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IN VITRO FORMATION AND ACTIVITY-DEPENDENT PLASTICITY OF SYNAPSES BETWEEN *HELIX* NEURONS INVOLVED IN THE NEURAL CONTROL OF FEEDING AND WITHDRAWAL BEHAVIORS

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Abstract—Short-term activity-dependent synaptic plasticity has a fundamental role in short-term memory and information processing in the nervous system. Although the neuronal circuitry controlling different behaviors of land snails of the genus *Helix* has been characterized in some detail, little is known about the activity-dependent plasticity of synapses between identified neurons regulating specific behavioral acts. In order to study homosynaptic activity-dependent plasticity of behaviorally relevant *Helix* synapses independently of heterosynaptic influences, we sought to reconstruct them in cell culture. To this aim, we first investigated in culture the factors regulating synapse formation between *Helix* neurons, and then we studied the short-term plasticity of *in vitro*-reconstructed monosynaptic connections involved in the neural control of salivary secretion and whole-body withdrawal. We found that independently of extrinsic factors, cell–cell interactions are seemingly sufficient to trigger the formation of electrical and chemical synapses, although mostly inappropriate—in their type or association—with respect to the *in vivo* synaptic connectivity. The presence of ganglia-derived factors in the culture medium was required for the *in vitro* reestablishment of the appropriate *in vivo*-like connectivity, by reducing the occurrence of electrical connections and promoting the formation of chemical excitatory synapses, while apparently not influencing the formation of inhibitory connections. These heat-labile factors modulated electrical and chemical synaptogenesis through distinct protein tyrosine kinase signal transduction pathways. Taking advantage of *in vitro*-reconstructed synapses, we have found that feeding interneuron–efferent neuron synapses and mechanosensory neuron–withdrawal interneuron synapses display multiple forms of short-term enhancement-like facilitation, augmentation and posttetanic potentiation as well as homosynaptic depression. These forms of plasticity are thought to be relevant in the regulation of *Helix* feeding and withdrawal behaviors by inducing dramatic activity-dependent changes in the strength of input and output synapses of high-order interneurons with a crucial role in the control of *Helix* behavioral hierarchy. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: mollusk, soma–soma cell culture, synaptogenesis, gap junctions, protein tyrosine kinases, short-term homosynaptic plasticity.

Land snails of the genus *Helix* have been extensively employed in behavioral and neurophysiological studies (Balaban, 1993, 2002; Chase, 2002) and the neuronal circuitry controlling different aspects of their behavior is at least partially known (e.g. Cottrell, 1977; Zhuravlev et al., 1988; Altrup and Speckmann, 1994; Koene et al., 2000; Malyshev and Balaban, 2002). In particular, several high-order giant interneurons activating and/or modulating complex behaviors such as feeding and body withdrawal have been identified and studied in detail (Cottrell and Macon, 1974; Balaban, 1979; Galanina et al., 1986; see for review Cottrell, 1977; Chase, 2000; Balaban, 2002). The behavioral choice of the animal can be influenced by the synaptic connections established by sensory neurons onto these interneurons that, in turn, are able to activate/modulate complex behaviors by acting synaptically and non-synaptically on multiple follower cells (Cottrell, 1977; Chase, 2000; Balaban, 2002).

Short-term activity-dependent synaptic plasticity is a widespread form of dynamic regulation of the strength of synaptic connections as a consequence of their previous activity, that has a crucial role in short-term memory and information processing in the nervous system (Abbott and Regehr, 2004). While this type of homosynaptic plasticity has been studied at synapses between enteroceptive sensory neurons and interneurons in the parietal ganglia of *Helix* snails (Logunov, 1985; Sokolov and Palikhova, 1999), little is known about the activity-dependent plasticity of other behaviorally relevant input and output synapses of high-order interneurons, such as those involved in the neural control of feeding (Cottrell and Macon, 1974; Altrup and Speckmann, 1994) and withdrawal behaviors (Malyshev and Balaban, 2002).

A particular advantage in studying synaptic plasticity in mollusks is given by the possibility of reconstructing *in vitro* synapses between individually identifiable neurons. This allows one to study the intrinsic properties of isolated neurons and circuits with known behavioral functions (Bulloch and Syed, 1992) and to unambiguously distinguish homosynaptic and heterosynaptic mechanisms of synaptic plasticity in a manner that is difficult to achieve in the intact nervous system (Bailey et al., 2000). However, except for the synapse be-

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CM, conditioned medium; DM, chemically defined medium; DMSO, dimethyl sulfoxide; EM, electron microscopy; EPSP, excitatory postsynaptic potential; FE test, Fisher's exact test; IPSP, inhibitory postsynaptic potential; K-Ac, potassium acetate; Lav A, Lavendustin A; Lav B, Lavendustin B; LDCV, large dense-core vesicle; NRTK, non-receptor protein tyrosine kinase; PSP, postsynaptic potential; PTP, posttetanic potentiation; RTK, receptor protein tyrosine kinase; SCV, small clear vesicle; soma–soma, soma to soma.

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tween the neurons C1 and B2 (Ghirardi et al., 1996), none of the other circuits with a known function in the control of *Helix* behavior has been successfully reconstructed *in vitro*, nor their plasticity has ever been studied in cell culture.

Helix neurons have been cultured using different technical approaches mainly aimed to obtain neurite outgrowth (Marom and Dagan, 1987; Green et al., 1990; Tiwari and Woodruff, 1992; Ivic et al., 1995), generally disregarding the analysis of synaptogenesis between the cocultured cells. Synapse formation between cultured *Helix* neurons contacting each other by regenerating neurites was obtained for the first time by using *Aplysia* hemolymph as a source of trophic factors promoting both neurite outgrowth and synapse formation (Ghirardi et al., 1996). More recent studies in other molluscan systems have shown that neurite outgrowth and synaptogenesis can be differentially regulated by trophic factors released from the nervous system (Feng et al., 1997; Munno et al., 2000). In addition, these factors can differentially affect inhibitory and excitatory synaptogenesis in *Lymnaea* (Feng et al., 1997; Magoski and Bulloch, 1998; Hamakawa et al., 1999) and can influence in opposite ways the formation of electrical connections in different molluscan species (Magoski and Bulloch, 1998; Szabo et al., 2004). To our knowledge, no study has yet analyzed the factors specifically involved in synapse formation between *Helix* neurons independently of those regulating neurite outgrowth. Moreover, almost nothing is known concerning possible differences in the regulation of the formation of different types of *Helix* synapses (i.e. electrical vs chemical; excitatory vs inhibitory).

Here, in order to study homosynaptic activity-dependent plasticity of behaviorally relevant *Helix* synapses independently of heterosynaptic influences, we sought to reconstruct them in cell culture. To this aim, we first investigated the factors regulating the formation of synapses between identified *Helix* neurons in culture and then we studied the short-term plastic properties of *in vitro*-reconstructed monosynaptic connections between feeding interneurons and efferent neurons involved in the neural control of salivary secretion, and between mechanosensory neurons and withdrawal interneurons activating body withdrawal.

EXPERIMENTAL PROCEDURES

Materials

All chemicals used in this study were purchased from Sigma (Milano, Italy), unless stated otherwise.

Animals

Juvenile and adult specimens of *Helix* snails (*H. pomatia*, *H. lucorum* and *H. aspersa*) were provided by local breeders and maintained inactive at a temperature of 7 °C. Before experimental procedures, the snails were kept in active state for at least 2–3 weeks in a climatic chamber regulating temperature, humidity and light according to a circadian cycle (16 h of light at 20 °C and 70% relative humidity/8 h of darkness at 17 °C and 90% relative humidity). During the activity period, snails were fed lettuce, carrots and water *ad libitum*. During surgical procedures, the snails were always anesthetized (see below) and all efforts were made to minimize the number of animals used and their suffering, in accordance with EC Directive (86/609/EEC).

Culture media

Different culture media were used according to the experimental requirements. 1) Chemically defined culture medium (DM), consisting of modified L15 Leibovitz medium with the addition of antibiotics and salts to obtain final concentrations appropriate for *Helix* (80 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 7 mM $CaCl_2$, 20 mM HEPES, pH 7.6) as previously reported (Ghirardi et al., 1996). 2) Ganglia-conditioned medium (CM), obtained after incubation of *Helix* nervous ganglia in DM (two to three ganglionic rings/ml for 72 h). CM was prepared following a modified version of previous protocols (Green et al., 1990; Feng et al., 1997). Briefly, under sterile conditions in a laminar flow hood, juvenile *H. aspersa* snails (1–3 g) were anesthetized by injection of isotonic $MgCl_2$ in the foot and dipped in 30% Betadine (Viatris, Milano, Italy) for 1 min. After rinsing in 5% ethanol, the anesthetized snails were pinned to a Sylgard base (Dow Corning, Wiesbaden, Germany). Under sterile conditions, the entire ganglionic ring (cerebral and subesophageal ganglia) was surgically removed. The ganglionic rings were repeatedly washed in 50 μM gentamicin in DM (six to seven passages of 15 min each) and then transferred to a Sigmacote-treated glass Petri dish containing DM (two to three ganglionic rings/ml), whose pH was adjusted to 7.9 to compensate for the slight pH reduction (0.2–0.3) observed after ganglia incubation. The Petri dish was kept in a moist chamber at 18 °C. After 72 h, the supernatant CM was removed from the Petri dish, aliquoted and stored at −20 °C until used for cell culture. 3) Heat-inactivated CM. CM was heat-inactivated by boiling at 100 °C for 20 min (Hamakawa et al., 1999).

Cell culture

Soma-to-soma ('soma–soma') cocultures of *Helix* neurons were obtained following a modified version of previous protocols (Ghirardi et al., 1996; Whim et al., 1997). The cell culture procedures were performed under sterile conditions in a laminar flow hood. The snails were anesthetized by the injection of isotonic $MgCl_2$ in the foot, deshelled and pinned to a Sylgard base. Buccal, cerebral and subesophageal ganglia were isolated and incubated for enzymatic digestion in protease type IX in DM (10 mg/ml) at 34 °C for 3 h (ganglia from snails weighing 1–4 g) or 4.5–6 h (ganglia from snails weighing more than 20 g). After digestion, the ganglia were repeatedly washed in DM (two to three times). To isolate the neurons, the ganglia were pinned to a Sylgard base in a Petri dish containing DM. The connective sheets covering the ganglia were removed with fine scissors and forceps to expose the neurons. Neurons were individually identified by their position in the ganglia and their size, removed by sharp glass microelectrodes and transferred to DM-containing plastic Petri dishes Falcon #1008 (Becton Dickinson, Franklin Lakes, NJ, USA) that had been pre-treated with 5% bovine serum albumin (BSA) (Whim et al., 1997; Szabo et al., 2004) to prevent cell-substrate adhesion. After 24–48 h under these non-adhesive conditions, the floating neurons retracted their neuritic arborizations, so that spherical somata devoid of any process were obtained. The somata were transferred to another BSA-treated Petri dish containing the desired culture medium and gently micromanipulated to form soma–soma pairs. In experiments analyzing synaptogenesis, the somata were paired in untreated Falcon #3001 dishes and allowed to adhere to the plastic substrate. We could not detect any difference among the synaptic connections formed under adhesive and non-adhesive culture conditions.

Nomenclature of the neurons

Three species of *Helix* snails are traditionally employed in neurobiological studies almost interchangeably, namely *H. pomatia*, *H. lucorum* and *H. aspersa*. The neurons employed in our experiments were isolated from *Helix* snails of the same species in