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Glutamate Induces Different Neuronal Conditioned Responses than ACPD When Used As a Locally Ionophoresed Unconditioned Stimulus in the Cat Motor Cortex

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Summary

Single unit recordings were made from the motor cortex of conscious cats with glass micropipettes that allowed ionophoretic application of 0.5 M glutamate in 2 M NaCl or 0.5 M ACPD (1S,3R-1-amino-cyclopentane-1,3-dicarboxylic acid, a mGluR agonist) in 2 M NaCl. Activity in response to a 70 dB click (1 ms rectangular pulse to loudspeaker) was studied before, during, and immediately after applying each agent locally as a paired US (90 nA current 570 ms after click for 300 ms in combination with glabella tap). A 70 dB hiss sound was presented 4.4 sec after the click as a discriminative stimulus (DS). CS and DS were presented 10 times initially (adaptation); then CS, US plus tap, and DS (approximately 10 times as conditioning); and then CS and DS (2-10 times to test post-conditioning). Glutamate potentiated the mean, early, 8-16 ms response to the click after conditioning (t=18.2, $p<0.0001$), but not the baseline activity which decreased from a mean of 17 spk/sec to 7 spk/sec (t=3.71, $p<0.001$). Baseline activity increased to 31 spk/sec when glutamate was applied during conditioning (t=3.30, $p<0.005$). ACPD reduced the intermediate, 64-72 ms response to the click after conditioning (t=8.18, $p<0.0001$), and potentiated the late 104-112 ms response (t=15.4, $p<0.0001$). Baseline activity was slightly increased after conditioning with ACPD. Saline did not potentiate the response to click. The results indicate that glutamate agonists that differ in their receptor affinities can induce different CRs when used as locally applied USs to condition neuronal responses to a click CS in the motor cortex of cats.

Key words

Conditioning • Learning • Pavlov • Metabotropic receptors • Excitatory amino acids

Introduction

Understanding the process of conditioning depends on an understanding of how conditioning works at the cellular level of the brain (Hebb 1949). Bureš and Burešová (1967) were among the first to recognize the need for direct investigation of single units of the brain to accomplish this[1]. It had not been established if single units were capable of undergoing conditioning individually or if this required a network of neurons working en masse. Bureš and Burešová applied glutamate with an ionophoretic current (10-50 nA) locally, at the site of recording within the brain, as a US to condition changes in single unit activity elicited in response to a paired acoustic CS. They demonstrated that a conditioned response to the CS could develop in units of the thalamus, reticular formation, parietotemporal cortex, hippocampus, and inferior colliculus. Later studies by Ovcharenko and Kotlyar (1978), Woody *et al.* (1984 1991), and Malgaroli and Tsien (1992) confirmed that

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putative transmitters such as glutamate could be used successfully to condition neuronal CRs as well as to produce LTP. Additionally, in Bureš and Burešová's studies, a depressed rather than increased response to the CS often developed after conditioning, and it was thought that the ionophoretic current might act as a US to contribute to this and other effects.

The present study addresses the question of whether glutamate agonists that differ in their receptor affinities can induce different neuronal CRs when used as locally applied USs. Comparisons were made between effects of glutamate and ACPD (an agonist specific for metabotropic glutamate receptors) as USs for conditioning changes in the activity of neurons of the motor cortex of awake cats in response to a click CS. Applications of saline were used to control for effects of the ionophoretic current.

Methods

Recordings were made from the pericruciate cortex of adult cats. The animals were prepared under sodium pentobarbital anesthesia (35 mg/kg) for subsequent awake intracortical recording as in earlier studies (Woody and Black-Cleworth 1973). During recording sessions the cats were comfortably restrained by placing their bodies in cloth sleeves. Their behavior was continuously observed, and the studies were discontinued if the animals gave any signs of discomfort. Recordings were led by Ag/AgCl wires to a Dagan 8100-1 amplifier with high input resistance and negative capacitance compensation, and stored on FM tape (recorded at DC - 4000 Hz band pass) for subsequent playback.

Single (1-2 MΩ resistance) or triple-barrel (1-2 MΩ drug; 10-40 MΩ recording) microelectrodes were used to record extracellularly (or with the latter intracellularly) before and after local extracellular ionophoresis of 0.5M glutamate (monosodium salt) or 0.5 M ACPD (1S,3R-1-amino-cyclopentane-1,3-dicarboxylic acid) in 2 M NaCl. Glutamate and ACPD were delivered extracellularly with ionophoretic currents of 90-100 nA passed through a Dagan 6400 constant current source. Previous studies have shown that this method reliably supplies effective dosages of ACh to cortical neurons in awake cats (Swartz and Woody 1979). In separate experiments ionophoretic applications of 1.5 M or 2 M saline were made at equivalent current strengths.

Cells were sampled by chance. Previous intracellular marking studies have shown that about 70 % of cells of the cat motor-sensory cortex studied in this manner are pyramidal cells of layers III and V (Sakai *et al*. 1978). Pyramidal tract neurons of layer V are preferentially activated at latencies of 40 ms or less by auditory stimuli similar to the click that was used as the CS (Sakai and Woody 1980).

Conditioning of the cells was accomplished rapidly in ten trials (10 s intertrial interval) by presenting tap 570 ms (or in some studies 340 ms) after the click followed 10 ms later (or in some studies 240 ms later) by a 300 ms period of ionophoresis of glutamate, ACPD or saline. A hiss of equal intensity to the click was presented as a discriminative stimulus (DS) 4.4 seconds after the click. Physical characteristics of the 70 dB click and hiss stimuli were similar to those used earlier for behavioral conditioning (Woody *et al.* 1970). The responses to click and hiss were evaluated before and after conditioning by means of histogram averages of the activity of all units of each group (Figs. 1 and 2). Changes in activity were evaluated by two-tailed differences of the means.

Results

The effects of glutamate were studied in 17 cells. The mean histograms of the activity recorded from all cells are shown in Fig. 1. Examples of unit recordings during similar procedures have been shown earlier (Figs. 1 and 2 of Woody *et al.* 1991). After pairing glutamate as a US with click, there was a potentiation of the early, 8-16 ms response to the click from 15±9 (SD) spk/sec to 64±5 spk/sec (t=18.2, df29, $p<0.0001$). In contrast, the baseline activity was reduced from a mean of 17±9 spk/sec to 7±5 spk/sec (t=3.71, df29, $p<0.001$), and the response to hiss diminished. During conditioning with glutamate the baseline activity increased transiently to a mean of 31±15 spk/sec (t=3.30, df32, $p<0.005$).

The effects of ACPD were studied in 21 cells. The mean histograms of their activity are shown in Fig. 2. After pairing ACPD as a US with click, there was a reduction of the intermediate, 64-72 ms response to the click from 25±3 spk/sec to 16+4 spk/sec (t=8.18, df39, $p<0.0001$) and a potentiation of the late 104-112 ms response from 11±3 spk/sec to 28±4 spk/sec (t=15.4, df39, $p<0.0001$). The baseline activity increased slightly after conditioning with ACPD (from 14±3 spk/sec to 16±4 spk/sec; t=1.82, df39, $p<0.10$), and there was an increase from 13±3 spk/sec to 32±4 spk/sec (t=17.3, df39, $p<0.0001$) in the late (112-120 ms) response to hiss

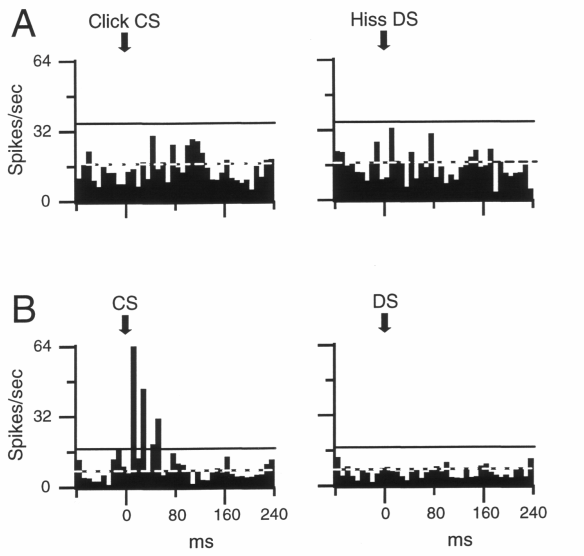

Fig. 1. *Histogram averages of spike activity before (A, n=17 cells) and after (B, n=14 cells) conditioning with glutamate as a US. Diagonal arrow points to a potentiation of the short latency response to the CS after conditioning. Data are the grand averages of the activity recorded from all cells. The click CS and hiss DS were presented at time 0 (vertical arrows). Calibrations of spike activity (spikes/sec) are as shown. Baseline levels of spike discharge shown by the dashed horizontal lines are the means of the activity in the period from 0 to 380 msec preceding delivery of the CS. Solid lines are drawn 2Z above the means of the baseline spike activity.*

equivalent to that to the click. During conditioning with ACPD the mean baseline activity remained stable at 15±4 spk/sec

Saline did not potentiate the response to click. After pairing with saline (n=20 cells), the response to click failed to exceed 2 S.D. of the baseline activity. Comparing the ten 32 ms bins in the 320 ms before click delivery with the forty 32 ms bins immediately following, the peak activity of the pre-click bins (21.7 spk/sec) exceeded the peak of the post-click bins (20.8 spk/sec). Baseline activity decreased slightly from 19±3 spk/sec to 17±3 spk sec (t=2.11, df38, p=0.04).

Discussion

Both glutamate and ACPD produced rapid, "cellular" conditioning of changes in unit activity of the motor cortex. Changes in response appeared after just 10 pairings of click, tap, and the ionophoretically applied transmitter agent.

Does glutamate act as a US?

The first question is whether glutamate acted as a US. There are three possibilities. One is that the changes developed from pairing the click and tap alone. Weighing against this is the observation that behavioral conditioning of an eye blink response requires 750-1000 pairings of the click CS and tap US for development of the CR (Woody *et al*. 1974). Also, earlier studies failed to find potentiated unit responses after ten click-tap pairings (Woody *et al*. 1984).

A second possibility is that the ionophoretic current acted as the effective US instead of glutamate. Bureš and Burešová (1967) noted that the nA ionophoretic current used to apply a chemical agent such as glutamate might sometimes act as a US, particularly to produce decreases in activity. Direct μA electrical stimulation of the motor cortex is an adequate CS, when paired with glabella tap-US, to produce a short latency

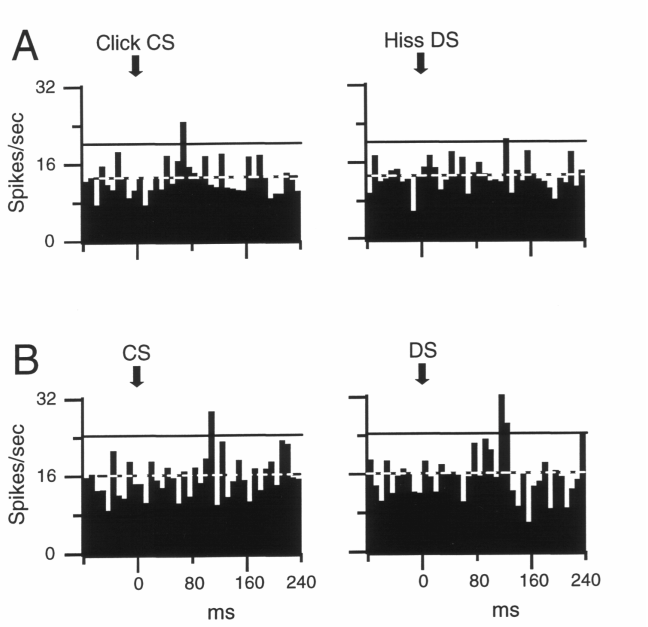

Fig. 2. *Histogram averages of spike activity before (A, n=21 cells) and after (B, n=20 cells) conditioning with ACPD as a US. Data are the grand averages of the activity recorded from all cells. The click CS and hiss DS were presented at time 0 (vertical arrows). Calibrations of spike activity (spikes/sec) are as shown. Baseline levels of spike discharge are shown by the dashed horizontal lines. Solid lines are drawn 2Z above the means of the baseline spike activity.*

blink CR (Woody and Yarowsky 1972). There is also strong evidence that weak, nA intracellular currents can affect neuronal conditioning (Voronin and Solntseva

1970), and that weak, nA extracellular currents can affect unit discharge properties (Brons *et al.* 1982). In the present experiments the changes produced by glutamate and ACPD were not produced by saline, and thus it may be inferred that these changes were not attributable to the ionophoretic current. However, one cannot exclude the possibility that changes over lengthier periods than those studied were produced (c.f. Bureš and Burešová 1967).

A third possibility is that glutamate acted as a US in conjunction with the tap. In earlier behavioral studies it was found that pairing of click, tap, and lateral hypothalamic electrical stimulation (HS) allowed blink CRs to develop rapidly after ten pairings (Kim and Woody 1983, Hirano *et al.* 1987, c.f. Voronin *et al.* 1975). Cells of the motor cortex that mediated this learned response were activated at short latencies by HS (Woody *et al.* 1983), and this activation could be reduced or prevented by local ionophoretic application of glutamate diethyl ester (GDEE), a glutamate antagonist (Cooper and Woody 1983). The onset latencies of the potentiated responses to the click CS in the behavioral studies were short as they were in the present study.

We suggest that local ionophoretic application of glutamate is a successful substitute for HS to produce rapid conditioning of increases in CS-evoked activity in cells of the motor cortex. Occupancy of NMDA receptors by glutamate allows Ca^{++} to enter cells when the Mg^{++} block of the receptors is removed by postsynaptic depolarization (Brown *et al.* 1988). The increase in calcium is thought to be needed for the induction of most forms of LTP and for the development of the long lasting postsynaptic changes following applications of acetylcholine or cGMP that mirror the changes found in neurons of the motor cortex after behavioral conditioning (Swartz and Woody 1979, Woody 1982).

2. Does cellular conditioning produce conditioned changes in unit activity?

The second question is whether the changes in activity produced by pairing click, tap, and glutamate represent conditioning. Bureš and Burešová (1967) noted early findings that units conditioned with somatosensory USs such as sciatic nerve stimulation (Yoshii and Ogura 1960) tended to develop CRs that waned or disappeared with further training (perhaps resembling overtraining behaviorally). Bureš and Burešová were able to produce stable, long lasting CRs to acoustic CSs paired with glutamate in 17 of 128 neurons, and transient changes in another 34. They recognized that the early development of transiently potentiated responses could be equivalent to conditioned arousal (see Bureš and Burešová 1967). The possibilities of conditioned arousal and sensitization should also be considered when evaluating the behavioral relevance of brief or moderately long lasting potentiation acquired after repeated presentations of paired or single stimuli, as in some forms of LTP.

The properties of the paired CS are also important. Ovcharenko and Kotlyar (1978) produced CRs readily, in 95 % of units of the somatosensory cortex, using glutamate as a US and electrical stimulation of the cortex as the CS. In pseudoconditioning controls only 17 % of the units they investigated developed potentiated responses.

In the present study the duration of the potentiated responses was not assessed, but the CR produced by glutamate was unlikely to represent simple sensitization since it appeared to be discriminative, with an increased response to the click CS and a diminished response to the backward paired hiss DS. The order of pairing of glutamate required for successful cellular conditioning was similar to the order of pairing of HS required for increasing the rate of behavioral conditioning (Kim *et al.*1983, Hirano *et al.* 1987), having to come after rather than before the paired CS and US. The CR also did not represent conditioned arousal since its latency after the CS was much shorter than that for classical arousal. Thus, instead, the outcome appeared to represent a form of cellular conditioning that closely paralleled what was found after rapid behavioral conditioning with the same CS. This conclusion is important because other investigators (Storozhuk *et al.* (1992) have shown that ionophoretic application of glutamate can potentiate responses to a CS in cortical units of behaviorally conditioned animals when given just before the CS. That potentiation appears to reflect direct, transient interactions between glutamate and acetylcholine (Metherate *et al.* 1988, Dykes *et al.* 1991).

3. Limitations of approaches to assessment of cellular conditioning

Bureš and Burešová (1967) recognized the limitations of the approach of Jasper *et al.* (1960, c.f. Woody *et al.* 1970) that compared responses in populations of neurons from the same regions in naïve and overtrained animals (e.g. the need for large numbers of samplings and the ambiguities surrounding the identities of the sampled units) versus the more direct approach of studying the development of conditioning in a single neuron. They also recognized the difficulty in

directly demonstrating that single neurons are intrinsically capable of undergoing conditioning.

Even when glutamate is applied in small amounts locally at a singly recorded unit, there are processes from other neurons as well as other cells within the diffusion radius (about 200 μm according to Ovcharenko and Kotlyar 1978), and their influence on the recorded unit cannot easily be ascertained. Interestingly, in the cat motor cortex, most neurons capable of being conditioned with glutamate as a US appear to have the ability to respond to glutamate. In one study of effects of glutamate as a US for conditioning (Woody *et al.* 1984), more cells that developed a CR responded to glutamate (59 %) with an increase in baseline activity than cells that did not (29 %, $X2=7.64$, $p<0.01$). However, it was not possible to establish if these were direct or indirect responses.

To avoid ambiguities about the direct involvement of individual neurons, a more far-reaching approach might be to study single neurons in complete isolation – e.g. cell culture. Here, however, in addition to possible developmental abnormalities and trauma during dissociation, the isolated cells would lack the usual tonic background inputs present in vivo, and likely rest at a hyperpolarized transmembrane potential. In short, each of the above approaches has its limitations as well as its advantages.

4. Different CRs with ionotropic and metabotropic receptor activation

The present findings add to growing evidence that the roles of ionotropic and metabotropic glutamate receptors differ in conditioning. Here, the difference was one of response to the same acoustic CS. Both glutamate and ACPD appeared to be effective USs for rapid conditioning, but when paired with click as a CS, the mGLUr agonist, ACPD, produced a much different pattern of mean activity in response to the CS than did glutamate. Glutamate potentiated short latency activity in response to click while ACPD potentiated late activity and actually reduced intermediate latency activity. Also, ACPD potentiated the response to the hiss DS as well as the click CS, a phenomenon more representative of sensitization than conditioning.

5. Relevance of motor cortex for mediation of short latency conditioning

It has been surprising to some that neurons of the motor cortex could be relevant for mediation of short, <20 ms latency, blink CRs, or that such CRs might obey the same laws of Pavlovian conditioning as longer latency CRs. Evidence for the latter was provided recently by Woody and Aou (1999). It was shown long ago that bilateral ablation of the motor cortex prevented acquisition of short-latency conditioned blinking (Fig. 1 of Woody *et al.* 1974). The impairment persisted despite extensive training for three months after surgery. Application of 25 % KCl to the rostral cortex also abolishes the conditioned blink response to click (but not the unconditioned blink response to glabella tap) in cats (Woody and Brozek 1969) and rabbits (Gutman *et al.* 1972). Of further relevance is the utility of a short latency CR for studies of information transmission between the cochlear nucleus, the motor cortex, and the muscles producing the blink CRs (Woody 1997).

6. A mechanism for mediating the cellularly conditioned increases in CS-evoked activity

The short latency (8-16 ms) neuronal CRs produced by glutamate are thought to be mediated by a decrease in a potassium A-current postsynaptically in the affected cells (Woody *et al.* 1991). Since postsynaptic electrical properties are reportedly unchanged in cortical cells after LTP (Markram *et al.* 1997), it is uncertain whether the cellular basis for conditioning is the same as for that form of LTP. Use of single electrode voltage clamp techniques to assess changes in currents within a space constant of the electrode tip has demonstrated changes in such currents after pairing click, tap, and glutamate (Woody *et al.* 1991). The currents were sensitive to aminopyridine (Woody *et al.* 1989), and were decreased by acetylcholine and cGMP (Woody and Gruen 1987). Not only will the probability of short latency discharge be increased by decreasing the magnitude of this rapidly activated and rapidly inactivated current, but the latency of spike initiation by slowly rising PSPs will be reduced (Pay and Woody 1999). Both outcomes would be ideal for exercising fine motor control adaptively.

Appendix

In 1967 Dr. Woody left his position as a Research Associate at the Laboratory of Neurophysiology, NIMH, NIH to spend a year as a Harvard Moseley Fellow with Dr. Jan Bureš at the Institute of Physiology. At the end of that period, Dr. Woody returned to NIH as a permanent Research Officer of the Laboratory of Neural Control, NINDS.

Dr. Woody was attracted by the exciting work of Drs. Bureš and Burešová that utilized putative

neurotransmitters as USs to study their effects directly in the brain. These scientists had created a remarkable interdisciplinary milieu at their laboratory in Prague in which exacting behavioral studies could be carried out while applying state-of-the-art computational analyses of concurrent recordings of neuronal activity, careful histological analyses of the brain performed with the assistance of Ms. Vaněčkova, and advanced biochemical anayses performed by Dr. Křivánek. Some of the analyses depended on devices designed by Dr. Bureš and constructed with his colleague, Mr. Tůma. Others derived from the award of a LINC (laboratory instrument computer), which proved to be the first fully operational LINC in Europe. There were many other stimulating colleagues, including Dr. Vyklický, who was studying the trigeminal system, and Dr. Brožek, who worked collaboratively on a series of studies with Dr. Woody. The kindness, hospitality, and generosity of this group was expressed to Dr. Woody and his wife, Pat, in such a manner that they returned to Prague often over subsequent years, maintained and broadened their ties with those in the Institute, and named their two children after Jan Bureš and Jarmila Křivánek.

Scientifically, the period in Prague from 1967-1968 was spent investigating whether field potentials of the facial nucleus could be used to assess the neural representations of conditioned and unconditioned eyeblink responses and how these representations changed after extinction of the CR. The outcomes were described in six reports.

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Reprint requests

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