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LONG-TERM POTENTIATION IN THE HIPPOCAMPUS OF THE ANAESTHETIZED RAT IS NOT ASSOCIATED WITH A SUSTAINED ENHANCED RELEASE OF ENDOGENOUS EXCITATORY AMINO ACIDS

L. ANIKSZTEJN,* M. P. ROISIN, R. AMSELLEM and Y. BEN-ARI
INSERM U29, Hôpital de Port-Royal, 123 Bld de Port-Royal, 75014 Paris, France

Abstract—The relationship between long-term potentiation of synaptic transmission and the release of endogenous glutamate and aspartate has been investigated in the CA1 region of the hippocampus and in the fascia dentata of the anaesthetized rat. A high-frequency train of electrical stimulation of afferent pathways produced a long lasting (>2 h) enhancement of the field excitatory postsynaptic potential in CA1 and of the population spike in the fascia dentata. In both regions, this was not associated with a significant long lasting increase in the release of glutamate and aspartate.

It is concluded that the maintenance of long-term potentiation is not associated with a sustained increase in the release of excitatory amino acids.

High-frequency trains of electrical stimulation produce a long lasting enhancement of synaptic transmission.[7] This long-term potentiation (LTP), which has been extensively studied in the hippocampus,[30] persists for weeks or months in the intact animal.[6,8,15] LTP is considered to be a physiological substrate of learning and memory processes[23,24] and as such has attracted considerable interest in its underlying mechanisms.

The contribution of pre- and postsynaptic processes to LTP has not been clarified. Using a push–pull technique, Bliss and coworkers[5,16] have provided evidence in favour of a presynaptic mechanism in the fascia dentata, i.e. a sustained enhancement of the release of glutamate and aspartate which are thought to mediate synaptic transmission in the perforant pathway.[14,29] However, to observe this enhancement, Bliss *et al.*[5] have compared one group of animals receiving control stimulation (0.033 Hz) to another group which received high-frequency stimulation; in fact there was no statistical difference in the release of excitatory amino acids in the same group of animals before and after the train (e.g. Fig. 3).[5] In contrast to the above, we found that the long-lasting potentiation induced by a phorbol ester[26] or by the mast cell degranulated peptide[11] is not associated with a sustained enhancement of glutamate and aspartate release[2] (see also Aniksztejn *et al.*, unpublished observations). Although the underlying mechanisms of the potentiation produced by a train of electrical stimulation and by these agents may be different (see for example Ref. 17), these observations suggest that the postsynaptic neurons are important in the maintenance of the LTP. This is in keeping with several other studies.[3,19,22,25,27]

In the present report, we have reexamined the relationship between electrically induced LTP and the release of endogenous glutamate and aspartate, using each animal as its own control. This work has been presented in brief elsewhere.[1]

EXPERIMENTAL PROCEDURES

Twenty-six adult Wistar rats weighing 200–300 g were used. They were anaesthetized with urethane (2 g/kg) and placed in a stereotaxic frame. A push–pull cannula was introduced in the stratum radiatum of CA1 (A = 4; L = 2.8) or in the dentate gyrus (A = 4; L = 2.3) of one hippocampus. The position of the cannula was adjusted so as to record the typical field potential evoked by the electrical stimulation of the commissural pathway or the perforant pathway respectively. A monopolar electrode (50 μm o.d.) was used for recording; its length was the same as the inner cannula and it was attached to the outer cannula (0.6 mm o.d.). A twisted bipolar electrode was implanted, either in the hippocampal commissure (A = 6.6, L = 1, H = 7) to stimulate the commissural pathway, or in the entorhinal cortex (A = 0, L = 4.2, H = 6.4) to stimulate the medial perforant pathway. Test stimuli (0.05 ms) were applied at a frequency of 0.033 Hz. Once the push–pull cannula was accurately placed, an oxygenated artificial cerebrospinal fluid was perfused, with the following composition (in mM): 126 NaCl; 3.5 KCl; 2 $CaCl_2$; 1.3 $MgCl_2$; 1.2 NaH_2PO_4; 25 $NaHCO_3$; 11 glucose, pH 7.4. The flow rate was 10 μl/min. The perfusion was continued for a stabilization period of 1 h. At the end of this period, samples were collected at 5-min intervals during 1 h (control period) and a high-frequency train (100 Hz for 1 s at the same intensity as during the control simulation) was applied. Samples were collected at 5-min intervals for an additional period of 2 h. The content of endogenous glutamate and aspartate was determined by high-performance liquid chromatography (HPLC).

*To whom correspondence should be addressed.

Abbreviations: EPSP, excitatory postsynaptic potential; HPLC, high performance liquid chromatography; LTP, long term potentiation; NMDA, *N*-methyl-*d*-aspartate; OPA, *O*-phthaldialdehyde.

High-performance liquid chromatography procedure

All chemicals were analytical reagent grade obtained from Carlo–Erba. *O*-Phthaldialdehyde (OPA) and amino acids were obtained from Sigma (Saint-Louis, MI). Standard solutions were made in methanol:water (1:4) at a concentration of 1.5 mg/ml. Aliquots of these solutions were stored at −20°C and diluted each day for calibration. OPA reagent was prepared by dissolving 50 mg of OPA in 1 ml methanol then diluting to 10 ml with 0.2 M sodium tetraborate (pH 9.5). Finally 40 μl of 2-mercaptoethanol was added. This reagent was stored at 4°C and every 4 days, 40 μl of 2-mercaptoethanol were added. The HPLC system consisted of two Beckman 114M pumps controlled by a Beckman 421A programmer, a 7010 Rheodyne injection valve fitted

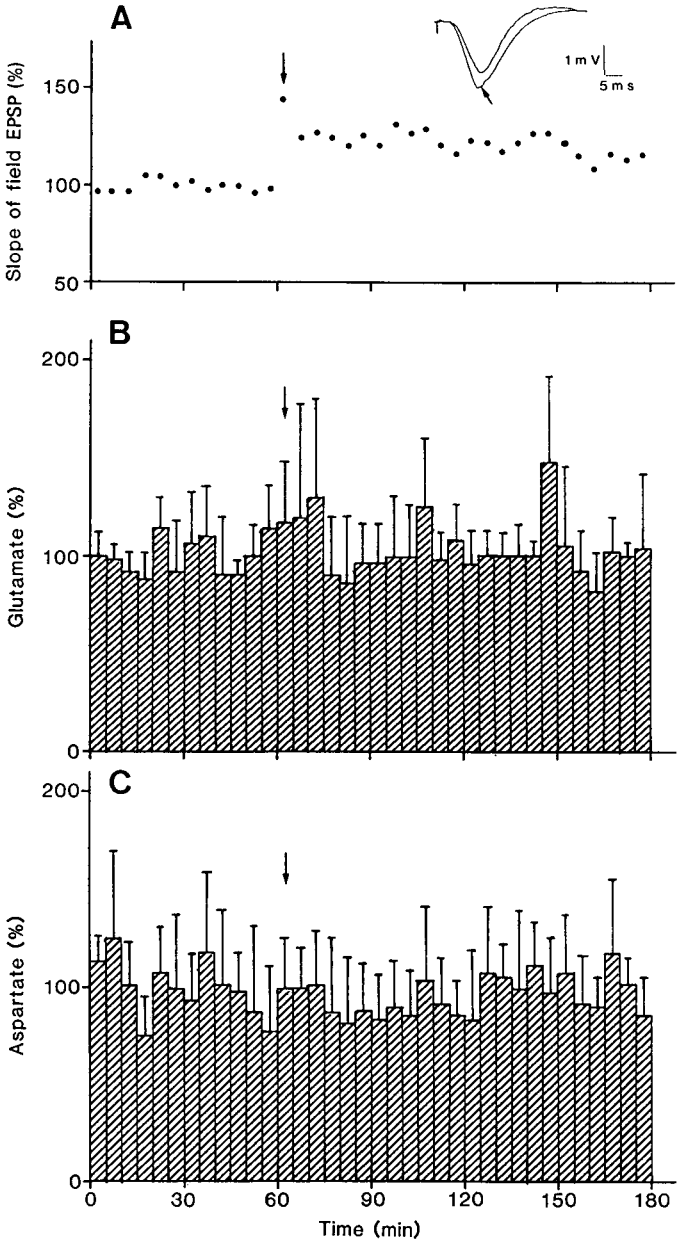

Fig. 1. Brief high-frequency stimulation of the commissural–Schaffer collateral pathway (100 Hz, 1 s) produces long-term potentiation of the field EPSP in the stratum radiatum of CA1 but no change in the release of glutamate and aspartate. (A) Long-term potentiation of the field EPSP in a typical experiment. The slope of the field EPSP evoked by the Schaffer–commissural stimulation (0.033 Hz, 0.05 ms duration) is indicated as a percentage of the 60-min control (pre-train) level. The traces represent the field EPSP just before the high-frequency stimulation and the potentiated EPSP (arrow) 1 h after the stimulation. One field EPSP was digitized every minute and five consecutives EPSPs were sampled to obtain the average shown in the figure. This EPSP therefore corresponds to one (5-min) sample used for HPLC. Release of glutamate (B) and aspartate (C) expressed as a percentage of the 60-min control period (% mean ± S.D. of mean) ($n = 8$). Note that there is some cyclic variation in the release of the two amino acids; however, the levels of glutamate and aspartate are not significantly different before and after the train ($P = 0.05$, Student's t-test).

with a 20 μl loop. A 4.6 × 75 mm column packed with Ultrasphere XL3μ ODS (Beckman), an XL ODS pre-column, was used for the separation of amino acids. The mobile phase was constituted by a mixture of sodium acetate (50 mM) pH 7 and methanol, the proportions of which were adjusted by the controller according to the following programme. Time 0: 20% methanol; time 1 min: 32% in 1.5 min; time 4 min: 80% in 2 min; time 7 min: 20% in 2 min. The flow rate was adjusted to 1 ml/min and the separation was monitored with a Kratos 950 Fluorimeter (excitation = 330 nm; emission = 418 nm). The chromatographic data were processed with a Shimadzu CR3A integrator. The derivatization was performed using one volume OPA for one volume of the perfusate collected and the mixture was injected after 60 s. A single run took 10 min to complete. At the end of each experiment, the rats were perfused intracardially with saline solution followed by 4% paraformaldehyde. Conventional histological procedures were done to localize the exact position of the cannula.

RESULTS

*CA*1 *region*

In control conditions, stimulation of the commissural pathway at 0.033 Hz evoked in stratum radiatum a negative field excitatory postsynaptic potential (EPSP). As shown in Fig. 1A the stimulation at 100 Hz for 1 s enhanced the field EPSP. The curve had a typical biphasic slope with a 40% increase over the first 5 min (frequency potentiation), and a subsequent 20% increase over the control persisting for 2 h; in eight rats which were used for release experiments (see below), 2 h following the train the slope of the EPSP was increased by 22 ± 10% ($\bar{X}$ ± S.D.). To accurately determine the effects of the train on the release of glutamate and aspartate, we used three methods of analysis as follows.

(1) In each rat, the time course of change in individual (5-min) samples (expressed in ng/samples) was studied in the 1-h control period and for 2 h after the train. We found no significant change in the release of glutamate and aspartate. Similar observations were made in the eight rats (not shown). However, in two cases there was a small increase in the release of amino acids which persisted (5–10 min) after the train.

(2) The release of glutamate and aspartate was then expressed as a percentage of the 1-h control period, and the values pooled for the eight animals. As shown in Fig. 1B and C there was no obvious long lasting increase in the release of amino acids. Table 1 provides quantitative data on the effect of LTP. Here, to reduce variability, three and six consecutive samples were pooled (15 and 30 min); clearly there were no significant changes in the release of glutamate and aspartate.

(3) We have also studied the release of glutamate and aspartate in a control group of animals in which the commissural–Schaffer collateral system only received test stimuli at a frequency of 0.033 Hz (n = 6). As shown in Table 1, in the control group the release was relatively stable during the 3 h of the control experiments; Table 1 also shows that there was no

Table 1. Release of aspartate and glutamate in CA1, in control animals (n = 6) and animals subjected to a brief high-frequency train after a 60-min control period (n = 8)

	Aspartate			Glutamate		
Time (min)	Control (n = 6)		LTP (n = 8)	Control (n = 6)		LTP (n = 8)
0–60	100 ± 24		100 ± 28	100 ± 33		100 ± 14
60–75	82 ± 27	train →	99 ± 22	83 ± 25	train →	121 ± 50
75–90	92 ± 57		87 ± 29	108 ± 56		90 ± 22
90–120	100 ± 46		86 ± 21	103 ± 36		102 ± 23
120–150	105 ± 65		100 ± 25	106 ± 38		107 ± 17
150–180	121 ± 62		96 ± 21	130 ± 53		98 ± 24
ng/sample	0.725 ± 0.140		0.725 ± 0.203	0.770 ± 0.260		0.875 ± 0.122

The values which were pooled in 15- or 30-min samples are indicated as a percent of the 60-min control period. The values expressed in ng/sample in the lower part of the table correspond to 100%.

Table 2. Release of aspartate and glutamate in the fascia dentata in control animals (n = 5) and animals subjected to a high-frequency stimulation after a 60-min control period (n = 7)

	Aspartate			Glutamate		
Time (min)	Control (n = 5)		LTP (n = 7)	Control (n = 5)		LTP (n = 7)
0–60	100 ± 30		100 ± 33	100 ± 25		100 ± 24
60–75	78 ± 38	train →	148 ± 79	91 ± 23	train →	119 ± 39
75–90	89 ± 53		99 ± 38	97 ± 34		100 ± 21
90–120	95 ± 51		90 ± 33	95 ± 30		96 ± 20
120–150	112 ± 44		112 ± 56	100 ± 30		115 ± 31
150–180	107 ± 55		100 ± 43	105 ± 30		100 ± 24
ng/sample	0.536 ± 0.161		0.545 ± 0.180	0.805 ± 0.201		0.835 ± 0.200

The table is organized as shown in Table 1.

significant difference between the control and stimulated (LTP) groups. There was, however, a small but insignificant difference in glutamate release in the first 15-min sample which followed the train (121 ± 50 and 83 ± 25).

Fascia dentata

Stimulation of the medial perforant pathway at 0.033 Hz evoked a negative population spike riding on top of a positive field EPSP. The high frequency train produced a large increase in the amplitude of

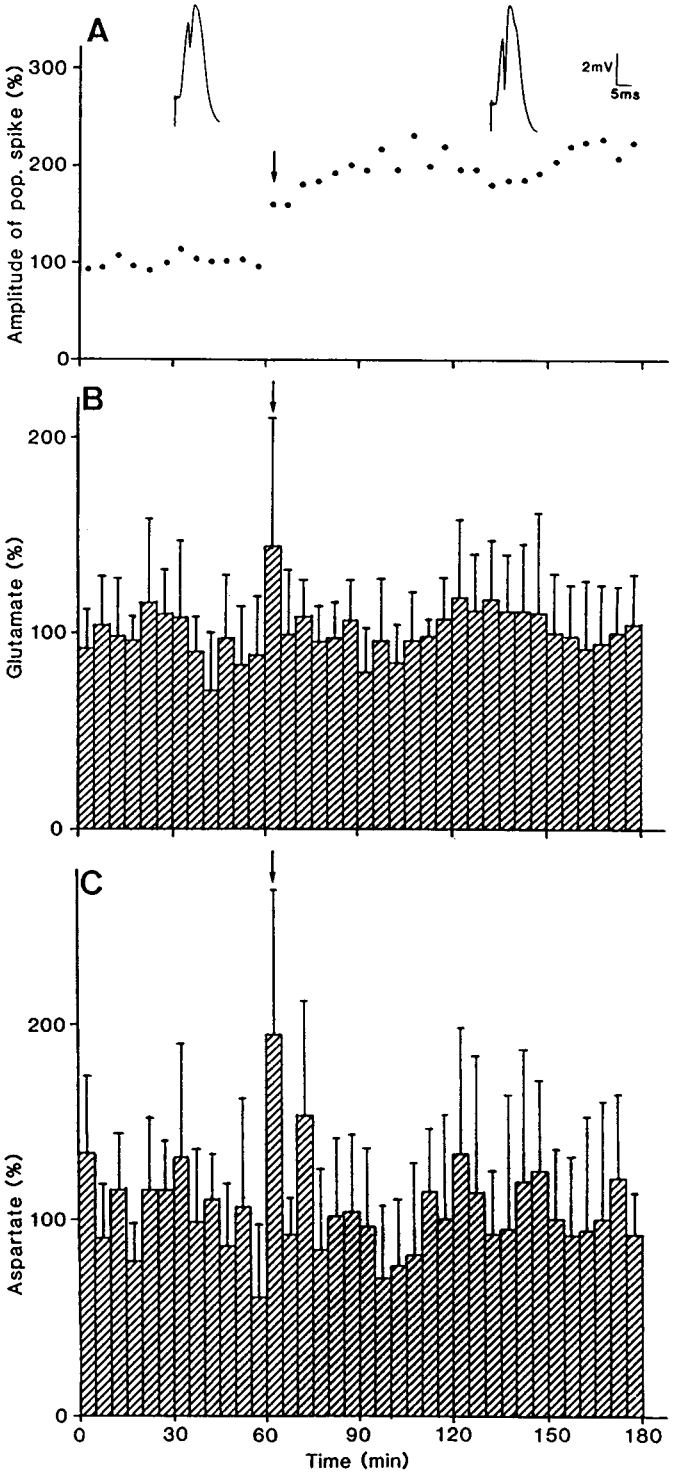

Fig. 2. The brief high-frequency stimulation of the medial perforant pathway (100 Hz for 1 s) produces long-term potentiation of the population spike in the fascia dentata. (A)–(C). Same presentation as in Fig. 1. The release of glutamate (B) and aspartate (C) is expressed as a percentage of the 60-min control levels ($n = 7$). The release of the two amino acids was not significantly different before and after the train. Note cyclic variation in the release of amino acids in particular for aspartate.

the population spike which persisted over 2 h after the train (Fig. 2A). The procedure to analyse the release of glutamate and aspartate was the same as that used for CA1. Again we found no significant increase in the release over time: (i) in each potentiated case ($n = 7$); (ii) when the values expressed as a percentage of the 1-h control period were pooled for the seven animals (Fig. 2B and C, Table 2); (iii) when the LTP group was compared to a control group ($n = 5$) (Table 2). As in CA1, there was a small but not statistically significant difference in the release of glutamate and aspartate in the first three samples (15 min) which followed the train. We found in fact a significant increase in glutamate and aspartate release in three rats; this only persisted, however, for 10–15 min.

DISCUSSION

The principal conclusion of the present study is that LTP in CA1 and in the fascia dentata is unlikely to be related to a long lasting enhancement of the release of glutamate and aspartate. In fact, in both regions, the release of glutamate and aspartate was not different 1–2 h after the train from the pre-train control period. We have occasionally seen a small increase in release in CA1 and in fascia dentata; this, however, concerned the first 10–15 min period which immediately followed the train. This both reflects the validity of the procedure to monitor the release of relatively small changes in depolarization induced release (see also introduction) and suggests that the long-lasting enhancement of the EPSP cannot be due to a sustained increase in the release of glutamate and aspartate by synaptic stimulation.

The reasons for the discrepancy between our results and those of Bliss *et al.*[5,16] are not clear. The experiments are virtually identical in both studies (including HPLC procedure); in fact, the concentrations of glutamate and aspartate found in the perfusate are in close agreement in both studies. The only differences between the study of Bliss *et al.*[5,16] and the present study are (i) the strain of rats (Sprague–Dawley versus Wistar) (differences in the hippocampus between these strains have been reported[31]) and (ii) the type of push-pull cannula used: "closed" system for Bliss *et al.*[5,16] and "open" system in the present study (for details see Ref. 10); the latter is less traumatizing than "the closed system" in that it avoids large changes in local pressure which may result in tissue damage. In our conditions we have never found blood in samples as was occasionally observed by Bliss *et al.*[5,16] In control and experimental rats, there was a stable release during the 3 h period, in contrast to the progressive reduction seen by Bliss *et al.*[5] However, in more recent experiments, Bliss and coworkers (unpublished observations) have found a stable release of excitatory amino acids during time and a sustained enhanced release after the train (as compared to the pre-train period).

Several recent experiments strongly favor postsynaptic mechanisms (e.g. Ref. 18). These include: (i) the long-lasting potentiation produced by intracellular injection of protein kinase C[20] or by combined brief intracellular depolarization and iontophoretic application of *N*-methyl-*d*-aspartate (NMDA)[21]; (ii) a delayed change in the postsynaptic sensitivity to excitatory amino acids after the train[13]; and (iii) the observation that a train of electrical stimulation produces a delayed release of proteins[4,9] which could be released after the train, to modulate the postsynaptic sensitivity (see also Ref. 12). However, studies by Bliss and coworkers[4] and in the present laboratory[9] indicate that the enhancement in proteins released occurs 1.5–2 h after the train, suggesting that they do not play a role in the early phase of LTP. However, Stanton and Sarvey have reported that blockers of protein synthesis completely block the induction of LTP.[28] Clearly, the sequence of events which produces LTP is not fully understood at present.

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