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SHORT COMMUNICATIONS

CONDITIONING FACTOR(S) PRODUCED BY SEVERAL MOLLUSCAN SPECIES PROMOTE NEURITE OUTGROWTH IN CELL CULTURE

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The ability to correlate the behaviour of neurones in intact ganglia with events in isolation as has been shown in the leech (Ready & Nicholls, 1979; Fuchs, Nicholls & Ready, 1981), may provide insight into the intrinsic properties of individual neurones.

The culture of molluscan neurones is relatively new and the exact procedures for the control of environmental conditions necessary for obtaining neurite outgrowth have not been uniformly established. In early experiments, Chen, von Baumgarten & Takeda (1971) isolated viable identified *Aplysia* neurones, but were not able to obtain neurite outgrowth. Geletyuk (1977), using dissociated neurones from *Lymnaea*, demonstrated for the first time the regeneration of neurites in cell culture. Outgrowth has been reported by Kaczmarek, Finbow, Revel & Strumwasser (1979) and Dagan & Levitan (1981) for neurones isolated from several ganglia in *Aplysia*.

Recently we reported (Wong, Hadley, Kater & Hauser, 1981) that isolated neurones from adult central ganglia of *Helisoma* required the presence of co-cultured, intact *Helisoma* brains or brain-conditioned medium in order for significant neuritic outgrowth to occur. The need for a growth-promoting factor was also suggested by Proshansky, Schacher & Camardo (1981), who found that addition of *Aplysia* haemolymph was required for optimal outgrowth of *Aplysia* neurones. This communication assesses the species-specificity of conditioned medium (CM) for *Helisoma*, *Lymnaea*, *Biomphalaria* and *Aplysia*.

To make CM, isolated brains (two central ganglionic rings per ml for *Helisoma*, *Biomphalaria* or *Lymnaea* or one entire CNS per 10 ml for *Aplysia*) were placed in serum-free 50 % Liebowitz medium (L-15) for 72 h with the salts appropriate for each species. For experiments with *Helisoma*, *Biomphalaria* or *Lymnaea*, salt concentrations of the media were: NaCl, 40 mM; KCl, 1·7 mM; $CaCl_2$, 4·1 mM; $MgCl_2$, 1·5 mM; 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, pH 7·3]. For *Aplysia*, salt concentrations were: NaCl, 460 mM; KCl, 10 mM; $CaCl_2$, 11 mM; $MgCl_2$, 55 mM; 5 mM-Hepes (pH 7·3). Isolated neurones for culture were obtained

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from *Helisoma*, *Biomphalaria*, *Lymnaea* and *Aplysia* basically as described previously (Wong *et al.* 1981). Neurones were dissociated by enzymatic digestion of dissected ganglia in 0·1 % trypsin for 1·5 h. Enzymatic digestion was stopped by using 0·1 % trypsin inhibitor for 15 min, and followed by desheathing of connective tissue and repeated trituration with plastic Pasteur pipettes. These mass-dissociated cells were transferred and cultured on polylysine-coated culture dishes in either defined medium or in CM.

To assess whether production was species-specific, CM produced by brains from each of the four species was placed in separate polylysine-coated culture dishes and plated with *Helisoma* neurones. For the dishes receiving *Helisoma*, *Biomphalaria* or *Lymnaea* CM, *Helisoma* neurones were plated directly since each medium contained the same salt concentrations. However, since the salt concentration for *Aplysia* CM is different from that of *Helisoma*, we took advantage of the fact that conditioning factor(s) binds to polylysine-coated surfaces (Wong *et al.* 1981). Thus *Aplysia* medium was discarded after covering the polylysine-coated surface for 24 h, and replaced with *Helisoma* defined medium before *Helisoma* neurones were plated.

Extensive neurite outgrowth of *Helisoma* neurones was obtained in the CM of each of the species tested (Fig. 1). As reported earlier (Wong *et al.* 1981), our primary criterion for outgrowth was a quantitative comparison of percentages of neurones sprouted (neurite length greater than the soma diameter) in defined medium *vs* experimental groups in CM. When the level of outgrowth was not significantly different on a quantitative basis, qualitative assessments determined whether outgrowth of the experimental groups was more extensive than in control groups (mostly spherical cells; Wong *et al.* 1981). Elaborate outgrowth, as in Fig. 1, was never seen in control dishes of defined medium.

Quantification of the growth-promoting activity (Fig. 2) showed that CM from *Helisoma*, *Biomphalaria* and *Lymnaea* brains significantly enhanced the outgrowth of *Helisoma* neurones ($P < 0{\cdot}05$). However, surface adsorbed material (SAM) produced somewhat variable results. While *Helisoma* CM was highly significant ($P < 0{\cdot}001$) in stimulating outgrowth, *Helisoma* SAM was significant to a lesser degree ($P < 0{\cdot}02$). Similarly, *Aplysia* SAM, despite clearly fulfilling our more qualitative criterion for outgrowth, was only significant at a level of $P < 0{\cdot}1$. One explanation for the low level of growth in *Aplysia* SAM is that the concentration of brains per unit medium for *Aplysia* was only a gross approximation, while that for *Biomphalaria* and *Lymnaea* was similar to that of *Helisoma* by virtue of similar animal sizes. An interesting possibility is that the phylogenetic distance between *Aplysia* and *Helisoma* may affect the ability of *Aplysia* CM to promote outgrowth in *Helisoma* neurones.

The above data show that outgrowth of *Helisoma* neurones can be promoted by a factor(s) from the brains of other molluscan species. This suggested the converse experiment. Because *Helisoma* CM has been extensively characterized (Wong *et al.* 1981; Barker, Wong & Kater, 1982), we used it to test whether a factor(s) was also necessary to promote the growth of neurones of other species. These experiments were complicated by the fact that the isolation procedures and growth conditions for neurones from different species are often different (cf. Dagan & Levitan, 1981; Mooney & Waziri, 1982). In two experiments in which we succeeded in growi

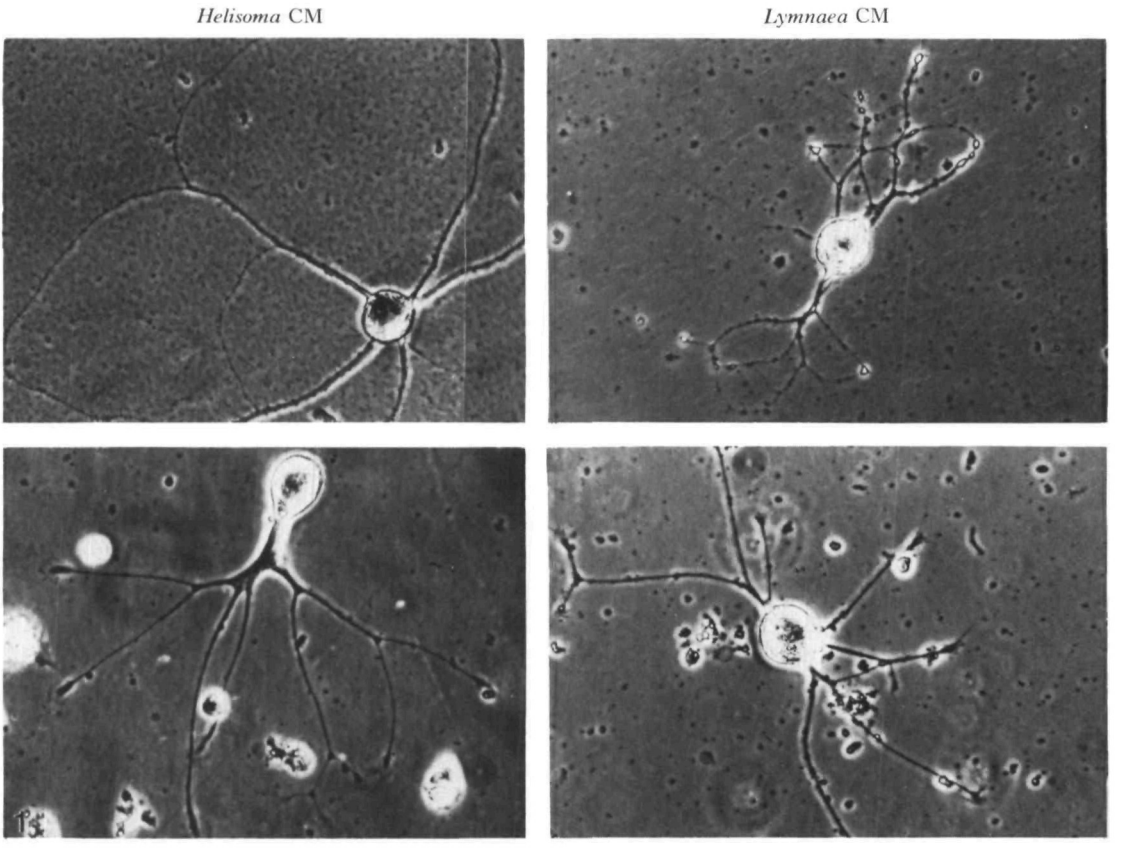

Fig. 1. Phase contrast photomicrographs of mass dissociated *Helisoma* neurones that were cultured in medium conditioned by central ganglia of either *Lymnaea*, *Biomphalaria* or *Aplysia*, as well as *Helisoma*. Neurones were initially plated as spheres on polylysine-coated culture dishes, in 72 h conditioned medium (CM) or surface adsorbed conditioned medium (SAM) and photographed 4 days after plating.

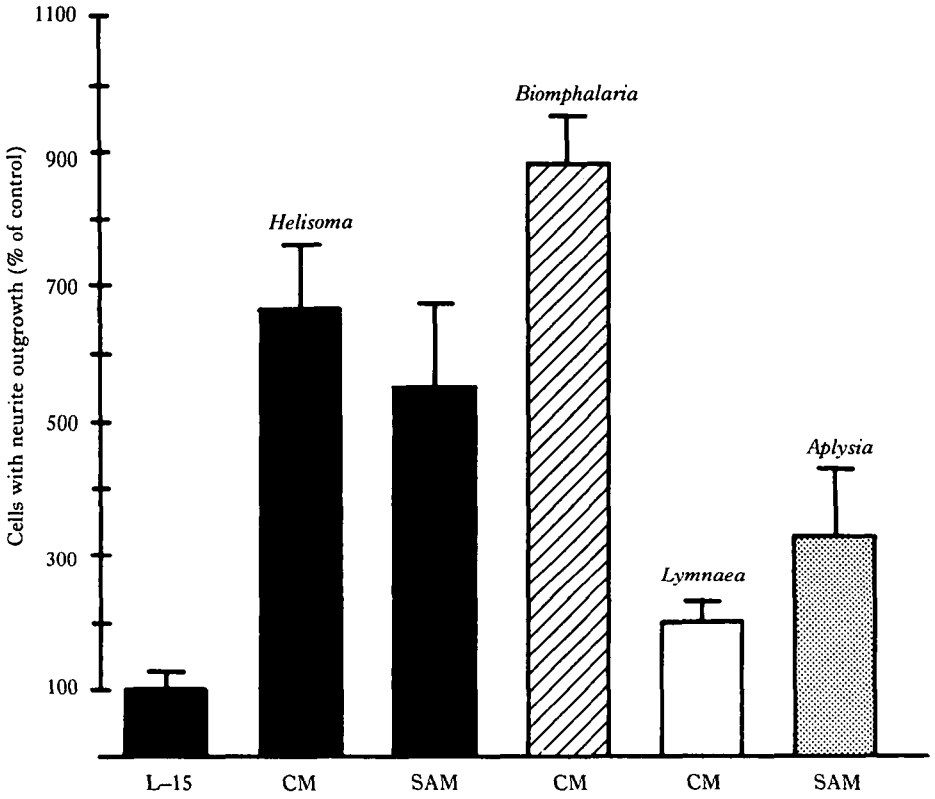

Fig. 2. Summary of species specificity of conditioned media. Cultures in conditioned medium (CM) or surface adsorbed conditioned medium (SAM) were scored as the relative percentage of adhering neurones which had neurites of a length greater than the soma diameter. Experimentals were compared to controls (defined L-15 medium) after 4 days in culture. Differences from control were significant for *Helisoma* CM ($P < 0{\cdot}001$) and SAM ($P < 0{\cdot}02$), *Biomphalaria* CM ($P < 0{\cdot}005$) and *Lymnaea* CM ($P < 0{\cdot}05$). The result for *Aplysia* SAM approached significance ($P < 0{\cdot}1$). Error bars on each histogram represent S.E.M. ($N = 3$–8).

neurones of *Biomphalaria* in *Helisoma* CM there was significant ($P < 0{\cdot}05$) and elaborate neuronal growth compared to defined medium. This growth was indistinguishable from the growth of *Helisoma* neurones in *Helisoma* CM or growth of *Biomphalaria* neurones in *Biomphalaria* CM. All attempts to grow *Lymnaea* (four experiments) and *Aplysia* (two experiments) neurones in *Helisoma* CM failed to produce any significant numbers of cells with neurite outgrowth ($P > 0{\cdot}1$). Nonetheless *Aplysia* and *Lymnaea* neurones plated on their respective CM did produce neurite outgrowth which was several times more extensive than that seen in control conditions. The number of such neurones was small, however, and not suitable for quantitative analysis. However, by our qualitative criterion these two species required CM for outgrowth. These collective observations demonstrate that for *Helisoma, Biomphalaria* and perhaps *Lymnaea* and *Aplysia*, not only can their ganglia produce CM, but additionally, their neurones, when isolated in cell culture, require a CM for elaborating complex neuronal geometry.

The differences in growth of isolated molluscan neurones *in vitro* may be attributed

to several factors: (1) the dissociation procedures used; (2) the presence of factors such as sera, haemolymph or CM which are added to defined medium; or (3) the ganglionic origin and type of cell that is isolated (e.g. bag cells *vs* buccal ganglia). It is not surprising that Chen *et al.* (1971) failed to observe any neurite outgrowth in their cultures since the culture media consisted only of sea water. In agreement with our finding that CM stimulates neurite outgrowth is that in all other studies, with the exception of Kaczmarek *et al.* (1979), the addition of sera (Kostenko, Geletyuk & Veprintsev, 1974; Geletyuk, 1977; Dagan & Levitan, 1981; Proshansky *et al.* 1981), haemolymph (Kostenko *et al.* 1974; Proshansky *et al.* 1981), or brain extract (Kostenko *et al.* 1974) may have enhanced neurite outgrowth. An additional possibility is that Dagan & Levitan (1981) and Kaczmarek *et al.* (1979) may have released a factor(s) directly into culture media when ganglia were dissociated in the culture dishes. The observation by Kostenko *et al.* (1974) that the vitality and outgrowth of neurones is always better in an unwashed suspension is consistent with this view. The factor(s) may be similar to the ones that are added to defined medium by other investigators (Kostenko *et al.* 1974; Wong *et al.* 1981; Proshansky *et al.* 1981). Taken together these observations suggest that within the central ganglia of gastropods there is an endogenous supply of related neurite growth-promoting factor(s).

Our results clearly indicate that for closely related species (e.g. *Helisoma* and *Biomphalaria*) the growth-promoting activity of CM may be highly conserved and cross-species active. The growth-promoting activity of *Lymnaea* CM may be active across species in respect to its ability to support growth of *Helisoma* neurones. However, no outgrowth was observed in the complementary experiment of growing *Lymnaea* neurones on *Helisoma* CM. Likewise, the growth-promoting activity in CM produced by *Aplysia*, an even more distantly related marine gastropod, is also diminished, and is not cross-reactive when *Aplysia* neurones are plated on *Helisoma* SAM dishes. The reduced growth of both *Lymnaea* and *Aplysia* may be related to the phylogenetic distance between *Helisoma* and these two species. In addition to the question of species-specificity of CM, we have shown that *Biomphalaria*, *Lymnaea* and *Aplysia* neurones also appear to depend on their respective CM for growth.

The results of this study demonstrate that: (1) *Helisoma*, and three other species of molluscs require a conditioning factor(s) for neurite outgrowth; (2) brains from each of four species contain, or are capable of producing, such a factor(s); and (3) the mechanism of action of CM for each species may be through a common component or mode of action present in each CM. These data taken together suggest that gastropod molluscs may be capable of regulating the extensiveness of neuronal outgrowth by factors produced by their own nervous systems.

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