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Developmental Dissociation of Excitability and Secretory Ability in *Aplysia* Bag Cell Neurons

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SUMMARY AND CONCLUSIONS

1. Despite the considerable progress made in understanding the role of electrical activity in triggering secretion, the developmental relationships between excitability and secretion are not well understood. The well-characterized bag cell neurons of *Aplysia* provide an advantageous system in which to investigate developmental interactions of these two key properties of neurons.

2. A prolonged afterdischarge triggers egg laying hormone (ELH) secretion in mature bag cell neurons. To investigate secretion in the developmental framework of excitability, we first examined whether immature neurons, which are incapable of the mature form of excitability (afterdischarge), contain ELH and whether this hormone is packaged in vesicles. We used immunoelectron microscopy to compare vesicular localization of ELH and to compare the size and density of ELH-containing vesicles in neurons from adult and juvenile *Aplysia*. This comparison revealed that immature neurons contain ELH in vesicles in the size range of secretory vesicles. However, they lack a class of large vesicles (>250 nm in diameter) that is characteristic of mature neurons.

3. To investigate whether the ELH contained in immature bag cell neurons could be secreted in response to electrical activity, we used the potassium channel blocker tetraethylammmonium (TEA) combined with nerve stimulation to depolarize neurons from both juvenile animals (ovotestes do not contain eggs) and from adult *Aplysia* (ovotestes contain eggs). Using radioimmunoassay, we have found that the duration and amount of ELH secreted from bag cell neurons from juvenile *Aplysia* in response to TEA does not depend on whether or not the cells can be induced to afterdischarge, and the amount and duration of ELH secreted from bag cell neurons of juvenile *Aplysia* (whether or not they afterdischarged) differed from those secreted by adult neurons. However, by normalizing for body size, we found that the final estimated hemolymph concentration of ELH would be similar in juvenile and adult animals.

4. We investigated the potential functional significance of secretion of bag cell hormones in juvenile *Aplysia* by attempting to bypass the bag cell neurons and directly activate downstream elements with extract from adult bag cell neurons (BCE), known to contain ELH and other peptides. We found that juvenile *Aplysia* exhibit at least one component of egg-laying behavior, cessation of locomotion, in response to BCE during a developmental period (as measured by weight) in which they normally would possess neurons incapable of afterdischarge. Thus developmental regulation of excitability in the bag cell neurons may prevent inappropri-

ate hormone release and subsequent premature expression of reproductive behaviors.

INTRODUCTION

The principal function of neuroendocrine cells is the regulated secretion of hormones (for review, see Schlegel and Mollard 1995). The development of stimulus-secretion coupling involves several complex steps that include the acquisition of excitability and the ability to synthesize, package, and release chemical messengers. Although the development of neuronal excitability has been studied in some detail (for reviews, see Ribera and Spitzer 1992; Spitzer 1991), the temporal relationships between differentiation of excitability and development of secretion are not well understood. The bag cell neurons of *Aplysia californica* provide a system that has several advantages for the investigation of the development of secretion relative to the differentiation of excitability: *1*) the neurons are relatively large and easily identified, even in juvenile animals (Leibowitz and Castellucci 1983; McAllister et al. 1983; Nick et al. 1993); *2*) mature neurons exhibit a well-described, stereotypical pattern of excitability, a regenerative afterdischarge, which is a period of prolonged depolarization and repetitive firing (15–30 min) (Kupfermann and Kandel 1970; for review, see Conn and Kaczmarek 1989) that triggers the release of peptide hormones; *3*) mature neurons contain very high concentrations of a peptide hormone, egg laying hormone (ELH), which triggers egg-laying behaviors (Bernheim and Mayeri 1995; Chiu et al. 1979); and *4*) bag cell neurons can be identified and their activity recorded early in development before they have the capacity to exhibit an afterdischarge (Nick et al. 1993; present study).

The developmental emergence of afterdischarge has been described previously (Nick et al. 1993, 1996). At early developmental stages (based on body weight), the normal, repetitive afterdischarge is not expressed (Nick et al. 1993). Developmental control of afterdischarge appears to be due to regulation of intrinsic properties of the bag cell neurons themselves. Detailed electrophysiological analysis revealed that specific ion currents are up-regulated (A-type potassium

and two calcium currents), whereas others are down-regulated (calcium-dependent and delayed-rectifier potassium currents) (Nick et al. 1995, 1996). The general pattern of ionic current expression is thus consistent with the progressive increase in excitability of the bag cell neurons observed during development.

In adult *Aplysia,* the release of peptide hormones from bag cell neurons occurs in response to an afterdischarge (for review, see Conn and Kaczmarek 1989). We have found that even though immature neurons are not capable of normal afterdischarge, prolonged depolarizations can be triggered in these cells by the potassium channel blocker tetraethylammonium (TEA) (Nick et al. 1993, 1996). This raised the possibility that secretion can be triggered by TEA-induced depolarization in the absence of afterdischarge and that this form of excitability could be used to study the development of hormone secretion in the bag cell neurons. This study focused on two major questions: *1)* at the developmental stage in which bag cell neurons do not show afterdischarge, do these cells contain ELH in secretory vesicles; and *2)* before the developmental emergence of afterdischarge, can ELH be secreted? We found that before the developmental onset of afterdischarge, bag cell neurons indeed contain ELH in vesicles. Moreover, at this developmental stage, the bag cell neurons are capable of secreting this hormone in response to a depolarizing stimulus. Our results show that secretion can occur in the absence of the normal afterdischarge, indicating that secretory ability in this system develops before the expression of the mature electrophysiological phenotype.

Aspects of this work have previously appeared in abstract form (Wayne et al. 1994).

METHODS

Animals

Wild-caught *A. californica* weighing 5.9–309.3 g were obtained from Marinus (Long Beach, CA). Animals that weighed >100 g contained orange-pigmented eggs in their ovotestes and thus were designated adult. Cultured juvenile *A. californica* weighing 7.7–15.6 g were obtained from the *Aplysia* Resource Facility (RSMAS, University of Miami). None of these cultured animals contained eggs and thus were designated juvenile. Animals weighing 11–20 g were selected because they are in a developmental transition period in which bag cell neurons from some *Aplysia* are capable of afterdischarge, whereas others are not (Nick et al. 1993, 1996). Neurons from *Aplysia* <11 g very rarely afterdischarge.

Animals were maintained in aquaria containing continuously circulating, aerated Instant Ocean (Aquarium Systems, Mentor, OH) at 15°C. *Aplysia* weighing >50 g were fed twice weekly with dried seaweed. Animals weighing <50 g were given ad lib access to laboratory grown macroalgae from the *Aplysia* Resource Facility. Experiments were performed between March and September, 1994.

Electron microscopy

Abdominal ganglia were dissected from anesthetized adult (256 g; *n* = 1) and juvenile (7.5 g; cultured; *n* = 1) *Aplysia,* fixed by immersion in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate containing 0.4 M sucrose for 1 h. The bag cell neurons from the juvenile (<11 g) were most probably incapable of afterdischarge (Nick et al. 1993, 1996). Investigation

of afterdischarge capacity was not possible, as electrical activity would have stimulated the release of hormone. After rinsing in the above-mentioned buffer, the ganglia were dehydrated in ethanol, substituted with propylene oxide, and embedded in Araldite plastic (CY212). Thin sections were collected on formvar and carbon-coated nickel grids and immunostained by the protein A-gold method as previously described (Moreira et al. 1989; 1991). Polyclonal antibodies to ELH were used at a 1:600 dilution in 0.02 M Tris-HCL, 0.85% NaCl, pH 7.4 [Tris-buffered saline (TBS)]. All grids were sequentially floated on drops of 1% bovine serum albumin (BSA) in TBS (30 min), primary antibody diluted in TBS (2 h), and protein A-gold (15 nm; BioCell Research Laboratories) diluted 1:20 in TBS (1 h). The grids were rinsed in TBS after each step and finally in distilled water before drying and staining with uranyl acetate and lead citrate. Negative controls of the reaction were carried out by incubation of grids following the same procedures using a nonimmune IgG or by omitting the primary antibody. The grids were observed and photographed in a JEOL 200 CX electron microscope operated at 80 kV. Morphometric and quantitative analysis of the immunolabeling was performed with a Zidas digitizing system (Carl Zeiss, Thornwood, NY) interfaced with a Macintosh II computer. Electron micrographs were taken at an initial magnification of ×10,000 and photographically enlarged to a magnification of ×26,000. Labeling intensities of the secretory vesicles were determined as gold particles/μm^2. Processing of the data was performed using Lotus 1-2-3 software for Macintosh.

Electrophysiology and sample collection

Animals were anesthetized by injection of isotonic $MgCl_2$ (50% bd wt) into the body cavity. Upon dissection, the presence or absence of eggs in the ovotestis was noted. The abdominal ganglion, together with the pleural-abdominal connectives, was dissected and placed in artificial sea water (ASW) containing (in mM) 460 NaCl, 55 $MgCl_2$, 11 $CaCl_2$, 10 KCl, and 10 Tris, pH 7.6. Both connectives were drawn into suction electrodes and action potentials were recorded extracellularly for 5 min to ensure preparation stability. All experiments were performed at 22°C.

In some experiments, intracellular records were obtained using 15 MΩ glass microelectrodes filled with 3 M KCl; the microelectrodes were used to penetrate the connective tissue sheath and record transmembrane bag cell neuron activity in immature ganglia that had been treated with collagenase Type IV (250 units/mL ASW; Sigma) for 30 min at 15°C. Intracellular signals were amplified using a Getting 5A amplifier (Iowa City, IA). In TEA (Sigma Chemical, St. Louis, MO), the bag cell neurons exhibited unique large amplitude, long-duration action potentials, which were recorded extracellularly during secretion experiments through suction electrodes and amplified using a Grass P5 Series preamplifier. Signals were monitored with a Tektronix 5111A oscilloscope (Lexington, MA) and stored on VCR tapes using a A/D VCR adaptor (Medical Systems, Greenvale, NY) and on chart paper using a Gould chart recorder (Valley View, OH).

After the 5 min stabilization period and every 5 min thereafter, perfusate samples were collected by exchanging the entire bath (1 ml volume) of ASW containing 2% Protenate (a human plasma protein fraction; Baxter Healthcare, Glendale, CA) and four protease inhibitors (ASW/PI): bacitracin, chicken egg white trypsin inhibitors Type II-O and Type III-O, and lima bean trypsin inhibitor Type II-L (each 25 mg/100 ml; Sigma Chemical). These protease inhibitors were included in the bath to limit degradation of ELH by proteases. Each 1-ml bath sample was boiled immediately to denature proteases and frozen at −80 to −20°C until analysis.

To assess the efficiency of recovery of ELH from the recording chamber, tests (*n* = 4) were run in which, as above, the ASW/PI medium was exchanged completely every 5 min. A 15-min baseline

period was followed by an infusion of 1 ml ASW/PI containing 300 ng/ml synthetic ELH, then sample collection continued for another 30 min. The efficiency of recovery was 53 ± 7% (mean ± SE).

For bag cell neurons from both adult and juvenile *Aplysia*, bath samples were collected as described above (every 5 min) during a 15-min baseline period and during 20 min of exposure to 25 mM TEA. After 2 min of TEA exposure, one connective was stimulated with 25-ms 40-V pulses at 6 Hz for 10 s using a Grass S88 stimulator (Quincy, MA). After the TEA period, samples were taken every 5 min for an additional 90 min. Intracellular recordings were not made during secretion experiments, but the activity of bag cell neurons was monitored extracellularly.

Radioimmunoassay

Concentrations of ELH in the medium were measured using the radioimmunoassay procedure described by Wayne and Wong (1994). For the 11 assays performed, the limit of detection was 0.92 ± 0.18 ng/ml (210 pM; 2 SD from buffer control values of 200 μL aliquots). The intraassay coefficient of variation of samples (i.e., the SD/mean of four identical control samples analyzed within each assay) containing 37 ± 2 and 77 ± 3 ng/ml averaged 12%, and the interassay coefficient of variation of these samples (i.e., the SD/mean of control samples across assays) averaged 22%. Baseline level of ELH was defined as all values within 2 SD of the mean of the three samples collected before stimulating the preparation. Assayed values >2 SD from the mean baseline value were defined as secretion above baseline.

Behavioral experiment

Before dissection, adult animals were anesthetized by injection of isotonic $MgCl_2$ (50% body weight) into the body cavity. Approximately one-third of one adult bag cell neuron cluster was homogenized in 100 ml ASW and injected directly into the hemocoele of a juvenile *Aplysia*, which was allowed to freely move about a 10-gal rectangular tank at 15°C. The number of centimeters traversed per minute was noted over a 15-min baseline period and a 2-h postinjection period. Control animals were injected with 100 ml ASW. Data were collected blind.

Statistical analysis

All data are presented as means ± SE. Statistical comparisons were made with an analysis of variance for the behavioral data, and a t-test for independent means for all other data. All probability values are two-tailed. Values were considered significantly different at $P < 0.05$.

RESULTS

Bag cell neurons from both adult and juvenile Aplysia contain ELH but possess a different vesicle complement

Immunogold staining using anti-ELH followed by Protein A-gold revealed that bag cell neurons from both adult *Aplysia* (which have eggs in their ovotestis) and juveniles (which do not possess eggs) contain ELH in dense core vesicles (Fig. 1). The juvenile *Aplysia* weighed <11 g and thus most probably contained immature bag cell neurons that were incapable of afterdischarge, which is the mature form of excitability (Nick et al. 1993, 1996) (for an example of an afterdischarge in adult neurons, see Fig. 3). Therefore these data suggest that bag cell neurons contain ELH before they developmentally express afterdischarge, the form of

electrical activity that normally stimulates secretion. In both developmental stages, intracellular organelles such as endoplasmic reticulum and Golgi cisternae were also labeled. Low nonspecific background staining was seen in negative controls (results not shown). Comparison of ELH immunogold micrographs (Fig. 1) revealed that immature bag cell neurons lack a class of large dense-core ELH-containing vesicles (>250 nm in diam), which have been described previously in mature bag cell neurons (Fisher et al. 1988); these large dense-core vesicles also were detected in the mature neurons in the present study (Fig. 1, *A* and *B*). We also observed a significantly greater density of small dense-core vesicles (<250 nm in diam) in immature bag cell neuron somata relative to mature somata [Table 1; $t_{(8)} = 16.73$; $P < 0.001$]. These small vesicles were evenly distributed in the cytoplasm in immature bag cell neurons. Thus these results show that bag cell somata from juvenile *Aplysia* contain a higher density of ELH-containing small dense-core vesicles (which are in the size range of secretory vesicles) than mature somata, but they lack bag cell peptide-containing large dense-core vesicles.

Bag cell neurons secrete ELH even when they lack the capacity for afterdischarge

Unlike mature bag cell neurons, which afterdischarge in response to a variety of stimuli, immature bag cell neurons are incapable of afterdischarge and are always quiescent. Moreover, immature bag cell neurons exhibit no spontaneous activity. These cells rarely, if ever, exhibit action potentials in response to either penetration of the membrane with a microelectrode (which triggers action potentials in various other neuronal subtypes and in mature bag cell neurons) or to electrical stimulation of the connectives, which stimulates an afterdischarge in mature bag cell neurons (Nick et al. 1993, 1996). In this study, we sought to examine neurosecretion, which is known to be induced by depolarization (del Castillo and Katz 1954). Therefore we artificially depolarized all preparations with the potassium channel blocker TEA (25 mM). TEA causes afterdischarge in mature bag cell neurons (Kaczmarek et al. 1982). However, TEA did not stimulate afterdischarge in immature bag cell neurons even when combined with connective stimulation, but instead caused prolonged depolarizations. These prolonged depolarizations occurred only in the presence of TEA. This form of excitability is illustrated in Fig. 2, which shows simultaneous extracellular recording of the pleural-abdominal connectives and intracellular microelectrode recording of individual bag cell neurons from a sexually immature *Aplysia*. In 25 mM TEA, immature bag cell neurons exhibit spontaneous prolonged depolarizations whose initiation is marked by a large amplitude, long duration spike in both connectives (Fig. 2) (see also Nick et al. 1993, 1996). This extracellular spiking allowed monitoring of bag cell neuron activity without the need for intracellular microelectrode recording. Upon drug washout, the neurons returned to their normal quiescent state.

The activity of bag cell neurons was recorded and ELH samples were collected from abdominal ganglia from both reproductively mature and immature animals. Secretion samples from abdominal ganglia of *Aplysia* that did not contain
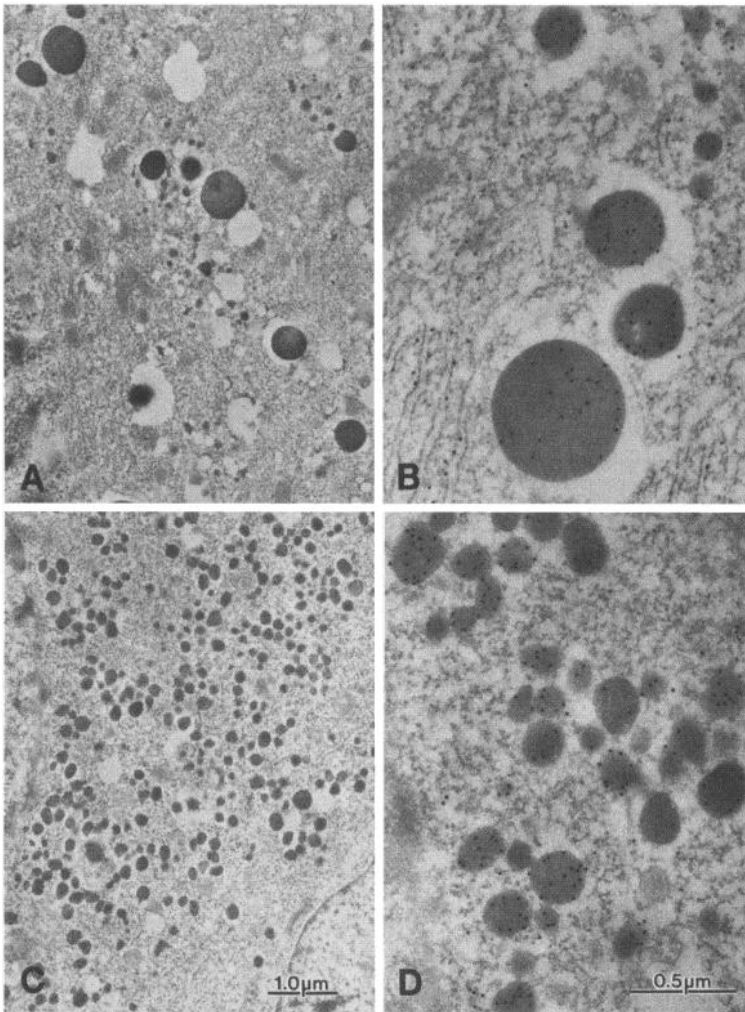
FIG. 1. Thin sections of bag cell neurons stained with anti-egg laying hormone (anti-ELH) followed by Protein A-gold. ELH is found in dense core vesicles in bag cell neurons from both adult (*A* and *B*) and juvenile (*C* and *D*) *Aplysia*. Bag cell neurons from juvenile *Aplysia* lack ELH-containing vesicles that are >250 nm in diam; these vesicles are found in mature bag cell neurons (*A* and *B*). Also, immature bag cell neuron somata have a greater density of vesicles <250 nm in diam compared with mature neurons. *A* and *C*: ×16,000; *B* and *D*: ×50,000.

eggs (i.e., were sexually immature juveniles) were divided into two groups: those that exhibited an afterdischarge in response to TEA and connective stimulation and those that did not. Afterdischarge occurred in all ganglia from adult *Aplysia*. Secretion data from the mature animals and both subgroups of juvenile animals were analyzed separately.

In response to connective stimulation in 25 mM TEA, bag cell neurons in ganglia from reproductively mature *Aplysia* generated an afterdischarge. An example of an afterdischarge in a mature bag cell neuron recorded extracellularly from both pleural-abdominal connectives is shown in Fig. 3*A*. The afterdischarge spikes of the bag cell neurons can be distinguished readily from other spikes in the connectives because they are of larger amplitude (2–3 times other spikes) and longer duration (50–100 ms) than other (non-bag cell) spikes seen in extracellular records of the connectives. Bag cell neurons secreted ELH during and following afterdischarge. Figure 3*B* reveals that the rate of release of ELH into the bath reached peak levels ~30 min after connective stimulation and subsequently declined, similar to previous descriptions (Loechner et al. 1990; Wayne and Wong 1994). The bulk of secretion occurred after termination of the afterdischarge, as previously reported (Wayne and Wong 1994).

TABLE 1. *Comparison of size and labeling of secretory vesicles in mature and immature bag cell neurons*

	Vesicle #/10 μm^2 Cytosol	Mean Cross-Sectional Area of Vesicles, μm^2	Vesicle Area/Cell, μm^2	Gold Particles Over Vesicles (#/μm^2 of vesicle)
Mature				
>250 nm diam	2.0 ± 0.2	0.16 ± 0.07	2.14 (3.5)	244 ± 33
<250 nm diam	1.5 ± 0.5	0.02 ± 0.08	0.2 (0.1)	300 ± 87
Immature				
Only <250 nm diam	67.0 ± 4.0	0.02 ± 0.01	4.4 (14.5)	200 ± 197

Values are means ± SE. Number of cells used was 5. Number in parentheses is percentage of cell area.
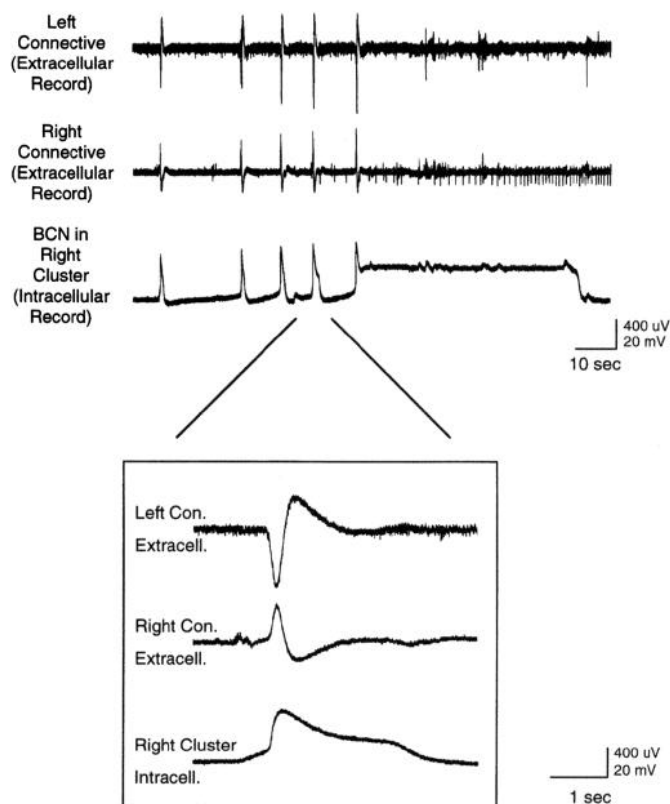
FIG. 2. Bag cell neurons incapable of afterdischarge exhibit a unique form of electrical excitability in the presence of tetraethylammonium (TEA). These are representative extracellular and intracellular recordings of bag cell neurons from a juvenile *Aplysia* that did not exhibit afterdischarge. *Inset:* portion of same data on an expanded time scale. Even shorter duration depolarizations are well over 1 s.

Some bag cell neuron clusters from animals that did not have visible eggs in their ovotestis (juvenile *Aplysia*) were also capable of afterdischarge (Fig. 4*A*). The mean duration of afterdischarge recorded extracellularly from juvenile animals was significantly shorter than the mean duration of afterdischarge recorded from ganglia from mature *Aplysia* [Table 2; $t_{(17)} = 4.86$; $P < 0.001$], as previously reported by Leibowitz and Castellucci (1983). Moreover, these bag cell neurons secreted less total ELH [$t_{(17)} = 7.16$; $P < 0.001$] for a significantly shorter duration [$t_{(17)} = 15.95$; $P < 0.001$] than those from reproductively mature animals (Fig. 4*B*; Table 2). Finally, the pattern of secretion was also different from mature neurons, in that the peak ELH secretion occurred significantly earlier in immature ganglia [$t_{(13)} = 3.23$; $P < 0.01$].

Some bag cell neuron clusters from reproductively immature animals did not show afterdischarge (Fig. 5*A*). However, as described above, these clusters did exhibit repeated prolonged depolarizations in 25 mM TEA (Nick et al. 1993, 1996). These bag cell neurons, which were incapable of afterdischarge, were still capable of secretion (Fig. 5*B*). Furthermore, there was no significant difference between these cells and bag cell neurons from animals of the same weight range that were capable of afterdischarge in terms of total ELH secreted [Table 2; $t_{(13)} = 0.47$; $P = 0.647$, NS], duration of secretion [$t_{(13)} = 0.65$; $P = 0.527$, NS], and time of peak [$t_{(6)} = 0.38$; $P = 0.717$, NS]. These data

thus show that capacity for afterdischarge is not a prerequisite for secretion of ELH in juvenile animals.

Differences in secretion profiles between mature and immature neurons are not attributable to rearing conditions

The differences in secretion that we have attributed to differences in maturity could in principle have resulted instead from differences in the source of the animals (wild caught vs. laboratory reared), because all of the adult animals used were wild caught and all of the juvenile animals used were laboratory reared. To address this question, ELH secretion data from ganglia from juvenile wild-caught and juvenile lab-reared animals of similar body weights were compared. The basic observation, described above (Fig. 5), that immature bag cell neurons lacking afterdischarge capacity nonetheless can secrete ELH was again observed. In fact, as shown in Fig. 6, bag cell neuron clusters from wild-caught sexually immature animals that did not afterdischarge actually secreted more ELH than bag cell neurons from lab-
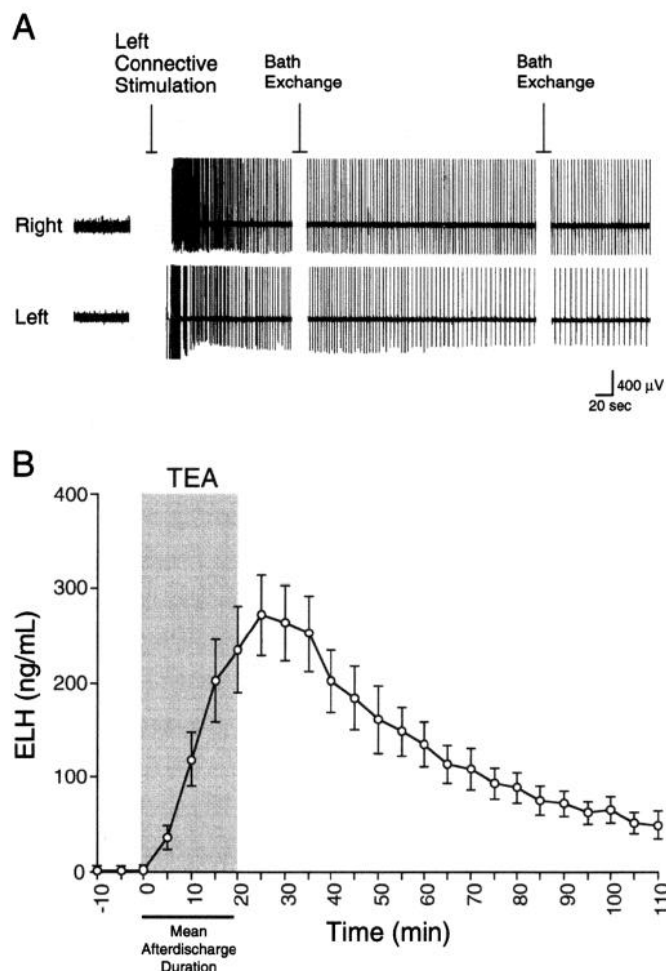

FIG. 3. Bag cell neurons from adult *Aplysia* are capable of afterdischarge and secretion of large amounts of ELH. *A*: representative extracellular recordings in TEA of bag cell neuron activity from both pleural-abdominal connectives from an adult *Aplysia* show repetitive spiking activity, an afterdischarge, after connective stimulation. *B*: group secretion data ($n = 10$) reveal pattern of ELH release from bag cell neurons from adult *Aplysia*.
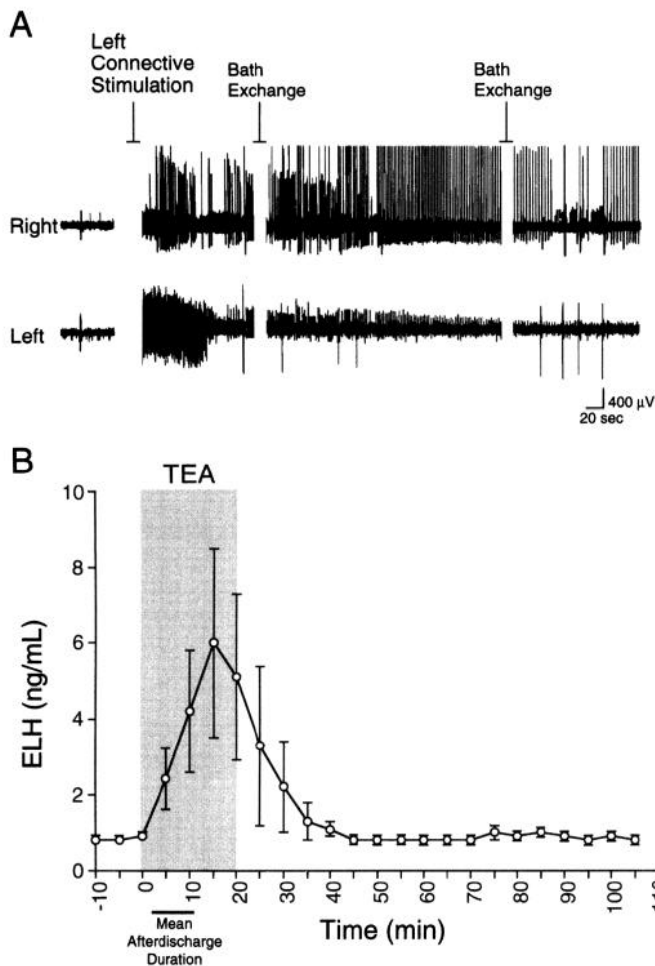
FIG. 4. Bag cell neurons from juvenile *Aplysia*, which were capable of afterdischarge secreted small amounts of ELH. *A*: representative extracellular recordings in TEA of bag cell neuron activity from both pleural-abdominal connectives from a juvenile *Aplysia* capable of afterdischarge. *A*: group secretion data (*n* = 9) reveal the pattern of ELH release from bag cell neurons from juvenile *Aplysia* that were capable of afterdischarge.
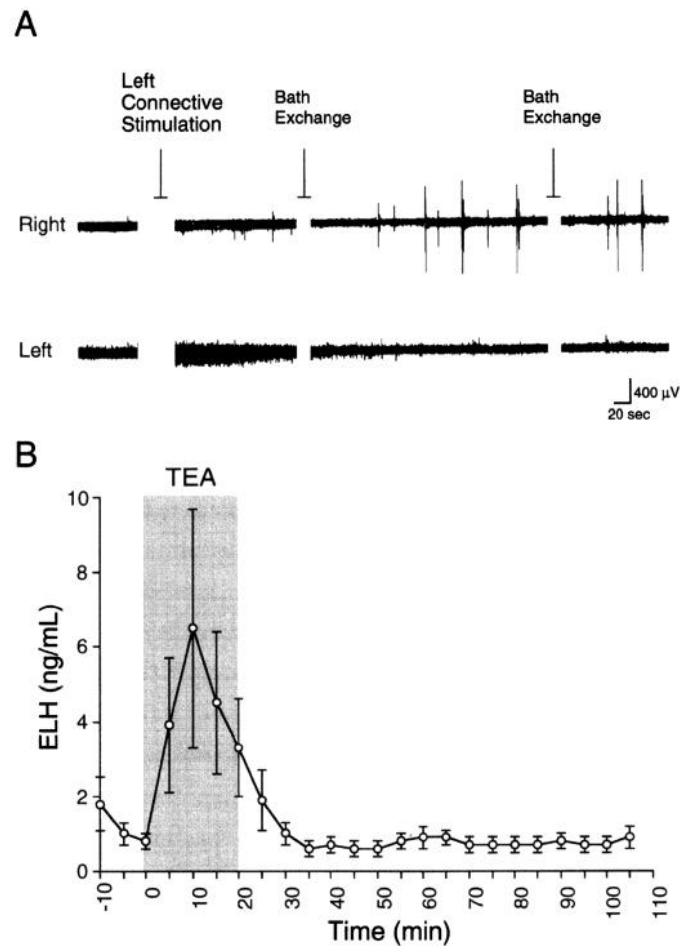
FIG. 5. Bag cell neurons from juvenile *Aplysia*, which were incapable of afterdischarge nonetheless were capable of secretion of small amounts of ELH, similar to amounts secreted by bag cell neurons from juvenile animals capable of afterdischarge (see Fig. 4). *A*: representative extracellular recordings in TEA of bag cell neuron activity from both pleural-abdominal connectives from a juvenile *Aplysia* incapable of afterdischarge. Large spikes in right connective are bag cell neuron spikes that initiate prolonged depolarizations (see Fig. 2). *B*: group secretion data (*n* = 6) reveal the pattern of ELH release from bag cell neurons from juvenile *Aplysia* that were incapable of afterdischarge.

reared juvenile *Aplysia* that did not afterdischarge [Table 2; $t_{(10)} = 2.71$; $P < 0.03$). Bag cell neurons from wild-caught *Aplysia* also secreted ELH for a longer duration [$t_{(10)} = 2.31$; $P < 0.05$]. However, the time of peak ELH secretion after TEA infusion was not significantly different between lab-reared and wild-caught animals [$t_{(6)} = 1.29$; $P = 0.244$, NS]. Moreover, as seen with ganglia from cultured juvenile *Aplysia,* bag cell neurons from wild-caught juvenile animals

still secreted much less total ELH than mature ganglia [$t_{(14)} = 5.63$; $P < 0.001$] for a shorter duration [$t_{(14)} = 16.60$; $P < 0.001$]. These results show that although there are some differences in secretion amount and duration between juvenile lab-reared and juvenile wild-caught animals, these ef-

TABLE 2. *Afterdischarge duration and ELH secretion*

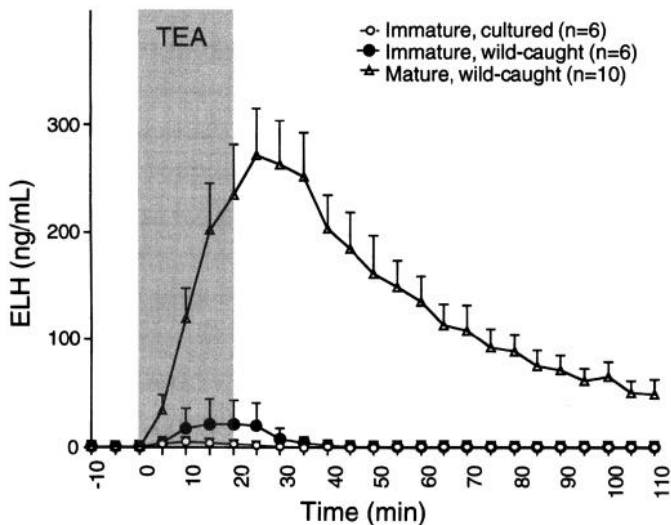| Source	Weight, g	N	Discharge Duration, min	Total ELH Secreted, ng	Duration ELH Secretion, min	Time of Peak ELH, min
Mature						
Wild-caught	234 ± 18	10	18.4 ± 1.1	3172 ± 416	109.5 ± 0.5	29.5 ± 2.8
Immature						
Cultured	11.9 ± 1	9	8.6 ± 1.7	23.4 ± 10.7	15.6 ± 6.2	15.0 ± 2.7
Cultured	9.4 ± 0.8	6	0	16.5 ± 20.0	10.0 ± 4.6	13.3 ± 3.3
Wild-caught	11.5 ± 1.6	6	0	103.8 ± 31.2	28.3 ± 6.4	18.0 ± 2.0

Values are means ± SE. ELH, egg laying hormone.

FIG. 6. Rearing conditions do not account for differences in secretion between bag cell neurons from adult and juvenile *Aplysia*. Mature bag cell neurons secreted much more ELH than immature neurons, regardless of rearing condition. However, some differences were observed: bag cell neurons from wild-caught juvenile animals that did not afterdischarge secreted significantly more ELH than neurons from cultured *Aplysia* of similar developmental status.

fects are minimal compared with the dramatic difference between either of these groups and adult animals (Fig. 6).

Juvenile Aplysia exhibit a component of egg-laying behavior in response to adult bag cell neuron extract

We investigated the possible functional consequences of premature hormone secretion in juvenile *Aplysia* by examining the behavioral effects of adult bag cell neuron extract (BCE) injection into the body cavity of juveniles. BCE contains ELH and other peptides and stimulates egg-laying behaviors in adult *Aplysia* (Kupfermann 1970). The component of egg-laying behavior we focused on was cessation of locomotion, a well-described behavioral feature of egg laying in adult animals (for review, see Conn and Kaczmarek 1989). Juveniles were injected with either BCE in artificial sea water (ASW; $n = 6$) or with ASW alone ($n = 5$). Both groups of animals exhibited similar locomotion during the 15-min baseline period [control: 0.22 ± 0.05 cm/min; BCE: 0.16 ± 0.04 cm/min; $F(1,9) = 1.23$; $P = 0.268$; NS]. However, during the postinjection period, BCE-injected *Aplysia* showed significantly less locomotion than controls [25–40 min postinjection; control: 0.24 ± 0.05 cm/min; BCE: 0 ± 0 cm/min; $F(1,9) = 27.602$; $P < 0.001$]. Thus BCE can induce at least one component of egg-laying behavior in juvenile animals that do not yet have the capacity for normal egg-laying.

DISCUSSION

This study shows that before the maturation of their electrophysiological phenotype, bag cell neurons express peptide hormone and have the capacity to secrete it, implying that a primary mechanism for ontogenetic regulation of stimulus-secretion coupling in this system is the developmental delay of electrophysiological maturation. Ultrastructural analysis

reveals that ELH is contained in vesicles in immature, as well as mature, bag cell neurons. Thus before the developmental appearance of afterdischarge, these neurons are already making peptide hormone and packaging it in vesicles. These results are consistent with those of McAllister and colleagues (1983), who found that bag cell neurons express the mRNA for ELH when they are first seen on the body wall, before their migration into the central nervous system. Unlike mature neurons, immature bag cell neurons do not possess ELH-positive vesicles that are >250 nm in diameter (large dense-core vesicles). These specific vesicles have been described previously as enriched for other peptides (bag cell peptides) derived from the ELH precursor protein relative to ELH. Fisher and colleagues (1988) suggested that because these large dense-core vesicles are soma-specific, they might shunt their contents to lysosomes for degradation. If this is indeed the case, immature bag cell neurons may lack these compartments because at this stage in development turnover of the ELH precursor may be very low.

Bag cell neurons from juvenile *Aplysia* secreted similar amounts of ELH in response to depolarization induced by the application of the potassium channel-blocker TEA regardless of whether they showed prolonged depolarizations or the mature electrical pattern, afterdischarge. We have shown previously (Nick et al. 1993) that repeated pleural-abdominal connective shock in the absence of TEA, which would be suprathreshold for afterdischarge in mature bag cell neurons, rarely evoked action potentials in immature neurons. The spikes that did occur were of low amplitude (~50 mV) compared with those of mature neurons (~70 mV). The fact that immature bag cell neurons, which show no spontaneous activity and rarely respond to connective stimulation with action potentials, nevertheless secrete hormone when depolarized by a potassium channel blocker shows that the apparatus for regulated secretion is in place before these neurons have the ability to activate it in response to normally occurring depolarization and afterdischarge.

The finding that the amount and duration of hormone secreted by juvenile ganglia, whether or not they are capable of afterdischarge, is much less than that from adult ganglia indicates a developmental difference in secretory capacity. Several explanations could account for this difference: *1*) bag cell neurons from juvenile *Aplysia* are much smaller and fewer in number than neurons from adults (McAllister et al. 1983). The amount of ELH produced may increase with cell volume or number. *2*) The duration of afterdischarge is significantly shorter in bag cell neurons from juvenile than from adult animals (Leibowitz and Castellucci 1983; present study). *3*) Bag cell neurons from juveniles may lack specialized secretory organs, which could be essential for secretion of large amounts of hormone over extended time periods. Previous work has shown that bag cell neurons from adult *Aplysia* have highly specialized neurosecretory endings (Frazier et al. 1967). In EM preparations, these endings were not seen in juvenile ganglia (Nick, unpublished observations). The present study has shown that bag cell neurons from juvenile animals have a high somatal density of vesicles that are in the size range of the small dense-core vesicles (<250 nm) described by Fisher and colleagues (1988). These vesicles may not be immediately releasable, but in-

stead may be awaiting shipment to developing secretory endings.

Although immature ganglia secrete far less hormone than mature ganglia, it should be noted that the hemocoele volume of juvenile *Aplysia* is also much smaller than that of adults. In fact, the ratio of total amount of ELH secreted to the weight of the animal is similar in wild-caught juveniles and adults: 9.0 ng ELH/(g body wt) and 13.6 ng ELH/(g body wt), respectively (see Table 2). Moreover, in other studies we have shown that ~6 ng ELH/(g body wt) is sufficient to stimulate egg laying in 50% of adult *Aplysia* (Wayne et al. 1996). Thus immature bag cell neurons appear capable of secreting enough hormone to achieve a concentration of ELH in the blood that is sufficient to elicit egg-laying behaviors in adults.

Developmental regulation of hormone secretion is essential for the appropriate timing of expression of different behaviors in a variety of diverse systems (Adkins-Regan et al. 1994; Huhtaniemi et al. 1991; Levine and Weeks 1989). In adult animals, ELH can induce several component behaviors of egg-laying, including cessation of locomotion and egg extrusion, when injected into the hemocoele (Arch and Smock 1977; Bernheim and Mayeri 1995). ELH also exerts widespread effects in the central nervous system (Mayeri et al. 1985). We have shown that juvenile *Aplysia* respond to mature bag cell neuron extract with at least one egg-laying behavior, cessation of locomotion. This quiescence could be costly because it exposes the animal to greater probability of predation and reduces time spent foraging for food and feeding (Susswein et al. 1983). Were bag cell neurons of juvenile *Aplysia* to afterdischarge, they potentially could trigger premature and costly reproductive behaviors in animals that do not yet contain eggs. Thus, precise developmental control of ELH secretion and/or the networks this peptide activates could be of considerable adaptive significance.

What might be the functional consequences of the early development of regulated secretion relative to electrical excitability? Several studies in other systems have indicated a role for secretion in neuronal pathfinding and/or synapse formation (Falls et al. 1993; Zheng et al. 1994). The bag cell neurons at the developmental stage addressed in the present study may be extending neurites and beginning to receive synaptic input (McAllister et al. 1985). It is possible that secretion of small amounts of ELH and/or other bag-cell peptides may be required in these processes. Release of large amounts of ELH, which could activate some components of egg-laying behavior in response to a prolonged afterdischarge, may be prevented by delayed biophysical maturation of the bag cell neurons. As the life-span of *Aplysia* is ~1 yr (Audesirk 1979), it may be essential that the animal begin to reproduce as soon as it possesses the body mass and reproductive apparatus to support egg production. Perhaps, at the stage examined in the present study, bag cell neurons are in fact rather mature except for a few rapidly regulated key elements that could enable afterdischarge. These elements may respond quickly to egg-producing capacity (e.g., through some growth factor) (see Nick et al. 1995), thereby enabling secretion of large amounts of ELH and, concomitantly, egg-laying behaviors, only in animals that are rapidly approaching reproductive maturity

or are already reproductively mature. In other studies, we have found that these key elements may be ion channels (Nick et al. 1995, 1996).

In summary, we have found that bag cell neurons contain ELH in vesicles and are capable of secreting this hormone before the developmental appearance of afterdischarge. Thus secretory ability develops before the expression of the mature electrophysiological phenotype. This suggests that final developmental control of peptide hormone release lies not with the secretory apparatus itself, but with the intrinsic cellular elements involved in neuronal excitability.

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