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Development of behavior and learning in *Aplysia*

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Summary. A set of fundamental issues in neuroethology concerns the neural mechanisms underlying behavior and behavioral plasticity. We have recently analyzed these issues by combining a simple systems approach in the marine mollusc *Aplysia* with a developmental analysis aimed at examining the emergence and maturation of different forms of behavior and learning. We have focussed on two kinds of questions: 1) How are specific neural circuits developmentally assembled to mediate different types of behaviors? and 2) how is plasticity integrated with these circuits to give rise to different forms of learning? From our analysis of the development of learning and memory in *Aplysia*, several themes have emerged: 1) Different forms of learning emerge according to different developmental timetables. 2) Cellular analogs of learning have the same developmental timetables as their respective forms of behavioral learning. 3) An analysis of non-decremented responses prior to the emergence of sensitization reveals a novel inhibitory process on both behavioral and cellular levels. 4) Sensitization emerges simultaneously in diverse response systems, suggesting an underlying general process. 5) A widespread proliferation of central neurons occurs in the same developmental stage as the emergence of sensitization, raising the possibility that some aspect of the trigger for neuronal proliferation may also contribute to the expression of sensitization.
Key words. Aplysia; development; habituation; dishabituation; sensitization; learning; locomotion; bursting neuron.

Introduction

One of the primary aims in neuroethology is to understand how natural behaviors can be explained in terms of their underlying neural mechanisms. In recent years a number of excellent model systems have been developed that have enabled significant advances in our understanding of the neural substrates of a variety of forms of behavior, especially in invertebrate animals. Two important themes have emerged from this work. First, the characteristic features of diverse behaviors, ranging from simple reflexes to complex fixed-action patterns, can be accounted for by the properties of specific neurons and specific neural circuits (for review see Getting [7] and Kandel [11]). Second, a fundamental property of many behaviors, that they can be modified by experience and learning, can be traced to particular forms of plasticity at specific loci within identified neural circuits (for review see Carew and Sahley [4]).

Among the preparations that have been useful in exploring these issues is the marine mollusc *Aplysia californica,* which offers several advantages for a neuroethological analysis of behavior and learning. Adult *Aplysia* exhibit a broad range of natural behaviors, including simple defensive withdrawal reflexes of the gill and siphon [17] and of the tail [29], all-or-none fixed acts such as the inking response [3], centrally programmed rhythmic behaviors such as locomotion [9] and feeding [30], and complex fixed-action patterns such as egg laying [15]. Moreover, many of these behaviors can be modified by a variety of forms of learning, including non-associative forms such as habituation, dishabituation, and sensitization, and associative forms, such as classical and operant conditioning [4]. Finally, because *Aplysia* has a relatively simple nervous system consisting of large pigmented neurons (some of which can be identified as unique individuals), it has been possible to specify critical aspects of the neural circuits underlying many of these behaviors [11].

In our laboratory in recent years, we have extended a simple systems approach in *Aplysia* by combining it with a developmental analysis aimed at examining the emergence and assembly of different forms of behavior and learning. This developmental approach provides a strategy for obtaining insights into the mechanisms underlying both behavior and learning by studying the emergence of particular neurons, circuits, and cellular mechanisms at the same time that particular forms of behavior and learning are being expressed during ontogeny. We have focussed on two principle types of questions: First, how are specific neural circuits developmentally assembled to mediate different types of behaviors in *Aplysia*? Second, how is plasticity integrated into these circuits to give rise to different forms of learning?

Aplysia provides a very useful preparation for combining a simple systems strategy with a developmental approach because, in addition to the thorough analysis of many behaviors and neural circuits in the adult [11], the development of *Aplysia* from embryo to adult has also been characterized in considerable detail [12–14]. It is therefore possible to examine the emergence and maturation of a variety of different behaviors, as well as their underlying neural circuitry, at a number of discrete identifiable stages of development. In Kriegstein's elegant work analyzing the development of *Aplysia* [13], he divided the animal's life cycle into 5 phases (embryonic, planktonic, metamorphic, juvenile and adult; fig. 1). Furthermore, on the basis of a number of external morphological criteria, he subdivided these phases into 13 discrete stages. We have focussed on the juvenile phase for our study of the development of behavior and learning in *Aplysia,* because it is during this phase that many of the response systems we are interested in first emerge. The juvenile phase lasts about 120 days and can be divided into four stages (stages 9–12). The approximate duration of each of the juvenile stages is shown in figure 1.

Development of behavior

In our analysis of the development of different forms of behavior, we have initially focussed on two response systems, 1) gill and siphon withdrawal, and 2) locomotion, because they provide representative examples of two broad classes of behavior, graded reflexes and cyclical motor programs, respectively. A comparison of these response systems is interesting for two reasons: First, the effector organs involved in the two behaviors emerge at different times during development: the siphon and gill in stages 9 and 10 respectively, the propodium and anterior foot about one week earlier in stage 8. Thus, by a comparative analysis of these two systems it may be possible to determine the degree to which the development of specific behaviors is dependent on the absolute age of the animal or the specific age of particular response systems. Second, both responses can be elicited by exogenous as well as endogenous triggers; it is thus possible to examine how these different types of response triggers are developmentally integrated into the neural circuits for each behavior.

A) Gill and siphon withdrawal

In adult *Aplysia,* the mantle organs (the gill, siphon and mantle shelf) exhibit two distinct types of coordinated withdrawal. One type is an exogenously-triggered graded defensive reflex mediated by a simple, well characterized neural circuit consisting primarily of about 20 sensory neurons, 10 identified motor neurons and a number of identified interneurons. The second type of withdrawal is an endogenously-triggered fixed-action pattern ('respiratory pumping') which is mediated by a somewhat more complex circuit involving two clusters of electrically-coupled interneurons (L25 and R25) that act as central command elements [2]. In the adult, one important characteristic of exogenously-triggered (reflex) withdrawal and endogenously triggered (spontaneous) withdrawal is that they each invariably involve *coincident* contractions of the gill and siphon.

The preparation we have used to study the development of the two types of withdrawal responses of the mantle organs is shown in figure 2. We found that reflex and endogenously-triggered withdrawal responses took on their adult form (*co-contraction* of the gill and siphon) according to very different developmental timetables. The *evoked reflex withdrawals* in-

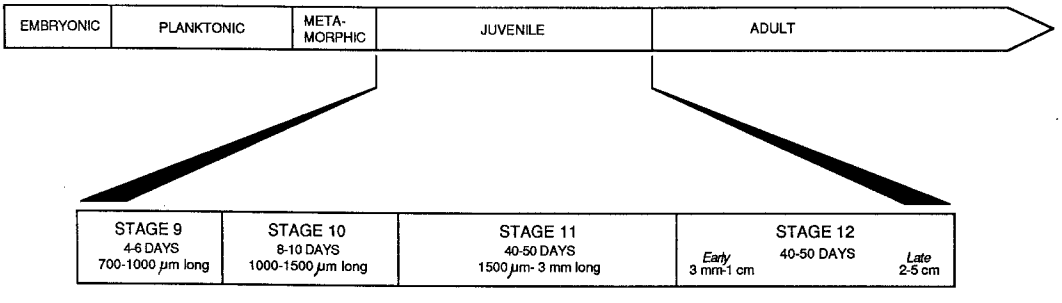

Figure 1. Phases in the development of *Aplysia.* The *Aplysia* life-cycle can be divided into five major phases: embryonic (lasting about 10 days), planktonic (lasting about 30 days), metamorphic (lasting about 3 days), juvenile (lasting about 120 days), and adult. We have focussed our attention on the juvenile phase, which can be subdivided into four stages based upon discrete morphological criteria. the approximate duration of each stage (in days) is shown, although this duration can vary substantially with water temperature and rearing conditions [13, 25].

variably exhibited coincident contraction of the gill and siphon as soon as the effector organs emerged in stage 10 (fig. 3). In contrast *spontaneous withdrawals* did not develop a high degree of coincidence until several weeks later, in the early adult stage (stage 13)[25]. These results suggest that the neural circuit required for the coincident contraction of the gill and siphon is present very early in development, but this circuit is primarily accessible to input from an exogenous trigger. Much later in development, endogenous triggers appear to gain progressively greater access to coordinating circuitry.

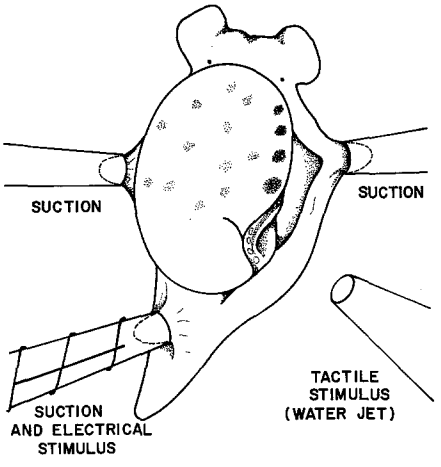

Figure 2. Preparation for the study of juvenile gill and siphon withdrawal. Juvenile *Aplysia* (a stage 10 animal is illustrated here) are suspended in seawater above the substrate by three suction pipettes: one attached to each parapodium, and a third, containing a wire electrode (for facilitating stimuli, see below) to the tail. Reflex withdrawals are elicited by quantifiable tactile water jet stimuli delivered to the siphon. The response is quantified by computing the net reduction in siphon area at the peak of contraction (for details see Rankin and Carew[23]).

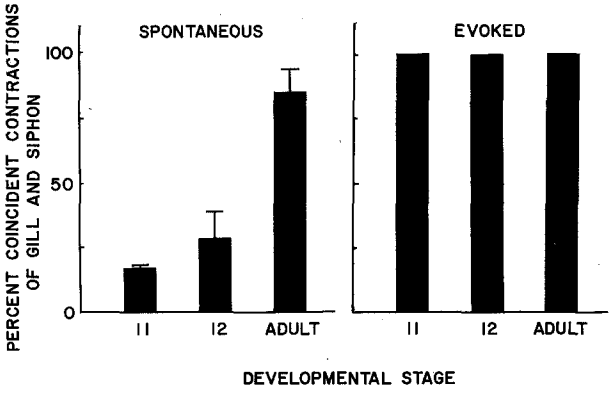

Figure 3. Development of gill and siphon co-contraction in spontaneous and evoked responses. Spontaneous gill and siphon withdrawals in juveniles begin as relatively independent events. As the animals mature, the amount of co-contraction progressively and significantly increases. By the young adult stage, most spontaneous withdrawals involve simultaneous contraction of the two organs. In contrast, evoked contractions invariably involve synchronous gill and siphon contraction throughout all of juvenile development, suggesting differential access of exogenous triggering input to coordinating circuitry that is already present quite early in development.

B) Locomotion

Locomotion in adult *Aplysia* is carried out by means of a series of discrete pedal waves (steps) that travel rearward along the length of the foot. The neural circuitry underlying this behavior has been partially characterized and appears to involve the coordination of triggering, oscillatory and effector circuits in the cerebral, pleural and pedal ganglia. Kriegstein and collegues[14] first described that young juvenile animals (stage 9) do not locomote by means of a pedal wave; rather they glide on cilia that line the length of the foot. One of the first questions of interest in examining the development of locomotor behavior, therefore, concerns the nature of the transition from ciliary gliding to pedal wave stepping. To address this question, Stopfer, Schuerman and Carew[28] quantified and analyzed the nature of the spontaneous locomotor behavior of animals at each stage of juvenile development. They found that from stages 9 to 12, spontaneous locomotion showed a clear and gradual transition from gliding to stepping locomotor programs. Even as late as the end of stage 12 (as long as 13 weeks after the onset of the juvenile phase), animals were not yet completely relying on the adult form of pedal wave locomotion.

The gradual and prolonged developmental transition in spontaneous locomotion from juvenile to adult forms raises interesting questions concerning the development of the underlying neural circuitry for locomotion. For example, does the gradual timecourse of emergence of the adult locomotor pattern reflect the gradual development of the neural circuit required for pedal wave locomotion, or are the circuits necessary for pedal stepping in place before the actual expression of the behavior? One way to address this question is to examine the development of exogenously-triggered (escape) locomotion and ask whether the form of locomotion used to escape a noxious stimulus is the same as that used in spontaneous locomotion. This type of experiment takes on added interest in light of our results concerning the development of gill and siphon withdrawal, which showed that endogenous and exogenous triggers appear to have differential access to a common effector system and that early in juvenile development, exogenous triggers may selectively access more mature forms of the motor program.

An analysis of the development of exogenously triggered escape locomotion[28] provided support for the prediction made on the basis of the development of gill and siphon withdrawal. Specifically, escape locomotion at each stage of juvenile development appeared to take on the form of locomotion typical of a later stage: young animals that only exhibited gliding during spontaneous locomotion could exhibit clear pedal wave stepping in response to a noxious stimulus. These results strongly suggest that juvenile *Aplysia* possess the neural circuitry necessary for mature pedal wave locomotion long before that circuitry is spontaneously used, and that input from exogenous triggers for locomotion have preferential access to the pedal wave circuitry early in development.

Taken collectively, our analysis of the development of gill and siphon withdrawal and of escape locomotion shows that endogenous and exogenous control over common effector systems develop according to different developmental timetables and that exogenous control appears to precede endogenous control. It will be of interest to see whether this developmental sequence is dictated in part by the complexity or organization of the neural circuitry required for these two kinds of triggering systems.

Neuronal development and differentiation

In order to relate developmental changes in behavior to developmental changes in specific neural circuits, it is impor-

tant to understand the overall pattern of neuronal development and differentiation in the central nervous system. Such an understanding could in turn permit an examination of the fundamental question of whether developmental changes in behavior can be attributed to the birth and incorporation of new neurons into new circuits, or whether particular instances of behavioral development reflect the differentiation or reorganization of neurons within previously existing neural circuits. In this analysis we have carried out three kinds of studies: 1) we have analyzed the pattern of neuronal development throughout the entire central nervous system (CNS); 2) we have examined the expression of a particular facilitatory neurotransmitter phenotype within the CNS; and 3) we have studied the development of intrinsic firing patterns within a single identified pacemaker neuron in *Aplysia.*

Development of neurons within the CNS

As a first step towards analyzing the development of specific neural circuits in the CNS of *Aplysia,* Cash and Carew[6] carried out a detailed study of neuronal development throughout the juvenile phase, by staining and counting the total number of cells in each ganglion of the CNS in each juvenile stage (stages 9, 10, 11, early 12, late 12, as well as early adult). Their results showed that there is a dramatic and highly non-linear increase in neuronal number during stage 12 (fig. 4). Prior to stage 12 (a period of about 60 days) there is relatively little change in cell number. Then in early stage 12 there is an approximate doubling of neurons, and in late stage 12 a further quadrupling of neurons. Thus, within this developmental stage (a period of about 60 days) there is roughly an eight-fold increase in neuronal cell number throughout the CNS. This overall pattern does not reflect a disproportionate increase in the number of neurons in any one ganglion because the same pattern of non-linear proliferation during stage 12 is reiterated in each ganglion. The widespread increase in cell number throughout the entire CNS suggests that there is system-wide trigger for the initiation of this striking neuronal proliferation. Moreover, this trigger is not likely to be related to the development of specific behaviors subserved by any particular ganglion, since the neuronal proliferation occurs long after most of the major behaviors exhibited by the animal are already developmentally expressed[6]. Although we have not yet established a direct relationship between the increase in neuronal number in stage 12 and changes in the functional output of specific neural circuits, our results suggest that stage 12 provides an important time window for the maturation or refinement of neural networks.

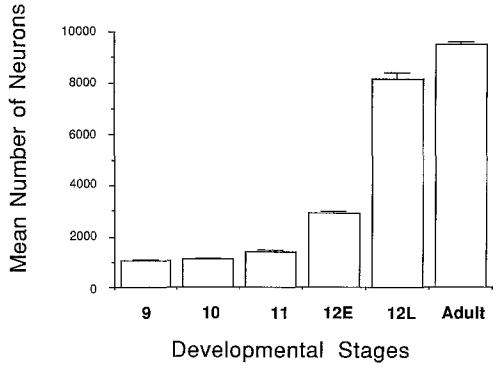

Figure 4. Development of neurons within the CNS. In early juvenile stages (stages 9–11) there is relatively little change in neuronal cell number throughout the CNS. In contrast, in early stage 12, there is an approximate doubling of neurons, and by late stage 12, a further quadrupling of neurons. This development pattern occurs synchronously throughout the CNS.

Development of neurotransmitter phenotype

In order to provide a more fine-grained analysis of the development of neural circuits within the CNS and to resolve some of the questions raised by the pattern of neuronal proliferation described above, we turned our attention to one functionally important aspect of neuronal differentiation – the expression of transmitter phenotype. Using immunocytochemical techniques to assay the expression of specific neurotransmitters, it is possible to trace the emergence and development of identified neurons that utilize a particular transmitter and are known to be important in mediating specific behaviors or forms of learning in the adult. As a first step in this analysis, we have used this technique to examine the patterns of serotonin immunoreactivity in each ganglion throughout the juvenile phase[21]. Serotonin (5-HT) is a particularly interesting neurotransmitter for this type of analysis because it has been shown in the adult to be capable of mediating heterosynaptic facilitation in the neural circuits underlying both gill and siphon withdrawal and tail withdrawal. 5-HT immunoreactive neurons were present in the abdominal, cerebral and pedal ganglia very early in development, as early as stage 9; in addition, extensive serotonergic fibers were observed in the neuropil of the pleural and buccal ganglia at this early stage[21]. The number of 5-HT immunoreactive cells in the abdominal, cerebral and pedal ganglia gradually increased throughout the juvenile phase.
It was also possible to identify a specific sub-population of 5-HT immunoreactive neurons in the cerebral ganglion that have recently been shown in the adult to produce heterosynaptic facilitation in the neural circuit for gill and siphon withdrawal[18]. Interestingly, these neurons have a very different pattern of development compared to all other immunoreactive cells. Rather than the gradual addition of neurons throughout juvenile development typical of other neurons, they appeared to achieve their normal adult complement of cells very early, by stage 10, and showed no further increase through development. It will be of interest to determine the functional significance of this pattern in the context of the development of different forms of learning, as will be discussed below.

Development of intrinsic firing patterns

The functional output of a neural circuit is determined primarily by two factors: 1) the strength and sign of synaptic contacts made within the network; and 2) the intrinsic firing patterns of the individual neurons themselves. For this reason, it is important not only to know how connectivity patterns are changing with the incorporation of new neurons, but also how the individual neuronal elements themselves are differentiating. With this type of analysis it may be possible to relate changes in intrinsic firing patterns of neurons to changes in the behavioral output of a particular circuit. An excellent candidate neuron for studying how intrinsic firing patterns develop is the identified cell R15 in the abdominal ganglion of *Aplysia.* In adult *Aplysia,* R15 (in the absence of any synaptic input) shows a characteristic parabolic bursting pattern with periods of accelerating spike

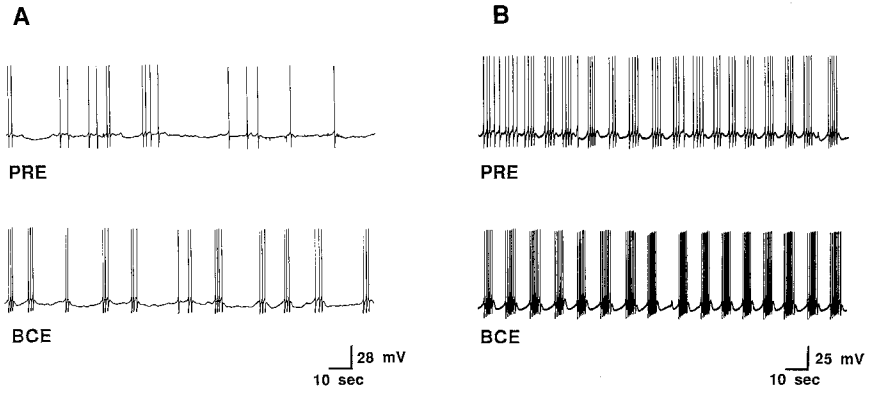

Figure 5. Bag cell extract induces or enhances burst patterns in juvenile R15. The adult burst pattern of the neuron R15 develops gradually through the juvenile phase, beginning with random spikes (mid stage 12) to fully mature parabolic bursts (adult). The application of bag cell extract (BCE), which contains neuropeptides known to initiate a complex fixed action pattern and thus modulate a number of behaviors, can induce or intensify burst patterns in R15 of immature animals.

A PRE: Intracellular recording from a mid stage 12 juvenile R15 showing a typical immature firing pattern of infrequent random action potentials. BCE: Application of the extract induced a significant clustering of spikes, more characteristic of the adult burst pattern.
B PRE: A typical late stage 12 immature burst pattern. BCE: The burst pattern is clearly intensified as a result of BCE addition. These results indicate that the ionic channels responsible for the adult burst pattern are present but not yet functionally expressed in juvenile *Aplysia*.

activity alternating with long interburst hyperpolarizations. The specialized membrane properties and ionic currents that mediate this pattern have been extremely well characterized (for review see Adams and Benson [1]). Moreover, it has been shown by Kupfermann and Weiss [16] that R15 may play an important role in maintaining osmotic balance in *Aplysia*.
From a developmental perspective an interesting question then concerns the ontogeny of R15's ability to exhibit its unique bursting activity pattern. How does this pattern develop and what affect does it have on the behaviors mediated by R15? Ohmori [22] first showed that R15 in juvenile animals does not fire in the parabolic burst pattern characteristic of the adult. We have confirmed and extended this finding and shown that there is a gradual developmental progression from single random spikes (mid stage 12), to small immature clusters of spikes (late stage 12), to the fully mature parabolic burst (stage 13) [19]. Moreover, we have shown that this developmental progression in the intrinsic membrane properties of R15 can be modulated by a specific family of peptides contained in the bag cells. The bag cells are a cluster of neurosecretory cells known to be involved in egg-laying.
Recording intracellularly from R15, we were able to demonstrate that in cases where R15 showed no endogenous burst pattern, firing only infrequent random spikes, bag cell extract was capable of inducing a burst pattern (fig. 5). Moreover, in cases where R15 showed an immature endogenous burst pattern, application of bag cell extract intensified the burst pattern [19]. Thus, the peptides contained in the bag cells are capable of advancing the developmental expression of the intrinsic firing pattern of R15. This observation shows that the ionic channels that mediate the normal burst pattern are present for some time before they are actually functionally expressed. Taken together with the data on neuronal proliferation in the CNS (fig. 4), our results suggest that mid to late stage 12 may provide a critical developmental time window for both the incorporation of new neurons into functional circuits and for the final differentiation of the intrinsic firing patterns of previously existing neurons. An important goal of our current work is to elucidate the causal relationships between the developmental changes we see in neuronal number, transmitter phenotype and intrinsic firing patterns, and developmental changes in the behavioral repertoire of the animal and the ability of the animal to express different forms of learning.

Development of learning

A fundamental question in neuroethology concerns the neural basis of behavioral adaptations that occur in response to changes in the environment. Learning, the modification of behaviors with experience, represents an important class of these adaptive behavioral changes. Recent work in a variety of invertebrate and vertebrate preparations has provided important insights into the neuronal mechanisms underlying learning [4]. For example, in the gill and siphon withdrawal reflex in adult *Aplysia* it has been possible to relate specific cellular and molecular changes at identified synapses to the behavioral expression of a variety of forms of learning. In our laboratory, we have approached the analysis of learning and memory from a developmental perspective in the hope of elucidating how these mechanisms are assembled from simpler components, and how they interact to produce more complex forms of learning. We have carried out these studies on both behavioral and cellular levels of analysis.

Behavioral analysis

1) *Siphon withdrawal*

In our initial experiments we focussed on the siphon withdrawal reflex because a great deal is already known about the cellular mechanisms of learning in this response system in the adult. We have examined the development of three forms of non-associative learning – habituation, dishabituation, and sensitization. Habituation, a decrement in responding produced by repeated presentation of a stimulus, was present in the siphon withdrawal reflex as soon as the effector organs emerged in stage 9 (fig. 6). Specifically, with repeated presentations of a tactile stimulus (water jet) to the siphon, the amplitude of the evoked siphon and gill contraction decreased significantly [23]. However, in order to produce habituation at this early stage, the stimuli had to be presented at a very rapid rate – a 1-s interstimulus interval (ISI); longer ISIs were not effective in producing habituation in stage 9. This observation is in marked contrast to habituation in the adult where ISIs as long as 2–5 min are sufficient to produce habituation. Interestingly, at progressively later stages of development, habituation could be produced with progressively longer ISIs. Stage 10 animals, for example, habituate to stimuli presented at 1 and 5 s ISIs, but not 10 s; stage 11

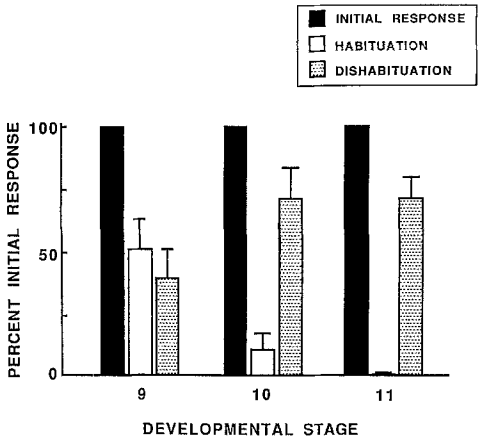

Figure 6. The development of learning in the siphon withdrawal system. Two forms of non-associative learning (habituation and dishabituation) are illustrated in three developmental stages. Black bars represent the (standardized) response amplitude to the initial water jet stimulus. Open bars indicate the response amplitude to the last in a series of habituating stimuli, expressed as a percentage of the initial response amplitude. Stippled bars represent the response amplitude following a dishabituating stimulus (strong tail shock) also expressed as a percentage of the initial response.

Animals of all stages habituate to the water jet stimuli, but habituation becomes increasingly more profound as the animals mature. Stage 9 animals show no dishabituation; their responses following tail shock are in fact slightly depressed compared to those prior to tail shock. By stage 10, however, dishabituation is clearly evident.

animals habituate to 1, 5, and 10 s, but not 30 s; and stage 12 animals habituate to 1, 5, 10 and 30 s [23]. These observations demonstrate that although habituation is present very early in development, it continues to mature throughout the juvenile phase.

Dishabituation is defined as the facilitation of habituated responses by the presentation of a novel or noxious stimulus. In examining the developmental emergence of this form of learning Rankin and Carew [23] found that, in contrast to habituation, dishabituation of siphon withdrawal was completely absent in stage 9 (fig. 6). Regardless of the intensity of the facilitating stimulus (an electric shock delivered to the tail) habituated responses could not be potentiated at this stage [23]. However, significant dishabituation in response to tail shock was evident in animals 4–7 days older, in stage 10, and persisted in stage 11 (fig. 6). These results suggest that while the critical machinery for habituation is present in the neural circuit mediating siphon withdrawal as early as stage 9, some essential component of the mechanism underlying dishabituation must be added to the circuit in the 4–7-day time window between stages 9 and 10 (see also Rayport [26], and below).

Our finding that dishabituation emerged developmentally at stage 10 raised several interesting questions concerning the relationship between dishabituation and another related form of non-associative learning – sensitization. Sensitization is defined as the facilitation of non-decremented responses by the presentation of a noxious stimulus. Historically, dishabituation and sensitization were thought to involve a common facilitatory mechanism since both decremented and non-decremented responses could be simultaneously facilitated by the presentation of a single noxious stimulus [5, 8]. Recent cellular evidence in adult *Aplysia*, however, has suggested that dishabituation and sensitization may involve different cellular mechanisms [10]. If, in fact, there is only one common mechanism underlying the two learning phenomena, one would predict that sensitization would also be absent in stage 9 and would emerge in synchrony with dishabituation in stage 10. Alternatively, if sensitization emerged at some time before or after the expression of dishabituation, this would argue that dishabituation and sensitization might be mediated at least in part by different mechanisms.

Rankin and Carew [24] compared the developmental emergence of sensitization and dishabituation in the siphon withdrawal reflex by examining the effect of a facilitatory stimulus (tail shock) on both decremented and non-decremented responses, in stages 11, early 12, and late 12. In all stages, habituated animals showed significant facilitation of response amplitude following tail shock (fig. 7), confirming our earlier finding that dishabituation emerges early in development [23]. However, in contrast to dishabituation, sensitization did not emerge until surprisingly late in juvenile development (late stage 12). In stage 11 and early 12, regardless of stimulus intensity, tail shock did not produce facilitation of non-decremented responses. In fact, in these stages in which sensitization was absent, tail shock produced modest but significant depression of response amplitude. In late stage 12 however, tail shock did indeed produce sensitization of the reflex [24] (fig. 7). These data demonstrate that, whereas dishabituation emerges in stage 10, sensitization does not emerge until at least 60 days later in late stage 12. Thus, we have shown that it is possible to developmentally dissociate these two simple forms of non-associative learning. This difference in their developmental timetables supports the hypothesis that the two processes are mediated at least in part by different mechanisms. It should now be possible to examine the circuit for siphon withdrawal in early and late stage 12 and attempt to causally relate specific biophysical or molecular changes in neurons within that circuit with the behavioral emergence of sensitization in that response system (see below).

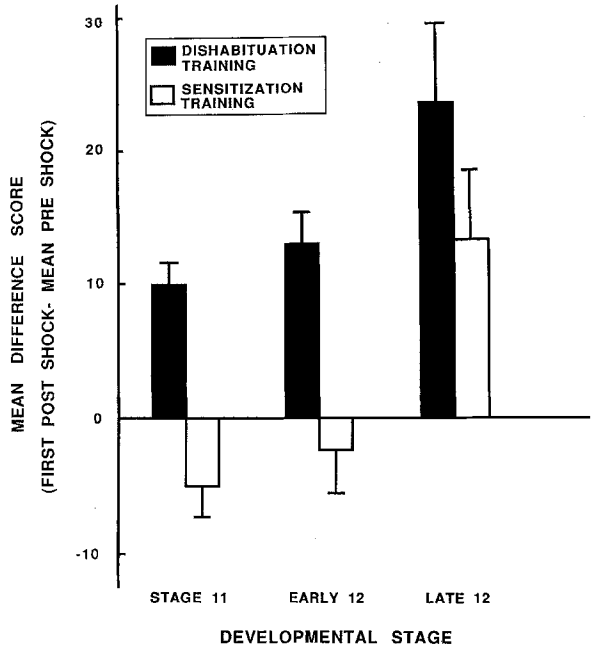

Figure 7. Emergence and maturation of dishabituation and sensitization. To permit comparison of the magnitude of dishabituation and sensitization the data are expressed in terms of a mean difference score (post shock – pre shock). There was a significant developmental increase in both dishabituation and sensitization across in three juvenile stages examined. Dishabituation was present in all developmental stages, but sensitization did not emerge until late stage 12. Prior to the emergence of sensitization, in stage 11 and early stage 12 sensitization training (tail shock) produces reflex depression.

2) Escape locomotion

In addition to examining the developmental emergence of many forms of learning within a single response system, it is also possible to take advantage of the ontogenetic approach to ask questions about the emergence of a single form of learning in many different response systems. Sensitization in *Aplysia* provides an opportunity to analyze this type of question, because many different response systems exhibit this form of learning. Thus it is possible to ask whether 1) sensitization emerges as an organism-wide process, affecting all the animal's behavioral responses simultaneously; or, alternatively, 2) whether it emerges as a form of learning that is added to each response system independently, as a function of the age of the response system itself. To address this question, we examined the developmental emergence of sensitization in the escape locomotion system [27] and compared it to the timetable for sensitization in the siphon withdrawal reflex. Escape locomotion provides an excellent system in which to make this type of comparison for several reasons: 1) it is expressed very early in development (as early as stage 8, unpublished observation); 2) it involves an entirely different and more complex effector system than siphon withdrawal; and 3) the response in the adult is known to be modulated by several different forms of both non-associative and associative learning.

Sensitization in the escape locomotion system was assessed in stages 10, 11, early 12 and late 12 by comparing the distance animals locomoted in response to a weak test stimulus both before and after presentation of a strong electric shock to the tail. Sensitization was absent in stages 10, 11, and early 12; animals receiving tail shock showed no facilitation of responding to the test stimulus and were not significantly different from controls (which received no strong shock). In contrast, in late stage 12 tail shock did produce significant sensitization. Sensitization, therefore, emerged in the escape locomotion system between early and late stage 12, at the same developmental stage as sensitization emerged in the siphon withdrawal reflex [27]. Taken together, these findings suggest that sensitization may develop as a unified organism-wide process that is expressed simultaneously in diverse response systems. It is especially intriguing that despite differences in ontogeny, response topography and underlying circuit complexity, both siphon withdrawal and escape locomotion first express sensitization in the same late stage of juvenile development. Moreover, it is at this same late stage of development that the number of neurons within the CNS shows a dramatic proliferation (fig. 4). Thus it will be of interest to determine whether a subset of the neurons born in stage 12 play a role in the expression of sensitization.

Cellular analysis

One of the primary goals of our work investigating the development of learning and memory is to relate the behavioral emergence of particular forms of learning to specific developmental changes occurring in the underlying neural circuit. A first step in this analysis is to establish that cellular analogs of these forms of learning can be identified and analyzed in the CNS at the same stages of development that the learning is first expressed. To carry out this analysis, we took advantage of the early appearance of the large mucous motor neuron R2 in the abdominal ganglion. R2 receives afferent input from the siphon and is readily identifiable throughout juvenile development. For these reasons, R2 allows us to monitor the emergence of plasticity in the reflex pathway for siphon withdrawal as non-associative learning is first expressed behaviorally.

The development of the cellular analog of habituation was first examined in this system by Rayport and Camardo [26]. Recording intracellularly from R2, they showed that as early as stage 9, repeated activation of the siphon nerve with brief electrical pulses produced a progressive decrease in the amplitude of the evoked complex excitatory postsynaptic potential (EPSP). These observations are consistent with the behavioral observations of Rankin and Carew [23] that habituation in the siphon withdrawal reflex is present as early as stage 9.

Rayport and Camardo [26] also found that the cellular analog of dishabituation (heterosynaptic facilitation of decremented EPSPs) was not present in stage 9, but emerged about 1 week later in stage 10. Specifically, activation of the pleuroabdominal connectives, which carry facilitating input from

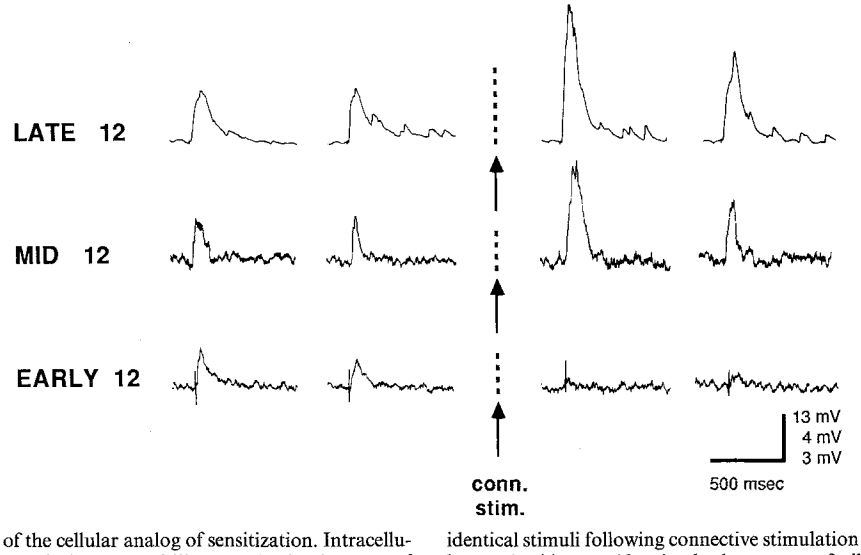

Figure 8. Development of the cellular analog of sensitization. Intracellular recordings from the identified neuron R2 illustrate the development of heterosynaptic facilitation of non-decremented synaptic responses (the cellular analog of sensitization). For each developmental stage examined, on the left side are shown EPSPs in response to siphon nerve stimuli delivered at non-habituating ISIs. On the right are shown responses to identical stimuli following connective stimulation are shown. EPSPs from late and mid stage 12 animals show strong facilitation, but those from early stage 12, in contrast, actually show inhibition following connective stimuli. These results parallel those of behavioral studies which indicate that sensitization emerges between early and late stage 12.

the tail to the abdominal ganglion, produced facilitation of decremented EPSPs in stage 10 but not in stage 9. Again, these observations are consistent with the behavioral findings of Rankin and Carew [23] which showed that dishabituation in the siphon withdrawal reflex emerged in stage 10.

More recently, Nolen and Carew [20] have examined the development of the cellular analog of sensitization (heterosynaptic facilitation of non-decremented EPSPs) in this system. Specifically, they examined the ability of connective stimulation to facilitate non-decremented synaptic responses in early, mid and late stage 12. They found that connective stimulation produced significant facilitation of non-decremented EPSPs in mid and late stage 12 (fig. 8). In contrast, connective stimulation produced no significant facilitation of non-decremented EPSPs in early stage 12 (fig. 8). In fact, in early stage 12, connective stimulation produced significant depression of EPSP amplitude. Thus there are two striking parallels between these cellular results and our previous behavioral observations: 1) behavioral sensitization does not emerge until late stage 12 of juvenile development; and the cellular analog of sensitization first emerges in mid to late stage 12; and 2) prior to the emergence of behavioral sensitization, tail shock produces significant reflex depression; and prior to the emergence of the cellular analog of sensitization, connective stimulation produces significant depression of the reflex synaptic input.

In summary, for each form of learning we have examined, habituation, dishabituation, and sensitization, the developmental emergence of its respective cellular analog occurs in close temporal register with the emergence of the behavioral expression of the learning (fig. 9). We are currently using these cellular analogs, as well as recording from individual cells in relevant neural circuits, to examine the biophysical and molecular mechanisms that are developmentally inserted into specific neurons and neural circuits to give rise to the expression of various forms of synaptic plasticity underlying learning.

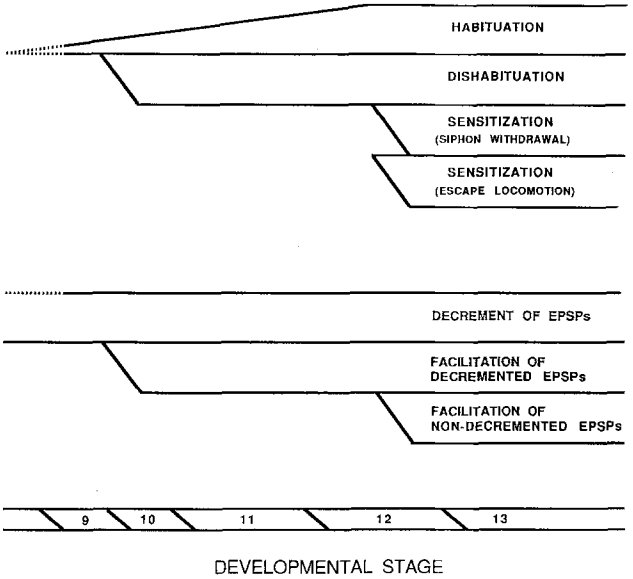

Figure 9. Development of different forms of learning and their respective cellular analogs. Habituation and its cellular analog (decrement of EPSPs) are present as early as stage 9; dishabituation and its analog (facilitation of decremented EPSPs) both emerge about seven days during stage 10; sensitization and its analog (facilitation of non-decremented EPSPs) emerge together several weeks later during stage 12.

Concluding remarks

In this paper we have discussed our use of development in *Aplysia* as an analytic tool with which to examine different classes of behavior, as well as different forms of learning and memory, as they emerge and are assembled during ontogeny. This approach has been a useful one, for it has provided a means for the developmental dissection and elucidation of sub-components of behavior and learning that were not readily apparent in the adult. Moreover, the analysis of parallel timetables for the emergence of different behavioral and cellular processes provides important clues as to the critical circuit properties and cellular mechanisms that may underlie the expression of different forms of behavior and learning in adult animals. Finally, this developmental analysis provides a powerful means of analyzing behavior and learning because it permits the investigation of early emerging processes in developmental isolation from later emerging ones. In this fashion the animal can serve as a functional mutant that transiently has certain capabilities, but developmentally lacks others that are present in the adult. Thus, using a developmental approach in identified neural circuits it may be possible to gain unique insights into mechanisms of behavior and learning by studying their developmental expression and assembly.

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The tuning of moth ears

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Summary. The simple ears of months have responded evolutionarily to the varied levels of selection pressure exerted upon them by insectivorous bats. While frequency-matched (syntonic) bats exert the primary pressure that determines where a sympatric moth's best frequency (BF) lies, there are bats that echolocate in mismatched (allotonic) bandwidths forming selection pressures strong enough to warrant increased secondary sensitivity at these frequencies. It is unknown what neural mechanism is used by these insects to broaden their audiograms but for some neotropical moths, external hearing aids provide a mechanical means of obtaining this sensitivity. Recent studies have uncovered social uses for auditory systems in certain moths and these requirements may provide additional selection pressure. Auditory conditions exist in certain moths that should provide a means to study the evolution of this sensory system from its mechanoreceptor origins to its degeneration in the absence of bat predation.
Key words. Moth auditory characteristics; defensive behaviour; bat echolocation frequencies; predation pressure; evolutionary adaptation.

Introduction

Most moths possess ears that alert them to the echolocation calls of hunting bats [29, 30]. Although simply designed, they provide the moth enough information about the bats in their environment to allow these insects a considerable advantage in staying alive [31].

Bats (Chiroptera) are the world's second largest order of mammals with over 900 described species and co-incident with this taxonomic diversity is a wide array of echolocation designs. Bats employ specialized forms of biosonar to accomplish their nocturnal tasks and there are specific relationships between their echolocation cells and foraging ecologies [3, 7, 14, 24]. Many bats are insectiovorous and the various communities of these predators around the world present considerably different selection pressures on moths. If the ears of moths are specifically tuned to the calls of bats, these pressures should be reflected in their auditory designs [13].

There are three lines of evidence that support the assumption that bat predation forms the main selective force on moth ears. First, moths, with some exceptions [1, 19, 32, 34], do not produce sounds (and therefore do not require ears) during their social interactions. Moths that use social sounds belong to geographically and taxonomically distant groups, and acoustic intraspecific communication likely represents a secondary adaptation for ears. Second, the neural simplicity of moth ears differs from those used for social communication. The multineuronal ears of Orthoptera appear to function in frequency discrimination [22, 26], a superfluous task for moths concerned only with detecting the presence of bats. Of interest in this regard is *Teleogryllus oceanicus*, a cricket that apparently uses a single interneuron for bat-detection [23]. Third, bats form the only source of nocturnal sound with frequencies matched to the sensitivities of moths' ears. For instance, figure 1 illustrates the spectra of sounds that could be construed to influence moths and indicates that bats alone generate the frequencies that moths are best able to hear.

Moth ears and defensive responses

Lepidoptera are either deaf or have independently evolved ears according to their superfamilial affiliation (table), a rule that has held, to date in all areas studied (nine countries, four continents). Although ears provide an effective anti-bat defence, the table suggests that not all Lepidoptera have adopted this particular strategy. The ear of a moth represents the