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RESPONSES OF THE NUCLEUS ACCUMBENS FOLLOWING FORNIX/FIMBRIA STIMULATION IN THE RAT.
IDENTIFICATION AND LONG-TERM POTENTIATION OF MONO- AND POLYSYNAPTIC PATHWAYS

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Abstract—The nucleus accumbens occupies a strategic position as an interface between limbic cortex and midbrain structures involved in motor performance. The fornix–fimbria carries limbic inputs to the ventral striatum, namely by way of fibers originating in the CA1/subiculum and projecting to the nucleus accumbens. It also carries fibers arising in the septal area that project to the hippocampal formation, and projection fibers to other areas of the rostral forebrain from Ammon’s horn. Electrical stimulation of this bundle causes characteristic field potentials both in the nucleus accumbens and in the subiculum. In rats, under halothane anesthesia, the responses evoked by fornix/fimbria stimulation in the nucleus accumbens consist of two main positive peaks (at 10 and 25 ms, referred to as P10 and P25, respectively). P10 represents monosynaptic activation. We hypothesized that P25 reflects the activation of a polysynaptic loop, i.e. a fornix–fimbria hippocampal loop in series with the fibers that arise in the subiculum and project to the nucleus accumbens. To test this hypothesis, we reversibly blocked the fibers projecting caudally to the hippocampus by a local anesthetic (lidocaine) and the glutamatergic transmission through the CA1/subiculum by a local injection of kynurenic acid. Both manipulations yielded a reversible depression of about 90% of the P25 component while P10 remained unaffected as expected. In concert a strong reduction (to 24–31%) of control values of the responses evoked in the subiculum was seen. The dynamics of the mono- and polysynaptic pathways differ markedly. The synaptic responses through both pathways are enhanced by paired-pulse stimulation, but the polysynaptic pathway is facilitated in a much stronger way.

Following a tetanus (50 Hz, 2 s duration) applied to the fornix/fimbria, the P10 component of the nucleus accumbens responses showed an immediate increase by a factor of about 2 followed by a phase of gradual decrement with half-decay time of about 10 min, after which a persistent long-term potentiation of about 25% above control level was maintained for the rest of the experiment (max 90 min). The P25 component showed a transient 10-fold potentiation with return to control values after about 10 min. In contrast to the P25 elicited by a conditioning stimulus, the P25 component elicited by a second stimulus delivered at an interval of 100 ms (test stimulus) showed a persistent long-term potentiation. This suggests that in the polysynaptic pathway responsible for the P25, long-term potentiation becomes visible only after the synergistic action of the mechanisms responsible for paired-pulse facilitation and those responsible for long-term potentiation.

In conclusion, the mono- and polysynaptic pathways differ in the expression of induced long-term potentiation. Stimulation of the fornix/fimbria fibers also elicited a long-term potentiation of the responses of the subiculum with a time course similar to that of the P10 of the nucleus accumbens.
Acb, but it is still unclear what causes the component with the longer latency.

One possible explanation is that there are two distinct groups of Fo/Fi fibers having different conduction velocities. However, the diameter of the myelinated fibers of the Fo/Fi shows merely gradual changes and no anatomical evidence for two clusters was reported, at least in the cat. Another possibility is that the P25 component is a manifestation of oscillatory events within the circuits of the Acb. However, a number of neurons showed an increased probability of firing only in relation to the P25 wave and not at earlier latencies. Furthermore, single units of the Acb do not display any sign of oscillations with a period of about 15 ms.

An alternative explanation is that the electrical stimulation of the Fo/Fi may activate a more complex, polysynaptic pathway. We hypothesize that Fo/Fi stimulation may elicit a volley that travels caudally, invades the hippocampal formation, reaches the CA1/subiculum where the fibers projecting to the Acb arise, and then activates the Acb.

Indeed, fibers arising from the septal area travel along the Fo/Fi and reach the subiculum in addition to CA3, CA1 and dentate gyrus. In addition, the Fo/Fi stimuli may activate antidromically axons of CA3 pyramidal neurons, and subsequently CA1 and subiculum neurons. We tested experimentally the possibility that polysynaptic circuits passing through the hippocampal formation may be responsible for the P25 component of the Acb responses to Fo/Fi stimulation by blocking the transmission through the pathway in a reversible manner. If such a polysynaptic pathway can be demonstrated, this would provide the possibility of investigating in parallel a mono- and a polysynaptic pathway in the same target area and, in particular, of directly comparing plastic properties in such distinct pathways.

In this respect we investigated the phenomenon of paired-pulse facilitation and of long-term potentiation (LTP), i.e. until 90 min after tetanic stimulation. The investigation of LTP in the hippocampal-accumbens pathway is of importance for two reasons: (i) it may permit extending the concept that limbic pathways have the capacity of expressing LTP also in a subcortical limbic target area; and (ii) it offers the possibility of comparing, in the same experiment, whether mono- and polysynaptic pathways respond similarly to a tetanus that can elicit LTP.

Along with evoked potentials recorded in the Acb, field potentials were recorded from the CA1/subiculum, in order to compare the LTP in the hippocampal formation with that in the Acb.

Some of the results have been presented in a preliminary form.

EXPERIMENTAL PROCEDURES

Surgery

The methods used to record field potentials were essentially similar to those described in a previous paper. In short, male Wistar rats (Harlan, CPB, Zeist, Holland) were kept under halothane anesthesia and respirated via a tracheal tube. Stimulation and recording electrodes (stainless steel wires, diameter 100 μm, insulated except at the tip) were placed stereotaxically in the Fo/Fi fiber tract (A:5.5, L: 0.9–1.9, V: 3.5; in mm, reference to inter-aural line, midline and cortical surface), the dorsal (A: 2.0, L: 1.2, V: 3.2) and ventral subiculum (A: 0.6, V: 6.0–7.0; entering the skull at L = 4.5 and penetrating into the brain down to L = 5.75) and the lateral aspects of the Acb (A: 8.8, V: 7.2; entering the skull at L = 3.3 penetrating down to L = 2.0), using coordinates of the atlas of Pellegrino et al. The adjustment of the depth of the electrodes was carried out under electrophysiological control. Positioning of the recording electrodes was optimized by stimulation of the Fo/Fi fibers until a maximal amplitude of the field potential was recorded. The electrodes were fixed to the skull using dental cement. Prior to electrode placement, guidance cannulae for the injection of various drugs by way of a 10-μl Hamilton syringe were aimed at the Fo/Fi fibers (A: 4.5, L: 1.5, V: 3.5), or at the hippocampal formation and were fixed to the skull. Small volumes of lidocaine (2% in 0.9% NaCl) or saline could be injected caudal to the stimulation electrode. The layout of these experiments is depicted schematically in Fig. 2. To suppress hippocampal activity, kynurenic acid (10 mM in 0.9% NaCl) was injected in the medio-dorsorostral to lateral-ventral-caudal axis of the CA1/subiculum area (entering the skull at A: 7.8 and I: 4.0 penetrating 8 mm into the brain to A: 1.2 and L: 6.5; injecting about 0.5 μl/mm over a distance of 6 mm). The effects of the drugs were verified electrophysiologically by stimulation with standard pulses in the Fo/Fi, and recording field potentials in the CA1/subiculum, both dorsal and ventral portions.

Stimulation and data acquisition

Evoked potentials were amplified and digitized by way of an interface (CED 1401) that was connected to an IBM-PC, sampled at a rate of 1000 samples/s, averaged (n = 16) and stored on hard-disk.

Standard experiments consisted of recording average field potentials in both the Acb and CA1/subiculum, evoked by Fo/Fi stimulation at low repetition rate (once every 7.2 s). In the experiments with lidocaine and kynurenic acid recordings from the ventral parts of the CA1/subiculum were also made. The stimulus consisted typically of two identical 0.2-ms paired pulses, at an interval of 100 ms and intensities ranging from 0.3–0.6 mA. The first stimulus of the pair is called the conditioning (C) pulse and the second the test (T) pulse. At the beginning of an experiment, responses were measured as a function of stimulus strength, yielding so called input–output (I/O) curves. Tetanic stimulation consisted of a train of 100 equidistant pulses, applied within a 3-s period at saturation intensity.

Histological processing

At the end of an experiment, under deep anesthesia, the stainless steel electrodes were marked by passing three 0.4-s blocks of 1 mA anodal current. The animal was perfused transcardially with saline, followed by 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) with ferrocyanide. The brain was quickly removed and postfixed. Thereafter, the brain was placed in the same buffer containing 30% sucrose. After at least one night, frozen sections (40 μm thick) were cut on a microtome; these were incubated for immunohistochemical staining as described by Voorn et al., in order to facilitate the demarcation of the different subdivisions of the Acb. Antibodies against enkephalin and substance P were used routinely. At each level, one slice was stained with Cresyl Violet. Characteristic placements of Acb, CA1/subiculum and Fo/Fi electrodes are shown in Fig. 1.
Off-line analysis

The moments of occurrence of both the positive and negative maxima of the field potentials were determined off-line. The parameter to quantify the P10 component was the mean of the amplitudes measured between N5-P10 and P10-N18 (Fig. 2, upper right), or in those cases where the N5 component could not be distinguished, the first sample showing a rise in amplitude after the artifact was used as reference. For the P25 component, only the amplitude difference N18-P25 was used because of the complexity and variability of the decay towards the late negative wave. For the CA1/dorsal subiculum (Fig. 2), the decaying phase was quantified, i.e. the amplitude difference between the positive component at 9 ms (P9) and the negative component at 14 ms (N14).

In the LTP experiments, the changes in amplitude of the different components of the evoked potentials were normalized by setting the pretetanus amplitudes at 100%. After normalization, the results of the LTP experiments obtained from different rats were pooled. The mean post tetanus values at a given point in time were compared with the pretetanus values using the Student’s sample t-test for paired comparisons. Also the correlation coefficients between the Acb and the CA1/dorsal subiculum parameters as a function of post tetanus time were computed.

RESULTS

Comparison of P10 and P25 components: dynamics and paired-pulse facilitation

The evoked potentials in the Acb after Fo/Fi stimulation consisted of several components (Fig. 2). As reported in a previous paper, strong paired-pulse facilitation (PPF) occurs for these responses when two identical stimuli are given at an interval of 100 ms. This was used as a standard protocol in the present experiments. Since the different components were much more pronounced following the test stimulus, we give a description of the peaks and troughs based on these responses. Directly after the stimulus artifact a first negative going peak was visible with a latency of 5 ms (N5). This was followed by a positive peak at
about 10 ms (P10). Next, a second negative deflection (N18) followed by a positive component at about 25 ms (P25) was seen. After the P25, sometimes a complex negative-going wave of long duration was seen. First we investigated whether the two positive components showed differences both in dynamic properties as well as in PPF phenomena. To this end, the responses to electrical stimulation with increasing stimulus intensity were studied in eight rats, yielding so called I/O curves. In all rats, a clear-cut P10 component developed in the responses both to the first, or conditioning, stimulus (P10 conditioning response, CR10) and to the second, or test response (TR10). The P25 was only present in the conditioning responses (CR25) at strong stimulation in five out of eight animals, whereas all animals displayed a TR25. In most cases, the absolute amplitudes of both the CR25 and TR25 remained lower than those of the CR10. From the weak and variable amplitude of the P25 in the CR and its clear-cut presence in the TR we conclude that this component exhibits a higher degree of PPF than the P10. This implies that different mechanisms are responsible for the generation of the P10 and P25 components. We compared the dynamic range for the CR and TR using the five experiments that showed both components. To compare the I/O curves for the P10 and P25 components both of CRs and TRs, we constructed normalized graphs, in which the maximal amplitude and the corresponding stimulation current were set at 100%. Figure 3 shows that at the relative stimulation intensity that resulted in a mean CR10 component with 50% of maximal amplitude, the mean P25 was only about 15%. However, both components were above 50% in the test response for the same relative intensity. A comparison of the I/O curves (Fig. 3) shows two main
Fig. 4. Left-hand side: this panel shows the effect of blocking the transmission in the Fo/Fi with lidocaine on the evoked potentials of the Acb and the CA1/subiculum (only TR are shown). (A) Examples of the signals before and after lidocaine injection. Calibration bar represents 250 μV, 30 ms. (B) Time-course of the amplitude of the Acb and CA1/dorsal subiculum (Sub) response evoked by fornix stimulation for two different volumes (0.8 and 1.6 μl) of lidocaine injected. For the P25, the amplitude was measured at a latency of 25 ms which could yield a negative value using the baseline as zero reference. Note the increased duration of suppression in both P25 and CA1/subiculum following the second application. Open circles, Acb P10; closed circles, Acb P25; X, CA1/dorsal subiculum. Right-hand side: this panel shows the effects of the injection of kynurenic acid in the CA1/subiculum on the evoked potentials of the Acb and the CA1/dorsal subiculum. (C) Examples of the field potentials before and after kynurenic acid injection. Scale bar = 250 μV, 30 ms. (D) Time-course of the amplitude of the Acb and the CA1/subiculum responses evoked by fornix stimulation. Open circles, Acb P10; closed circles, Acb P25; X, CA1/dorsal subiculum.
results: (i) that both the CR25 and the TR25 have a higher threshold than the corresponding CR10 and TR10; and (ii) that the paired-pulse facilitation was much more pronounced for the P25 than for the P10 component, for the relative intensities above 20%.

Reversible blockade of pathways mediating evoked field potentials in the nucleus accumbens and CA1/ subiculum

Intracerebral application of lidocaine

Injection sites. In five rats, the injection of lidocaine reduced the responses in the CA1/subiculum substantially. Histological reconstruction of the injection sites for these successful experiments revealed that the needle tip was placed either in the Fo/Fi-bundle or in the rostral pole of the hippocampal formation, and involved the fimbria. Application of lidocaine in more medial aspects of the rostral hippocampus, i.e. CA3/ fascia dentata had no effect on the field potentials.

Evoked potentials in nucleus accumbens and CA1/ subiculum As indicated above, the P10 and P25 components of the Acb were both clearly present, but the latter was clearer in the response to the test pulse. For this reason, the quantification reported below was based on the test responses, where both P10 and P25 were present. An example of the changes following an injection of 0.8 \( \mu \)l lidocaine is depicted in Fig. 4 (left-hand side). The P10 component was depressed transiently to 74% of control, whereas the P25 dropped dramatically to about 3% of the control amplitudes. Recovery was clearly present, but not always complete (range 70–116%). In line with the P25 reduction the attenuation of the dorsal and ventral CA1/subiculum response was down to 24% (range 14–30% of the control values), and to

![Fig. 5. Examples of the signals following paired-pulse stimulation in the Fo/Fi before and after tetanic stimulation. Recordings are made simultaneously from the Acb (left) and the dorsal part of the CA1/subiculum (SUBd) (right). Note the enhancement of the responses to the test (T) stimulus with respect to those of the conditioning (C) response. In the lower trace the positive components that could be distinguished, are indicated for the test response. Scale bar = 250 \( \mu \)V, 40 ms.](image-url)
approximately 50 min after which recovery of the P25 and CA1/subiculum-evoked potentials could be seen. Eventually recovery up to 85% of control was present. The time-course of the reduction and recovery of the field potentials in CA1/subiculum and in the Acb (P25 component) is very similar, which confirms the results of the lidocaine experiments, showing the strong link between these two phenomena.

These results indicate that the P10 component, which was only slightly affected during the pharmacological manipulation, reflects the monosynaptic activation of the Acb neurons, whereas the P25 component depends on the integrity of a polysynaptic loop through the hippocampal formation.

**Long-term potentiation in the nucleus accumbens**

The stimulus intensity selected for low-rate stimulation during LTP experiments was the current that evoked a CR10 at half-maximal or intermediate amplitude. Examples of average evoked potentials, recorded from a site in the Acb where the depth profile showed optimal response amplitudes, are shown in Fig. 5 (upper trace).

The tetanus was given only once and the intensity of the pulses was near saturation level. In the following sections, we describe first the results concerning the conditioned and test response for each of the components of the field potential in the Acb, and second, those of the dorsal subiculum.

![Graphs showing LTP effects](image-url)
Conditioned responses in the nucleus accumbens after tetanization. Immediately following the tetanus, the P10 component was enhanced, and the P25 component became evident (Fig. 5, middle trace). For the group of animals \((n = 6)\) the time-course of the P10 enhancement is shown in Fig. 6A. About 2 min post tetanus, a doubling of the amplitude was observed. This initial rise gradually declined within 20 min to a level of about 25% above baseline values and remained rather constant for the further duration of the experiment. This enhancement was significantly different from baseline until about 80 min post tetanus. It can be concluded that the monosynaptic inputs of the Acb increased their efficacy following tetanic activation for a duration of at least 60 min.

The other component of the Acb CR response, the P25, increased strongly (Fig. 6B). This effect could be dramatic since in many cases, the P25 was insignificant during the pre-tetanus period (Fig. 3). However, within about 15 min, the amplitude of this component returned to the pre-tetanus values. Therefore, we cannot state that LTP of this component occurred.

Test responses in the nucleus accumbens after tetanization. The P10 of the test response showed a significant increase in comparison with the baseline values for at least 60 min. This component presented an initial rise of about 35% after the tetanus followed by a decline to a level around 10% (Fig. 6D), which nevertheless was significantly larger than baseline values for 60 min after the tetanus.

Regarding the P25 component (Fig. 6E), the TR showed an increase up to a mean value larger than 200% of control and stayed for the observation period above about 150%. However, the variance of this component was relatively large. Nonetheless clear LTP of this component was found.

Since the polysynaptic component depends on the integrity of the pathways through the hippocampal formation, it was of interest to compare the long-term effects measured in the Acb to those simultaneously obtained from the subicular cortex/hippocampal formation.

Effects of a tetanus on the evoked potentials in the CA1/subiculum area. For the CA1/subiculum, the same group of animals was analysed. Electrical stimulation was the same as for the Acb responses. Directly after the tetanus, the P9–N14 component rose by 90%, declining within 20 min to approximately 40% above control level. The enhancement above baseline was maintained during the rest of the experiment (Fig. 6C). The CRs and TRs showed approximately the same behavior. For both the CR and TR, we can state that LTP was established.

The CR time-course showed a strong similarity to that of the P10 component of the Acb responses. To evaluate whether the long-term changes in P10 amplitude of the Acb and that of the CA1/subiculum were related, we calculated the correlation coefficient, for the amplitudes measured after the tetanus (11 time-points), between the amplitude of the P10 Acb component and that of the CA1/subiculum (correlation coefficient was 0.97, \(t = 12.68, \text{d.f.} = 9, P < 0.001\)).

**DISCUSSION**

Identification of the components of the accumbens evoked response

The field potentials that were recorded from the Acb showed two characteristic positive waves with peak latencies at 10 and around 25 ms. These values are in good agreement with those reported previously. In the latter paper, it was argued on the basis of recordings of unit-activity that both waves reflect excitatory activation of the neurons in the Acb. Increases in firing at two different latencies have been described also for the rat, the rabbit, and the cat.

Following the injection of lidocaine in the Fo/Fi or kynurenic acid in the CA1/subiculum area, the component occurring at 25 ms was strongly depressed, or disappeared completely, whereas the component peaking at 10 ms was not, or only slightly affected. In concert, the amplitudes of the evoked responses in CA1/subiculum were also reduced, and the process of recovery had a strikingly similar time-course to that of the P25 wave of the Acb. The concurrent, reversible changes in both brain structures strongly indicate that pathways passing through the hippocampal formation are responsible for the generation of the P25 component in the Acb. An additional experimental argument that indicates that the P25 component does not arise simply in the Acb due to recurrent activity in local circuits is the fact that in slices of the Acb studied in vitro the response evoked by stimulation of the fornix fibers does not show such late components.

In order to justify the conclusion that the P25 wave is actually caused by the activation of a polysynaptic pathway through the hippocampal formation, a question to be addressed is whether indeed, following lidocaine injection, the transmission through this structure is blocked. To this end, it is of importance to know whether the field potential in CA1/subiculum, as reported in the present study, reflects locally evoked neuronal events in the CA1/subiculum. This is most likely since the evoked potential recorded from the hippocampal formation following Fo/Fi stimulation changed in polarity when recordings were made at different depths within the CA1/subiculum area (cf. Fig. 1C in Ref. 2). Similar response types have been recorded in different subfields of the hippocampus. Furthermore, McNaughton and Miller described a clear positivity at 8 ms following medial septum stimulation, that is accompanied by a population spike. Individual spikes could be recorded with latencies of 10 ms or more. It should be added that...
complementary pathways may also be involved in these polysynaptic responses since neurons in the entorhinal cortex send fibers to the Acb, and may be activated by way of subicular inputs. The demonstration that stimulation of the Fo/Fi leads to mono- and polysynaptic responses in the Acb is of interest since it allows to study well defined responses generated in the same target structure of the hippocampal formation that are mediated by two different pathways, which have different dynamics and respond in characteristic ways to paired-pulse stimulation.

Long-term potentiation

We describe here, for the first time, that LTP can be elicited by stimulation of fornix-fimbria both in the Acb and the CA1/subiculum. The fact that we used a paired-pulse paradigm allowed us to consider the effects of the tetanus on the CR and on the TR.

First, the monosynaptic P10 component of the CR in the Acb showed a clear LTP. However, the polysynaptic P25 wave showed only a decremental potentiation that decayed to baseline levels within 15 min. In the CA1/subiculum, LTP was found with a similar time course as that of the P10 component of the Acb.

Second, for the test responses, the P10 component showed a gradually declining LTP between 30 and about 60 min post tetanus, whereas the P25 component showed a LTP of long duration.

Two of these effects should be put in evidence: (i) that the P10 component showed LTP both for the CR and the TR; and (ii) that the P25 component showed clear LTP only for the TR. This indicates that, in order for LTP of the P25 to be evident, this component has to be strongly facilitated by the conditioning stimulus. It is thus interesting that the tetanus, as such, is not sufficient to elicit LTP of the P25 component.

Thus there is a considerable difference in the capacity to manifest LTP for the monosynaptic response, as reflected in the P10 component, and for the polysynaptic response (P25). It is likely that the LTP of the P10 component is a local phenomenon that depends on the synaptic modifications at the level of the Acb. In contrast, it is possible that LTP of the polysynaptic response (P25) depends not only on local circuits within the Acb, but also on the pathways through the hippocampal formation, taking into consideration the fact that the P25 component only occurs if the pathway through the hippocampal formation-subiculum to the Acb is intact. At this moment we cannot explicitly indicate where the main "locus" of LTP of the P25 component is situated along the pathways mentioned above. In this respect, it is important to note that in our experiments, the stimulation of the Fo/Fi may activate septo-hippocampal pathways. McNaughton and Miller described LTP of the responses of the fascia dentata after tetanic stimulation of these pathways. In addition, tetanization of the septal area results in long-term enhancement of responses recorded from CA1 and subicular subfields. Furthermore, the Fo/Fi tetanization may stimulate CA3 axons. In this way, LTP may be induced in CA1 pyramidal cells through the collaterals of these axons, the Schaffer collaterals and also in the subiculum. It is of interest to mention that recently LTP has been described in freely moving cats for the pathways from the amygdala to the Acb. The duration of enhanced amplitudes was about 1 h, whereas a decline was seen. Concerning the possible mechanisms responsible for the LTP phenomena, we can only indicate two facts that make it likely that in this system NMDA receptors may play an important role, like in the hippocampal area CA1. One is that the subiculum-acumbens pathway uses excitatory amino acids (L-Glu, t-Asp) as neurotransmitter and the second is that N-methyl-D-aspartate (NMDA) receptors are present in the Acb as revealed by binding studies in vitro and electrophysiological experiments. Moreover, LTP in individual neurons was found to be present in slices of the Aeb, following tetanic stimulation of accumbens inputs. After bath-application of APV, a selective NMDA antagonist, no LTP is expressed. The possibility that NMDA receptors play a similar role in both hippocampus and Acb is supported by the fact that the time-course of LTP of the P10 component resembles that of the responses recorded from the dorsal subiculum within the same experiment.

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REFERENCES


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