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Event-related potentials in an invertebrate: crayfish emit ‘omitted stimulus potentials’

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Summary

Electrical signs of neural activity correlated with stimuli or states include a subclass called event-related potentials. These overlap with, but can often be distinguished from, simple stimulus-bound evoked potentials by their greater dependence on endogenous (internal state) factors. Studied mainly in humans, where they are commonly associated with cognition, they are considered to represent objective signs of moderately high-level brain processing.

We tested the hypothesis that invertebrates lack such signs by looking in the crayfish *Procambarus clarkii* for a class of OFF-effects shown in humans to index expectancy. Disproving the hypothesis, we find, using chronic, implanted preparations, that a good omitted stimulus potential is reliably present. The system learns in a few cycles of a regularly repeated light flash to expect one on schedule. Omitted stimulus potentials are found in the protocerebrum, the circumesophageal connective and in the optic tract – perhaps arising in the retina, as in vertebrates. These potentials can be very local and can include loci with and without direct visual evoked potentials in response to each flash. In some loci, the omitted stimulus potential has a slow wave component, in others only a spike burst. Omitted stimulus potentials are more endogenous than visual evoked potentials, with little dependence on flash or ambient light intensity or on train duration. They vary little in size at different times of the day, but abruptly fail to appear if the ambient light is cut off. They can occur during walking, eating or the maintained defense posture but are diminished by ‘distraction’ and are often absent from an inert crayfish until it is aroused.

We consider this form of apparent expectation of a learned rhythm (a property that makes it ‘cognitive’ in current usage), to be one of low level, even though some properties suggest endogenous factors. The flashes in a train have an inhibitory effect on a circuit that quickly ‘learns’ the stimulus interval so that the omitted stimulus potential, ready to happen after the learned interval, is prevented by each flash, until released by a missing stimulus.

Key words: event-related potential, local field potential, expectation, endogenous, emitted wave, time-locked cognitive wave, crayfish, *Procambarus clarkii*.

Introduction

Within the class of electrical signs of neural activity (Bullock, 1997) is a subclass, the event-related potentials (ERPs), that overlaps with the evoked potentials (EPs). ERPs, in our usage, are more endogenous, that is, more dependent on the state of the animal and the brain. EPs, as we use the term, are in general determined more by the stimulus strength, rise time, duration, modality and locus. ERPs, known mainly from studies on the human scalp, are considered to be signs of moderately high-level processing and, from their association with conscious experiences (e.g. ‘There’s one!’ or ‘What’s that?’), are sometimes called ‘cognitive waves’ (Bullock et al., 1994).

A general goal of the present work is to uncover grades of complexity among the phyla and classes of animals with respect to the physiology of nervous systems (Bullock, 1984; Bullock and Başar, 1988). A specific goal is to ask whether an advanced invertebrate can produce ERPs when subjected to a stimulus paradigm similar to a standard one in the vertebrate literature. Some types of ERP can be found among vertebrates from rays to humans, suggesting that simple neuronal circuits have been preserved through many evolutionary steps.

We chose to look for the omitted stimulus potential (OSP), a type of ERP emitted when an expected stimulus does not come (Bullock et al., 1990, 1993, 1994; Karamürsel and Bullock, 1994, 2000; Prechtl and Bullock, 1994). The extensive literature from human subjects is listed in Bullock et al. (1990, 1994). Such ERPs have been studied elsewhere only in mammals, almost entirely from scalp recordings on human

subjects. The OSP is particularly suitable for study because the relevant feature of its causation is the absence of a physical event. It is, in fact, a special form of OFF-response consisting of a burst of spikes and/or slow waves that may develop after a train of stimuli with constant interstimulus intervals (ISIs). The feature that distinguishes OSPs from other types of OFF-response is their time-locked occurrence when measured from the due-time of the first missing stimulus following a train of stimuli. The latency is well defined and consistent following one ISI after the last stimulus in a train, as though the system were expecting something on schedule. Can we find such an electrical event in an advanced invertebrate? Shimozawa et al. (1977) had found single interneurons in crayfish that emit a special OFF-burst, which they called an entrained response, with properties corresponding to our OSP. Since the test for OSPs is a learned, accurate expectation of timing, and we were unaware of the study of Shimozawa et al. (1977), we hypothesized that crayfish lack such an ERP. Here, we report that this hypothesis is disproved since crayfish show a good, reliable OSP.

Two types of OSP are known in humans, 'fast' and 'slow' (Bullock et al., 1994). The first type is elicited by relatively fast stimulus trains (best >4 Hz), occurs relatively early after the due-time of the first missing stimulus (approximately 50–150 ms), does not require directed attention and is known in humans, reptiles and fish. The second type of OSP appears after slower trains (0.3–1 Hz), after a longer latency (usually peaking at 500–800 ms), requires attention and is known only in mammals. The slow OSPs are recorded from the cerebral cortex and are believed to index a high level of processing. For extensive literature on humans, see Bullock et al. (1994). The fast OSPs in turtles and rays have been shown to arise in the retina and do not require directed attention or brain processing (Bullock et al., 1990; Prechtl and Bullock, 1994).

Contrary to our hypothesis, we find it is possible to elicit from the crayfish brain a burst of spikes and a slow wave that meet the time-lock to due-time requirement for a fast-type OSP response. Under our conditions, directed attention is not required, the response peak is in the early range and we consider that the OSP does not index a high-level 'mental' event even though it is relatively more endogenous than the visual evoked potential (VEP).

The crayfish is a mainly crepuscular animal, but we find it rarely possible to elicit OSPs at night or when the background is dark; hence, its ethological significance is not obvious. Nevertheless, the central state is important in modulating its probability, amplitude and latency; the OSP in crayfish is more facultative than the VEP.

Preliminary reports of this work have appeared previously (Hernández et al., 1999; Ramón et al., 1999).

Materials and methods

Experiments were performed on more than 60 crayfish, *Procambarus clarkii*, of either sex and a minimum size of 10 cm from rostrum to uropod, obtained from commercial dealers. The principal technical features of the study were recording from unanesthetized animals, using implanted semimicroelectrodes on the surface of the brain and wideband filters passing both spikes and slow potentials. The advantages of such recordings are discussed in Bullock (1997, 1999).

Animals were chronically implanted through a small dorsal hole just above the brain with up to four Teflon-coated platinum fine-wire electrodes (diameter 0.1 mm, occasionally 0.125 or 0.15 mm; with only the cut end uninsulated). These were placed on the brain surface, avoiding injury to the dorsal blood vessel and cranial muscles, as described previously (Hernández et al., 1996; Serrato et al., 1996). A stainless-steel guide tube was the common reference electrode. Crayfish live for long periods with such an implant, walking unrestrained about their container. Such electrodes can record spikes or slow waves for months; muscle action potentials were sometimes troublesome but usually unambiguously distinguishable from neural activity. Small differences in position, e.g. two electrodes close together, could alter the picture greatly, including the amplitude of 'spontaneous' neural background spiking and the appearance of VEPs. Some experiments were performed with animals free to move around a small tank containing approximately 3 cm of water. In other experiments, a piece of cork was cemented to the dorsal carapace and this was clamped to suspend the animal in deeper water to prevent its pereipods from touching the bottom of the tank. In both cases, the eyes were above the water surface when the animal was upright.

Stimulation was delivered as trains of light flashes of 10 μs duration at frequencies between 3 and 50 Hz. Flashes were delivered by a reflector–diffuser lamp, 13 cm in diameter, connected to a Grass photic-stimulator (model PS22) placed 20–30 cm in front of the crayfish. The maximum intensity given by the manufacturer for a new unit, at 25 cm and in the center of the illuminated area, is 0.024 lm s cm^{-2} per flash, falling by 25 % at a repetition rate of 12 Hz. Physiologically, the maximum flash was not maximal in terms of the electroretinogram. The stimuli used in different experiments were reduced from the maximum by various amounts, usually to 1/16th or less. One or two flashes were omitted from the train or, more commonly, we terminated the train and looked for a response beyond that expected from the last flash. Simply ending the stimulus train allows time for observation of activity long after the last stimulus and uncontaminated by new responses to new stimuli. A typical experiment involved five trains of 1 s duration containing light flashes at different repetition rates. The 1 s trains were delivered every 10 s.

Recording of extracellular potentials employed Grass preamplifiers (P-15) with filters passing from 0.3 or 30 or 100 Hz to 3 kHz followed by digitization at 2 or 8 kHz and storage in a computer for off-line analysis. Typical recordings were 3 s long and consisted of 1 s of pre-stimulus control, 1 s during the stimuli and 1 s after the stimulus train. Analyses were performed with 'DataWave' (Longmont, CO, USA), 'Igor' (WaveMetrics, Inc. Lake Oswego, OR, USA) and 'Brain Wave Tools' (M. C. McClune, University of California, San Diego, Department of Neurosciences).

Experiments were performed in rooms with both window and artificial illumination and an air temperature usually between 20 and 25 °C, but the water bath could be set from 10 to 22 °C.

Gross electrode localization was assessed during *post-mortem* dissection. In most cases, it was found that electrode tips rested on one side of the protocerebrum, although in some cases an electrode was found in the region between the proto- and deutocerebrum or on the optic tract or circumesophageal connective. Passing a suitable current through an electrode on the surface of the small brain made a rather diffuse lesion and did not usually show a well-demarcated brain region.

It is not self-evident how to measure the 'strength' of a response such as the OSP, in the literature on brain activity termed an 'emitted' response to an internal state, particularly when the data are not confined to spike activity in each of many classes of single units but consist of both spikes and slow potentials of clusters of units. Documenting raw waveforms allows visual integration of numbers, spacing and voltage of peaks and valleys and, for most purposes, categorization as strong and weak is sufficient, whether in single trials or in averages. In addition to displaying the wave form of responses, we quantified their area or power by computing the mean and standard deviation of the root-mean-square voltage (V_{rms}) over the whole pass-band of the recording filters, obtained from a defined 200 ms epoch measured from the 'due-time' of the first missing flash after the end of a regular train. Usually, these V_{rms} values were averaged for at least five successive identical trials. This estimator includes the whole burst of spikes and slow waves defined as OSPs together with the spontaneous activity during this period. We therefore avoided this method for recording with unusually high background activity. For observations studying the daily variations in OSP, we also measured the V_{rms} of a segment 200 ms long, in response to hourly sets of five trains 10 s apart, using the same stimulation frequency throughout the experiment. When possible, efforts were made to record only when the animal was motionless and the background electrical activity was low. However, the background can fluctuate widely even during the 3 s period usually recorded, and the reliability of attempting to measure and subtract it was judged too low to be justified.

Results

The spontaneous background electrical activity recorded with semimicroelectrodes on the surface of the brain from a quiescent crayfish is dominated by spikes of different sizes. The spikes are brief, like single-unit action potentials, their intervals are irregular and this ongoing activity varies widely from locus to locus in 'busy-ness' or integrated amplitude and frequency. When the animal is quiet or held, distinct clusters or bursts of spikes are seldom seen. Slow waves (>10 ms wide) are also seen but are prominent in only a few electrode locations.

Visual evoked potentials

In response to a single, brief flash of light, some electrode loci record a spike burst and/or a slow wave that stands out above the spontaneous background activity. The recording takes a variety of forms, depending on the locus (Figs 1B,D, 2, 3, 4B). In favorable sites, this VEP consists of a spike burst that frequently starts with the largest spikes. The envelope of the spikes has a nearly triangular shape, it is fast rising and slow falling, and it decreases to the basal level approximately 100 ms after its initiation. The usual onset latency of this response is approximately 5 ms, under our conditions, with light flashes well above threshold. Some recordings show one or more late bursts at latencies of approximately 100 ms and even longer. The form and dynamics of the VEP depend strongly on the ISI. OSPs are usually distinguishable from VEPs since they are mainly seen at flash rates at which VEPs are greatly reduced or at loci from which VEPs are absent.

Stimulating with trains of pulses at 3 Hz, each flash, at good loci elicits a spike burst that finishes before the following stimulus. As the train frequency is increased to 7 Hz, the bursts are reduced in amplitude and duration and, at a frequency of approximately 12 Hz, they begin to overlap with the burst elicited by the next stimulus. Frequencies in the range 10–20 Hz do not usually produce obvious VEPs at each flash, but some bursts, irregular in size and duration, can be seen after some stimuli. The diverse forms of VEP in different loci appear to have distinct dynamic properties, intensity and ambient light adaptation functions, but these have not been systematically studied. OSPs cannot be mistaken for most of these forms.

Omitted stimulus potentials

After a train of flashes at a frequency above approximately 4 Hz, there is usually, in suitable electrode loci, a new form of response, unlike the VEP, with particular properties justifying the name omitted stimulus potential (OSP). The principal and defining property is a consistent latency, if measured from the due-time of the first missing stimulus (Fig. 1C). This means that the latency after the last stimulus is one ISI plus this consistent value. The value is usually consistent within 15 % for a given preparation and set of conditions. Its value varies with certain conditions. For example, at approximately 11 °C, the peak of the burst occurs between 100 and 150 ms in different preparations, and at approximately 22 °C it occurs between 35 and 70 ms, but it is close to the same value in a given preparation. Possibly, the recording site plays a role, but we cannot be certain of that. The OSP is robust and repeats hundreds of times without decline or habituation if, for example 1 s trains are used at 10 Hz with an interval of approximately 10 s between trains. It has been seen in virtually every suitable preparation (well above 40 preparations in the three laboratories). It can be either a burst of spikes from several units or a slow wave or both, depending on the locus (Figs 1–5). The spike burst form of OSP consists of up to 10–20 large, brief spikes with an amplitude usually well above that of most spontaneous spikes. If the amplifier output goes to a loudspeaker, the OSP after each train within the appropriate conditioning ISI range produces a characteristic sound.

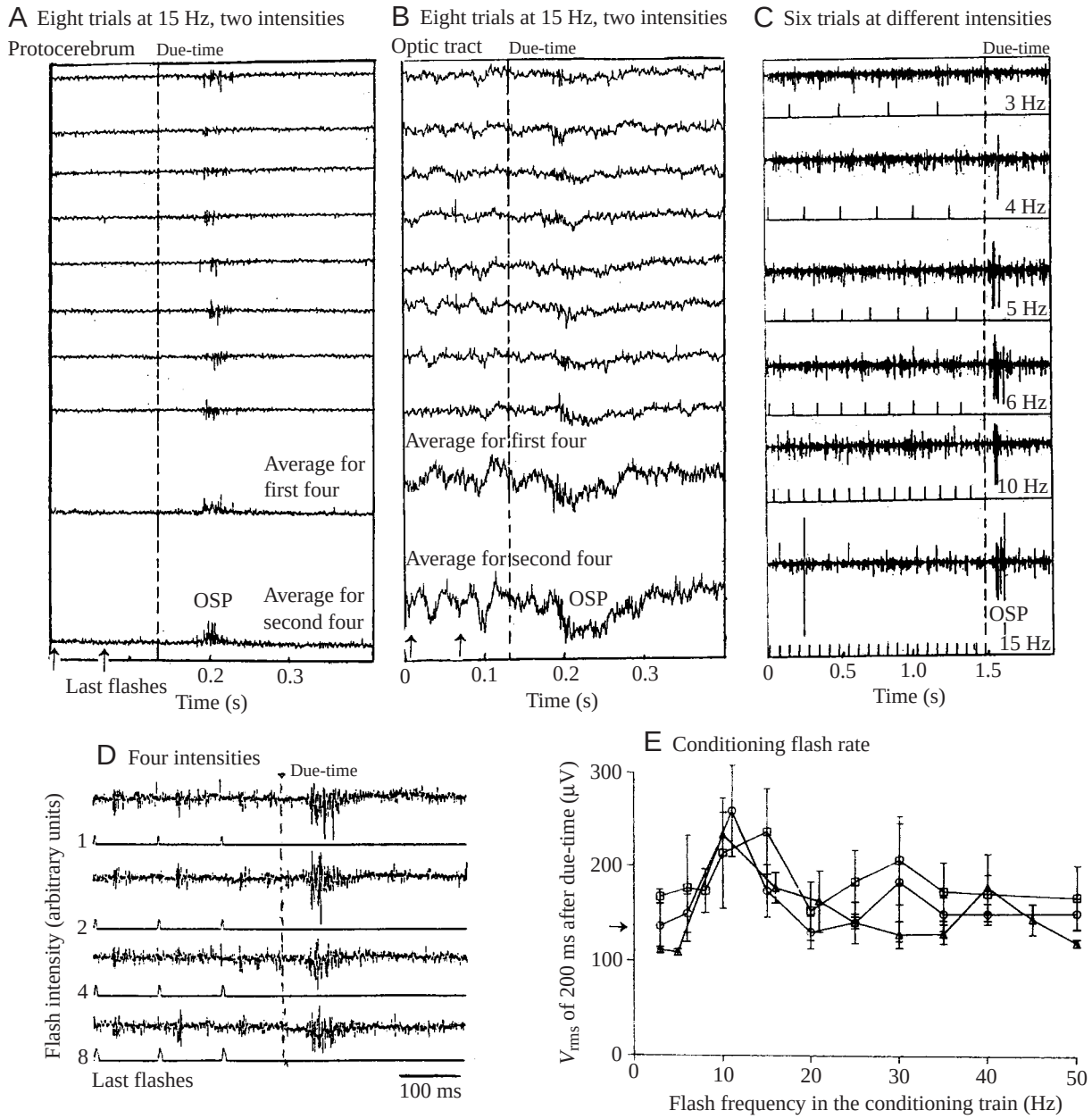

Fig. 1. Omitted stimulus potentials (OSPs) from crayfish. (A) Eight successive trials (single sweeps) from the protocerebrum, a locus that gives no visual evoked potentials (VEPs) and mainly spike bursts for OSPs. The last two flashes (arrows at bottom) of a 1 s train at 15 Hz are shown. Trials 1–4, from the top, with a low flash intensity of 1 (arbitrary units proportional to lumens); trials 5–8 at a high flash intensity of 16. (B) Simultaneous recording from the optic tract at a locus that shows slow waves larger than spikes, both VEP and OSP. Averages of the optic tract recordings 1–4 and 5–8, below, reveal the consistent slow negative wave approximately 40 ms wide and peaking at approximately 75 ms after the due-time of the first missing flash (vertical dashed lines). At some electrode loci, this wave is much more conspicuous than it is here. Note that the spike burst, without a slow wave, in the protocerebral locus (A) coincides with a spike burst in the optic tract (B). Both recordings were filtered to pass 1–2000 Hz. The recordings in A and B illustrate the robustness of the OSP and the small effect of the intensity of the conditioning stimulus train. (C) Effect of frequency of conditioning flashes. This recording was taken from the protocerebrum of another preparation and shows single sweeps after trains at different flash rates. Responses are aligned by the due-time of the first missing flash. Note the near constancy of the OSP latency, if measured from the due-time. At 3 Hz, no OSP is seen, and a single spike occurs in some trials at 4 Hz. At values above 15 Hz, some preparations continue to emit a burst at this time, whereas others do not. The constant latency from a given locus, within a given preparation, shown here, is the hallmark of the OSP. Its value varies with temperature and possibly with locus. (D) Recordings from another preparation showing the lack of effect of flash intensity over a range of 8:1 (same units as in A and B). Note the VEPs after each flash. These are of the small spike burst type, with long latency. (E) Effect of stimulus frequency on the amplitude of the OSP, estimated by the root-mean-square voltage (V_{rms}) during the 200 ms beginning with the due-time. The arrow marks the mean V_{rms} under the prevailing conditions, without stimulation. The increased V_{rms} beginning somewhat below 10 Hz is attributable mainly to the spike burst OSPs. Data are from three preparations, each shown by an individual symbol; values are means ± standard deviations (N=6).

The slow-wave type of OSP is distinct from the electroretinogram (ERG). That widespread response begins with a latency of a few milliseconds after each flash, whereas the OSP always begins many tens of milliseconds after the last flash (the ISI plus at least 35 ms). In recordings with a slow-wave OSP (Figs 1B, 2B), the ERG, if present, ends shortly after the last flash. Some electrode loci are favorable for showing VEPs in response to each flash, some for showing ERGs and some for showing OSPs; a few show two of the three responses. Given several ISIs and intensities, there is usually no ambiguity about which kind of response is visible. OFF-responses that do not meet the constant latency criterion for an OSP are not normally seen in the stimulus regimes we used, i.e. low-frequency flashes in short trains.

The possibility that a small movement of some muscle accompanies or follows the OSP could not be ruled out but, since the OSP is seen in a few places on the brain and disappears with a small movement of the electrode, we judge that it is not itself a muscle action potential.

Flash frequency and number within the conditioning train

Omitted stimulus potentials become larger as flash frequency increases between approximately 3–5 Hz and 10–15 Hz, depending on the preparation (Fig. 1E). Under our conditions, lower frequencies produce no OSP. Frequencies higher than approximately 15 Hz have commonly produced no OSP in La Jolla experiments, whereas in the Campeche laboratory OSPs typically continue up to 50 Hz, the upper limit tested. We have not identified a difference between these laboratories that explains this discrepancy. The difference is not quite consistent since a number of La Jolla experiments also showed good OSPs up to 35–50 Hz.

The occurrence of OSPs has only a small dependence on the train duration, and only a few flashes at the appropriate frequency are sufficient to elicit it (Fig. 4A). Spike bursts with the characteristics of OSPs can be seen after a very short train, 3–4 flashes at frequencies of 10–15 Hz, although they consist of fewer spikes than those elicited after more extensive conditioning. Some cases are borderline and difficult to classify, especially if the level of spontaneous activity is considerable, which varies with electrode locus. Fig. 4A shows V_{rms} values for different numbers of flashes in trains at either 10 Hz or 15 Hz. The V_{rms} increases above four flashes until it levels off or shows a second rise above 12 flashes.

Intensity of conditioning flashes

A good OSP can be seen after trains of almost any flash intensity. Given the crude measurement of response amplitude, especially of bursts of spikes from several units, we did not attempt to quantify the intensity function. Fig. 1A shows an apparent effect of a 16-fold difference in intensity on the averages of four trials, but we cannot assert that this is representative; some experiments showed no apparent effect (Fig. 1D). We cannot compare this dependence quantitatively with that of VEPs, which have commonly not been present in the loci favorable for OSPs and, if they are present, cannot follow the repetition rate in any but the slowest OSP tests. Furthermore, VEPs are themselves heterogeneous among the loci that show them; some are early and brief, others late and

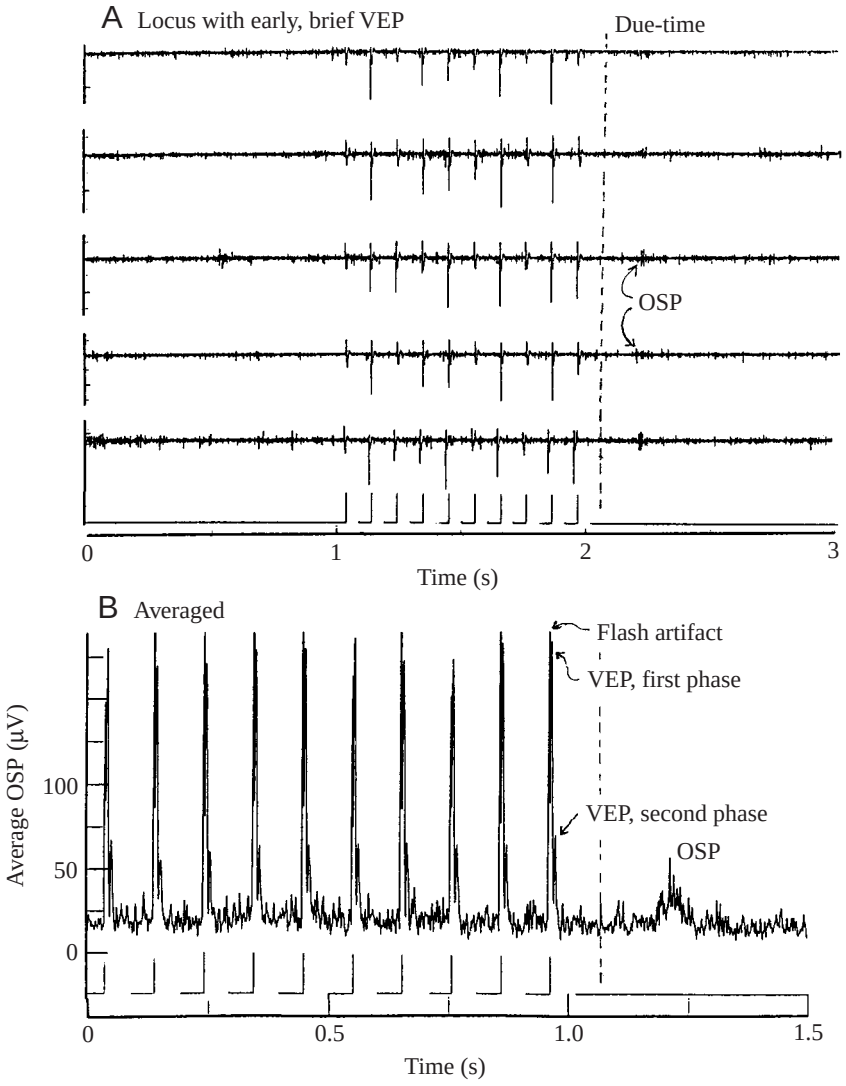

Fig. 2. The early, brief type of visual evoked potential (VEP). (A) Five successive single sweeps (the 21st to 25th of a series) with 1 s trains of 10 Hz flashes eliciting early biphasic VEPs following the stimulus artifact (variable because of the digitizing rate). Note the small spike burst type of omitted stimulus potential (OSP) approximately 140 ms after the due-time. (B) The same sweeps averaged after rectifying to avoid negative-going and positive-going spikes cancelling each other. The slow wave average OSP is thus the result of summing spikes in this case.

protracted. It is, however, our impression that the amplitude of some VEPs is relatively much more exogenous, i.e. influenced by intensity, than is that of the relatively endogenous OSPs.

Background light and diurnal rhythm

Background light conditions have some, but a very non-linear, influence; in the range between daylight and near darkness, they have almost none. Fig. 5 suggests this by the extremely discrepant curves of OSP V_{rms} and ambient light level over a 24 h period.

To distinguish a possible time-of-day effect from the ambient light effect, we compared four quite different levels of ambient light at the same time of day. Trains of flashes of fixed intensity were delivered under four conditions: with approximately 800, 100, less than 5 and between 0 and much less than 1 lx (midday window light plus room lights, window light only, crayfish covered with a 'leaky' black drape and crayfish under an opaque cover, respectively). We compared the means of the V_{rms} values of the 200 ms following the 'due-time' at the end of trains. These values represent OSP plus background activity, which cannot be reliably subtracted. The V_{rms} values show three apparent levels of OSP amplitude. At the two higher levels of illumination, they are not significantly different, in spite of an eightfold difference in ambient light intensity. Values at both higher levels are different from that at the fourth level, i.e. complete darkness. Examination of the raw data on the single sweeps shows that OSPs are usually completely absent when trains of flashes, whether bright or dim, are delivered in complete darkness. The third condition, very dim light, gives an intermediate V_{rms}. Examination of the raw data shows that the OSPs, when they occur in dim light, are nearly all equal but are graded in probability of occurring on every trial. Averaging makes the V_{rms} intermediate in amplitude. Since values for the V_{rms} vary in different brain loci, we have not obtained a sufficient sample to measure the trend quantitatively, but it appears fair to conclude that OSPs are not greatly dependent on background illumination except for a major non-linearity between darkness and some rather dim ambient threshold. Time of day remains a major candidate determinant that has yet to be tested.

The possibility that OSPs are modified with a diurnal rhythm under natural light/dark cycles was tested by recording from tethered animals every hour for 5 days. V_{rms} values under prevailing light (window light only) alternate daily between a maximum around midday and a minimum when there is no OSP at all, from approximately 20:00 to 05:00 h (corresponding approximately to local daylight hours). The mid-day maximum was also seen in three other preparations. To compare the variations in V_{rms} amplitude with the daily changes in background light, we measured the latter every hour for 24 h. The light intensity under our conditions is shown in Fig. 5. There is little relationship between light level and OSP during the day. However, from 20:00 h until the next morning at approximately 06:00 h, light levels were below 10 lx and OSPs seldom occurred.

Temperature

Initial studies were conducted both at Campeche and La Jolla; OSP latencies recorded at La Jolla were significantly longer than those recorded at Campeche. Suspecting that this might be due to different prevailing temperatures, we estimated the temperature effect, measuring in the bath close to the animal's head. The temperature of the brain may differ

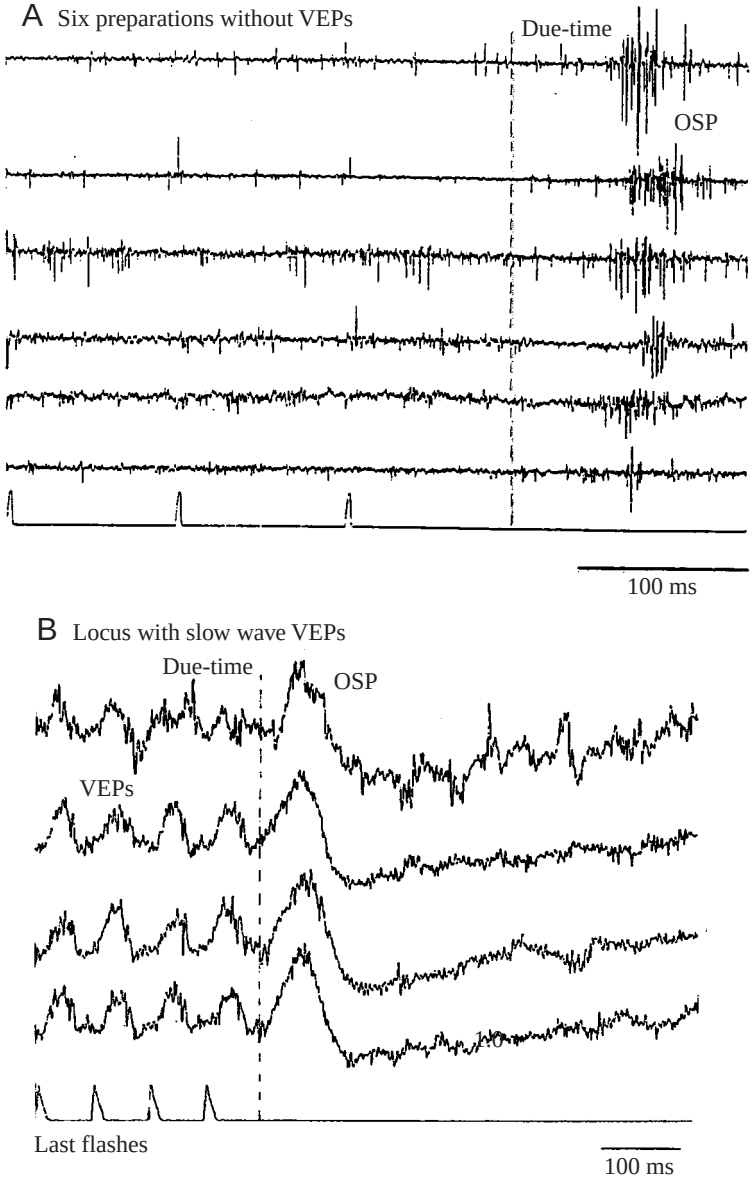

Fig. 3. Variations among preparations. (A) Single sweeps from six animals. The loci in each case gave no visual evoked potential (VEP) but a good omitted stimulus potential (OSP), appearing as a spike burst, and all having a similar latency after the due-time. The OSP is seen whether the stimulus is in the middle of a train or at its end. (B) Some loci (here demonstrated from a seventh preparation) show slow waves overshadowing the spike bursts, in this case also giving VEPs. Four successive trains are shown, all at 15 Hz, with only the last four flashes in single sweeps.

somewhat from this, since the head was not always immersed completely and the water and air temperatures were quite different at low bath temperatures. The latencies of OSPs at 11 °C ranged between 100 and 170 ms, in different preparations, while those obtained at 20–25 °C were between 40 and 80 ms, in some preparations down to 30 ms. The experimental rooms were not temperature-controlled, and this source of variation together with inconstancy in the degree of immersion might account for the range of latencies at each nominal temperature. The range is only between preparations; each one maintains its latency within narrow limits. It appears, therefore, that this factor can account for at least the majority of the difference between the two laboratories. Other factors such as source, condition and acclimation of animals could not be standardized.

Brain regions

Good sites for recording the OSP can give robust and consistent responses, without habituation, many scores of times. These sites can be quite local. Small differences in electrode position can lead to a loss or a restoration of the OSP. OSPs can be seen in electrode loci that show good VEPs and in loci that show no VEPs. We cannot localize well the fairly large electrode tips, usually on the surface of the brain, but most data come from some part of the protocerebrum. OSPs are also well seen on the optic tract, which connects the optic ganglia and brain, and on the circumesophageal connectives between the brain and subesophageal ganglia. The optic tract is the only site that quite frequently shows slow wave OSPs (Figs 1B, 3B). We have not yet been able to cut the optic tract and record on the retinal side of the cut, thus excluding centrifugal influences, as was done in rays (Bullock et al., 1990).

State of the animal

As stated in the Introduction, a characteristic of ERPs is their dependence on the state of the animal and/or the brain. To look for evidence of endogeny, we altered the state in several ways. One, already described above, was the removal of ambient light, which caused the prompt loss of the OSP within the few seconds of a short conditioning flash train. Dark adaptation does not restore OSPs, but a very modest ambient illumination restores them within a similar period of a few seconds.

Under our experimental conditions, crayfish left for long periods without stimulation commonly fall into a ‘dormant’ state in which they are ‘resting’ on one side. Under these conditions, a regular train of light pulses fails to elicit an OSP, although VEPs are normal. If, after several failures, the animal is aroused by gently stroking it with a probe, OSPs can be consistently elicited. This behavior has been seen on numerous occasions, but it depends on the animal first becoming unresponsive, and we have been unable to produce this state at will. However, a condition perhaps similar is sometimes seen following tethering a crayfish. In some of our animals, clear OSPs were abolished when a free

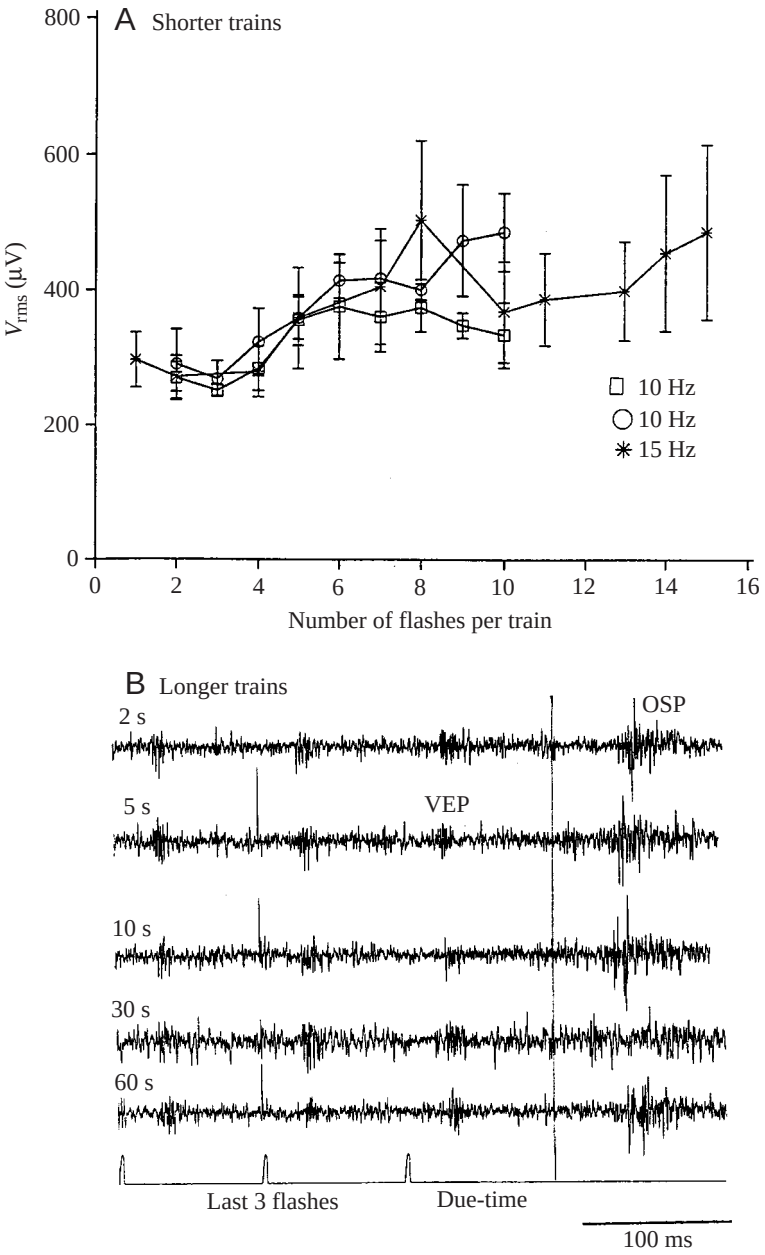

Fig. 4. Effect of conditioning time. (A) Short trains of stimuli. Root-mean-square voltage (V_{rms}) values (mean ± S.D., N=6) of 200 ms following the due-time of the first missing flash at the end of trains of various numbers of flashes at 10 Hz (squares and circles) and 15 Hz (asterisks). Since omitted stimulus potentials (OSPs) cannot confidently be identified after trains of only three or four flashes, V_{rms} values for such trains measure essentially the spontaneous activity. Values are higher for trains containing more than five flashes and reach a maximum at around eight flashes. In this preparation, the values increase again after 12–14 flashes. (B) Longer trains of stimuli. Trains of 9 Hz of various durations, showing only the last three flashes. This recording locus showed a late spike burst type of visual evoked potential (VEP) and a more vigorous OSP, essentially uninfluenced by the duration. The OSP after 30 s is anomalous.

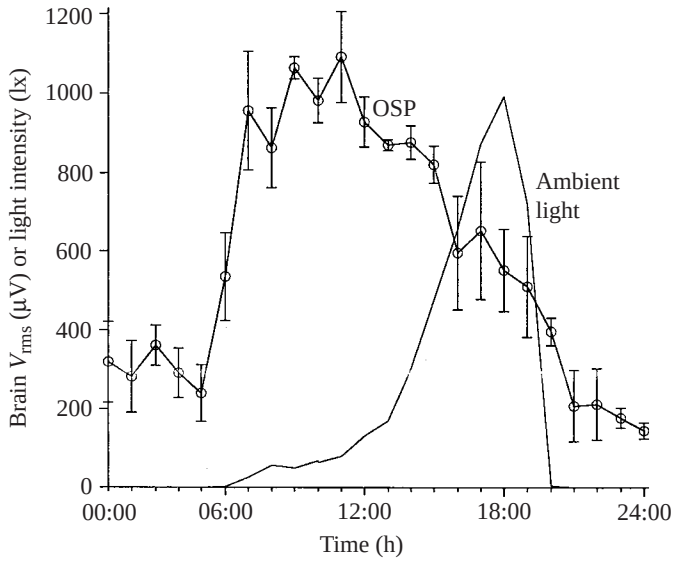

Fig. 5. Effect of time of day under natural laboratory light on root-mean-square voltage (V_{rms}) values (mean ± S.D. of 200 ms following the due-time of the first missing flash) at different times of the day. Values are averages of 5 days of recordings and show the diurnal fluctuation. After basal (spontaneous) values of approximately 300 μV, there is a clear increase in V_{rms} during daylight hours (06:00–18:00 h) with a return to basal levels after 20:00 h. A comparison with the light intensity values (shown in lx) in the experimental room averaging the same 5 days of recording (thin line) shows the extreme non-correspondence between omitted stimulus potential (OSP) and prevailing light. The skewness of the room light is due to the sun shining through the window near sunset.

animal was tethered and were elicited again upon release. Wine and Krasne (1972) have shown that in tethered crayfish the tail-flip escape response is abolished, indicating an active inhibition of the circuit, including the giant fiber system. Others of our animals, such as that in Fig. 5, gave clear OSPs during 5 days of recording even when tethered. These observations suggest differences in the tethering or in degrees of sensitivity to it.

A consistent OSP can disappear with conditions that suggest distraction, as with moving visible objects or gentle touch. The OSP, however, can occur during walking, eating or the maintained defense posture. We have seen an increase in the robustness of the light-induced OSP on two occasions when a piece of food was dropped near the animal. These animals may have been in the semi-dormant state described above, but we could not repeat this observation at will, putting it in a class with many episodic behaviors.

Discussion

Omitted stimulus potentials can be recorded at the surface of the crayfish brain after the animal has been exposed to short trains of light flashes at frequencies of 5–15 Hz or higher. We believe this is the first report of an ERP in an invertebrate, using that term, not in its widest sense for any external event (synonymous with EP), but in the narrower sense of a relatively endogenous potential that accompanies what would be called a mental event in humans. Perhaps the closest parallel among familiar responses of invertebrates would be some of the higher-order units, requiring, for example, visual movement combined with some other features (Shimozawa et al., 1977). ERPs are distinguished from simpler EPs in response to direct stimuli. This distinction is both subtle and arbitrary, but rests on the properties of both stimulus and response. The adequate stimulus for an ERP is its relationship to previous stimuli, the absence of a stimulus at a time when it could have been expected in the present case. The response is relatively endogenous, i.e. it is determined less by the intensity, distribution or duration of the light than by the recent history and the state of arousal of the animal. These subjects have been reviewed elsewhere [see chapters 9 and 11 in Bullock (1993)] (see also Bullock, 1997), as has the history of OSPs in relation to the initial findings in non-mammalian subjects (Bullock et al., 1990, 1993; Karamürsel and Bullock, 1994; Prechtl and Bullock, 1994).

Most previous studies of the OSP wave (Bullock et al., 1994) were performed on the human long-latency type (peaking at 400–800 ms) with single stimuli omitted from a regular series, using long ISIs of 1–3 s. A prominent feature of these late OSPs is the requirement for the subject to be attentive. In the present case, an OSP of the short-latency type (usually 50–150 ms), without any control of attention, occurs best with a short ISI (in the vicinity of 100 ms). Since the OSP latency is in the same range, it is convenient to terminate a train of stimuli to avoid the complication of a fresh EP during the protracted duration of the OSP. The crayfish OSP has characteristics similar to those of the fish, reptile and human ‘fast’ OSPs. While the human has both types of OSP response (Karamürsel and Bullock, 2000), only the ‘fast’ type is known in non-mammalian species, partly because, if the ‘slow’ type existed, its detection would require training of the animals.

The OSP at the end of a train of stimuli is a kind of OFF-response distinguished by being time-locked to the due-time of the first omitted stimulus. The OSP can be further distinguished from other ‘ordinary’ OFF-responses whose latency varies with the duration and intensity of the light and, in our preparation, requires continuous light for some time or sustained flashing above the presumed flicker fusion frequency.

The OSP has attracted attention because it acts as though the system has a temporally specific expectation, which in humans is considered a moderately high cognitive process. It has been explained (Bullock et al., 1990) as being due to some mechanism (probably a central neuronal circuit) that, after a few stimuli at a regular interval, learns the ISI and prepares a discharge with a fixed latency. This is really just descriptive inference since, if the next stimulus arrives on schedule, the discharge is suppressed and a VEP is evoked. If the stimulus fails to arrive on schedule, and if other permissive conditions prevail, the discharge is not suppressed and the OSP is emitted after its characteristic latency.

The OSP in crayfish exhibits some unusual features whose

significance is not yet understood. One is the possibility that the response following trains of 15 Hz or less is not the same as that following trains of higher frequency. This is not established by the present work but is suggested by a few La Jolla experiments in which the latencies of the ostensible OSPs were longer following trains in the range 35–50 Hz. We found that OFF-responses other than OSPs, i.e. not conforming to its definition, under our regimes, were rare. In many La Jolla experiments, the OSP was activated only by stimuli between 5 and 15 Hz (11–15 °C) and failed to appear after shorter ISIs. In other experiments, a good response continued for stimuli up to at least 50 Hz and showed the characteristic latency of OSPs after the due-time. These responses conform to the definition of an OSP, but discriminability becomes poor as the ISI becomes much shorter than the characteristic latency. The possibility of interaction between OSPs and other 'ordinary' OFF responses, which may depend on the gradation of flicker fusion, would seem reasonable but cannot be assessed on the present evidence.

Another feature requiring further characterization is the reduced or missing OSP in the absence of ambient light. In darkness, OSPs seldom appear after a stimulus that would produce a robust one under both low and high light levels. This is still true after a period of dark adaptation and using weak flashes. This effect was shown under two conditions: (i) giving test trains during the daytime with the crayfish covered by opaque material and (ii) every hour during the night (20:00–06:00 h).

The absence of an OSP when the ambient light level is low raises an ethological question because the crayfish is generally crepuscular, with circadian rhythms of locomotion and social activity that peak at dusk and dawn (F. Ramón and J. Hernández-Falcón, unpublished observations). Higher social and food-seeking activity should require higher levels of brain activity, but the hours when OSPs are absent seem to overlap with those of peak behavioral activity. Possibly vision is not the major sense at these times. The conclusion, on other grounds, that the OSPs in this animal are not an indication of higher cognitive processing may lessen the apparent ethological discrepancy.

The OSP is graded from the minimal response of a single spike, as seen at a given locus, to a robust burst of 5–20 spikes of large amplitude compared with those in the spontaneous background activity. Certain recording loci have shown a graded slow potential component with or without spikes; most loci show only spikes. The OSP shows a small dependence on the duration and intensity of the stimulating light and that dependence, like the one on ambient light levels, is extremely nonlinear.

Factors that affect the robustness of the OSP underline its relatively endogenous character. The OSP is reduced or prevented by other stimuli that we would classify as distracting. It is often absent when the animal appears to be somnolent and does not appear after gentle prodding or touching the carapace or appendages. However, a stroking touch or vibration of the chamber water may arouse the animal into a startle response, and a good OSP will then follow an appropriate train of stimuli. Modalities other than vision should be investigated as well as other behavioral states.

Our limited evidence that the crayfish OSP arises in the retina raises the question of whether its modulation, suppression or enhancement is due to a centrifugal influence from the brain upon the retina. Experiments with the isolated eye stalk and with a reversible block of the optic tract would be valuable in answering such questions.

The fact that crayfish OSPs are similar to the 'fast' type seen in vertebrates, although not homologous, suggests that analogous neuronal circuits are in place in invertebrates and behave similarly to those in vertebrates. We regard the OSP, although operationally equivalent to an expectation, to be a very early process, both in time (reflex-like latency) and in pathway (starting in the optic tract, but not yet proven to be independent of centrifugal impulses, as has been shown in fish). This does not imply that crayfish lack cognitive capacities. Further tests with ERPs, for example with 'odd-ball' tasks of different levels of abstraction, combined with operant conditioning to manifest directed attention would contribute to our understanding of this phenomenon.

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