Pharmacology Biochemistry & Behavior, Vol. 12, pp. 533–541. Printed in the U.S.A.

Amnestic Potency of Proline Analogs Correlates With Anti-Spreading Depression Potency

ANTHONIE VAN HARREVELD[1], ARTHUR CHERKIN[2] AND JOEL L. DAVIS[2]

[1]*Division of Biology, California Institute of Technology, Pasadena, CA 91125 and*
[2]*Psychobiology Research Laboratory, Veterans Administration Medical Center, Sepulveda, CA 91343*

Received 21 September 1979

VAN HARREVELD, A., A. CHERKIN AND J. L. DAVIS. *Amnestic potency of proline analogs correlates with anti-spreading depression potency.* PHARMAC. BIOCHEM. BEHAV. 12(4) 533–541, 1980.—L-Proline and some of its analogs have been shown to prevent spreading depression (SD) in the chick retina at relatively low concentrations and to impair memory processing without provoking toxic or electrophysiological disturbances. Both effects are hypothesized to be caused by inhibition of the effects of glutamate released into the extracellular space. L-Proline, its D-enantiomer, six proline analogs including two homologs (L-azetidine-2-carboxylic acid and DL-pipecolic acid), and five other compounds were examined for their effects on spreading depression and their amnestic and electrophysiological effects. L-Proline, L-baikiain, DL-3,4-dehydroproline, and L-4-hydroxyproline all reduced the incidence of SD in the chick retina and proved to be amnestic. D-Proline, L-pyroglutamic acid, L-azetidine-2-carboxylic acid, DL-pipecolic acid, L-glutamic acid diethyl-ester, L-isoleucine and L-norleucine neither depressed SD nor caused retrograde amnesia. L-Prolyl-L-proline and L-glutamine did not depress SD at low concentrations but had significant amnestic effects. None of the listed compounds induced EEG disturbances. Implications for memory mechanisms are discussed in the light of these results.

Spreading depression Conditioning Retrograde amnesia Memory trace Glutamate L-Proline
L-Proline analogs

A LONG lasting facilitation of selected synapses is believed to be involved in establishing a memory trace. Such a facilitation could be caused presynaptically. There is evidence, however, for the involvement of post-synaptic structures in the long lasting facilitation of responses of dentate granular cells to perforant path stimulation [3, 4, 15]. It was suggested on theoretical grounds [26] that swelling of dendritic spines would enhance the efficacy of synapses on these spines. The swelling of spines and their stalks by the uptake of water and electrolytes results in a decrease of the longitudinal electrical resistance of these structures, facilitating the electrotonic spread of the post-synaptic depolarization caused by activation of the spinal synapses. Indeed, stimulation of the perforant path was found to cause swelling of dendritic spines of dentate granular cells [16,39]. There is some evidence that conditioning causes a similar spine swelling [17].

Swelling of dendritic structures by the uptake of extracellular fluid occurs also during asphyxiation of central nervous tissue and during spreading depression (SD), as suggested by an increase in tissue impedance [31,32] and demonstrated electron microscopically using appropriate fixation methods [40,41]. There is evidence that this swelling is caused by a release of glutamate into the extracellular space [36] which increases the Na^+ permeability of the dendritic plasma membranes, disturbing the Donnan equilibrium across the membrane and resulting in the diffusion of NaCl accompanied by water into the tissue elements involved [31, 32, 33]. The postulated (and demonstrated, [17]) spine swelling during conditioning can be expected, at least initially, to be caused by a similar release of glutamate in the extracellular space. Since this spine swelling is of long duration (at least one day; [16,39]) glutamate may trigger other processes, probably involving protein synthesis, which ensure a more enduring enlargement of the spines [38].

Assuming that glutamate is involved in SD as well as in the formation of a memory trace then both these processes can be expected to be affected by the glutamate antagonist L-proline [37]. Indeed, this has been demonstrated for SD in the isolated chicken retina [35]. The effect of L-proline on SD in this preparation is a biphasic one, reducing the incidence of this phenomenon markedly at low (2 mM) concentrations, causing less reduction at higher concentrations (5 and 7 mM) and again inducing marked inhibition at still higher concentration (10 mM and higher). This complicated effect has been explained by the postulate that the retina can under appropriate conditions entertain two mechanisms for SD, one caused by a release of glutamate from the intracellular compartment, the other by a release of K^+ [34]. The inhibiting effect of L-proline at low concentration (2 mM) would be due to a competition of released glutamate and L-proline for glutamate receptors on dendritic plasma membranes. The binding of L-proline at 5–7 mM to the glutamate receptors would cause a moderate release of K^+ into the extracellular space, promoting K^+-based SDs, and a more marked release at higher L-proline concentrations (10 mM and more), preventing SD [35].

An amnestic effect of L-proline administered intrave-

nously [38] or by cranial injection [9] was demonstrated in newly hatched chicks subjected to a one-trial conditioning procedure. Furthermore, the effects were investigated of some proline analogs (D-proline, L-baikiain and L-azetidine-2-carboxylic acid) on spreading depression in the retina [8,35] and on the one trial avoidance conditioning procedure [7,8]. A comparison of the effects of these four amino acids on spreading depression and on memory formation seemed to indicate that those compounds which inhibited spreading depression at low (2 mM) concentration (L-proline and L-baikiain) had amnestic properties, in contrast to the other amino acids (D-proline and L-azetidine-2-carboxylic acid), which did not inhibit SD at this concentration and did not cause amnesia. In the present investigation the effects of four additional proline analogs on SD in the chicken retina and their amnestic potency after one-trial conditioning were investigated in order to extend the comparison between the inhibition of SD and amnestic potency. Four other amino acids and one dipeptide were investigated in the same way, all thirteen compounds are listed in Table 1. The possibility that the amnestic effect might be due to covert brain seizures was ruled out in each case by electrophysiological studies of each compound at doses used in the amnesia studies.

EFFECTS ON RETINAL SPREADING DEPRESSION

METHOD

The retinas of 3–4 week old White Leghorn chicks were used. The eye was cut in the equatorial plane and the front part of the eye and the vitreous body were removed. This left the retina *in situ* in the posterior half of the eye. The eyes were bathed in a physiological salt solution (PS) containing 150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 15 mM glucose. To this solution the compound to be investigated was added in suitable concentrations. All solutions were kept at 32°C. Spreading depression was observed under a binocular microscope. When not developing spontaneously it was elicited by pinching the edge of the retina with fine forceps. This is a highly effective stimulus, initiating both glutamate- and K^+-based SD under adequate conditions [34]. Spreading depression develops under the conditions of the experiment as a sharp black line (increased transparency of the retina revealing the underlying black choroidea) which spreads concentrically from the stimulated area at a rate of 1.5 to 2.5 mm/min. Inside the dark line is a lighter zone, and inside this light band the transparency is again greater, showing the dark choroidea. At the center of the latter area the retina recovers, regaining its pre-spreading depression appearance [24,34].

The half eyes were kept for 6–7 min in the physiological salt solution (PS) at 32°C to allow the retinas to recover from SD initiated during the preparation of the eye. The retinas were then transferred to PS containing the compound at the concentration to be investigated and placed under the binocular microscope. The retina was checked for SD at delays of 1, 2.5 and 4 min after transfer to the test solution. This had to be done under minimal illumination since some of the compounds investigated (e.g. L-proline, [35]) tend to make the retina respond with SD to strong light stimuli. Retinas which showed no SD after 4 min were stimulated after 5 min and 15–20 sec later examined under stronger light to ascertain the presence of SD. A period of 5-min exposure to the solution was chosen because investigations on the rabbit retina [1] suggested that the concentrations in the bathing solution and in the retinal extracellular space of molecules of the size of the compounds tested in the present investigation are almost completely equilibrated within that time.

Retinas which showed SD 2.5 or 4 min after transfer were kept in the test solution. The SD observed at these times may continue during the remainder of the test period and thus not be arrested by the test compound, or they may die out before reaching the edge of the retina showing that the compound in the same concentration used, inhibits SD.

Some retinas showed SD in progress when tested after 1 min. Such SDs could have been elicited by mechanical stimulation during the transfer of the retina from the PS to the test solution. Also some of the compounds investigated tend to initiate SD (e.g. L-glutamine). Retinas showing SD 1 min after transfer to the test solution were returned to the PS. After 5 min they were then again transferred to the test solution and checked for SD after 1, 2.5 and 4 min. Since the refractory period of SD is 7–8 min such retinas usually do not show SD after 1 min. They were checked after 2.5 and 4 min and when not showing SD after these periods, stimulated after 5 min as described above.

At least 8 retinas were used to determine the effect of each concentration of the compound under investigation.

RESULTS

L- and D-Proline

Figure 1 shows the effect of L-proline in concentrations of 1 to 10 mM on SD in the chicken retina. As described previously [35], this amino acid in low concentrations (2 and 2.5 mM) decreased the incidence of SD markedly (down to 20%). Higher concentrations (5 and 7 mM) had a smaller effect (the incidence rose to 80% at 5 mM). At still higher concentrations the inhibiting effect of L-proline became more pronounced again. As reported previously, the relatively high incidence of SD at 5 mM L-proline can be reduced markedly by decreasing the K^+ concentration of the bathing solution from 3 to 1 mM. This observation supports the concept that SD in 5 mM L-proline is K^+-based [35]. In all the experiments reported in the present paper a PS containing 3 mM KCl was used.

The effect of L-proline is stereospecific. D-Proline has no effect (Fig. 3), confirming previous observations in which this D-amino acid was applied in concentrations up to 20 mM [8]. The absence of SD in one retina of 8 tested, when stimulated after being subjected to 10 mM D-proline, is not to be considered as an inhibiting effect of this amino acid. Occasionally a single retina does not show SD after stimulation under the conditions of the present experiments. The most likely explanation is that the retina was refractory after an unnoticed SD during the 5-min test period.

Since some of the compounds tested were available only in the racemic form, it was of interest to examine the effect of equimolar mixtures of L- and D-proline. The effects of such solutions in a number of concentrations were compared with the effect of L-proline alone in the same concentrations. The effects of the latter enantiomer and of the racemic mixture were quantitatively the same, justifying the investigation of DL-compounds in twice the concentration as that of the L-form.

DL-3,4-Dehydroproline

This compound, which has a double bond in the 3,4 position of the five-membered proline ring, was investigated in concentrations between 1 and 20 mM on the assumption that

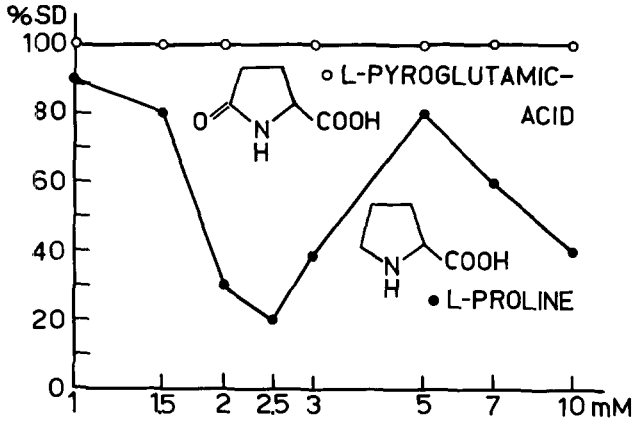

FIG. 1. The percentage of retinas showing SD after having been subjected to L-proline and L-pyroglutamic acid in various concentrations for 5 min is plotted against the concentrations of the compound on a logarithmic scale.

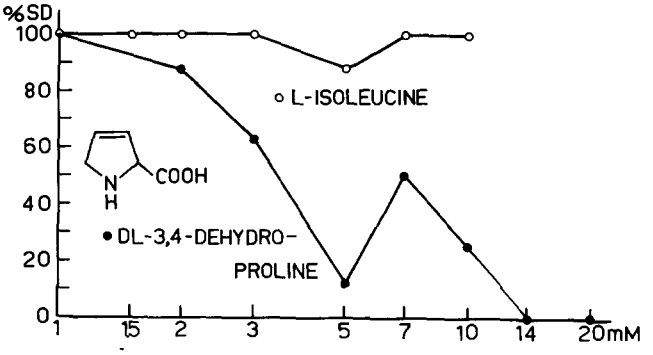

FIG. 2. The percentage of retinas showing SD after exposure to DL-3,4-dehydroproline and L-isoleucine in various concentrations. The L-enantiomer of 3,4-dehydroproline is present in one-half the concentrations plotted.

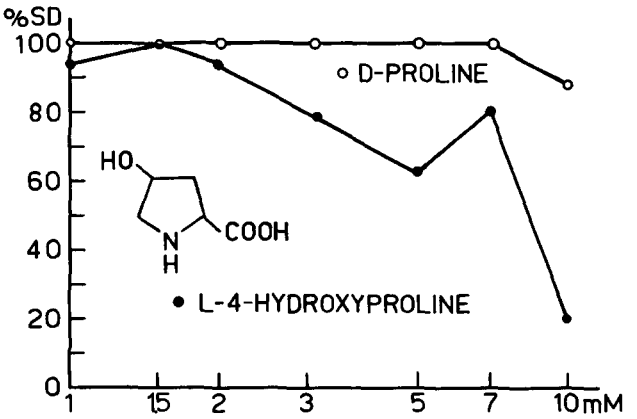

FIG. 3. The percentage of retinas showing SD after exposure to L-4-hydroxyproline and D-proline in various concentrations.

only the L-component is active. Figure 2 shows a biphasic effect on the retinal SD similar to that observed with L-proline. Pronounced reduction of SD (down to 13%) was found at 5 mM, twice the concentration at which retinas treated with L-proline showed a similar effect (Fig. 1). The maximum increase of the incidence of SD at higher concentrations (up to 50%) occurred at 7 mM, which corresponds to a similar maximum with L-proline at 5 mM. Spreading depression was completely suppressed at 14 and 20 mM. Although the effect of DL-3,4-dehydroproline resembles that of L-proline at half the concentration, this compound has a more potent inhibiting effect at concentrations of 14 and 20 mM than does L-proline at 7 and 10 mM.

L-4-Hydroxyproline

Figure 3 shows the effect of this compound on SD. A similar relationship was found for L-4-hydroxyproline as for L-proline. However, the inhibiting effect at low concentration is not very pronounced and is maximal at 5 mM (incidence of SD down to 63%). Because occasional shipments of chicks react atypically to L-proline [35] this experiment was carried out on two shipments with the same result (N for each point is 16).

L-Pyroglutamic Acid (L-5-Oxoproline)

In contrast to the compounds mentioned above, L-pyroglutamic acid had no effect on SD (Fig. 1).

L-Baikiain

Whereas the compounds examined above are characterized by a five-membered ring, L-baikiain has a six-membered ring with a double-bond in the 4–5 position. Figure 4 shows, as reported previously [35], that this compound has a pronounced effect on SD which resembles that of L-proline. A marked reduction of the incidence of SD (down to 13%) was produced by 3.5 mM L-baikiain. At higher concentrations (7 mM) the incidence of SD increased again to 83%. The inhibiting effect at a concentration of 10 mM was not marked.

DL-Pipecolic Acid

In contrast to L-baikiain this compound, which has also a six-membered ring, but lacks the double bond, had no effect on SD (Fig. 4) when applied in double the concentration (racemic mixture) used with L-baikiain.

L-Azetidine-2-Carboxylic Acid

This lower homolog of proline has a four-membered ring structure. Its effect was investigated previously in a PS containing 1 mM KCl [35]. A repetition of this examination with a PS containing 3 mM KCl yielded similar results (Fig. 5). Up to a concentration of 2 mM there is no effect, at higher concentrations the incidence of SD becomes increasingly smaller. There is no increased incidence of SD at 5–7 mM of the compound.

L-Isoleucine and L-Norleucine

L-Isoleucine has been used as a "control" amino acid without effect in memory experiments [9,38]. As shown in Fig. 2, it had no effect on SD in the retina up to the maximum concentration (10 mM) examined. L-Norleucine (up to 10 mM) was also without effect.

L-Glutamic Acid Diethylester

This compound is generally considered to be a glutamate antagonist [11, 19, 20, 22, 23, 27, 28, 29, 42]. Glutamic acid diethylester failed to inhibit the glutamate effect on the toad

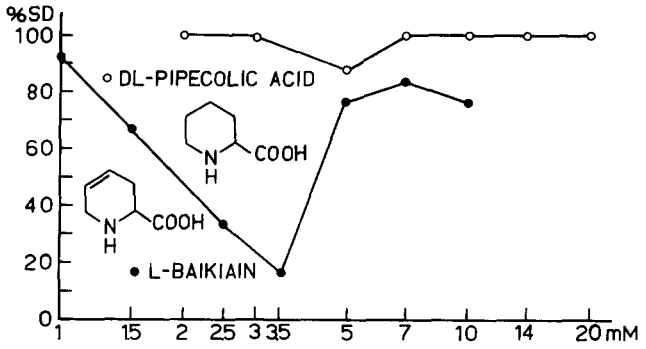

FIG. 4. The percentage of retinas showing SD after exposure to L-baikiain and DL-pipecolic acid in various concentrations.

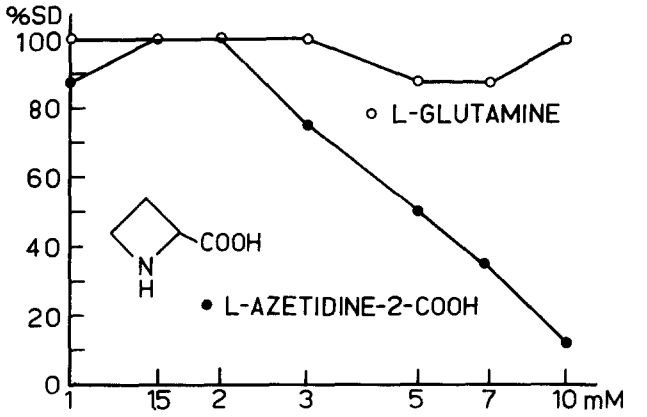

FIG. 5. The percentage of retinas showing SD after exposure to L-azetidine-2-COOH and L-glutamine at various concentrations.

spinal cord, however [2]. In the present experiments this compound in concentrations up to 10 mM did not inhibit SD in the chicken retina, as noted previously [37]. However, at higher concentrations an inhibiting effect developed, and at 40 mM this compound suppressed SD in all 8 retinas examined.

Since glutamic acid diethylester tends to hydrolyze, yielding glutamic acid which might affect the inhibiting effect of the ester, the glutamate content of the ester sample was determined by chromatography. The content was found to be smaller than 0.01%. However, after hydrolysis for 2 hr at 32°C in a 10 mM solution, a glutamate content of 0.1% was found, corresponding to 0.01 mM glutamate in the 10 mM solution of the ester. Since this is less than 1/10 of the threshold concentration causing transparency changes in the retina [36] it seems unlikely that this would have interfered with the action of the ester.

L-Glutamine

Topically applied glutamine initiates SD in rabbit and rat cortex [5,30]. Also in the chicken retina a tendency to promote SD was noticed. As shown in Fig. 5, after exposure to glutamine concentrations of 10 (and even 20) mM for 5 min SD was not inhibited.

L-Prolyl-L-Proline

This compound had no effect on SD up to 10 mM.

DISCUSSION

In order for a compound to affect the ion permeability of a membrane, a specific membrane configuration—the receptor—due to its steric and other properties has to accommodate the compound which becomes bound to the receptor. Specific groups or configurations of the compound may then cause a change in permeability. It was postulated that both L-glutamate and L-proline can be accommodated by the glutamate receptor. From differences in the inhibiting effect of $MgCl_2$ on L-glutamate-based and on L-proline-induced SDs it was concluded that the affinity of the glutamate receptor for L-glutamate was about 3 times as great as that for L-proline [35]. Furthermore, it was suggested that both L-glutamate and L-proline increase the Na^+ permeability of the receptor, but that the effect of L-glutamate is considerably greater than that of L-proline. Given a favorable ratio of the concentration of L-proline in the bathing solution and of L-glutamate released during SD, the competition of these compounds for glutamate receptors may result in such a reduction of glutamate-receptor complexes that the change in membrane permeability becomes insufficient for the propagation of SD. This mechanism would account for the inhibition of SD at a concentration of 2 mM L-proline in the bathing solution [35].

As the proline concentration is increased, more and more glutamate receptors bind with this compound, resulting in a gradually increasing enhancement of the Na^+ permeability of the membrane. Ultimately, the sodium pump will become unable to counteract the resulting influx of Na^+ ions. Sodium ions will enter partly with chloride ions and water (for osmotic reasons) resulting in swelling of the tissue elements involved, and sodium ions will partly replace intracellular potassium ions which move into the extracellular compartment [31, 32, 33]. The enhanced K^+ concentration in the extracellular space can be expected to promote K^+-based SD, accounting for the enhanced incidence of SD at 5 to 7 mM L-proline [34].

At still higher concentrations of L-proline the extracellular K^+ concentration may become so high that no visible SD, depending on the uptake of $K^+$by the Müller fibers [25,35], occurs. Furthermore, the accumulation of NaCl in the cellular elements leads to swelling and opaqueness of the retina, indicative of tissue damage as is seen after exposure to excitatory amino acids [35].

Of the proline analogs tested, some (D-proline, L-pyroglutamate, DL-pipecolic acid) had no effect on SD. It has to be assumed that their configuration makes it impossible for them to be accommodated by the glutamate receptor.

Of the proline analogs which affect SD, the inhibition of SD at low concentrations varied considerably. DL-3,4-Dehydroproline and L-baikiain depressed SD as much as L-proline at low concentrations. It has to be assumed that the affinity of the glutamate receptor for these compounds is at least as great as that for L-proline. L-4-Hydroxyproline shows only a small depression at 3–5 mM, suggesting that the affinity of the glutamate receptor for this compound is low. A small increase of the incidence of SD at 7 mM may be due to K^+-based SDs promoted by released potassium. At high concentrations L-4-hydroxyproline suppresses SD as markedly as does L-proline.

L-Azetidine-2-carboxylic acid also does not markedly inhibit SD at low concentrations (2–3 mM) and thus can be expected to have a low affinity for the glutamate receptors. It fails to increase the incidence at 5–7 mM concentrations of

this compound. The observation of opaqueness of retinas bathed in 10–20 mM L-azetidine-2-carboxylic acid [8] comparable with that caused by L-proline [37] suggests that this compound increases the membrane permeability for Na^+, which may cause the reduction of SD at high concentration.

A study of space-filling molecular models of the proline analogs did not suggest an explanation of the striking differences found in their effect on SD in the retina.

None of the other amino acids investigated had an effect on SD. The occasional failure to respond with SD to stimulation after 5-min exposure to the compound may, as mentioned above, be due to refractoriness at that time after an unnoticed SD. This can be expected especially with compounds which tend to promote SD, such as glutamine, because the transparency changes of SD developing before the refractory period (7–8 min) has fully passed is not easy to detect, especially since the possibility of the retina to respond with SD to strong illumination necessitated the use of dim light during checking for SD.

EFFECTS ON MEMORY RETENTION

METHOD

Neonatal White Leghorn cockerels (strain DeKalb XL, Pace/Setter Products, Alta Loma, CA) were housed individually in disposable white cartons, 8.5 cm in diameter, 18.5 cm tall. The temperature in the cartons was 33.5±1.0°C, the ambient relative humidity was 43±3%, the light level was 115±55 lux, and the masking white noise level was 76 dB (0.0002 dynes/cm^2). At the time of training the chicks were 44±12 hr old and had been acclimated to their individual cartons for 2 hr. They remained in the cartons and were not fed or watered throughout the experiments.

The trial was one-trial avoidance conditioning, utilizing suppression of the chick's spontaneous tendency to peck at small bright objects. The training target was a 3-mm stainless steel bead made aversive by dipping it into liquid methyl anthranilate just prior to each training presentation. During the experiments, the bead was passed through a 3-cm central aperture of the transparent plastic cover of the carton and hand-held approximately 1 cm in front of the chick's beak. A timer was started when the chick oriented to the bead, typically within 0.5 sec. Ten sec later, the bead was withdrawn. The latency of the first peck and the total number of pecks in the 10-sec period were recorded.

Intracranial injections were given 1 min after the start of the 10-sec training period. Each chick was removed from its carton and restrained in a headholder pre-calibrated to guide the 27 ga needle of a Hamilton microliter injection syringe into each forebrain hemisphere. A detailed description of the headholder and demonstration of its use in placement of solutions into the lateral ventricles has been reported [14]. Each chick received 10 μl/hemisphere of amino acid solution. In all cases the isotonic 300 mM solutions were buffered (if necessary) to pH 7.2±0.2 with $NaHCO_3$ crystals. All amino acids were purchased in the purest available grade from Sigma Chemical except for the following: DL-3,4-dehydroproline, L-baikiain, L-isoleucine, L-norleucine, L-glutamine (Calbiochem) and L-prolyl-L-proline (Bachem).

Retention of the avoidance response was tested 24 hr later using the uncoated dry bead. Reduced avoidance scores (percentage of chicks that do not peck during the 10-sec retention test) and increased peck scores (mean square root of pecks emitted in 10 sec) indicate impaired memory retention. We have found avoidance scores to be less sensitive than peck scores although the two scores are highly correlated [6]. Both scores are presented, to permit comparison with publications from other laboratories using the avoidance score. Illness reduces peck rate and increases avoidance scores. Therefore, when a compound causes illness, we get an underestimate of the amnestic effect, because the latter increases peck rate and reduces avoidance.

Experimenters neither knew which amino acid they were injecting after conditioning, nor during the final test which amino acid the chicks had received 24 hr earlier.

RESULTS

L-Proline and D-Proline

Several publications [8, 9, 18, 38] have described the amnestic nature of L-proline in chicks. At a dose of 6.0 μmols per chick (Table 1) data from many experiments lead us to conclude that L-proline produces retrograde amnesia in the paradigm used. The 6-μmol dose produced a maximal amnestic effect; increased doses lead to increased mortality, especially with D-proline [10]. Retention scores of chicks injected with D-proline did not differ significantly from those of uninjected controls or controls injected with L-isoleucine (Table 1). A comparison (t-test) between the proline stereoisomers shows them to differ significantly ($p<0.0001$).

DL-3,4-Dehydroproline

This amino acid produced retrograde amnesia similar to that caused by L-proline but at a smaller dose of 3.0 μmols [12].

L-4-Hydroxyproline

Table 1 shows L-4-hydroxyproline to be weaker than L-proline in amnestic potency. A comparison with D-proline, run as a concurrent control, shows that chicks injected with L-4-hydroxyproline produced significantly more peck responses on the test day ($p<0.04$).

L-Pyroglutamic Acid (L-5-Oxoproline)

This amino acid produced no amnesia, as compared with D-proline controls ($p>0.2$).

L-Baikiain

L-Baikiain caused retrograde amnesia similar to that produced by L-proline, but at the smaller dose of 1.5 μmols.

DL-Pipecolic Acid

This racemic amino acid, a higher homolog of proline, proved to be without amnestic properties when compared with D-proline ($p>0.4$) even at twice the dose. In fact, early data gave indication of slight memory enhancement but this could not be duplicated.

L-Azetidine-2-Carboxylic Acid

Previous work [8] indicated this lower homolog of proline to be non-amnestic when compared with D-proline controls.

L-Isoleucine and L-Norleucine

L-Isoleucine exhibited no amnestic activity when compared with D-proline and has been used as a non-amnestic control in memory experiments [38]. L-Norleucine also yielded no amnestic effect but its lower water solubility pre-

TABLE 1

RETENTION SCORES IN CHICKS 24 HR AFTER ONE-TRIAL AVOIDANCE TRAINING AND INTRACEREBRAL INJECTION OF SOLUTIONS OF L-PROLINE AND RELATED COMPOUNDS (10 μl PER BRAIN HEMISPHERE)

Compound	Dose (μmoles/chick)	N	Peck score $\sqrt{P}$	Peck score Std. dev.	Avoidance (%)	Inhibition of spreading depression*
1. L-Baikiain	1.5†	59	1.51§	1.23	30.5§	+
2. DL-3,4-Dehydroproline	3.0†	49	2.48§	1.35	12.2§	+
3. L-Proline	6.0	304	1.59§	1.49	34.5§	+
4. L-4-Hydroxyproline	6.0	48	1.20‡	1.48	52.1	(+)
5. L-Pyroglutamic acid	6.0	50	0.92	1.41	64.0	–
6. L-Azetidine-2-carboxylic acid	12.0	29	0.96	1.42	52.0	–
7. DL-Pipecolic acid	12.0	29	0.62	0.94	62.1	–
8. D-Proline	6.0	296	0.77	1.19	56.1	–
9. L-Prolyl-L-proline	6.0	82	2.09§	1.48	23.2§	–
10. L-Glutamine	6.0	89	1.26§	1.38	42.7‡	–
11. L-Glutamic acid diethylester	6.0	30	0.76	1.30	56.7	–
12. L-Isoleucine	6.0	134	0.71	1.07	60.5	–
13. L-Norleucine	1.6	20	0.70	0.98	60.0	–
14. No injection	0	50	0.72	1.09	60.0	

*Inhibition of SD at low concentration is indicated by +.

†Higher doses (6.0 μmols) show high lethality.

‡$p<0.05$; §$p<0.001$ (t-test for peck score; χ^2 test for avoidance). All comparisons are with pooled D-proline controls.

vented injection in concentrations greater than 80 mM, limiting the dose to 1.6 μmols per chick.

L-Glutamic Acid Diethylester

This compound at a dose of 6 μmols did not produce amnesia when compared with a concurrent D-proline control ($p>0.9$).

L-Glutamine

As observed previously [38], L-glutamine produced amnesia compared with D-proline run as a concurrent control ($p<0.0001$) and when compared with pooled D-proline (N=296) data ($p<0.0008$).

D-Glutamine

The D-isomer was strongly amnestic when compared with the D-proline data ($p<0.0001$) but caused severe seizure spiking.

L-Prolyl-L-Proline

This dipeptide proved to be the second most potent amnestic compound in this study; comparison with a concurrent L-proline group showed a significant increase in peck responses ($p<0.0001$). It appears unlikely that the amnestic effect was due to hydrolysis of the peptide since 12 μmols of L-proline was not significantly more amnestic than 6 μmols [7]. Note that L-prolyl-L-proline did not inhibit SD in chick retina. We are now investigating this compound's mechanism of action.

DISCUSSION

Increased pecking activity could result from processes unrelated to amnesia. Three lines of evidence indicate that the injection of L-proline does not increase the rate of pecking per se. If L-proline were to increase the peck rate via a mechanism unrelated to its amnestic properties, the training-to-injection intervals should have no effect when the chicks are tested at 24 hrs. We found, however, that injection of L-proline 1 min after training does increase the peck rate, whereas the rate decreases as the interval increases to 63 or 239 min [9]. Also, L-proline did not increase the peck rate of untrained chicks presented with the target 24 hr after injection. Finally, the procedure described in this paper involved the presentation of a novel stimulus 1 min after retention testing. This "discrimination" control indicates that the increased peck rates to the training target were not concomitant with increased peck rates to a novel target, in experiments where proline analogs showed amnestic activity.

The mean square root of pecks after injection of L-proline and its three analogs with amnestic properties differed significantly from that after administration of D-proline (Table 1). However, L-proline (6 μmols) and L-baikiain (1.5 μmols) and the racemic compound DL-3,4-dehydroproline (3 μmols) were clearly more efficient in increasing the peck score than was L-4-hydroxyproline (6 μmols). Also, the avoidance scores for L-proline, L-baikiain, and DL-3,4-dehydroproline, but not that for L-4-hydroxyproline were significantly different from that for D-proline. The analogs with strong amnestic properties resemble L-proline in their ability to reduce the incidence of SD at low concentrations (Table 1; Figs. 1, 2, 4). The lower amnestic property of L-4-hydroxyproline may be compared with the lower SD de-

pressing potency of this compound in low concentrations (Fig. 3).

Of the nine remaining compounds, seven had no significant amnestic effects nor did they inhibit SD at low concentrations (Table 1). Finally, two compounds did not inhibit SD at low concentrations but had significant amnestic effects without producing EEG abnormalities. The most potent was L-prolyl-L-proline, the other was L-glutamine. Since these two compounds do not inhibit SD at low concentrations, it is suggested that they interfere with memory by a mechanism which is different from the one postulated for the proline analogs.

ELECTROPHYSIOLOGY

METHOD

Electrodes were implanted in chicks chosen randomly from the shipments described in the previous section. Each chick had active bilateral recording electrodes stereotaxically positioned in right and left ectostriata, under the comb (indifferent), and in dorsal neck muscle (ground). Under halothane anesthesia, the electrode assembly, with miniature amphenol recording connections, was embedded and fixed to the skull with Grip dental acrylic. Active recording electrodes were insulated to 0.5 mm of the tip. Recording was begun approximately 24 hr after surgery, always between 1300–1600 hr. Electrode locations were histologically verified.

Electrophysiological screening and amplifier adjustments to be used in the experiment were determined during the adaptation period. During this period recording leads were fastened and each chick was placed in a 8.5-cm dia.×21.0-cm deep carton. The electrical activity was recorded on 6 channels of a Grass Model 7B polygraph and a Consolidated Electrodynamics VR 330 FM tape recorder. Two of the polygraph channels were used to monitor monopolar-EEG activity from each ectostriatum referenced to the comb, and a third channel recorded bipolar activity between hemispheres. Three EEG amplifiers were set at a band pass of 1 to 75 Hz (−3 dB points). The fourth channel recorded the same bipolar activity at a band pass of 10–75 Hz. For the last recording channel, multiple unit activity (MUA) recorded bipolarly from the ectostriatum was led from an input probe to a Grass Model P511 H preamplifier (−3 dB band pass points at 300 Hz to 3KHz); this activity was subsequently selected by high-pass filtering (−3 dB at 500 Hz: −2 dB at 100 Hz) with a David Kopf Model SFA 12 spike filter, and then was integrated with a David Kopf Model C10 DC converter. MUA was integrated above the threshold of system noise (10 μV) to a peak count of 1 K/sec. The integrated EEG and MUA were smoothed with low-pass filtering at 3 and 0.5 Hz, respectively.

Upon completion of a 15-min adaptation period, each chick was removed from the recording carton and manually restrained in a head holder precalibrated to guide the 27 ga needle of a Hamilton microliter injection syringe into the posterior corpus striata. A total of 10 μl/hemisphere of the amino acid was quickly injected through a hole in the acrylic recording assembly posterior to the ectostriatal electrodes. Within 5 sec after the injections, a 10-min postinjection recording period commenced.

RESULTS

None of the compounds listed in Table 1 induced EEG seizures or isoelectricity. In contrast, EEG analysis showed that 5 of 9 chicks injected intracranially with 6.0 μmols of D-glutamine (3.0 μmols/hemisphere) emitted a mean of 641 spikes recorded from both bilateral ectostriatal electrodes within 10 min after injection. The criteria for counting seizure spikes were that a spike be $\geq$400 μV peak to peak on a 1–75 Hz bipolar channel, noncoincident in left and right monopolar channels, and have a rate of occurrence of greater than 1 per 4 sec in a seizure epoch. This frequency of spike production is in the amnestic range described by Herz, Spooner, and Cherkin [21] for a potent chemoconvulsant amnestic agent, flurothyl (Indoklon®; hexafluoro-diethyl-ether). The D-isomer of glutamine was strongly amnestic when compared with the D-proline data ($p<0.0001$). However, presence of high-amplitude EEG spikes indicated that the amnesia was produced by a modification in CNS activity unrelated to glutamate release.

DISCUSSION

In the chick model, severe brain seizures induced by agents such as flurothyl [21], pentylenetetrazol and CO_2 (unpublished) do not cause amnesia unless they also produce a large number of seizure spikes. Such severe seizures spread through the striatum [21]. Therefore, electrodes placed on the ectostriatum for reasons of both convenience and avoidance of damage to striatal tissue provide an adequate control against the possibility of amnesia induced by electrophysiological disturbances.

Neither seizure spiking nor isoelectricity was observed in ectostriata after injection of any compound in Table 1. Note that the ability of this system to detect brain seizure activity was confirmed by recordings made after D-glutamine injection. Electrophysiological recording of multiple-unit activity and integrated EEG activity showed that D-glutamine was the only compound of those examined to produce ectostriatal seizures. We conclude, therefore, that the amnesia produced by the compounds listed in Table 1 are not the result of a seizure-related mechanism.

GENERAL DISCUSSION

The formation of a memory trace was postulated to be due to a glutamate mediated swelling of dendritic spines, resulting in facilitation of synapses in specific neuronal pathways in the brain. Such a mechanism can be expected to be counteracted by an appropriate glutamate antagonist such as L-proline, as was indeed observed. Memory will in this view also be counteracted by SD which, although causing spine swelling, does this in a general way, interfering with the exclusive facilitation of the specific pathways necessary for the formation of a memory trace. Spreading depression then could be considered an uncontrolled, general manifestation of a mechanism which in its normal, circumscribed form is instrumental in memory formation.

The postulated glutamate action on dendritic structures, which in SD causes the dendritic depolarization necessary for the propagation of this phenomenon, and in conditioning the spine swelling resulting in the synaptic facilitation of specific pathways, suggests that the effects of L-proline and its analogs on these activities should be correlated. Indeed a close correspondence was found between the amnestic potency of 7 proline analogs and their ability to inhibit SD at low concentration which is a measure for the competition of L-glutamate and the proline analog for glutamate receptors. Analogs which did not affect SD had no amnestic effect.

L-4-Hydroxyproline which reduced the incidence of SD only slightly at low concentrations, was the least potent of the amnestic L-proline analogs.

Interesting is the effect of L-azetidine-2-COOH, which does not have a biphasic effect on the retina but causes a decline in the incidence of SD, starting at a concentration of 3 mM (Fig. 5). This homolog of L-proline has no amnestic effect. It was suggested that L-azetidine-2-COOH has a low affinity for the glutamate receptors, apparently hardly affecting their binding with L-glutamate. However, when bound to the receptor it seems to be able to cause an increase of Na^+ permeability. From the lack of an amnestic effect of L-azetidine-2-COOH it can be concluded that the amnestic property of L-proline and some of its analogs is not due to their effect on the ion permeability of plasma membranes but on the competition of glutamate and the amnestic compound for glutamate receptors.

Of the other compounds investigated, L-isoleucine, L-norleucine and L-glutamic acid diethylester had neither an amnestic effect nor affected retinal SD. L-Glutamine was found to lack an inhibiting effect on SD, yet it proved to be amnestic. Discrepancies of this kind are to be expected, since a compound which is not a glutamate antagonist may have an effect on some other process in the chain of events involved in the laying down of a memory trace. In the case of L-glutamine the tendency of this compound to elicit SD, which has a well established amnestic effect, may be responsible for the effect on memory. The amnestic effect of D-glutamine is probably related to the EEG changes (spike activity) observed. For the similar discrepancy between the effects of L-prolyl-L-proline on SD and on memory no obvious explanation presents itself.

Gibbs *et al.* [18] proposed an alternate mechanism for the amnestic effect of L-proline. They ascribed this effect to an excessively high level of a single amino acid (L-proline) in the brain which would impair protein synthesis necessary for long-term memory. It seems possible that L-proline has a specific effect on protein synthesis, for instance by competing with other amino acids for carrier mechanisms, a property not shared by five other amino acids (L-azetidine-2-COOH, L-isoleucine, L-norleucine, L-pyroglutamate and DL-pipecolic acid) which injected in similar amounts did not affect conditioning. Such an interpretation would require a high level of specificity of the protein synthesis involved in memory formation. The azetidine-2-COOH results are particularly relevant because this lower homolog resembles L-proline so closely as to be incorporated into protein in place of L-proline in chick embryos and human fibroblasts, yet it does not impair memory [8].

Also the explicit dependence of the amnestic effect and the low concentration SD inhibiting effect on the molecular structure of L-proline and its analogs does not agree well with the mechanism proposed by Gibbs *et al.* [18]. The correspondence of the action on these two processes strongly suggests that the target of L-proline and its amnestic analogs is the same in SD as in conditioning. This is supported by the similarity of the L-proline concentration in the brain of chicks injected intraperitoneally with an amnestic dose of L-proline, and the L-proline concentration causing the low concentration reduction of the incidence of SD in the retina. From an overall concentration of free L-proline in the chick brain of 0.75 mM, after an injection of an amnestic dose, an extracellular concentration of about 3 mM was estimated [38] which is similar to the concentration, 2.0–2.5 mM, inhibiting SD in the retina. Since it seems highly unlikely that protein synthesis is involved in the effects of L-proline on the retina, the above reasoning strongly suggests that also the amnestic effect is not due primarily to an impairment of protein synthesis during conditioning, but to the antagonistic action of L-proline on the effects of L-glutamate on dendritic plasma membranes.

ACKNOWLEDGEMENTS

We thank Mary W. Garman for excellent technical assistance and to John Racs for the chromatographic analysis of the glutamic acid diethylester used. Supported in part by the Medical Research Service of the Veterans Administrations and by Grant NIMH IROI MH30279.

REFERENCES

1. Ames, A. and F. B. Nesbitt. Intracellular and extracellular compartments of mammalian central nervous tissue. *J. Physiol., Lond.* **184:** 215–238, 1966.
2. Bailey, P. A. and J. W. Phillis. The interaction of four putative glutamate antagonists with glutamate and their effects on the toad spinal cord. *Gen. Pharmac.* **7:** 283–287, 1976.
3. Bliss, T. V. P. and A. R. Gardner-Medwin. Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit, following stimulation of the perforant path. *J. Physiol., Lond.* **232:** 357–374, 1973.
4. Bliss, T. V. P. and T. Lømo. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol., Lond.* **232:** 331–356, 1973.
5. Bureš, J. Some metabolic aspects of Leão's spreading depression. *J. Neurochem.* **1:** 153–158, 1956.
6. Cherkin, A. Pharmacologic manipulation of memory processing in the neonate chick model. *Ergebn. exp. Med.* **17:** 459–473, 1974.
7. Cherkin, A. Retrograde amnesia induced in chicks by L-proline is stereospecific. *Soc. Neurosci. Abstr.* **2:** 426, 1976.
8. Cherkin, A. and A. Van Harreveld. L-Proline and related compounds: Correlation of structure, amnesic potency and antispreading depression potency. *Brain Res.* **156:** 265–273, 1978.
9. Cherkin, A., M. J. Eckhardt and L. K. Gerbrandt. Memory: Proline induces retrograde amnesia in chicks. *Science* **193:** 242–244, 1976.
10. Cherkin, A., J. L. Davis and M. W. Garman. D-Proline: stereospecificity and sodium chloride dependence of lethal convulsant activity in the chick. *Pharmac. Biochem. Behav.* **8:** 623–625, 1978.
11. Curtis, D. R., A. W. Duggan, D. Felix, G. A. R. Johnston, A. K. Tebēcis and J. C. Watkins. Excitation of mammalian central neurones by acidic amino acids. *Brain Res.* **41:** 283–301, 1972.
12. Davis, J. L. and A. Cherkin. Retrograde amnesia in chicks and mice induced by 3,4-dehydro-DL-proline, a proline analog. *Pharmac. Biochem. Behav.* **10:** 643–645, 1979.
13. Davis, J. L., L. K. Gerbrandt and A. Cherkin. Retroactive amnesia induced in chicks by the proline analog L-baikiain, without EEG seizures or depression. *Physiol. Behav.* **21:** 653–658, 1978.

14. Davis, J. L., D. T. Masuoka, L. K. Gerbrandt and A. Cherkin. Autoradiographic distribution of L-proline in chicks after intracerebral injection. *Physiol. Behav.* **22:** 693–695, 1979.
15. Douglas, R. M. and G. V. Goddard. Long-term potentiation of the perforant path-granule cell synapse in the rat hippocampus. *Brain Res.* **86:** 205–215, 1975.
16. Fifkova, E. and A. Van Harreveld. Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. *J. Neurocytol.* **6:** 211–230, 1977.
17. Fifkova, E. and A. Van Harreveld. Changes in dendritic spines of the dentate molecular layer during conditioning. *Soc. Neurosci. Abstr.* **4:** 257, 1978.
18. Gibbs, M. E., K. T. Ng and A. L. Richdale. L-Proline inhibition of long-term memory function. *Neurosci. Lett.* **6:** 355–360, 1977.
19. Haldeman, S. and H. McLennan. The antagonistic action of glutamic acid diethylester towards amino acid-induced and synaptic excitations of central neurones. *Brain Res.* **45:** 393–400, 1972.
20. Haldeman, S., R. D. Huffman, K. C. Marshall and H. McLennan. The antagonism of the glutamate-induced and synaptic excitations of thalamic neurones. *Brain Res.* **39:** 419–425, 1972.
21. Herz, M. J., C. E. Spooner and A. Cherkin. Effects of the amnestic agent flurothyl on EEG and multiple-unit activity in the chick. *Expl Neurol.* **27:** 227–237, 1970.
22. Lowagie, C. and H. M. Gerschenfeld. Glutamate antagonists at a crayfish neuromuscular junction. *Nature, Lond.* **248:** 533–535, 1974.
23. MacDonald, J. F., A. Nistri and A. L. Padjen. Neuronal depressant effects of diethylester derivates of excitatory amino acids. *Can. J. Physiol. Pharmac.* **55:** 1387–1390, 1977.
24. Martins-Ferreira, H. and G. De Oliveira Castro. Light-scattering changes accompanying spreading depression in isolated retina. *J. Neurophysiol.* **29:** 715–726, 1966.
25. Mori, S., W. H. Miller and T. Tomita. Müller cell function during spreading depression in frog retina. *Proc. natn. Acad. Sci. U.S.A.* **73:** 1351–1354, 1976.
26. Rall, W. Cable properties of dendrites and effects on synaptic location. In: *Excitatory Synaptic Mechanisms,* edited by P. Anderson and J. K. S. Jansen. Proc. 5th Internat. Meeting of Neurobiologists, 1970, pp. 175–187.
27. Spencer, H. J. Antagonism of cortical excitation of striatal neurons by glutamic acid diethyl ester: Evidence for glutamic acid as an excitatory transmitter in the rat striatum. *Brain Res.* **102:** 91–101, 1976.
28. Spencer, H. J., V. K. Gribkoff, C. W. Cotman and G. S. Lynch. GDEE antagonism of iontophoretic amino acid excitation in the intact hippocampus and in the hippocampal slice preparation. *Brain Res.* **105:** 471–481, 1976.
29. Stone, T. W. Blockade by amino acid antagonists of neuronal excitation mediated by the pyramidal tract. *J. Physiol., Lond.* **257:** 187–198, 1976.
30. Van Harreveld, A. Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle. *J. Neurochem.* **3:** 300–315, 1959.
31. Van Harreveld, A. Brain tissue electrolytes. In: *Molecular Biology and Medicine Series,* edited by E. E. Bittar. Washington, DC: Butterworths, 1966.
32. Van Harreveld, A. The extracellular space in the vertebrate central nervous system. In: *The Structure and Function of Nervous Tissue,* Vol. IV, edited by G. H. Bourne. New York and London: Academic Press, 1972, pp. 447–511.
33. Van Harreveld, A. Pharmacology of the vertebrate retina. *Prog. Neurobiol.* **8:** 1–43, 1976.
34. Van Harreveld, A. Two mechanisms of spreading depression in the chicken retina. *J. Neurobiol.* **9:** 419–431, 1978.
35. Van Harreveld, A. Effects of L-proline and some of its analogs on retinal spreading depression. *J. Neurobiol.* **10:** 355–365, 1979.
36. Van Harreveld, A. and E. Fifkova. Glutamate release from the retina during spreading depression. *J. Neurobiol.* **2:** 13–29, 1970.
37. Van Harreveld, A. and E. Fifkova. Effects of amino acids on the isolated chicken retina,and on its response to glutamate stimulation. *J. Neurochem.* **20:** 947–962, 1973.
38. Van Harreveld, A. and E. Fifkova. Involvement of glutamate in memory formation. *Brain Res.* **81:** 455–467, 1974.
39. Van Harreveld, A. and E. Fifkova. Swelling of dendritic spines in the fascia dentata after stimulation of the perforant fibers as a mechanism of post-tetanic potentiation. *Expl Neurol.* **49:** 739–749, 1975.
40. Van Harreveld, A. and F. I. Khattab. Changes in cortical extracellular space during spreading depression investigated with the electron microscope. *J. Neurophysiol.* **30:** 911–929, 1967.
41. Van Harreveld, A., J. Crowell and S. K. Malhotra. A study of extracellular space in central nervous tissue by freeze-substitution. *J. Cell. Biol.* **25:** 117–137, 1965.
42. Wheale, H. V. and G. A. Kerkut. The antagonistic action of L-glutamate diethyl ester on the excitatory post-synaptic membrane of the crab neuromuscular junction. *Comp. Biochem. Physiol. (Comp. Pharmac.)* **51:** 79–81, 1975.