An improved method for recording responses from single retinotectal axon arborization in frog

A. Kuras,
N. Gutmanienė

Laboratory of Neurophysiology,
Institute for Biomedical Research,
Kaunas University of Medicine,
9 Mickevičiaus Street,
Kaunas LT-3000, Lithuania

The method of recording responses from terminal arborization of a separate retinotectal axon in frog, previously developed in our laboratory, was improved by introducing a stimulating multi-channel metallic electrode and a recording low noise carbon-fibre microelectrode. The use of the multi-channel electrode for threshold electrical stimulation of the frog retina made the whole process of searching for the "all-or-none" type response in the tectum easier, because it enabled us: a) to change the site of excitation without displacing the electrode; b) to ensure the stimulation of several separate neighbouring ganglion cells by employing different pairs of the stimulating channels, and thus, the registration of the responses from overlapping terminal arborizations of their axons with the same microelectrode. The low noise carbon-fibre microelectrode with the longer recording tip allowed us: a) to record the "all-or-none" type response without averaging, and b) to increase the stability of the registration, *i.e.*, to eliminate the effect of brain pulsation on the recording.

Key words: threshold stimulation, "all-or-none" response, carbon-fibre microelectrode

INTRODUCTION

The mechanism of chemical transmission in the central nervous system is rather complicated [1]. There are also possibilities for the interaction between the systems of various neurotransmitters, especially when many neurons or nerve fibers are activated. Therefore, it is preferable to investigate synaptic transmission on the level of synapses of an individual axon. For this purpose, synaptic potentials evoked by activation of a single presynaptic neuron are recorded both intra- [2–5] and extracellularly [6–13].

The method of extracellular recording of synaptic potentials evoked by activity in single presynaptic neuron, *i.e.*, recording the responses from terminal arborization of a separate axon, was independently introduced by Mendell and Henemann [14] and by Gutman with co-authors [15–17]. In our laboratory this method was developed for the recording of the mentioned responses from the frog tectum, the main visual area of anurans. Presynaptic action potentials and synaptic potentials from terminal arborizations of single retinotectal fibers were recorded by stimulating a separate ganglion cell in the retina visually [6, 17] or electrically [9–11]. A conventional 2-channel electrode for bipolar electrical stimulation was used. The change of the site of excitation was possible only by displacing the electrode itself. This caused the danger of injuring the retina's tissue. In addition, relatively small terminal action potentials and synaptic responses have to be recorded with low noise (in the wide range of frequencies) microelectrodes. The noise of the previously used Gesteland's metallic electrode [18] and the ordinary Armstrong–James and Millar carbon-fibre microelectrode [19] contained slow waves which impair their use for recording synaptic potentials. To improve the method, we substituted the bipolar stimulating electrode by the multi-channel one and modified the ordinary carbon-fibre microelectrode for simultaneous recording of small fast and slow potentials from nerve fiber terminal arborization. The aim of this paper is to show the advantages of the introduced modifications of the method and to demonstrate the possibility of prolonged electrical stimulation of a separate neuron in the retina and of stable recording of signals from the terminal arborization of an activated nerve fiber.

METHODS

Animal surgery. Experiments were done *in vivo* with adult *Rana temporaria* frogs. Frogs were anesthetized by cooling [20] in a refrigerator until their movements ceased and then immobilized completely by an intramuscular injection of 0.2–0.3 mg d-tubocurarine and

by subsequent injections of 0.1 mg every 20–30 min during the experiment. For local anesthesia, 5% solution of novocain (0.25–0.5 ml) was injected subcutaneously in both sites of the surgical entry (regions above the tectum and around the eye to be operated on). The sclera of the eye was infiltrated with the same anaesthetic. The dorsal tectum was exposed in the way described by Maturana et al. [21]. The skin above the tectum was cut off by scissors, and the skull and the *dura mater* were trepanned. The *pia mater* was eliminated under the microscopic control with a tungsten wire sharpened to 1–2 μm. The retina, contralateral to the opened tectum, was prepared by the method of George and Marks [22]. The upper eyelid, the nictitating membrane, and the sclera were excised. The lens and the hyaloid were sucked off. The eyeball cavity was filled with the Ringer's solution (in mM: 117 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1.2 $NaHCO_3$, and 0.17 $NaH_2PO_4 \cdot 2H_2O$, pH 7.2–7.3). The exposed dorsal tectum was perfused with the same solution. The oral cavity of the frog was ventilated with a moistened mixture of O_2 (95%) and CO_2 (5%). All recordings were done in the dark. Temperature in the recording chamber varied from 12 to 18 °C in different experiments and was constant during a separate one. The experiments were approved by Animal Care and Use Committee of the State Food and Veterinary Service of Lithuania (No. 030).

Stimulation and recording. A special six-channel electrode and the block of two five-channel electrodes [23] were constructed for the minimal electrical stimulation of the naked retina, that is, stimulation of a single retinal ganglion cell or its axon. The channels were tungsten wires insulated along all their length to the very tips 15 or 40 μm in diameter with the distances between the centers 50–250 μm and the resistance to the direct current of 180–250 kΩ. Single pulses of the square-waved current (1–3 V, 20–60 μs) were applied to the retina at a rate of 0.5 Hz through one pair of stimulating channels.

The Gesteland's metallic microelectrode, previously used by us for recording [9], was replaced with a modified carbon-fibre microelectrode having a recording tip 10–30 μm long [24, 25]. The recording tips were formed by the methods of 'local boiling' or 'dosed burning'. The intrinsic electric noise of our just made carbon-fibre microelectrode, immersed in saline, did not contain slow components that are observed in the noise from a usual carbon-fibre microelectrode [19, 26]. The noise of improved carbon-fibre microelectrodes was characterized by a root mean square (RMS) noise level of 3–5 μV and by peak-to-peak values to about 30 μV over the bandwidth of 2 Hz to 5 kHz [25].

Signals from the amplifier were sampled at 10.4 kHz. Single stimuli triggered sweeps or responses that were collected as averages of 4–10 sweeps on line were stored in the computer for later analysis. The frequency pass band was 2 Hz–5 kHz.

RESULTS

The stimulating six-channel electrode or the block of two five-channel electrodes was placed on the nasoventral quadrant of the naked retina. At the beginning, a mass response was evoked over a relatively large area of the dorsal tectum (areas 1, 3, 4, 5, and 6 according to Ewert et al. [27]) by delivering a stimulating pulse of a moderate strength (5–10 V, 100 μs) through several pairs of channels of the stimulating electrode. The site of the occurrence of the maximal evoked potential was searched for according to the data of a retinotopic projection [27]. When such a site of recording of the biggest evoked potential was found on the tectum surface, the recording carbon-fibre microelectrode was inserted to a depth of 200–300 μm where a current sink that reflects excitatory monosynaptic activities derived from myelinated optic fibers is maximal [28, 29]. Then, by varying the pairs of channels, stimulation through one pair of channels, which evoked the largest mass response in the site of insertion of the microelectrode, was searched for. This maximal response signified the best correspondence between the projection of activated fibers and the point of registration. The mass response was similar to the one recorded by Chung et al. [28] and consisted of two waves

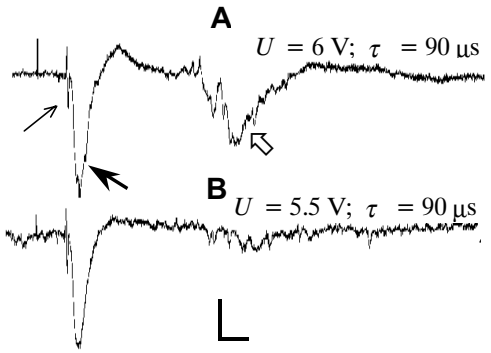

Fig. 1. (A) The mass response following achievement of the best correspondence between the projection of activated fibers and the point of recording by selecting the most suitable pair of the stimulation channels. The recording was carried out at the depth of 220 μm of the tectum. Such a response consists of waves with different latencies. The short (thick black arrow) and the long (open arrow) latency waves are associated with the activity in myelinated and nonmyelinated retinotectal fibers, respectively. Thin arrow points to the presynaptic compound potential of myelinated fibers. (B) The mass response with a considerably decreased long latency wave after the intensity of the stimulating pulses was lowered. At the begining there are artefacts of stimulation. Calibration marks indicate the same in both parts: vertical, 100 μV, horizontal, 10 ms, positive up

with different latencies (Fig. 1 A). The long latency wave, associated with the activity in nonmyelinated retinotectal fibers, considerably decreased (Fig. 1 B) and disappeared after the intensity of the stimulating pulses was lowered. Next, the stimulating pulse's strength and duration were gradually diminished until the threshold response of "all-or-none" type was recorded, that is, when the response failed on about half of the stimulation trials (Fig. 2 A). The depth of insertion of the microelectrode was adjusted by ±10–20 µm to attain a maximal amplitude of the response. This "all-or-none" type response consisted of the terminal action potential (AP) and of the synaptic potential (SP), which arose from the synaptic currents generated synchronously at the synapses made by the activated individual optic fiber [6, 7, 17]. In most cases the size of APs and SPs did not vary when the duration of the stimulating pulse was increased by 10–20 µs above the threshold value (Fig. 2, B and C). Further increasing the pulse duration above the threshold value evoked a jump-like increase of both pre-and postsynaptic components of the response in some trials (Fig. 2, D and E). Turning the stimulation intensity down the threshold value (by 5–10 µs) led to an ab-

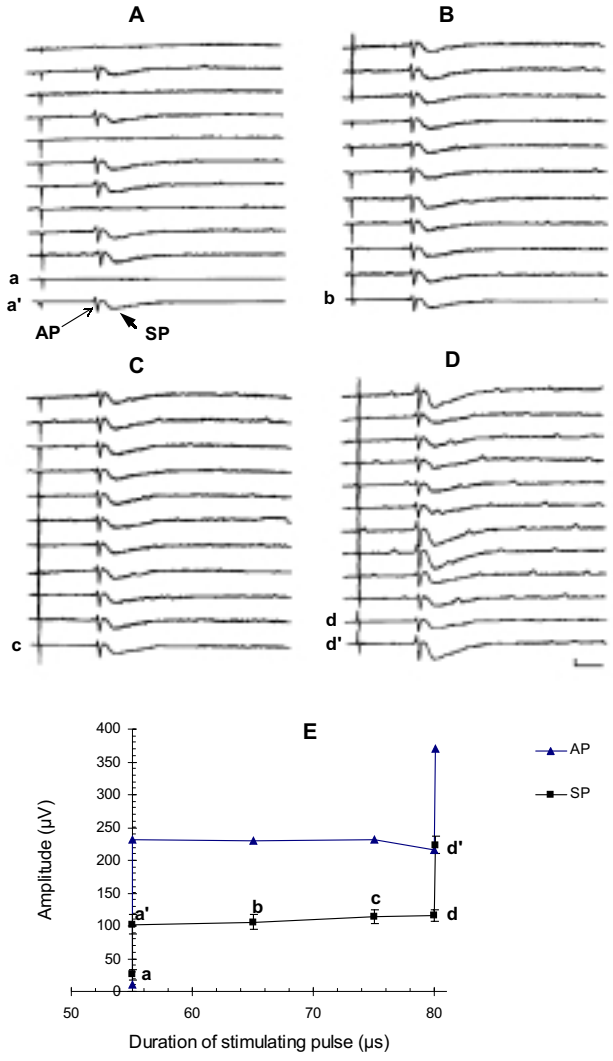

Fig. 2. Registration of tectal responses at threshold (A) and suprathreshold (B–D) stimulation of the retina. Non-averaged sweeps are shown in the order in which they occurred, the average sweeps are shown below and indicated by letters. Failures (from A) and sweeps with increased responses (in D) were averaged separately (see (a) and (d') records, respectively). Calibration marks: vertical, 100 µV, horizontal, 5 ms, positive up. (E) Dependence of amplitudes of the presynaptic and postsynaptic responses on the duration of the stimulating pulse. Error bars signify S.E.M

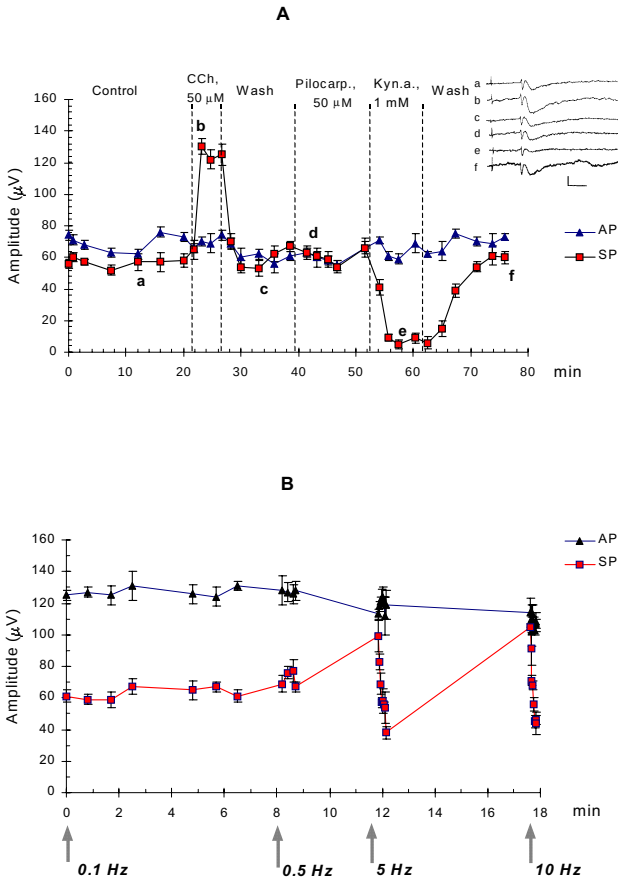

Fig. 3. The time course of the AP and SP during an examination of the effects of pharmacological agents (A) and of the frequency modulation of SPs (B). Carbamylcholine chloride (CCh), a nonspecific agonist of acetylcholine receptors, pilocarpine hydrochloride (Piloc), a muscarinic agonist and kynurenic acid (Kyn.a), a nonspecific antagonist of glutamate receptors, were applied on the tectum with a subsequent washout. Note the augmentative action of the CCh on the SPs and the depression of the latter by the glutamate receptor antagonist, kynurenic acid, in (A). Inset: the examples of averaged responses at the points indicated by the letters a–f. Calibration marks mean: vertical, 100 µV, horizontal, 5 ms, positive up. (B) The evident changes in the transmitter release occurred at different stimulation rates, while the presynaptic AP underwent only slight alterations. Each point represents the average of 2–7 sweeps of successful trials (out of 10 totals) of the threshold stimulation in (A) and of 5 sweeps in (B)

rupt disappearance of both the AP and the SP in all stimulating trials. The nerve tissue protecting threshold stimulation could be delivered to the retina within hours and the stable response, with apparent changes of its components to the action of pharmacological or physiological factors, could be recorded in the tectum (Fig. 3). Following the described above general criteria of minimal stimulation, the registration of the APs (46–188 μV) and the SPs (28–219 μV) from 56 individual retinotectal axon arborizations (layer F according to Potter [30]) in 35 frogs was achieved with the aim to investigate the n-cholinergic modulation of glutamate release [13].

The axons of retinal ganglion cells terminate in the superficial layers of the tectum, and terminal arborizations of axons of the neighbouring ganglion cells overlap there [21]. By employing 2 to 3 different pairs of the stimulating channels, 2–3 separate ganglion cells could be stimulated in the retina and thus, the responses from overlapping terminal arborizations of their axons could be recorded in the tectum with the same microelectrode (Fig. 4).

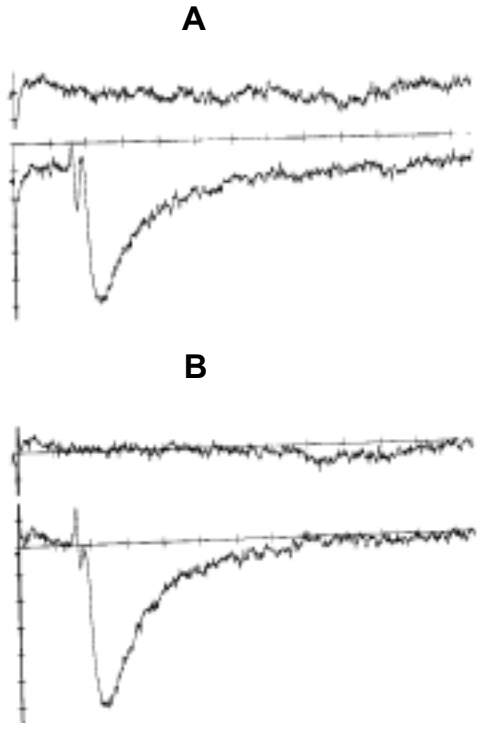

Fig. 4. Non-averaged "all-or-none" type responses recorded with the same microelectrode from overlapping terminal arborizations of two retinotectal axons. Neighbouring ganglion cells were stimulated through two pairs of channels of the 5-channel electrode. (A) The three-phase AP shows [30] that the registration took place in the proximal part of the terminal arborization of one axon. In (B) the AP is two-phase, *i.e.* the microelectrode was at the same time in another terminal arborization distal part. The units on the vertical gradual scale mean 20 μV and on the horizontal scale 5 ms

The use of a multi-channel electrode for bipolar stimulation allowed us also to diminish the artefact of the electric stimulus effectively by joining additional channels to the anodic one [23].

DISCUSSION

The search for "all-or-none" type response always begins from detecting a mass response on the tectum's surface. It is quite easy to detect a mass response evoked over a large area of the tectum. By using the multi-channel electrode it was possible to evoke such response by delivering a stimulating pulse through several pairs of channels at a lower current in comparison with that delivered through a two- or monochannel electrode. Thus, the retina's tissue was preserved from injury that would occur with a high density current. This is an important advantage when prolonged stimulation is needed.

The registration of the AP and SP from individual retinotectal axon arborization was achieved following the general criteria of minimal stimulation. The large resistance of each stimulating channel ensured that the stimulating currents (under 50 μA) were sufficiently stable when a stimulus was applied to the retina. Voltage changes by 0.1 V caused steps as low as 1 μA. For even a more sensitive dosage of stimulation intensity, the duration of the stimulating pulse was shortened by steps of 2–5 μs. Thus, single retinal ganglion cell stimulation was verified: by the "all-or-none" response elicited by graded changes in stimulus intensity, by an abrupt disappearance of all responses when stimulation intensity was gradually turned down, by occurrence of the response in all stimulating trials when the threshold stimulus intensity was slightly increased, and by a recruitment of similar potentials at still higher stimulus intensities [8, 9, 22]. If the response did not follow the minimal stimulation criteria, the site of insertion of the microelectrode in the tectum was adjusted and the pair of stimulating channels was changed. There are 30 variants for bipolar stimulation for a 6-channel electrode. The use of a block of two five-channel electrodes expands further the possibilities to vary the area of excitation.

The efficient artefact reduction provided by the usage of a multi-channel electrode eliminates the need for electronic subtraction technique for stimulus artefact suppression.

Carbon-fibre microelectrodes produced by our technology [25] were used for recording. The intrinsic electric noise of our just made carbon-fibre microelectrodes, immersed in saline, did not contain slow components. Therefore, these carbon-fibre microelectrodes fit well the purpose of simultaneous recording of fast action and slow synaptic potentials. An increase of the length of the recording tip reduces further the impedance and the intrinsic noise of the microelectrode

and makes it possible to isolate small signals, *i.e.* to record the responses from individual retinotectal axon arborization without averaging (Figs. 2 and 4). The length of the recording tip (20–30 μm) of the most frequently used carbon-fibre microelectrodes was comparable with the vertical dimension of retinotectal terminal arborization [32]. This enhanced the stability of recording due to elimination of the effect of brain pulsation on the registration.

Thus, the use of the multi-channel electrode for threshold electrical stimulation of the frog retina made the whole process of searching for the "all-or-none" type response in the tectum quite easy, because it enabled us: a) to change the site of excitation without displacing the electrode; b) to ensure a prolonged stimulation of a single ganglion cell and stimulation of several separate neighbouring ganglion cells by employing different pairs of stimulating channels and thus the registration of responses from overlapping terminal arborizations of their axons with the same microelectrode. The low noise carbon-fibre microelectrode with a longer recording tip allowed us: a) to record the "all-or-none" type response without averaging, and b) to increase the stability of the registration, *i.e.* to eliminate the effect of brain pulsation on the recording. We suggest an improved method of recording the pre- and postsynaptic potentials from terminal arborizations of separate nerve fibers for investigation of synaptic transmission in the CNS on the level of single axon synapses.

Received
15 May 2002

References

1. Vizi ES. Pharmacol Rev 2000; 52(1): 63–89.
2. Miles R. J Physiol 1990; 428: 61–77.
3. D'Angelo E, De Filippi G, Rossi P, Taglietti V. J Physiol 1995; 484: 397–413.
4. Ali AB, Thomson AM. J Physiol 1998; 507(15): 185–99.
5. Feldmeyer D, Lubke J, Silver RA and Sakmann B. J Physiol 2002; 538(3): 803–22.
6. Гутман А, Курас А. Биофизика 1974; 19: 894–8.
7. Witpaard J, Ter Keurs HEDJ. Vision Res 1975; 15: 1333–8.
8. Luscher H-R, Ruenzel PW, Henneman E. J Neurophysiol 1983; 50: 1045–58.
9. Курас АВ, Хусаиновене НП. Нейрофизиология 1981; 13: 643–6.
10. Курас А, Хусаиновене Н. Нейрофизиология 1984; 16: 829–35.
11. Курас А, Хусаиновене Н. Нейрофизиология 1986; 18: 45–55.
12. Блистрабас Р, Гутман А, Курас А и др. Нейрофизиология 1989; 21 (6): 756–65.
13. Kuras A, Gutmanienė N. Visual Neuosci 2001; 18: 549–58.
14. Mendel LM, Henneman E. J Neurophysiol 1971; 34(1): 171–87.
15. Гутман А. Материалы VI Всесоюзной конференции по электрофизиологии центральной нервной системы. Ленинград, 1971: 91–2.
16. Гутман А. Биофизика внеклеточных токов мозга. Москва, 1980: 184 с.
17. Груодис Ю, Гутман А, Курас А и др. Докл. АН СССР 1972; 204: 1246–9.
18. Gesteland RC, Howland B, Lettvin JI, Pitts WH. Proc I. R. E. 1959; 47(11): 1856–62.
19. Armstrong-James M, Millar J. J Neurosci Methods 1979; 1: 279–87.
20. King WM, Schmidt JT. Visual Neurosci 1993; 10: 419–37.
21. Maturana HR, Lettvin JV, McCuloch WS, Pitts WH. J Gen Physiol 1960; 43: 129–75.
22. George SA, Marks WB. Exp Neurol 1974; 42(3): 467–82.
23. Kuras A., Gutmanienė N. J Neurosci Methods 1997; 75: 99–102.
24. Блистрабас Р, Курас А, Хусаиновене Н. Физиол журн (СССР, им. ИМ Сеченова) 1990; 76(3): 418–21.
25. Kuras A, Gutmanienė N. J Neurosci Methods 1995; 62: 207–12.
26. Блистрабас Р, Курас А, Хусаиновене Н. Физиол. журн. СССР им. ИМ Сеченова 1989; 75(7): 1019–23.
27. Ewert J-P, Hock FJ, Wietersheim A von. J Comp Physiol 1974; 92: 343–56.
28. Chung SH, Bliss TVP, Keating MJ. Proc Roy Soc 1974; 187 (1089): 421–47.
29. Nakagawa H, Matsumoto N. Prog Neurobiol 2000; 61: 1–44.
30. Potter HD. J Comp Neurol 1969; 136: 203–32.
31. Mallart A. Pflug Arch 1984; 400: 8–13.
32. Potter HD. J Comp Neurol 1972; 144: 269–84.

A. Kuras, N. Gutmanienė

PATOBULINTAS VARLIŲ PAVIENIO RETINOTEKTALINIO AKSONO ABORIZACIJOS ATSAKŲ UŽRAŠYMO METODAS

S a n t r a u k a

Atsakų nuo pavienių varlės retinotektalinių aksonų terminalių registravimo metodas buvo patobulintas įdiegiant stimuliuojantį daugiakanalį metalinį elektrodą ir registruojantį mažatriukšmį anglinį mikroelektrodą. Daugiakanalio elektrodo panaudojimas varlės tinklainės slenkstiniam elektriniam dirginimui palengvino slenkstinių atsakų tektume paiešką, nes leido: a) keisti dirginimo vietą neperkeliant elektrodo, b) dirginti kaimynines ganglijines ląsteles pro skirtingas elektrodo kanalų poras, taip pat tuo pačiu mikroelektrodu registruoti atsakus nuo šių ląstelių aksonų terminalių, iš dalies persiklojančių tektume. Mažatriukšmiu angliniu mikroelektrodu su ilgu nuvedančiuoju galiuku buvo galima: a) registruoti slenkstinius atsakus be vidurkinimo ir b) padidinti registravimo stabilumą, pašalinant smegenų pulsacijos poveikį registravimui.