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SHORT COMMUNICATION

DIFFERENTIAL TRACER COUPLING BETWEEN PAIRS OF IDENTIFIED NEURONES OF THE MOLLUSC *LYMNAEA STAGNALIS*

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Electrical coupling is a common means of cell-to-cell communication in both neuronal and non-neuronal tissues (Lowenstein, 1985). Within the nervous system, many electrically coupled neurones exhibit dye coupling (Bennett, 1973; Stewart, 1978; Glantz and Kirk, 1980; Spencer and Satterlie, 1980; Fraser and Heitler, 1993); however, some electrically coupled cells do not dye-couple (Audesirk *et al.* 1982; Murphy *et al.* 1983; Berdan, 1987; Robinson *et al.* 1993; Veenstra *et al.* 1993). Electrical coupling and dye coupling, often considered in parallel, are in fact two different parameters that can vary independently (e.g. Audesirk *et al.* 1982; Perez-Armendariz *et al.* 1991). The giant identified neurones of pulmonate and opisthobranch molluscs have frequently been used for studies of neuronal communication and its plasticity (Winlow and McCrohan, 1987; Bulloch, 1989). In the present study, we explored the relationship between electrical and tracer coupling in both strongly and weakly coupled pairs of molluscan neurones. Specifically, we examined electrically coupled, identified neurones in a freshwater pond snail, *Lymnaea stagnalis* L., and tested for tracer coupling with Lucifer Yellow CH and biocytin. The cells examined were the strongly electrically coupled neurones, visceral dorsal 1 (VD1) and right parietal dorsal 2 (RPD2) (Boer *et al.* 1979; Benjamin and Pilkington, 1986), and the weakly coupled neurones, left buccal 1 (LB1) and right buccal 1 (RB1) (Benjamin and Rose, 1979). The use of these particular neurones made it possible to compare electrical coupling with tracer coupling in the molluscan central nervous system (CNS).

All experiments were performed on laboratory-bred *Lymnaea stagnalis* (Mollusca, Pulmonata), maintained as previously described (Ridgway *et al.* 1991). The CNS was dissected from mature animals (16–18 mm shell length) and pinned to the silicone rubber (RTV 616 GE) base of a recording dish in normal saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Hepes,

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pH 7.9). Following removal of the outer connective tissue sheath, a small Pronase crystal (Sigma, type XIV, P-5147), held by forceps, was carefully applied to specific ganglia; this treatment softened the inner sheath and facilitated microelectrode penetration. The CNS was then rinsed several times at 5 °C in normal saline.

Lucifer Yellow was ionophoretically injected and the preparations were processed according to methods modified from Syed and Winlow (1989). Briefly, the tips of glass microelectrodes (WPI, no. 200) were filled with 4 % w/v Lucifer Yellow CH (Molecular Probes, L-453) dissolved in 0.1 % lithium chloride, and the electrode was back-filled with 0.1 % lithium chloride (final resistance 30–70 MΩ). Individual neurones were impaled and the dye was injected with constant (0.5–1.0 nA) hyperpolarizing current for 20–60 min. After staining, the preparations were left in normal saline overnight at room temperature (18–20 °C) to allow the dye to spread. They were subsequently fixed for 3–4 h in 3.7 % v/v formaldehyde diluted in phosphate buffer (132.3 mmol l^{-1} Na_2HPO_4 and 25.2 mmol l^{-1} NaH_2PO_4, pH 7.3). The preparations were then dehydrated in a series of ethanol washes: 50 %, 70 %, 90 % and 100 % (2×30 min), defatted in dimethylsulphoxide for 15 min and then cleared and mounted in methyl salicylate.

The procedure for intracellular injection of biocytin was modified from that of Horikawa and Armstrong (1988). Typically, the tips of microelectrodes were filled with biocytin (Molecular Probes, B-1592) dissolved at 4 % w/v in 50 μl of sterile deionized water. The electrodes were backfilled with 0.75 mol l^{-1} KCl and 0.15 mol l^{-1} Tris buffer (pH 7.6) (final resistances were 30–70 MΩ). Biocytin was injected by superimposing a 1–2 s hyperpolarizing rectangular pulse on a slightly suprathreshold constant depolarizing current for at least 20 min so as to create alternate depolarizing and hyperpolarizing (1–2 nA) currents. Following injection, the preparations were left overnight in saline to allow the tracer to spread and then fixed as for the Lucifer Yellow protocol. The preparations were washed several times in phophate-buffered saline (PBS; 137 mmol l^{-1} NaCl and 50 mmol l^{-1} Na_2HPO_4, pH 7.4) to remove excess fixative and then placed in a streptavidin–conjugate solution (1:100 dilution, streptavidin–BODIPY-FL conjugate, Molecular Probes, S-2642, and 4 % v/v Triton X-100 in PBS) overnight in darkness. The preparations were rinsed several times in PBS to remove excess conjugate solution, dehydrated, defatted and cleared, and mounted as for the Lucifer Yellow protocol. Preparations were viewed with a Zeiss Universal microscope using epifluorescence and photographed using negative (Kodak Tri-X, 400 ASA) film.

Electrical coupling was measured by impaling neurones with bridge-balanced microelectrodes having resistances of 10–30 MΩ when filled with a 0.75 mol l^{-1} KCl solution. Hyperpolarizing current pulses, 0.5–1 nA for 2 s, were injected into each cell and the responses of both cells recorded. The coupling coefficient, or coupling ratio, between neurones was calculated and averaged (Berdan, 1987).

Tracer coupling of biocytin (conjugated to streptavidin–BODIPY) was observed between the strongly electrically coupled neurones, VD1 and RPD2, in all preparations tested (Fig. 1; Table 1). Conversely, we observed that neurones VD1 and RPD2 were not dye-coupled with Lucifer Yellow, an observation similar to that previously reported by Audesirk *et al.* (1982). Both electrical coupling and tracer coupling of biocytin were reciprocal between neurones VD1 and RPD2. However, in the weakly electrically

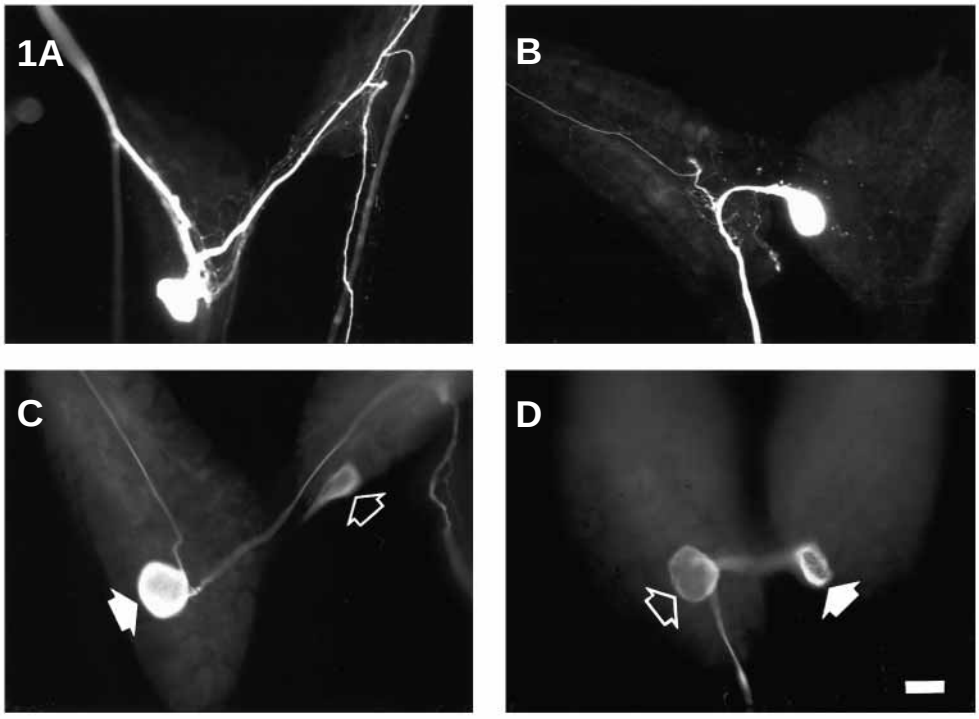

Fig. 1. A test for tracer coupling between identified neurones VD1 and RPD2 of *Lymnaea stagnalis*. No dye coupling is detectable between the cells when Lucifer Yellow is injected into either (A) VD1 or (B) RPD2. However, tracer coupling is evident when biocytin is injected into either (C) VD1 or (D) RPD2. In C and D, the filled arrow indicates the cell injected with biocytin, whereas the open arrow indicates the dye-coupled cell. Scale bar, 100 μm.

coupled neurones LB1 and RB1, no tracer coupling with either biocytin or Lucifer Yellow was detected (Fig. 2; Table 1).

Gap junctions permit cell-to-cell communication by allowing the passage of small molecules (Bennett, 1966; Hertzberg *et al.* 1981). Although Lucifer Yellow has commonly been used as a tracer to demonstrate dye coupling, it does not cross all gap junctions (Dermietzel and Spray, 1993). As mentioned above, neurones VD1 and RPD2 showed little evidence of tracer coupling in a previous study, positive coupling with

Table 1. *Electrical coupling and tracer coupling between two pairs of identified* Lymnaea stagnalis *neurones*

Neurone pair	Mean coupling coefficient	(*N*)	Tracer coupling	
			Biocytin (*N*)	Lucifer Yellow (*N*)
VD1/RPD2	0.677±0.026	(27)	+ (8)	– (9)
LB1/RB1	0.040±0.001	(6)	– (7)	– (4)

Values are mean ± S.E.M.

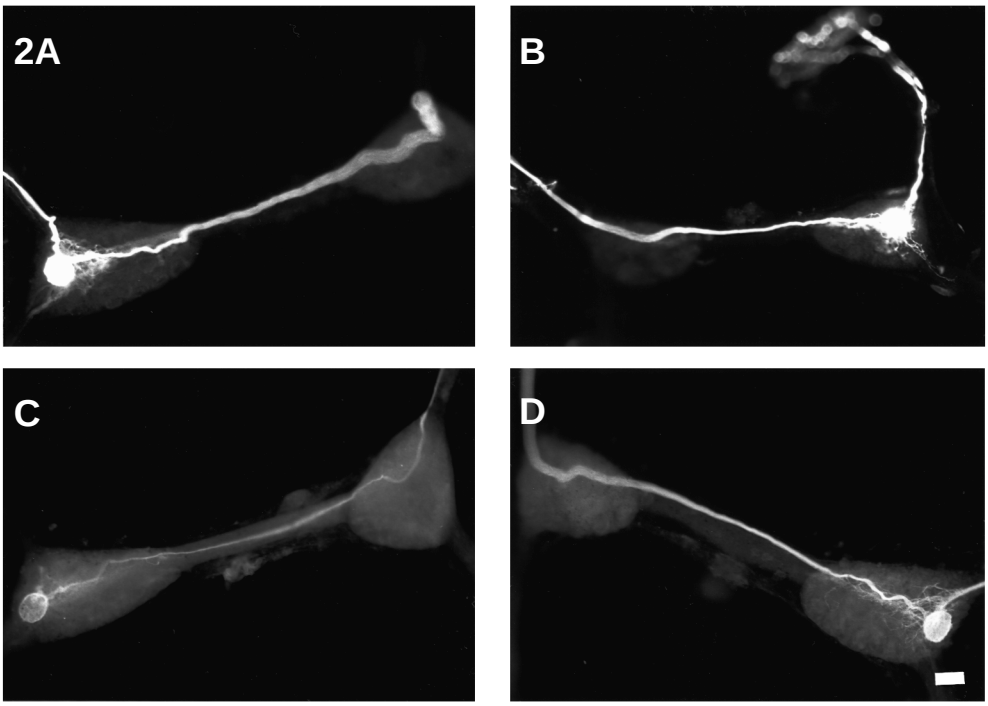

Fig. 2. A test for tracer coupling between identified neurones LB1 and RB1 in *Lymnaea stagnalis*. There is no dye coupling between the cells when Lucifer Yellow CH is injected into either (A) LB1 or (B) RB1. Similarly, coupling is not observed when biocytin is injected into either (C) LB1 or (D) RB1. Scale bar, 100 μm.

Lucifer Yellow being observed in only 1 of the 41 preparations tested (Audesirk *et al.* 1982). Additionally, there was no correlation between the amount of dye coupling observed and either the length of time allowed for dye diffusion or the magnitude of the coupling coefficient. However, in the closely related pond snail *Helisoma trivolvis*, Murphy *et al.* (1983) demonstrated that the strength of electrical coupling between identified neurones LB4 and RB4 (homologues of LB1 and RB1 in *Lymnaea stagnalis*; Bulloch and Ridgway, 1994) was the factor determining whether dye coupling occurred with Lucifer Yellow. No dye coupling was observed in neuronal pairs with coupling coefficients less than 0.50, while dye coupling always occurred when coupling coefficients were greater than 0.70.

Our results with *Lymnaea stagnalis* are consistent with the finding of Murphy *et al.* (1983) in that neurones LB1 and RB1, which are weakly electrically coupled (Table 1), are not tracer-coupled (Fig. 2; Table 1). However, it is unusual that neurones VD1 and RPD2, which are strongly electrically coupled (Table 1), do not exhibit tracer coupling with Lucifer Yellow (Fig. 1; Table 1). Estimates of the neurone VD1 to neurone RPD2 junctional conductance are 45–50 nS (for animal size used here, see Benjamin and Pilkington, 1986; Wildering *et al.* 1991). According to Dermietzel and Spray (1993), Lucifer Yellow dye coupling is generally undetectable if the junctional conductance is below 1–2 nS. Therefore, it is surprising that Lucifer Yellow does not cross between

neurones VD1 and RPD2, and this observation suggests the existence of a different type of gap junction between these neurones.

Our data do not preclude the possibility that Lucifer Yellow becomes bound within *Lymnaea stagnalis* neurones. Additionally, for both pairs of neurones examined, tracer coupling might exist but be too weak to detect against background. Despite these uncertainties, our data indicate that Lucifer Yellow may not be optimal for the detection of dye coupling in some preparations. In the case of biocytin, presumably the specific form of gap junction present in neurones VD1 and RPD2 allows the passage of this tracer because of its size, conformation or lack of charge. Tracers such as biocytin and Neurobiotin (a molecule related to biocytin) have molecular masses of 373 Da and 323 Da, respectively, and are uncharged, while Lucifer Yellow is slightly larger with a molecular mass of 457 Da and is negatively charged. In this context, it has been reported (Flagg-Newton *et al.* 1979) that more electronegative molecules are less permeable to movement through gap junctions than are more electroneutral molecules of similar molecular mass.

There are many variables that can influence the measurement of coupling coefficient: for example, the distance between the cells, the number of non-junctional conductances through which current can be shunted, the surface area of the cells and the diameter of the axons (Berdan, 1987). Thus, weaker electrical coupling does not necessarily indicate fewer gap junctions. This may be true for neurones LB1 and RB1, in which the electrotonic distance from the soma to the synapse appears to be great, perhaps facilitating transmembrane current shunting. However, in neurones VD1 and RPD2, the synapse is electrotonically close to the cell bodies, i.e. the access resistance to the synapse is small compared with the input resistance (Benjamin and Pilkington, 1986; Wildering and Janse, 1992).

There have been other reports which contrast tracer coupling with biocytin and Lucifer Yellow. Biocytin and Neurobiotin, but not Lucifer Yellow, produced strong tracer coupling in the retina (Vaney, 1991). More recently, Peinado *et al.* (1993) demonstrated that, in slices of rat neocortex, Neurobiotin was transferred more readily to coupled neurones than was Lucifer Yellow. This difference in the ability to cross gap junctions was attributed to differences in size or charge between the tracers.

Recently, Veenstra *et al.* (1993) reported that NA2 cells transfected with genes for two different types of connexin protein formed gap junctions displaying differences in dye coupling but no differences in electrical coupling. Their study provides the first evidence that the type of connexin determines the tracer permeability of the gap junction. Futhermore, differential expression of connexin protein, and corresponding differential tracer coupling, has recently been observed between astrocytes and oligodendrocytes of rabbit retina (Robinson *et al.* 1993). Differential tracer coupling between pairs of molluscan neurones may indicate the existence of a family of connexins in this phylum. Unfortunately, no invertebrate connexins have yet been cloned and this hypothesis cannot yet be directly tested.

Several dyes and tracers are used to study the morphology of neurones. Differences in the properties of these substances (e.g. size, net charge, fluorescence efficiency) may make some of them more suitable than others for different types of studies, including

studies on different species. For instance, mammalian gap junctions can pass molecules of up to 1600 Da, whereas in arthropods passage of molecules of up to 3000 Da has been reported (Berdan, 1987). Consequently, careful selection of tracer type, based upon these physical criteria, must be considered. Lucifer Yellow has been widely used to study cell morphology, but perhaps biocytin, with a slightly lower molecular mass and lacking charge, may be a better tracer for studies examining both cell morphology and dye coupling. Many groups of electrically coupled cells occur in invertebrates, especially cells involved in coordinating particular types of behaviour, and this technique may be valuable in tracing these coupled systems.

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