >16,384) to homologous HIV-1_{IIIB} virus by independent neutralization assays (Table 1 and Fig. 2A). For comparison, neutralizing titers displayed by most sera from HIV-infected individuals range from <1:100 to 1:1,000, and the most potent type-specific antiserum examined (serum 505, Fig. 2A) displayed neutralizing titers of <1:4,096 in the same assays. Octameric p127 induced NA titers to HIV-1_{IIIB} virus that progressively exceeded those induced by p127-BSA, with increases from fourfold at 37 wpi to 32-fold at 48 wpi (Table 1), which were far greater than the relative increases in ELISA titer.

The length of the peptide chain in an octameric structure was proportional to its ability to elicit antibodies to p127 (Table 2). However, only when the HIV-1_{IIIB} V_3 epitope was presented in a potential PND loop structure (*1, 2, 4, 6*), with residues GPGR (7) located in a central position, did the peptides elicit native gp120-reactive antibodies (127g′, 127h′, and 127). Additionally, appreciable neutralizing titer could be elicited only by octamer 127 and not octamers 127g′ nor 127h′, suggesting that the sequence of amino acids 297 to 309 of HIV-1_{IIIB} gp120 enhances the biologically relevant antigenicity of that determinant. Extension of the HIV-1_{IIIB} PND sequence on the COOH-terminal of p127 did not improve the antigenicity of the octamer (*17*).

Table 2. Relative immunogenicity of octamer p127 analogs. Two guinea pigs per immunogen were immunized by the protocol described in Fig. 1. Sera from a total of 20 bleedings were evaluated by three criteria for immunogenicity. Data from week 17, representative of the complete course of immunization, are presented for comparison. ELISA titers were scored as in Fig. 1; titers to gp120 and neutralization titers were scored as in Table 1.

Peptide	Amino acid sequence (7)	ELISA	Protein immuno-blot	Neutral-ization
127a′	TRKSIRIQRGPGR	2.8	<1	<4
127b′	CTRPNNNTRKSIRIQRGPGR	3.8	<1	<4
127g′	RIQRGPGRAFVTIGK	3.5	2.5	<4
127h′	TRKSIRIQRGPGRAFVTIGK	4.0	3.5	20
127	QSVEINCTRPNNNTRKSIRIQRGPGRAFVTIGK	4.5	4.5	512

The relative binding affinities of a set of nested peptides to antibodies elicited by octameric p127 were evaluated by antigen-limited ELISA (*11*). The increased binding affinity at 165 wpi was seen only with the full-length peptide, which may indicate that late-appearing conformational antibodies are important. Peptides 267c and 267d,

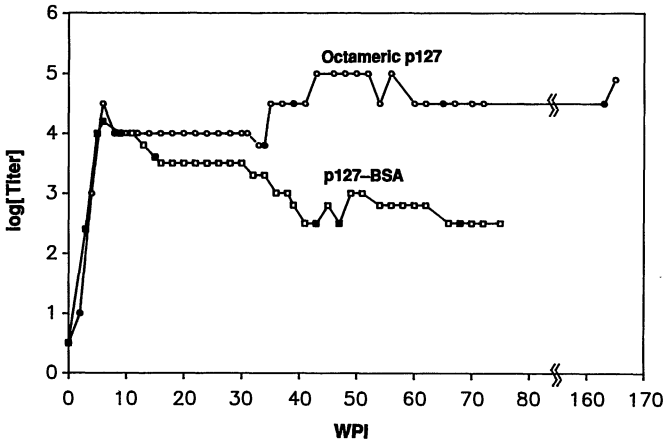

Fig. 1. Kinetics of guinea pigs' antibody response. Dunkin Hartly randomly bred female guinea pigs (two per immunogen) were immunized at week 0 with 100 μg of octameric p127 or p127-BSA in 0.5 ml of phosphate-buffered saline mixed with 0.5 ml of complete Freund's adjuvant. After 3 weeks, each animal was boosted with an identical dose of the immunogen mixed with 0.5 ml of incomplete Freund's adjuvant. Booster injections were given at the points shown by solid symbols. Guinea pig number 84, immunized with octameric p127 (circles), and guinea pig number 43, immunized with p127-BSA (squares), are shown as examples. The animals were periodically bled (*21*) as shown. ELISA titers were determined on microtiter plates coated with monomeric peptide p127 (5 μg/ml). Bound antibody was detected with horseradish peroxidase–conjugated goat antiserum to guinea pig immunoglobulin G (Biomeda, Foster City, California). The titers are the reciprocal serum dilution at which A_{492} was 1.0, extrapolated by linear regression through the linear absorbance range (reliability of regressions ≥0.97). SE of 1.0 estimated titer at A_{492} was ≤2.7% for points after 15 weeks.

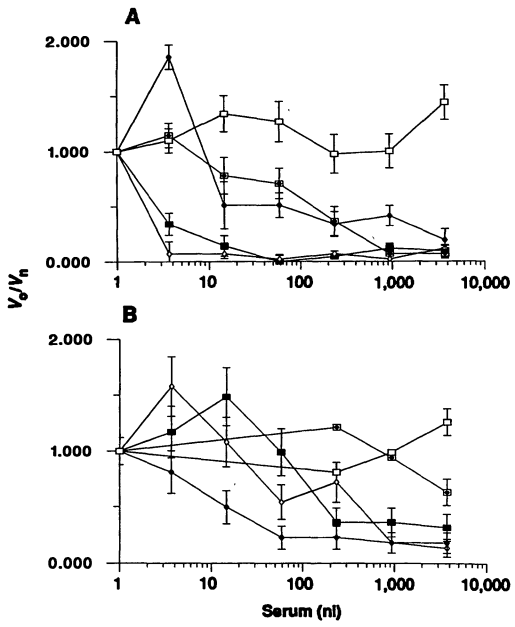

Fig. 2. (**A**) Neutralization of 50 syncytia-forming units (sfu) of HIV-1_{IIIB} by serially diluted antisera ranging from 1:16 (3750 nl) to 1:16,384 (3.6 nl), expressed as the fraction of surviving virus (V_o) divided by input virus (V_n). Virus and sera mixtures, incubated first for 45 min at 4°C and for 15 min at 20°C, were incubated for 2 hours with HT4-6C HeLa $CD4^+$ cells (the cells were first treated with DEAE-dextran) seeded 24 hours before into 48-well plates at a density of 4×10^4 cells per well, as described (*22*). Neutralization plates were incubated for 5 days, fixed in methanol, stained, and scored (*22*). Shown are neutralization titrations of three antisera to octameric p127 that include two from guinea pig number 84 obtained at 148 and 165 wpi (filled squares and open diamonds, respectively) and one collected from guinea pig number 83 at 70 wpi (filled diamonds) and the titration of goat antiserum to affinity-purified HIV-1_{IIIB} gp120 (filled circles in open squares; 505) and normal guinea pig serum (open squares). (**B**) Cross-neutralization of 50 sfu of HIV-1_{MN} by the antisera in (A). Error bars represent the standard error of syncytial counts for averages of duplicate experiments.

Table 3. Relative antibody-binding affinities and immunogenicities of linear epitopes on octameric p127.

Peptide	Amino acid sequence (7)	Concentration (μg/ml)*			
		6 wpi		165 wpi	
267a	QSVEINCTRPNNN	1.0	(300)	0.2–1.0	(300)
267b	CTRPNNNTRKSIR	>5	(10)	>5	(<10)
267c	TRKSIRIQRGPGR	0.04–0.2	(3000)	0.04	(1000)
267d	RGPGRAFVTIGK	1.0–5.0	(30)	1.0–5.0	(300)
p127	QSVEINCTRPNNNTRKSIRIQRGPGRAFVTIGK	0.04–0.2	(3×10^4)	0.008	(1×10^5)

*The antigen-limited concentration was the peptide-coating concentration at which a 10^{-2} dilution of guinea pig serum to octamer p127 gave an absorbance (A) at 492 nm of 1. ELISA titers, shown in parentheses, were determined as in Fig. 1, at peptide-coating concentrations for solid-phase antigen of 5.0 μg/ml.

which contain the conserved GPGR (7) sequence (*4, 6*), showed appreciable binding to the octamer p127–elicited antibodies (Table 3). Peptide 267c, which contains upstream flanking sequences to GPGR, also displayed greater binding affinity, consistent with the importance of these upstream sequences in eliciting HIV-1_{IIIB}–specific NAs (*2, 4, 18, 19*). The appreciable avidity of the more distal peptide 267a is consistent with a contribution of further upstream sequences [amino acids 297 to 302 (QSVEIN) (7)] to the overall immunogenicity of octamer p127 (Table 2). Although this analysis is based only on the HIV-1_{IIIB} sequence and may not be predictive of the optimal octamer for all HIV isolates, octamer p200 (an HIV-1_{MN} analog of octamer p127) and a hexavalent mixture of PND octamers (octamer p127, octamer p200, and four analogous V_3 determinants from isolates RFII, SC, WMJ2, and SF2) elicited a similar sustained ELISA titer and NA titer (Table 1).

By 70, 148, and 165 wpi, antisera to octamer p127 also displayed significant cross-neutralization titers to HIV-1 isolates RFII and MN (Table 1 and Fig. 2B). Antiserum to octamer p127 from animal number 83 at 70 wpi had an NA titer of >1:1024 to HIV-1_{MN}, whereas a standard antiserum to gp120 of HIV-1_{IIIB} (serum 505) failed to produce ≥50% inhibition of HIV-1_{MN} above a 1:4 dilution (Fig. 2B). Octamer p200 elicited a similar cross-reactive NA response (Table 1).

The use of a hexavalent mixture of V_3 determinants accelerated the kinetics for development of a broadened NA response (Table 1). Of practical importance is that alum, an adjuvant approved for human use, was substituted for the Freund's adjuvants in the hexavalent vaccine without diminishing the immune response. Studies with multivalent preparations are being extended to nonhuman primates and to determinations of NAs to patient-derived primary isolates for a more useful prediction of the immune response in humans.

Our results show that a high-titer, persistent (>3.5 years) NA response to an HIV-1 laboratory isolate is elicited when the PND is presented as an octameric synthetic antigen. The positive attributes of the octavalent immunogens should greatly facilitate the development of a subunit AIDS vaccine.

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25 June 1991; accepted 11 September 1991

Modulation of the Time Course of Fast EPSCs and Glutamate Channel Kinetics by Aniracetam

CHA-MIN TANG,* QING-YAO SHI, ALEX KATCHMAN, GARY LYNCH

It is generally accepted that glutamate serves as the neurotransmitter at most excitatory synapses in the mammalian central nervous system (CNS). Synaptic release of glutamate may trigger a fast and a slow excitatory postsynaptic current (EPSC). The slow EPSC is mediated by *N*-methyl-D-aspartate (NMDA) receptor channels, whereas the fast EPSC is mediated by non-NMDA receptor channels. The nootropic agent aniracetam selectively and reversibly slows the desensitization kinetics of non-NMDA channels and lengthens their single-channel open times. Aniracetam also modulates the kinetics of the fast EPSC in a manner that mirrors its action on the kinetics of the non-NMDA channels. These results support the hypothesis that the properties of the non-NMDA glutamate channels rather than the rate of neurotransmitter clearance are the primary determinants of the kinetics of the fast EPSC in the mammalian CNS.

IN THE MAMMALIAN CNS, SYNAPTIC currents evoked by the synaptic release of glutamate can be separated into two distinct components, the fast EPSCs and the slow EPSCs, which are respectively mediated by the non-NMDA and the NMDA subtypes of the glutamate receptor channels (*1–4*). The mechanisms that determine the time course of these two EPSC components are important for understanding synaptically mediated phenomena in the mammalian CNS. Although the time course of the slow EPSC is determined by the long burst kinetics of the NMDA channels in response to a brief exposure to glutamate (*5*), the mechanism underlying the time course of the fast EPSC is not well understood. In the experiments described here a drug that acts selectively on the non-NMDA receptor channel, aniracetam, was used to test whether modulation of the kinetics of the non-NMDA channel alone would effect an equivalent change in the time course of the fast synaptic current.

Aniracetam belongs to a class of nootropic (cognitive-enhancing) agents whose mechanisms of action are poorly understood (*6*). Ito and co-workers, using the oocyte expression system, showed that aniracetam enhances currents mediated by non-NMDA receptors but not those associated with NMDA and γ-aminobutyric acid (GABA) receptors (*7*). The mechanism of this selective enhancement, however, remains unknown.

Under conditions that minimize the conductance through NMDA channels (*8*), step application of glutamate (500 μM) elicited a rapidly activating current that rapidly decayed despite the maintenance of a constant glutamate concentration. Figure 1A illustrates the ensemble-averaged currents recorded from an excised outside-out patch before and during application of aniracetam (1 mM). Aniracetam prolonged the rate of desensitization from 6.9 to 13.8 ms (trace marked *). In 17 other membrane patches aniracetam prolonged the rate of desensitization (±SD) from 5.5 ± 1.9 to 12.5 ± 5.5 ms. In a hippocampal slice, we evoked fast EPSCs in a CA1 neuron by stimulation of Schaffer commissural pathways (*9*) (Fig. 1B). In the presence of aniracetam (trace marked *) the rate of current decay changed from 6.5 to 10.9 ms (6.9 ± 0.5 to 11.4 ± 1.0 ms in four experiments). A secondary, less pronounced, and more variable effect of aniracetam was a modest increase in the initial current amplitudes. In both excised membrane patches and the brain slice the onset of aniracetam's actions was rapid (within tens of milliseconds in the case of excised patches) and easily reversible.

Miniature EPSCs (mEPSCs) are synaptic currents evoked by the spontaneous release

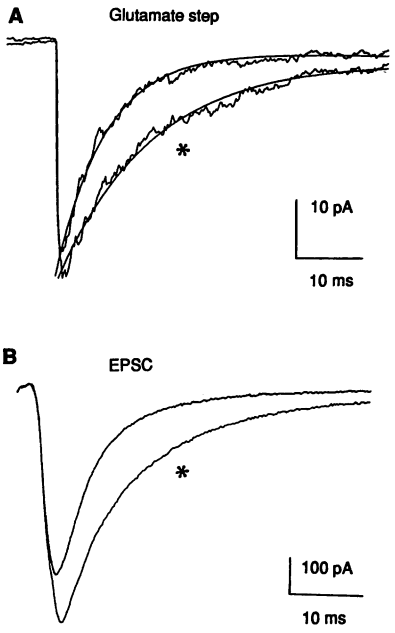

Fig. 1. Comparison of aniracetam's effects on non-NMDA glutamate channel desensitization kinetics (**A**) with its effects on the fast EPSC evoked in a CA1 neuron by stimulation of Schaffer commissural pathways in a rat hippocampal slice (**B**). In (A) the currents represent the ensemble average of recordings from ten consecutive step applications of glutamate (500 μM) on a single outside-out membrane patch. Holding potential, −80 mV in (A) and −70 mV in (B).

C.-M. Tang, Q.-Y. Shi, A. Katchman, Department of Neurology, University of Pennsylvania and the Graduate Hospital, Philadelphia, PA 19146.
G. Lynch, Bonney Center for Learning and Memory, University of California, Irvine, CA 92717.

*To whom correspondence should be addressed.