PHYSIOLOGY

Specificity of Postsynaptic Excitations of Different Sensory Modality in Neurons of Edible Snail during Learning

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The development of sensitization in edible snails during intracellular injection of oligonucleotides specifically inhibiting C/EBP transcription factors results in selective suppression of synaptic facilitation in responses of LPl1 neurons evoked by chemical stimulation of the head. At the same time, facilitation of the responses to tactile stimulation of the head or foot developed as in control sensitized animals. The data are interpreted from the viewpoint of P. K. Anokhin hypothesis that integrative function of cerebral neurons involved in construction of biologic functional systems is based on peculiar pre- and postsynaptic chemical processes selectively projected onto the genome of neural cell.

Key Words: *mollusk; neuron; nociceptive sensitization; postsynaptic plasticity; C/EBP*

According to P. K. Anokhin hypothesis proposed in 1974, integrative activity of cerebral neurons involved in construction of biological functional systems is based on specialized pre- and postsynaptic chemical processes selectively projected onto neural cell genome. Some problems relating to chemical mechanisms of selective mediation of the reactions of various sensory and biological modalities were studied by Anokhin' followers [9] and other researchers [2,8,10,12]. The results of these works indicate the development of fine postsynaptic processes in cerebral neurons, which include changes in activity of cyclic nucleotides, calcium ions, nitric oxide, and various protein kinases accompanied by complex molecular-genetic processes. However, molecular specificity of postsynaptic processes underlying participation of brain neurons in the formation its systemic activity was little studied [3,9].

During the development of nociceptive sensitization in LPl1 and RPl1 command defensive neurons of edible snails, facilitation of synaptic transmission is developed, which is specific in matter of stimulation site and stimulus modality, and which depends on translation and transcription process [3,6]. Induction of facilitation of chemosensory inputs in command neurons from head chemoreceptors depends on activity of glutamate (NMDA), serotonin, and probably κ- and/or ε-opioid receptors [3,5]. Intracellular injection of cAMP induced facilitation only in the responses to chemical stimulation, but produced no effect on responses to mechanical stimulation of the head and foot [3]. However, snail neurons are characterized by mutual interaction between the receptors to serotonin, NMDA, κ-opioid receptors, and adenylate cyclase [8, 12,13]. Therefore, stimulation of these receptors could change the level of cAMP in LPl1 and RPl1 neurons, although other mechanisms of intracellular mediation of stimulation of these receptors are also possible.

In addition, we showed that protein kinase C is involved into mechanisms of induction of synaptic facilitation in tactile receptors of the head, an alternative sensory input to LPl1 and RPl1 neurons. The development of sensitization under the action of antagonists to protein kinase C repressed facilitation in sensory inputs from the mechanoreceptors of snail head,

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while facilitation of the responses to stimulation of chemoreceptors of the head and mechanoreceptors of the foot developed in the same way as in controls [4]. During sensitization, met-enkephalin, a stimulator of μ- and/or δ-opiate receptors, selectively repressed facilitation of the responses of the command neurons to the signals from head mechanoreceptors [5], which probably resulted from inhibition of proteinase C activity.

Thus, it can be hypothesized that the long-term changes in the responses of snail command neurons during learning are specifically controlled by various postsynaptic molecular-genetic mechanisms. However, direct experiments are needed for confirmation of the hypothesis on the specific neurochemical "projection" of postsynaptic processes onto the neuron genome.

To test this hypothesis, we studied participation of the transcription factors C/EBP (CCAAT-enhancer-binding protein) in the postsynaptic processes controlling sensory-specific firing of edible snail neurons during the development of nociceptive sensitization, which is a simple form of learning.

MATERIALS AND METHODS

The experiments were carried out on defensive command neurons LPl1 of semi-intact preparations of edible snail *Helix lucorum* [4]. Before surgery, the animals were anesthetized by cooling in a water-ice bath for 30-40 min or by injection $MgCl_2$ (100-150 mg dissolved in 2-3 ml distilled water). The clamshell was removed, and the anterior aspect of the foot was dissected along the midline excluding its rostral part. Then the snails were placed into a chamber filled with paraffin. A silicon ring (200 μl) was fixed around the subglottal complex of ganglia (CNS) and this area was perfused with physiological saline at a rate of 300 μl min. Electrical activity of neurons was recorded by routine electrophysiological technique.

Sensitization was produced by three applications of 10% quinine (100 μl) to the rostral part of the head. The interval between applications was 15 min. The head was washed with physiological saline 2 min after each sensitizing stimulus.

To test the responses of neurons to sensory stimuli, the mechanical (tactile) and chemical (quinine, 0.5%) stimuli were used. Quinine (600 μl) was applied onto the rostral part of the head for 30 sec. Two minutes after the end of application, the head was washed with physiological saline. The tactile stimuli were applied to the head or median part of the foot with the help of electromechanical device. The test stimuli were presented every 15-20 min for 1 h before sensitization and on minutes 120-150 after its development. We previously showed that facilitation of synaptic transmission attained a stable level on minutes 120-150 after the development of sensitization and lasted for 24 h [3]. In responses triggered by sensory stimuli, we assessed the area of slow excitatory postsynaptic potentials (sEPSP), which is characteristic of command neurons.

To suppress C/EBP activity, double-chain oligonucleotides were used, which corresponded to both chains of ERE binding sites identified in mammals (ERE: gatca-tatta-ggaca-tgcgg), and C/EBP-binding series of Aplysia mollusk (BS2 C/EBP: gatcc-ggcac-tattg-cgcaa-tctca-agcta) [10,11]. It is suggested that these oligonucleotides bind to C/EBP proteins produced during sensitization and suppress their activity. ERE mutant oligonucleotide (random nucleotide sequence gatcc-atatg-cggac-atgcg) was used as the control [10]. These factors were synthesized by V. F. Kobozev on a SAM-102I automatic synthesizer (Bio-

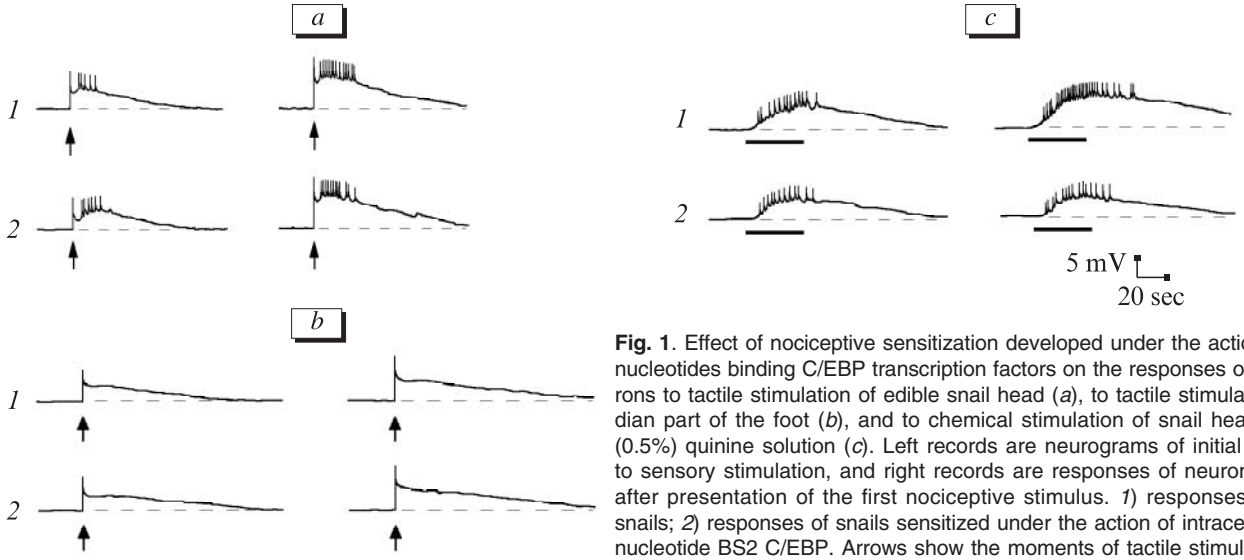

Fig. 1. Effect of nociceptive sensitization developed under the action of oligonucleotides binding C/EBP transcription factors on the responses of LPl1 neurons to tactile stimulation of edible snail head (*a*), to tactile stimulation of median part of the foot (*b*), and to chemical stimulation of snail head by weak (0.5%) quinine solution (*c*). Left records are neurograms of initial responses to sensory stimulation, and right records are responses of neurons 120 min after presentation of the first nociceptive stimulus. *1*) responses of control snails; *2*) responses of snails sensitized under the action of intracellular oligonucleotide BS2 C/EBP. Arrows show the moments of tactile stimulation. Horizontal bar (*c*) shows period of chemical stimulation with weak quinine solution.

sset). Microelectrode containing one oligonucleotide (300 μg/ml in 1 M potassium acetate, 10 mM Tris HCl, pH 7.5) was introduced into the cell immediately before injections of the substances (1 h before sensitization). Ten minutes after the end of sensitization procedure, the injection was stopped and the microelectrode with oligonucleotide was removed from the cell. In addition, immediately before presentation of each sensitizing or sensory stimulus, the microiontophoresis injection of oligonucleotide was interrupted for 3 or 2 min, respectively. The substances were injected by microiontophoretic current of negative polarity (-8 nA).

In each experiment, the data were normalized to the value of the parameter measured before the injection of substances in non-sensitized snail or before the development of sensitization in sensitized snails. Then the data were averaged and expressed in percentage to initial values. The results were analyzed statistically using Student's *t* test.

RESULTS

Application of quinine (0.5%) to snail head triggered sEPSP in LPl1 neurons, the area of sEPSP was 309±37 arb. units (*n*=47). The areas of sEPSP caused by tactile stimulation of the head and foot were 260±22 (*n*=49) and 148±19 arb. units (*n*=45), respectively.

On minutes 120-150 after the onset of the development of sensitization in LPl1 neurons subjected to control electric current of -8 nA or injected with BS2, C/EBP, ERE, or ERE mutant, the areas of sEPSP induced by tactile stimulation of the snail head surpassed the control values measured before sensitizing procedure by 57±11% (*n*=12), 67±17% (*n*=16), 51±12% (*n*=13), and 53±14% (*n*=10), respectively (Fig. 1). Under the same conditions, the areas of sEPSP induced by tactile stimulation of the snail foot increased by 37±9% (*n*=12), 48±15% (*n*=12), 33±11% (*n*=8), and 41±12% (*n*=7), respectively. On minutes 120-150 after the onset of sensitization in LPl1 neurons stimulated with electric current alone (-8 nA), or treated with BS2 C/EBP, ERE, or ERE mutant, the areas of sEPSP induced by chemical stimulation of snail head changed by 93±17% (*n*=12), 7±13% (*n*=14), -11±9% (*n*=19), and 102±21% (*n*=10), respectively (Fig. 2).

Our data suggest that during the development of sensitization under the action of oligonucleotides ERE or BS2 C/EBP, facilitated responses of LPl1 neuron to tactile stimulation of snail head and foot did not differ from those in control snails injected with ERE mutant

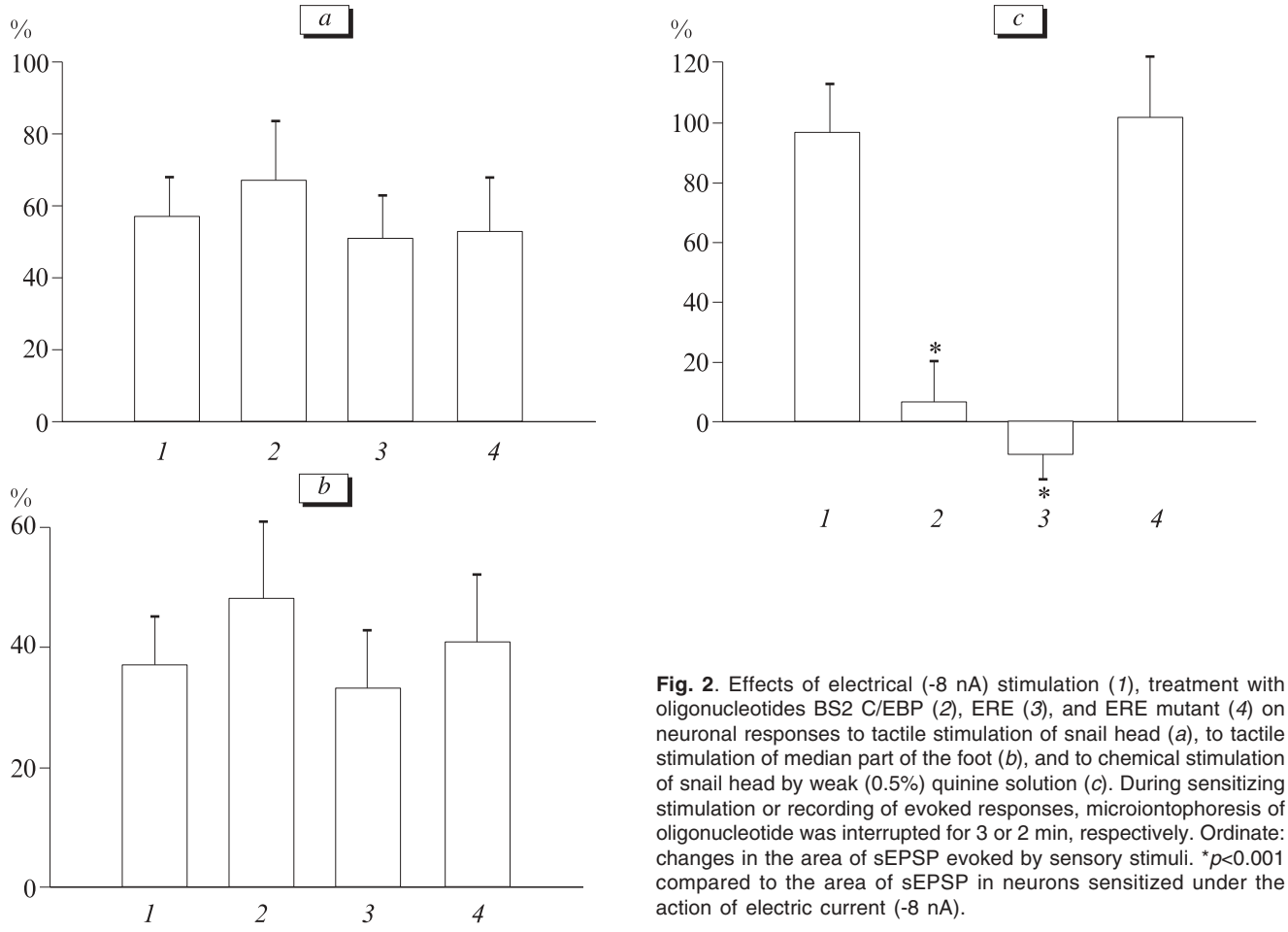

Fig. 2. Effects of electrical (-8 nA) stimulation (*1*), treatment with oligonucleotides BS2 C/EBP (*2*), ERE (*3*), and ERE mutant (*4*) on neuronal responses to tactile stimulation of snail head (*a*), to tactile stimulation of median part of the foot (*b*), and to chemical stimulation of snail head by weak (0.5%) quinine solution (*c*). During sensitizing stimulation or recording of evoked responses, microiontophoresis of oligonucleotide was interrupted for 3 or 2 min, respectively. Ordinate: changes in the area of sEPSP evoked by sensory stimuli. *$p<0.001$ compared to the area of sEPSP in neurons sensitized under the action of electric current (-8 nA).

or subjected to control electric current of -8 nA. At the same time, the responses induced by chemical stimulation of snail head recorded in sensitized snails treated with ERE or BS2 C/EBP were considerably lower than those in control neurons ($p<0.001$) and did not differ from the initial values.

The experiments showed that injection of oligonucleotides inactivating C/EBP proteins into command neurons LPl1 responsible for defensive behavior during the development of sensitization selectively inhibited facilitation of neural responses induced only by chemical stimulation of the snail head. Thus, the effect of these oligonucleotides is synapse-specific, because they did not affect synaptic facilitation in LPl1 inputs from tactile receptors of the head and foot.

It is known that learning is accompanied by expression of immediate-early genes [1,12]. Expression of these genes is controlled by preexisting protein transcription factors, which are activated by extracellular signals via different protein kinase systems. The protein products of the early genes are also transcription factors regulating expression of effector genes involved in the mechanisms of neural plasticity. C/EBP is one of the early genes, whose rapid and short-term expression is triggered by extracellular stimuli [12, 14,15]. Some members of C/EBP family are activated by cAMP-dependent transcription regulators CREB (cAMP-response element-binding protein). C/EBP can bind to ERE (enhancer responses element) of the *c*-fos early gene and to ERE of some late genes [2,12]. These data suggest that cAMP activates CREB-dependent genes (including C/EBP) in LPl1 neurons.

In various animal species, C/EBP is involved in neural mechanisms of long-term memory [10,14,15]. However, previous studies revealed no functional selectivity of C/EBP towards synaptic connections characterized by specific morphological, functional, or neurochemical features. This work is the first to show that during learning, immediate-early gene and the expressed transcription factors C/EBP are selectively involved in the mechanisms of postsynaptic reactions of the command neurons of edible snail. The present data provide experimental basis for the views on selective projection of certain synaptic inputs onto neuron genome.

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