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LEARNING: THE *APLYSIA* MODEL SYSTEM

by

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ABSTRACT

Both associative and non-associative conditioning of the defensive gill withdrawal response have previously been demonstrated in the intact Aplysia californica and in the motor neurons of its isolated nervous system. The increased behavioral response has been attributed to activity dependent amplification of presynaptic facilitation at the central sensory/motor neuron synapses. A direct correlation between the behavioral learning and facilitation at these synapses has not, however, been demonstrated.

By utilizing a semi-intact preparation of Aplysia, in which the gill and its motor neurons could be monitored simultaneously, the aim of this study was to determine whether facilitation of the central sensory-motor synapses is necessary and/or sufficient for the behavioral learning.

Preparations that received paired presentations of conditioned (CS) and unconditioned (UCS) stimuli were more likely to show facilitated gill and motor neuron responses to the CS than those receiving unpaired presentations of stimuli. The in vitro preparation could, therefore, be associatively conditioned.

The motor neuron responses of some preparations became facilitated although the responses of their gills did not change. The gills also demonstrated enhanced responses, in the absence of EPSP facilitation, in two preparations. No correlation existed between the response of the gill motor neuron and that of the gill. This suggests that

facilitation of the sensory motor neuron synapse is not sufficient and may not be necessary for conditioning of the gill withdrawal reflex.

When motor neurons were depolarized before and after training, the elicited gill withdrawal to the same number of action potentials significantly decreased in the control group. There was no significant difference in this gill response before and after classical conditioning.

It seems that some decremental process occurs at a site peripheral to the motor neuron cell bodies during exposure to the conditioned and unconditioned stimuli. A facilitatory mechanism, in addition to and distinct from presynaptic facilitation at the central sensory/motor synapse, opposes this habituation and is more likely to do so following associative conditioning than non-associative conditioning. This mechanism tends to occur at a later stage during training than central presynaptic facilition and may involve a change in the peripheral nervous system or in central neurons having a direct influence in the periphery.

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Learning has been defined in numerous ways; such as "..a relatively permanent increase in response strength that is based on reinforcement and that can be made specific to one out of two or more arbitrarily selected stimulus situations" (Miller, 1967). This sort of definition, however, is restrictve, and includes only associative learning, ignoring the important forms of non-associative learning such as sensitization and habituation. Thorpe's (1963) definition may be preferable: "..(a) process which manifests itself by adaptive changes in individual behavior as a result of experience; (or)..the organization of behavior as a result of individual experience" since it includes, in addition to associative phenomena, those processes which cause a change in an animal's behavior that are not necessarily linked by temporal or spatial association.

In fact, it has become impossible to precisely define learning as more remote forms of adaptive change are added to the repertoire. To complicate matters, 'learning' has now been demonstrated to take place when stimulation of the central nervous system is substituted for the usual external stimuli (Olds, 1969, Woody and Yarowsky, 1972, Tsukahara et al., 1981, Hawkins et al., 1983); Thorpe's more general definition may not include such changes, especially when they do not correlate with overt motor responses, or behavioral adaptations.

One of the goals of this thesis was to determine whether one particular example of such 'neural learning' (that demonstrated in

Aplysia californica), represents learning by the behavioral definition. A tentative biological basis of learning should be demonstrated as at least sufficient and necessary for the behavioral expression of learning before it can be accepted as a feasible neuronal analogue of behavioral plasticity. The importance of demonstrating the necessity and sufficiency of any cell biological model of learning, and the guidelines that should be followed, have been stressed in detail by Rose (1981).

II. Types of Behavioral Plasticity

There are many types of behavioral plasticity, including such diverse phenomena as imprinting (Lorenz, 1935), latent learning (see Rashotte, 1979) and insight learning (Kohler, 1925). The following brief discussion however, has been restricted to habituation, sensitization (non-associative learning) and one type of associative learning (classical conditioning) as these are of direct relevance to this thesis.

a. Habituation

Habituation is defined as a "response decrement to repeated stimulation" (Harris, 1943). It has also been classed as a type of learning by Thorpe (1963); specifically, learning not to respond to a repeating stimulus. Habituation is commonly distinguished from 'receptor adaptation', in which the decrement can be attributed to decreased responsiveness in sensory receptors to the stimulus, as in adaptation of the pacinian corpuscle to a constant pressure (see Harris, 1943). In the same review Harris also stressed that effector fatigue is not responsible for habituation.

b. Sensitization

Sensitization is considered as a more complex mechanism than habituation, and more evolutionarily advanced (Razran,1971). The ability to demonstrate habituation has also been shown to appear earlier in development (Rayport and Kandel, 1986). It was argued by Wells (1967) to be an adaptive phenomenon which developed before the evolution of associative learning mechanisms and that the machinery of associative learning developed from this pre-existing adaptive mechanism. In sensitization, the response to one stimulus is enhanced as a result of presentation of another stimulus, usually a strong or novel one. This facilitation is not, however, associative and does not require any specific temporal pairing of the two stimuli. Thus, sensitization can lead to adaptive changes and in many lower animals, where equipment for sensory analysis and information storage is relatively simple, this may be the most important means by which accumulated experience increases the chances of individual survival.

Dishabituation (the reversal of habituation) is often considered to be a type of sensitization (Groves and Thompson, 1970), and the underlying mechanisms are believed to be the same in many systems, such as the crayfish escape response (Krasne and Glanzman, 1986). However, in certain systems, such as the unicellular ciliates Spirostunum and Stentor, they may involve distinct mechanisms, (Eisenstein et al., 1982) and a recent report has suggested that two distinct mechanisms may be involved in these two processes in Aplysia (Hochner et al., 1986).

c. Classical Conditioning

Pavlov's (1927) early experiments on dogs best illustrate the phenomenon of classical conditioning. A normal dog's reaction to meat powder (the unconditioned stimulus or UCS) is salivation (the unconditioned response or UCR). Following several presentations of a bell (the conditioned stimulus or CS) paired with meat powder, Pavlov found that the bell by itself came to elicit a conditioned response (CR), which was similar to the normal response to the meat powder: salivation. During classical conditioning training, the CS and UCS are presented to the animal with no dependence on the animal's response and the animal learns an association between these two stimuli.

Classical conditioning resembles sensitization in that the response to a stimulus in one pathway is enhanced by activity in another. However, whereas sensitization leads to the indiscriminate enhancement of responses to a variety of stimuli, classical conditioning leads to the selective enhancement or acquisition of responses to stimuli that are temporally paired with the UCS.

In experiments concerned with demonstrating classical conditioning, a control group is necessary in order to discriminate the associative effects (based on the contiguity of the stimuli) from the more general, non-associative effects of sensitization. The control group receives either randomly or specifically unpaired presentations of the CS and UCS. Any differences between the responses to the CS in the conditioned group, when compared to the control group, can then be attributed to associative effects, beyond any non-associative effects that may occur.

As well as being specific to the paired CS, classically conditioned responses tend to be more persistent and more pronounced than those

produced by sensitization (Brons and Woody, 1980, Carew et al., 1981, Crow, 1983,). This distinction, however, often becomes blurred: A greater amount of sensitization training in Aplysia, for example, can lead to larger responses that can persist for at least 7 days (Frost et al., 1985).

Groves and Thompson (1970) have suggested a dual-process theory of learning in which a repetitive stimulus evokes both decrementing and incrementing processes, and that these processes are independent. The behavioral outcome would therefore reflect a balance of incremental and decremental factors. In training situations where a strong noxious or appetitive UCS is presented, the incremental effects of sensitization and classical conditioning would usually be expected to outweigh the decremental effects of habituation. As Pavlov noted in 1927, sensitization would be expected to result from the presentation of any novel stimulus and habituation from any repeated stimulus; both conditions occur during CS-UCS pairing. The contents of this thesis will demonstrate that both occur during exposure to a non-associative or an associative training protocol, but habituatory aspects are more apparent after non-associative training.

III. Models of Learning

In order to understand the biological basis of behavioral plasticity it is necessary (but not sufficient) to understand the nervous system and the cells that comprise it. To be considered a suitable model, not only must an animal demonstrate the ability to learn, it must also provide a nervous system that is suitable for electrophysiological, biochemical and anatomical analysis. Both of these criteria have been

fulfilled, to a greater or lesser extent, in a selection of 'higher' organisms, which can exhibit an enormous capacity for plasticity but that also have an immensely complex nervous system, and in a selection of 'lower' organisms that exhibit a necessary minimum capacity for learning but that also provide a relatively simple nervous system. These two types of preparations can be divided into vertebrate and invertebrate 'model' systems, respectively.

a. Vertebrate Models of Learning

The study of vertebrate models has yielded much information on the neural sites of plasticity, but little on the underlying mechanisms involved.

The spinal reflex system has proved particularly useful for the study of mechanisms mediating habituation (Thompson and Spencer, 1966). In the isolated Bullfrog spinal cord, a decreased release of neurotransmitter from the presynaptic terminals mediates the habituation of motor neurons to stimulation of lateral collateral fibres (Glanzman and Thompson, 1979). Habituation of the hindlimb flexor response in the spinal rat also appears to be mediated by synaptic depression (decreased release of neurotransmitter) from presynaptic terminals of interneurones within the spinal cord (Groves and Thompson, 1973). MacDonald and Pearson (1979) found that, if the rat's nervous system is left intact, presentation of sufficiently intense cutaneous stimulation will bring about the recruitment of additional supraspinal habituatory mechanisms in the form of increased output from inhibitory interneurones. This latter report indicates that habituation of the reflex can normally be mediated by more than one mechanism, synaptic depression and a gradual

build-up of inhibition both play a role. It also emphasizes the need to verify observations made in reduced preparations with similar studies in intact, behaving animals.

Successful classical conditioning of reflex leg flexion in spinal dogs was reported as early as 1938 (Shurrager and Culler, 1938). It was not until Patterson et al. (1973) had demonstrated successful conditioning of the spinal cat, however, that localization of these adaptive changes was attempted. In Patterson's protocol pulses (the CS) are delivered to the peroneal sensory nerve of the hind leg and paired with a strong shock to the ankle (the UCS). After approximately 20 trials the activity of the motor nerve that innervates the same hind leg is increased (the CR). The same interneuron pools which appear to be involved in sensitization and habituation (Groves and Thompson, 1970) have been suggested to mediate the classical conditioning (Patterson, 1976, Patterson et al., 1982). The processes responsible for the effects remain obscure as they do for nonassociative learning in this system and the role of motor neurons in these response increases has not been determined.

The cat spinal cord preparation provides a promising neural model for the analysis of associative learning. It exhibits the parametric features characteristic of learning in the intact mammal and presents possibilities for the analysis of underlying mechanisms. As with the rat habituation model though, supraspinal mechanisms would no doubt be involved in the simplest associative changes occurring at the spinal cord level, if they normally occur <u>in vivo</u> at all.

A well developed behavioral model for the analysis of cellular mechanisms of associative conditioning is that of conditioned tachycardia in the pigeon (see Cohen and Randall, 1984). After pairing the CS (retinal illumination) with the UCS (foot-shock) a reliable cardioacceleratory response (the CR) of predictable dynamics emerges (Cohen and Goff, 1978). One of the many advantages that this system offers is that the route followed by the sensory, motor and interneuronal pathways mediating the conditioned behavior has been meticulously mapped (Cohen, 1980). Once the relevant neuronal circuitry is understood the plastic properties of various components of the pathway can be investigated. Recordings from the extrinsic cardiac nerves indicated that a decrease in vagal activity and an increase in sympathetic activity mediate the CR (Cohen, 1980). These changes could reflect modifications local to the motor areas or changes occurring considerably earlier in the visual pathways. No change in retinal output is observed after conditioning although modification of output has been seen as peripherally as the avian lateral geniculate nucleus (LGN) (Cohen, 1984). The LGN neurons receive convergent UCS information from the locus coereleus (Cohen, 1984) and this input is necessary for associative modification of the LGN (Broyles and Cohen, 1985); whether it is necessary for associative behavioral modification has not been reported. Also, although pairing light with stimulation of the locus coereleus is sufficient to produce enhanced light evoked discharges in geniculate neurones (Gibbs et al., 1983), it has not been demonstrated as sufficient to support behavioral changes. The conclusion reached by Cohen and Randall (1984) is that most, if not all, of the central relays within the pathways mediating this CR show modification with training.

Mammalian studies of cardiac conditioning have demonstrated changes in the amygdala (Applegate et al., 1982) and hypothalamus (Francis et al., 1981). The role that these structures play in classical conditioning has not been determined. Considering the present state of knowledge on the loci of changes mediating cardiac conditioning, it seems unlikely that elucidation of the underlying mechanisms of these changes is imminent.

Tsukahara and his colleagues approached the task of identifying the cellular basis of behavioral plasticity from a different angle. Firstly they analyzed a modifiable system in the central nervous system, the red nucleus (RN) (Tsukahara et al., 1975, Tsukahara, 1981). They then reconstructed the modification of behavior that is mediated by the pathway containing the RN (Tsukahara et al., 1981). It was found that lesions of the cerebellar afferents, which synapse on the somata of the RN neurones, induced sprouting of the cortical inputs which normally synapse on the apical dendrites of the RN cells. Electrophysiological (Tsukahara et al., 1975) and ultrastructural (Murakami et al., 1982) evidence indicates that sprouts of the cortico-rubral fibres form active synapses on the more proximal portions of the soma dendritic membranes (the area that was previously occupied by the cerebellar inputs).

Having demonstrated that synapses within the RN are capable of considerable plasticity, Tsukahara et al. (1981) demonstrated classical conditioning of the cat forelimb-flexion response by pairing electrical stimulation of the central cerebral peduncle (the CS) with cutaneous paw shock (the UCS). This conditioning protocol was also found to cause greater CS-evoked activity in the RN cells (Oda et al., 1981). It

should be noted that few unpaired control animals were included in either of these studies (two cats and one cat respectively) and, although these controls did not show enhanced behavioral or cellular responses, a larger sample size would normally be required to distinguish changes due to non-associative learning from those due to the classical conditioning protocol.

The primary site for the neuronal changes following conditioning appears to be the cortico-rubral synapses in the red nucleus. After conditioning, as with lesion-induced changes, a new fast-rising component was found in the cortico-rubral dendritic EPSP (Tsukahara, 1984). Sprouting and morphological changes at these synapses have been suggested as the cellular basis of the learned response (Tsukahara, 1984). Tsukahara's model for the cellular basis of classical conditioning in this system is based on an analogy with previous lesion experiments and, as yet, there is no ultrastructural evidence from classically conditioned preparations to support the model. Further, there are other possible explanations for the electrophysiological changes observed after conditioning; the possibility of changes in presynaptic neurotransmitter release, for example, has not been addressed. Although much of the evidence is inconclusive at present, the red nucleus provides an interesting model for a morphological basis of behavioral plasticity.

The short latency (20-50 msecs.) eyeblink CR of the cat has been utilized by Woody and his collaborators since the 1960s (Woody and Brozek, 1969a, 1969b) when the pathway from the orbicularis oculi muscles was traced centrally to the cortical region which controlled the

movement. An intact rostral cortex is required for acquisition or retention of the CR after paired presentations of the CS (a click) and the UCS (glabella tap) (Woody and Brozek, 1969b, Woody et al., 1974). Increased excitability and incidence of cortical neurons projecting to the facial nuclei was also observed to correlate with conditioning (Woody and Black-Cleworth, 1973). Cortical cells, however, were also found to be as excitable just after exposure to UCS-only training as they were after CS-UCS paired treatment. It was only when long-term retention (2-38 days post training) results were compared that cells from CS-UCS animals were found to be significantly more excitable than UCS-alone animals (Brons and Woody, 1980). This latter study also revealed that the excitability persisted even after the CR had been extinguished by CS-alone presentations. Due to these findings Woody (1982) has suggested that the cortical neuron changes represent a neurophysiological correlate of latent facilitation. This type of facilitation does occur in cats; following repeated presentations of glabella tap a facilitation of the rate of acquisition of the CR is observed (Matsumura and Woody, 1982). The changes in excitability appear to arise from changes in membrane conductances intrinsic to the cortical neurons, and it is believed that the increased membrane resistances are mediated by cGMP and/or Ca^{2+}-calmodulin-dependent protein kinase (Woody et al., 1978, 1984). The discovery that such second messengers may be involved in an aspect of cellular mediation of classical conditioning is in accordance with similar findings in other model systems (Occorr et al., 1986, Baudry and Lynch, 1984, Acosta-Urquidi et al., 1984, Livingstone et al., 1984). The motor cortex does

not appear to be the only site involved during associative learning of the click-elicited CR. Neural excitability has also been found to increase in the auditory association cortex with a greater magnitude of change in CS-receptive neurons, furthermore this change is only induced by associative training and is not mediated postsynaptically (Woody et al., 1976). Although this area of the cortex is not necessary for conditioning to occur (Woody et al., 1974), removal of these areas does seem to reduce discriminability between CSs and other stimuli (Diamond and Neff, 1957). Woody (1984) suggests that more than one cellular mechanism mediates Pavlovian conditioning. The movement that is conditioned appears to depend on a postsynaptic mechanism represented in the neurons of the motor cortex, while the associative feature of this conditioning may depend mainly on the mechanism influencing CS reception which is represented in the association cortex, but must also be represented subcortically. Other stages and mechanisms are also likely to be involved.

The conclusions reached from the studies on cardiac conditioning in the pigeon and from Woody's eyeblink conditioning studies make it clear that complex changes in behavior that result from associative learning are likely to be mediated by more than one cellular mechanism; the contents of this thesis will serve to emphasize this point yet again.

The model systems described above have produced information on regions of the brain that are necessary and sufficient for associative learning, but few clues have emerged as to the actual cellular basis of the observed changes. Preparations that have been further reduced such as the hippocampal slice and the isolated sympathetic ganglion offer a

less complex, more restricted environment that is more amenable to cellular analysis.

Synaptic mechanisms have been investigated in particular detail in the bullfrog sympathetic ganglion. Repetitive stimulation of preganglionic cholinergic fibres results in the generation of a long lasting depolarization, the slow EPSP (sEPSP), which is mediated by muscarinic postsynaptic receptors (Koketsu, 1969). If the eighth spinal nerve is stimulated repetitively, a late slow EPSP (ssEPSP), which is mediated by LHRH, can be recorded in the postsynaptic cells (Schulman and Weight 1976, Jan and Jan, 1982). A single electrical stimulus to the preganglionic fibres elicits a fast EPSP (fEPSP), which results from the action of Ach on nicotinic postsynaptic receptors. Fast EPSP amplitudes and their efficiency in generating action potentials are enhanced by stimulation of the muscarinic slow EPSP pathways or the peptidergic late slow EPSP pathway (Schulman and Weight, 1976). The primary mechanism involved in this long lasting potentiation of synaptic transmission is a postsynaptic decrease in a voltage dependent K^+-conductance (Adams et al., 1982, Jan and Jan, 1982).

Results from the study of mammalian sympathetic ganglia have indicated similar mechanisms of plasticity to those of the amphibia ganglia (see Libet, 1984 for review). In addition a long term enhancement (LTE) of the muscarinic cholinergic slow EPSP by dopamine has been demonstrated (Libet and Tosaka, 1970), this heterosynaptically mediated facilitation can last for hours. The LTE is believed to be mediated by increased cAMP levels in the postsynaptic cell, via D-1 receptor types (Libet, 1984). Superfusion of cGMP over the ganglion has

been found to reverse LTE, but only if it is applied within 15 minutes or so following the onset of LTE (Libet et al., 1975). Libet (1984) has suggested an interesting analogy between these temporal characteristics of LTE and the stages of memory induction, consolidation and retention, inferring that cGMP interferes with the consolidation process but not the retention process.

The precise site and type of change that occurs during LTE is not known. There is no evidence that a temporal convergence of the 'test' (a muscarinic agonist) and 'induction' (dopamine) synaptic inputs is necessary for LTE. Its potential importance may therefore be to provide an understanding of nonassociative learning, particularly as there has been increasing evidence that dopaminergic systems are involved in a variety of learning and memory phenomena (Beninger, 1983).

The highly plastic hippocampus has been exploited as a model system for studies of habituation, short term facilitation, long term facilitation and, more recently, associative facilitation. Low frequency stimulation of the perforant path (PP) induces habituation of the EPSPs (to low intensity stimulation) and the population spike (to high intensity stimulation) recorded in the dentate granule cells (Teyler and Alger, 1976). Habituation of the dentate gyrus (DG) may involve decreased presynaptic neurotransmitter and/or decreased receptor sensitivity at the dendritic spines (Harris et al., 1979). Ca^{2+} involvement is suggested by the fact that increased extracellular calcium reduces the degree of observed habituation, inferring that it is the magnitude of calcium influx during repetitive stimulation that is

normally the limiting factor in neurotransmitter release (Turner and Miller, 1982).

Following repetitive stimulation of the entorhinal cortex at a moderate frequency (about 5 sec^{-1}) a potentiation of the EPSP in the dentate granule cells is observed. This potentiation is referred to as posttetanic potentiation (PTP) and typically lasts only seconds to minutes. In addition, it appears to involve an increase in the probability of presynaptic transmitter release (McNaughton, 1982). Another form of hippocampal short term potentiation is that which occurs to the second of a pair of electrical pulses separated by a brief interval, ie. paired pulse potentiation (PPP) (Lomo, 1971, Alger and Teyler, 1976). PPP can only be induced by homosynaptic conditioning pulses and has also been suggested to be mediated by an increased release of transmitter quanta (Creager et al., 1980). Due to the short duration of these forms of potentiation, the most that they may be able to provide is an explanation for short term memory. Smithson and Brown (1985) have, however, suggested a possible function for mechanisms such as PPP in retrieval processes.

A much more longer lasting example of an increased response was found in the hippocampus by Bliss and Lomo (1973). This long-term potentiation (LTP) of synaptic transmission results from brief trains of high frequency stimulation (>5 sec^{-1}) to any one of several excitatory hippocampal pathways. The DG, CA1 and CA3 cell populations are each capable of such facilitated postsynaptic responses. Furthermore, LTP can persist for days and weeks in intact animals (Bliss and Gardner-Medwin, 1973). As with short-term potentiation, LTP can also be

demonstrated in the in vitro hippocampal slice (Alger and Teyler, 1976), thereby facilitating its investigation by biochemical, electrophysiological, morphological and pharmacological methods (Lynch and Schubert, 1980). LTP and PTP can occur independently at the same hippocampal synapses and do not share a common mechanism (McNaughton, 1982). The precise locus of the change that mediates LTP has not been conclusively determined, and it seems likely that more than one mechanism and locus are involved (Bliss and Dolphin, 1984).

A number of investigators have suggested that LTP reflects primarily postsynaptic changes, initiated by increased Ca^{2+} levels (Eccles, 1983, Lynch and Baudry, 1984). Calcium is hypothesized to unmask glutamate receptors via a membrane associated proteinase; calpain I (Baudry and Lynch, 1984). The glutamate antagonist, AF3, prevents LTP (Dunwiddie et al., 1978) and increased glutamate binding follows LTP induction (Baudry et al., 1980). Together with the demonstration that small changes in postsynaptic dendritic spine morphology occur during stimulation conditions that induce LTP (Desmond and Levy, 1983), and that injection of the calcium chelator, EGTA, into postsynaptic membrane prevents LTP (Lynch et al., 1983), these observations strongly suggest a postsynaptic locus for LTP. The alternative site of change is the presynaptic terminal. Increased resting and evoked release of glutamate has, in fact, been demonstrated following LTP (Skrede and Malthe-Sorenson, 1981). Confirmation of the role of the postsynaptic site still requires the demonstration that the response of the postsynaptic membrane to applied transmitter is enhanced during LTP; so far such attempts have proved negative (Turner et al., 1982).

It is unclear what kind of information storage and what kind of behavioral change might best be subserved by homosynaptic LTP. There have, however, been recent demonstrations of associative LTP (Barrionuevo and Brown, 1983, Kelso and Brown, 1986). The mechanisms underlying these temporally dependent changes are not yet understood, though they may confer more credibility to the suggestion that LTP is a general mechanism involved in associative learning. There is also evidence that serotonergic input from midbrain raphe nuclei and, to a lesser extent, noradrenergic input from the locus coeruleus, can modulate LTP (see Abraham and Goddard, 1984). This is interesting in light of the putative action of monoamines in other model systems (for example, Tempel et al., 1984, Libet, 1984).

b. Invertebrate Models of Learning

Despite the successful identification of the loci of neural changes involved in learning by many of the groups mentioned above, the complexity of the vertebrate nervous system presents a number of problems for questioning the cellular and molecular basis of these changes.

The easily accessible "simple" nervous systems of invertebrate preparations have been utilized for a number of years to investigate mechanisms that may be involved in learning at the behavioral, cellular and molecular level. Research on invertebrate preparations has provided the means to localize learning induced changes to individual neurons.

The majority of evidence pertaining to the cellular mechanisms underlying behavioral plasticity, and particularly associative learning, has been obtained from studies on arthropods and molluscs. These have

recently been reviewed by Carew and Sahley (1986) and a more detailed review of the current state of research on gastropod molluscs has been formulated by Mpitsos and Lukowiak (1985). Although examples of behavioral plasticity can be found in every phylum (see Corning et al, 1973a, b), this discussion is restricted to those invertebrate preparations that have provided clues to the cellular basis of the behavior modification that they display.

Investigations utilizing the fruit fly Drosophila melanogaster have exploited its well developed genetic methodology. Flies can be classically conditioned to avoid specific odors that have been paired with shock (Quinn et al., 1974) and specific colors that are paired with mechanical shaking (Menne and Spatz, 1977) and to extend or flex their legs to avoid shock (Booker and Quinn, 1981). Learning deficits for these conditioned responses have been observed in a number of mutants. 'Dunce' and 'Rutabaga' mutants, for example, learn but then rapidly forget, indicating a retention deficit. 'Dunce' is lacking one of two enzymes that hydrolyze cAMP, which tends to lead to an increase in levels of cAMP in the cell (Byers et al., 1981). 'Rutabaga', on the other hand, is defective in calcium-calmodulin-activated cyclase activity (Livingstone et al., 1984) which would tend to cause a decreased level of cAMP activity in the cell. The observation that these two different mutants display the same behavioral deficit while the effects of their biochemical deficiencies are opposite implies, as Livingstone suggests (Livingstone et al., 1984), that learning does not depend on specific basal levels of cAMP, but rather the ability to modulate cAMP appropriately and/or in critical neurons. A mutant that

lacks dopa-decarboxylase, ('DdC'), shows deficient acquisition of learning but normal retention, as exemplified by flies with moderate lesions of the gene (Tempel et al., 1984). The dopa-decarboxylase enzyme is necessary for the synthesis of dopamine and serotonin, and it has been suggested that one or both of these neurotransmitters are essential for learning, but not for memory, in Drosophila (Tempel et al, 1984). Habituation and sensitization are also retarded in mutants that show associative learning deficits (Duerr and Quinn, 1982) which suggests that nonassociative and associative learning may involve common cellular mechanisms in this system. Research on Drosophila is in accordance with other systems mentioned in this discussion, in that both monoamines and second messengers have been implicated as playing pivotal roles in memory acquisition and retention.

Robust associative learning has been demonstrated in the gastropod mollusc Pleurobranchea. These animals can be conditioned to withdraw from squid homogenate, which is normally an appetitive stimulus, by delivering electrical shocks to the oral veil each time the slug does not withdraw from, or makes a biting movement towards the squid (Mpitsos and Collins, 1975). In later experiments Mpitsos et al. (1978) justified the exploitation of Pleurobranchea as a model for investigating the neurophysiological basis of learning by demonstrating that the CRs could be generated rapidly (after only one trial) and that the conditioned behavioral changes were much larger than those observed in control animals and were amenable to quantitative measurement. More recently, discriminative chemosensory learning has been reported in this preparation (Mpitsos and Cohan, 1985a), indicating a specific

suppression of feeding to a particular stimulus that has been paired with shock; this is a good indication that associative conditioning is occuring. The central control of feeding in Pleurobranchea has been analyzed in great detail (see Davis, 1985 for review) and neural correlates of associative chemosensory aversion learning have been identified. Intracellular recordings from whole-animal preparations have revealed differences in the responses of a group of "feeding command neurons" (the paracerebral neurons or PCNs) to infusions of squid over the oral veil. The PCNs of previously conditioned animals displayed an intensive barrage of IPSPs in response to squid homogenate, this is in contrast to the normal excitation that the PCNs of control (unpaired) or naive animals displayed. Interestingly, the responses of PCN cells from untrained but satiated animals were identical to those of the trained animals (Davis and Gillette, 1978) indicating, perhaps, a neural correlate of 'motivation' (Davis, 1985). Although the synaptic input to the PCNs is indistinguishable for associatively trained and food satiated whole animal preparations, this is not the case when the nervous system is removed. In nervous systems isolated from previously trained animals, the PCNs continue to show a barrage of IPSPs when food stimuli are delivered to the attached oral veil. The PCNs in brains isolated from satiated animals, however, display excitatory responses to food stimuli (Kovac et al., 1985). This latter finding suggests that some non-associative causes of changed feeding motivation (eg. satiation) may originate from peripheral sites (such as the gut) and can be separated from those factors controlling associative changes in motivation which may be intrinsic to the central nervous system.

It has been suggested that the PCNs themselves do not represent the site of neuronal change underlying associative learning in Pleurobranchea. A set of interneurones (Int-1) make monosynaptic inhibitory connections on the PCNs and these in turn receive excitatory input from a second set of interneurones (Int-2). Food stimuli produce longer depolarizing responses in the Int-2s of conditioned animals when compared to controls, resulting in a greater eventual inhibition of the PCNs (London and Gillette, 1986). The mechanisms by which these interneurones change their responsiveness to previously paired food stimuli are unknown. More recently, a direct role for the postsynaptic PCNs in the mediation of learning has been implicated. The excitatory response of these cells to direct application of Ach is suppressed in previously conditioned animals. The mechanisms involved in this postsynaptic change have also not been determined (Morielli et al., 1985).

Research on Pleurobranchea has revealed that there is a large amount of variability in the motor patterns of different animals (Mpitsos and Cohan, 1985b). As a result of this observation Mpitsos has suggested that there is distributed function among and within the various motor elements involved in Pleurobranchea feeding behavior (Mpitsos and Lukowiak, 1985). It seems, therefore, that similar motor patterns can produce different behaviors, and that extensive variability of the motor pattern produces similar adaptive behaviors. This suggests that a particular behavior may not be identifiable from examination of the motor pattern in the isolated nervous system, but requires concurrent monitoring of the behavior itself, by the use of a semi-intact preparation. Similar problems will be discussed later in reference to

the Aplysia preparation. Mpitsos and Cohan (1985b) have suggested that such variability does not arise by accident, but is an integral part of neural processing that allows animals to respond adaptively to certain environmental stimuli. The same authors have also proposed that neural circuits can 'self-organize' to meet demands imposed by the environment, such as those present during associative training. Whether such 'self-organizing' processes occur in the Pleurobranchea nervous system and whether they play any role in associative learning is purely speculative at this point.

Using a chemosensory aversion-learning paradigm, Gelperin (1975) first demonstrated that the garden slug, Limax maximus could be trained to avoid a particular food stimulus. Sahley et al (1981a) later demonstrated that only one trial of paired carrot odor and quinidine (a bitter taste) is sufficient training to induce selective avoidance of carrot odor on later tests. In addition, more complex forms of conditioning, ie. blocking, second order conditioning and UCS pre-exposure effects have proven successful with this learning paradigm (Sahley et al., 1981b). This suggests that the associative learning characteristics seen in Limax may be functionally similar to those seen in vertebrates. Interestingly, slugs will also selectively avoid eating a diet if an essential amino acid is removed (Delaney and Gelperin, 1983). The mechanism by which such a deprivation acts as a reinforcer is not clear at all. However, similar effects of post-ingestive cues have been reported numerous times in rats (Zahler and Harper, 1972), reinforcing the suggestion that common principles may govern vertebrate and invertebrate learning.

An *in vitro* preparation consisting of the cerebral ganglion, buccal ganglion and lips (Gelperin et al, 1978) has been utilized for the analysis of neural events underlying *Limax* chemosensory aversion learning. A co-ordinated pattern of motor neuron activity (the feeding motor program or FMP) can be elicited reliably if food extract is applied to the lips and can be recorded extracellularly from buccal nerve roots. This pattern corresponds to feeding behavior in the intact animal and is therefore considered to be the neural correlate of feeding (Gelperin et al., 1978). Successful conditioning of this semi-intact preparation has been demonstrated by pairing a food stimulus (CS) and a bitter taste (UCS) to the lips. The resultant CR is a selective suppression of the FMP to the taste paired with the UCS (Chang and Gelperin, 1980). It should be noted that such training did not lead to learning in 100% of the preparations; only 22% showed an impressive suppression of FMP, 29% showed a fair degree of learning and 49% showed no evidence of learning. The fact that Gelperin and Culligan (1984) reported that only 12% of intact animals performed poorly when tested after paired training suggests that comparisons between semi-intact and whole animal findings should be made tentatively. In addition, other differences exist between the whole animal and the isolated nervous system. The CR that is tested after training in the intact animal is locomotion to a preferred odor, whereas a correlate of feeding is measured in the *in vitro* preparation. Furthermore, an odor-taste association is used to modify the intact animal's behavior whereas a taste-taste association modifies the FMP in the isolated nervous system.

Gelperin and Culligan (1984) reported suppression of FMP in the isolated brains of animals that have been previously conditioned. In

this study, though, only 50% of the brains from successfully trained animals showed an impressive suppression of the FMP response to the paired CS. This low correlation between behavior and neural responsiveness indicates that the learned behavior can be expressed via alternative or additional mechanisms to those currently being investigated by Gelperin and his co-workers (see Gelperin et al., 1984).

One of the major setbacks in the determination of the neural basis of Limax learning has been that the cells involved in controlling the feeding behavior and the putative transmitters involved are only presently being identified (Gelperin et al., 1984, Cooke and Gelperin, 1985). It was demonstrated recently (Sahley et al., 1986) that slugs fed on choline-enriched diets showed similar rates of acquisition of chemosensory aversion learning to slugs fed on choline-deficient diets. Retention of the CR in the former group was, however, enhanced. Previous work had indicated that elevated dietary choline enhances blood choline levels and cholinergic transmission in Limax (Barry and Gelperin, 1982a,b) and that acetylcholine applied to the cerebral ganglion will trigger the FMP in the semi-intact preparation (Cooke and Gelperin, 1985). The findings of Sahley et al. (1986) may suggest that acetylcholine has a role to play in retention of this learning. A control group, fed on a normal choline diet, was not included in this study so that it is not clear whether the observed difference is due to an enhancement of memory by the choline enriched diet or to a deterioration of memory by the choline deficient diet, although the authors suggest, based on comparison with other studies, that the former is the case. Generalized effects on motivation, motor control and sensory input may also be expected from these manipulations. It is

noted by the authors, for example, that the high-choline slugs weighed, on average, nearly twice as much as the low-choline slugs. Such a difference could be caused by a high level of feeding motivation in the high-choline slugs, leading to greater food intake, or by a low level of motivation in the low choline group (pertinent observations were not recorded).

The demonstration that Limax can undergo higher forms of associative learning and that it may utilize the common neurotransmitters, such as Ach, to modulate these behaviors, lends greater credence to the use of such invertebrates to identify common cellular mechanisms of learning. Progress in the elucidation of the neuronal substrates underlying this particular behavior modification has unfortunately been slow, despite the development of an elegant semi-intact preparation.

Using diffuse light as the CS and high velocity rotation as the UCS, Crow and Alkon demonstrated associative suppression of phototaxis in Hermissenda crassicornis. This nudibranch mollusc normally displays positive phototaxis but 50 paired trials per day for three days brings about long-lasting (up to 7 days) increases in latency to enter a spot of light (Crow and Alkon, 1978). More recent observations (Lederhendler et al., 1986) have shown that contraction of the foot is also conditioned following paired CS and UCS presentations. Foot contraction is similar to the UCR to rotation and this behavioral change more closely resembles traditional Pavlovian conditioning. Conditioning in Hermissenda is much more successful when light is a reliable predictor of rotation; the addition of extra light-alone or rotation-alone presentations between pairings causes an attenuation of learning (see

Farley and Alkon, 1985)). Such 'contingency sensitivity' is also demonstrated by vertebrates and indicates that the mode of information processing may be the same in both. Pre-exposure of Hermissenda to the UCS before training, however, does not affect the animal's ability to learn; ie. it does not display latent inhibition (see Farley and Alkon, 1985). This indicates that, with this particular task, Hermissenda is not capable of such advanced forms of learning as those demonstrated by Limax (Sahley et al., 1981).

The eyes of Hermissenda each contain five photoreceptors, two 'type A' and three 'type B' photoreceptors. It has been proposed that the type B cells are the primary sites of neural change which mediate both acquisition and retention of the behavioral learning (see Alkon, 1984a). Crow and Alkon (1980) found that the spontaneous activity of dark-adapted B receptors was significantly greater in animals that had received associative training several hours previously. The B photoreceptors from these animals also had higher input resistances and more depolarized resting potentials. One to two days after training the cells are no longer depolarized, but they do show greater long-lasting depolarizations (LLDs) and increased activity in response to light presentations while maintaining an increased membrane resistance (West et al., 1982). Extinction of the behavioral CR also appears to be correlated with reversal of these changes in the type B cells (Richards et al., 1984). Similar changes in the B cells can be induced in the isolated nervous system, with the eyes and statocysts attached. Alkon (1976a, 1980) showed that paired illumination and rotation also produced a suppression of activity in type A photoreceptors. These correlational

observations, however, do not provide evidence for any causative role for type B or type A photoreceptor changes.

Farley et al. (1983) attempted to demonstrate that these cellular changes are the cause of the learned behavior. In this study rotation (the UCS) was replaced with direct depolarization of the type B cells and conditioning was carried out in situ in nervous systems of relatively intact animals. Light and depolarization were paired for five trials and the effects of the conditioning were observed on behavior. Phototaxis was found to be significantly more suppressed in conditioned animals than in controls. The paired cells also showed a significantly increased input resistance. The UCS in the in situ situation does not actually represent the rotation UCS, but rather mimics the rebound excitation on release from inhibition which is proposed to occur in the B cells at termination of the UCS. Also, only five training trials were used for the in situ training, whereas the behavioral training involved 50 training trials on each of three consecutive days; according to Crow (1983), only non-associative changes are evident in intact animals after 5 to 10 trials. Clearly there are inconsistencies between the two procedures; these were not discussed by Farley et al (1983)

The model that has been proposed for the mechanisms underlying these changes in the B type cells has been described in detail (Alkon, 1984a,b). Briefly, training is believed to produce a cumulative depolarization in B cells. This is derived from rebound excitation at the termination of rotation due to release from inhibition from caudal cells. The cumulative depolarization is thought to cause an increase in intracellular calcium accumulation in the type B cells (Connor and

Alkon, 1984). The elevated calcium, in turn, causes prolonged reduction of an early voltage-dependent K^+ conductance (I_A) (Alkon et al., 1982a). A decrease in this current causes increased membrane resistance and increased depolarization and is thought to be the basis for retention of the learned behavior (Alkon, 1984a). Inactivation of a calcium dependent K^+ current (I_C) and an increase in the voltage dependent Ca^{2+} current are also thought to be involved in this increased excitability (Alkon et al., 1982b, Alkon, 1984b). Ca^{2+}-calmodulin-dependent phosphorylation (mediated by protein kinase C) has been implicated as the means of K^+ channel inactivation (Neary et al., 1981, Farley and Auerbach, 1986, Acosta-Urquidi et al., 1984). Grover and Farley (1984) showed, however, that B cells can still exhibit pairing-specific cumulative depolarization when I_A and I_C are blocked and when there is no Ca^{2+} in the bathing solution. This questions the roles of decreased I_A and I_C, as well as that of Ca^{2+}.

The literature on Hermissenda associative learning is full of such inconsistencies. Alkon (1975), for example, reported no change in either A or B photoreceptors after associative conditioning, in 1976 (Alkon, 1976a) he observed differences in A cells, but not in B cells. Crow and Alkon (1980) found that the responses of all of the B type cells changed with associative learning. Farley et al. (1983), however, found changes in only two of the three B type receptors in each eye and Alkon et al. (1982b) found that only one B type cell displayed changes.

The change in phototactic behavior is proposed to be directly mediated by increased type B cell excitability. Increased B cell activity would cause greater B cell inhibitory input onto type A cells, the latter are believed to be responsible for mediating phototaxis through their connections to interneurones and motor neurons (Alkon, 1984b). This series of interactions would lead to greater suppression of the neurons involved in initiating and sustaining movement in intact associatively trained animals. The spontaneous firing frequency of type B cells in the dark is also increased in conditioned animals (Crow and Alkon, 1980). This would predict a suppression of locomotion in the dark which is not, however, observed in intact trained animals (Crow and Offenbach, 1979).

From an objective viewpoint, the behavioral and cellular mechanisms of Hermissenda learning do not seem to be compatible. Crow (1985b) has attempted to examine the photoreceptor responses at times that are more appropriate to the behavioral test procedures, ie. after five minutes of light adaptation. In this case, conditioned animals showed significantly reduced type B cell responses when compared to random controls. This finding is in direct contradiction to the reports discussed above. An alternative model, whereby conditioning results in diminished B cell responses, which are directly translated into reduced phototaxis, has been proposed (Crow, 1985a).

The proposals by Alkon and by Crow both implicate the primary photoreceptor as the major site of neural change underlying the associative behavioral modification. It is possible that both of their proposed mechanisms contribute to learning. Conditioned modifications of sensory systems have also been observed in vertebrates; the visual

system of pigeons (Cohen, 1982) and the auditory system of cats (Ryugo and Weinberger, 1978), for example. Unlike mammalian photoreceptors, the primary photoreceptors of Hermissenda are involved in a number of integrative processes (see Alkon 1976b), and it is feasible that they are involved in associative conditioning. Alkon (1984a) considers that the photoreceptors are the only site involved in, and causative to, the behavioral response; this is in contrast to predictions based on vertebrate models, as noted in the earlier discussion of work carried out by Cohen and by Woody. In view of the fact that the role of the photoreceptors themselves has not yet been conclusively demonstrated, it seems intuitively reasonable to assume that additional mechanisms are likely to be involved.

Alkon's claim (1984a) that the work carried out on Hermissenda classical conditioning has elucidated the only known biophysical mechanisms for associative learning in invertebrates or vertebrates may be somewhat premature considering the degree of inconsistency and controversy surrounding conditioning of this nudibranch mollusc.

Behavioral plasticity in the opistobranch mollusc Aplysia californica has been studied in great detail. This has resulted in an extensive accumulation of information concerning the underlying mechanisms of habituation and sensitization as well as associative learning. The defensive withdrawal reflex of the mantle organs has received the most experimental attention in the study of all three of these forms of behavior modification (see Kandel et al., 1983, Mpitsos and Lukowiak, 1985). In addition, sensitization and classical conditioning of the tail withdrawal reflex - another defensive response - has provided

evidence for cellular mechanisms which are similar to those proposed for modification of the mantle organ withdrawal response (Walters et al., 1983, Walters and Byrne, 1983). Aplysia have also been shown to be capable of a different form of associative learning, operant conditioning (ie. the animal is only rewarded or punished when it behaves appropriately): This was demonstrated by Susswein et al. (1986) who have shown that Aplysia can learn not to respond to foods previously found to be edible and by Cook and Carew (1986) who demonstrated successful modification of a head-waving response. These latter two studies may prove useful in the future for studying the cellular mechanisms underlying a different form of associative learning, as of now though, such mechanisms have not been identified and only those studies concerned with the defensive reflexes will be discussed here.

Like all opistobranch molluscs, Aplysia has a mantle cavity on its dorsal surface that houses a delicate gill. The gill lies under a protective sheet of skin, the mantle shelf, which terminates at the rear of the cavity in a fleshy, funnel-shaped spout, the siphon. A light tactile stimulus to the siphon or gill elicits a brisk, defensive withdrawal reflex of all the mantle organs; the gill, siphon and mantle shelf (Kupfermann and Kandel, 1969, Peretz, 1970). These organs also carry out spontaneous respiratory pumping movements during which the siphon, gill and parapodia contract. This contraction brings about a circulation of fresh seawater through the mantle cavity and out of the siphon (Byrne and Koester, 1978).

A significant advantage of studying the defensive reflexes of these mantle organs is that their neural control has been extremely well characterized (Kupfermann et al., 1974)). The siphon and the gill are

characterized (Kupfermann et al., 1974)). The siphon and the gill are each innervated by the central and peripheral nervous systems. The central innervation is derived entirely from neurons in the abdominal ganglion (AG), via the branchial, ctenidial and siphon nerves. A few identified neurons are known to innervate the gill; L7, LDG1, LDG2, $L9_1$, $L9_2$ and RDG (Kupfermann et al., 1974), only the first three have been shown to directly innervate the gill musculature (Carew et al., 1974). Each of these motor neurons will bring about distinct, identifiable contractions of the gill when depolarized (Kupfermann et al., 1974). Using intracellular injections of cobalt chloride, Winlow and Kandel (1976) showed that L7 sends axons to the gill via all three nerves, the morphology of the other motor neurons has not been similarly characterized. It has been determined electrophysiologically, however, that LDG1 sends axons via the branchial and ctenidial nerves, and that LDG2 innervates the gill via the ctenidial nerve only (Kupfermann et al., 1974). In addition, Lukowiak (1977a) has suggested that L9 innervates the gill via a small branch of the siphon nerve. The central siphon motor neurons have also been identified and characterized (Perlman, 1979). The gill motor neuron L7 has been noted to be a siphon motor neuron (Kupfermann et al., 1974). However, withdrawals of the siphon were not observed in response to depolarization of L7 in the course of the studies discussed in this thesis and Perlman (1979) reported that stimulating L7 only produced a movement at the base of the siphon.

Three clusters of siphon mechanoreceptors (the LE, RE and RF clusters.) have been identified within the AG (Byrne et al., 1974, Byrne,

1980) and these make both polysynaptic (via interneurones) and monosynaptic connections with the gill and siphon motor neurons.

The peripheral nervous system of the gill is complex. There is an extensive network of neurons between and within the muscle bundles of the gill and along the branchial nerve itself (Peretz and Estes, 1974) as well as a small gill ganglion that is located within the branchial nerve, at the point where it enters the gill. The gill ganglion of Aplysia californica contains peripheral gill motor neurons as well as cells whose axons remain intrinsic to the ganglion (Colebrook and Lukowiak, 1985). The functions of these latter cells are not yet known. The neurons in the gill peripheral network are thought to include mechanoreceptors, muscle receptors and muscle regulatory neurons (Peretz and Estes, 1974). The siphon nerve also contains peripheral cell bodies which have been characterized by Bailey et al. (1979).

The spontaneous pumping movements of the mantle organs are controlled by a command group of neurons (called the interneuron II network) that are located in the abdominal ganglion (Peretz, 1969, Kupfermann et al., 1974, Byrne and Koester, 1978). The spontaneous gill movement (SGM) generates a maximum contraction of the gill (Carew et al., 1979) and can be used as a standard against which to measure reflex responses as an index of behavioral state (Lukowiak, 1980, Ruben et al., 1981). During an SGM, the RDG and LDG cells receive excitatory input, the L7 and L9 cells, however, receive inhibitory input (Kupfermann et al., 1974) and then undergo rebound excitation.

The gill and siphon withdrawal reflexes to siphon stimulation are closely interconnected. They share the same mechanoreceptors and

interneurones (Hawkins et al., 1981a) and they are both capable of habituation, sensitization and classical conditioning.

Pinsker et al. (1970) first demonstrated habituation of the GWR in Aplysia with repeated stimulation of the siphon or gill. The reflex recovered within 130 minutes and could be dishabituated rapidly with strong brush strokes to the neck. Using semi-intact preparations, Kupfermann et al. (1970) measured a decrease in the excitatory responses of the gill motor neurons as the GWR habituated. The decreased responsiveness of the motor neurons and of the gill is believed by these investigators to be due to homosynaptic depression of neurotransmitter release from the central sensory neuron terminals. This hypothesis is supported by the demonstration that monosynaptic EPSPs evoked in L7 by an action potential in a sensory neuron decreased over three trials (Castellucci et al., 1970), even when neuronal excitability was decreased by the addition of high Ca^{2+} and Mg^{2+} concentrations to the bathing medium. A similar decrease in the EPSPs can be observed when the relevant neurons are isolated in culture (Rayport and Schacher, 1986). It has been demonstrated by Castellucci and Kandel (1974) that the number of quanta of transmitter released by the sensory neuron declines with the decrementing EPSP. Klein et al. (1980) reported a decrease in the voltage-dependent Ca^{2+} current measured in the sensory neuron soma. They proposed that this was due to an inactivation of Ca^{2+} current which directly caused a reduced amount of neurotransmitter release. Recently, however, Gingrich and Byrne (1985) concluded by mathematical modelling of the sensory neuron release process, that this synaptic depression cannot be fully accounted for by the observed

Ca^{2+} current kinetics and that a large component of synaptic depression is due to a reduction in the pools of releasable transmitter. Bailey and Chen (1983) found structural evidence to back up the physiological findings; the sensory neuron terminals of habituated animals had shorter and proportionally fewer active zones and fewer vesicles than controls.

No change associated with habituation could be detected in the input resistance of the motor neuron (Castellucci and Kandel, 1974). Furthermore, GWRs elicited by direct depolarization of L7 were identical before and after the presentation of habituating stimuli, and stimulation of L7 alone was not sufficient to induce habituation (Kupfermann et al., 1970). The authors suggested that postsynaptic changes do not contribute to the decrement in response amplitude. This evidence is not conclusive, however, because both sensory and motor neuron measurements were taken from the cell body; changes in Ca^{2+} current at the sensory neuron terminals and changes in input resistance at the motor neuron dendrites can only be inferred from such recordings. In addition, Lukowiak and Peretz (1977) and Jacklet and Rine (1977) reported, in direct contrast to the findings of Kupfermann et al (1970), that repeated depolarization of L7 resulted in habituation of the evoked GWR. Such depolarization can actually lead to enhancement, stability or depression of the evoked GWR depending on the ITI, impulse frequency and duration of individual trials (Lukowiak, 1977b). Synaptic depression and facilitation at the motor neuron terminals appears to supplement the plastic properties of the central sensory-motor neuron synapses.

Using a semi-intact preparation of Aplysia, with the abdominal ganglion removed, Peretz (1970) reported that the gill was still capable

of habituation, spontaneous recovery and dishabituation. The peripheral gill ganglion was reported to be necessary for this behavior (Peretz and Moller, 1974). More recently, however, a study in which the gill was completely isolated from both the central and peripheral ganglia has shown that the nerve plexus alone is capable of mediating habituation (Ruben and Lukowiak, 1982). It is not known how much of a contribution these peripheral loci make to habituation in the intact animal. Recently, (Goldberg, 1983, Goldberg and Lukowiak, 1984) it has been shown that habituation of gill withdrawals to siphon stimulation is transferred to the gill-evoked withdrawals, and vice versa, ie. stimulation of one receptive field affects the ability of another receptive field to activate the same reflexes. Similarly the two areas demonstrate cross-field dishabituation; siphon stimulation will dishabituate gill-evoked gill withdrawal and vice versa (Goldberg, 1983). Both transfer of habituation and cross-field dishabituation can be generated by the PNS in the absence of the CNS and by the CNS in the absence of the PNS (Goldberg, 1983). It has been proposed by Goldberg that heterosynaptic mechanisms contribute to habituation of these behaviors and that the PNS plays a major role in mediating reflex behaviors while the CNS expresses the behavioral state of the animal and modulates the PNS.

Studies revealing the complex interactions that appear to occur between the PNS and CNS have been examined in detail by Mpitsos and Lukowiak (1985). The CNS can exert both facilitatory and suppressive influences on the PNS (Peretz and Howieson, 1973, Peretz et al., 1976). The PNS is also capable of modifying the CNS input; repeated stimulation of efferent nerves to the gill in the absence of the CNS brings about a

decrement in gill contractions, a tactile stimulation to the siphon was found to cause an increase in the subsequent GWR (Jacklet et al., 1975, Lukowiak and Peretz, 1977). As the CNS was absent, such an increment of the CNS-evoked habituated response could only have been mediated by the PNS.

Initial studies on this system appeared straightforward in that a clear-cut 'monosynaptic depression' model of habituation seemed capable of accounting for observed behavioral phenomena. A more thorough investigation of various components of the system, however, has revealed multiple additional sites that may be involved in the behavioral decrements.

Considering the degree of interaction that seems to occur between the CNS and the PNS, both during normal reflex behaviors and during their modification by habituation and dishabituation, it would seem very likely that such interactions are involved in other forms of behavioral modification such as sensitization and associative learning. The current popular model for both sensitization and classical conditioning of the Aplysia defensive withdrawal reflex, however, involves mechanisms located entirely within the CNS at the same sensory-motor neuron synapses that mediate part of habituation (Hawkins et al., 1983, Kandel et al., 1983, Camardo et al., 1984).

The response of gill motor neuron L7 to a tap to the siphon or an action potential elicited in a siphon sensory neuron increases after a dishabituating stimulus to the neck (Castellucci et al., 1970, Kupfermann et al., 1970). The same synaptic connections involved in habituation can therefore be made more effective by activation of a second sensitizing pathway, this is referred to as heterosynaptic

facilitation (Kandel and Tauc, 1965). Quantal analysis has revealed an increase in the number of quanta released from the sensory neurons (Castellucci and Kandel, 1976). There also appears to be an increase in the density of active zones and vesicles in synapses of long-term sensitized animals (Bailey and Chen, 1983). Together with the demonstration that the postsynaptic input resistance - as measured in the cell body - does not change over the course of sensitization (Carew et al., 1971), these observations implicate the presynaptic sensory neuron terminals as the neural site of sensitization. Changes in the receptor sensitivity of the postsynaptic cell cannot be estimated as the neurotransmitter(s) at these synapses has not been identified.

The changes underlying this increased presynaptic release of neurotransmitter have been analyzed in detail. All of the ionic events that are believed to occur during sensitization have been observed in isolated nervous systems. To bring about presynaptic facilitation, Klein and Kandel (1978) used stimulation of the connectives-the nerves that would normally carry the sensitizing input to the AG. These authors also applied 5HT or injected cAMP into the sensory neurons. Both of these treatments can induce presynaptic facilitation (Brunelli et al., 1976). Klein and Kandel voltage clamped the sensory neuron during this sensitization training and reported that the action potential broadened due to an increased inward Ca^{2+} current, produced by a decreased opposing K^{+} current. The broadened action potential leads to an increase in the release of transmitter. All of the molecular events underlying sensitization are presumed to occur in the central sensory neuron terminals (Klein and Kandel, 1980). This model proposes

that terminals of the facilitatory neurons release 5HT on to the presynaptic terminals of the sensory neurons, this causes an increase in adenylate cyclase within the cell leading to a greater cAMP concentration in the terminals. Cyclic AMP is thought to activate a cAMP-dependent protein kinase which phosphorylates a membrane protein; the I_s (serotonin-dependent K^+) channel or a protein associated with it. As this K^+ current probably contributes to repolarization, the decrease in its conductance leads to a prolonged action potential and, therefore, a larger inward Ca^{2+} current and neurotransmitter release.

At least part of the source of facilitatory input to the sensory neurons has been identified as a cluster of neurons in the AG, called the L29 cells. Stimulation of the connectives is thought to activate the L29 cells and bring about facilitated neurotransmitter release (Hawkins et al., 1981b). Selective stimulation of individual L29 cells facilitates the synapse between the sensory cells and gill motor neurons as well as broadening the sensory neuron action potential and causing an increased calcium current (Hawkins, 1981).

Despite the convincing effect of 5HT on synaptic facilitation, Ono and McCaman (1984) and Kistler et al. (1985) failed to demonstrate the presence of 5HT in the L29 terminals. 5HT-immunoreactive fibres from unidentified cells were observed nearby, suggesting that the sensory neurons receive input from 5HT-containing cells but the L29 cells are not the source. The molluscan neuropeptides SCP_A and SCP_B have recently been found capable of a similar facilitatory effect by a closure of the

'serotonin sensitive' K^+ channel (Abrams et al., 1984). The L29 cells do not contain these peptides either (Kupfermann et al., 1984).

Because the studies implicating these molecular changes have all been carried out on isolated nervous systems, with no reports of simultaneous behavioral changes, the events that have been observed can only be considered as correlative to behavioral changes and not causative.

5HT has been demonstrated to play a more convincing role in sensitization of the tail withdrawal reflex in Aplysia. Walters et al. (1983) demonstrated simultaneous enhancement of the tail withdrawal reflex, tail motor neuron activity and the monosynaptic EPSP from the tail sensory neuron to the motor neuron. These enhancements followed either a shock to the tail or superfusion of the pleural ganglion with 5HT. Sensitization of the tail sensory neurons has been proposed to involve a similar 5HT-sensitive cAMP-mediated decrease in K^+ conductance (Walsh and Byrne, 1984a, 1984b, Occorr et al., 1986). SCP_B may also have similar effects to 5HT in this system (Occorr and Byrne, 1985). In the study of Walters et al. (1983) a semi-intact 'split-foot' preparation was utilized. This is the only report, until now, in which cellular changes and behavioral changes have been monitored simultaneously during tail-shock induced sensitization. It would be of interest to compare the variability in the behavioral and in the neuronal responses, as the model would predict them to be similar. Unfortunately, the authors do not include standard error bars in their only figure where group data for both cellular and behavioral changes are reported (Walters et al., 1983, Figure 3).

The present state of knowledge concerning sensitization in this system, then, is: "The physiological mechanism for sensitization is presynaptic facilitation" (Camardo et al., 1984)

By pairing a tactile stimulus to the siphon (the CS) and a strong shock to the tail (the UCS), Carew et al (1981) demonstrated that the gill and siphon withdrawal reflexes of Aplysia can be associatively conditioned. Classical conditioning has been claimed to reflect an amplification of the same process that underlies sensitization (Kandel et al., 1983). It should be noted, however, that a different UCS was used in the sensitization experiments described above. In their study, Carew et al. (1981) reported that animals receiving at least 15 paired stimuli demonstrated significantly longer durations of siphon withdrawal to the siphon tap within 15 minutes after training. Acquisition curves were included in this report. However, as with the study of Walters et al (1983) no standard error bars were included and the amount of variability between animals cannot be determined. The effects of sensitization (the control group) were found to have a 'delayed onset', they were not evident until 30 minutes after training. This latter demonstration poses problems for the proposed cellular mechanisms of associative learning (see below), as the effects of sensitization would be predicted to be manifest at least simultaneously to, if not before, the effects of classical conditioning. The observation of a delayed onset may suggest that other mechanisms are required before the behavioral change is expressed. Sensitization produced by headshock has little or no delay in onset (Pinsker et al., 1970); the two sensitizing stimuli are clearly not effectively the same.

Carew et al. (1981) also reported that paired training, when compared to unpaired training, induced conditioning of the gill withdrawal reflex. This latter demonstration is important as all later behavioral reports involved only the siphon withdrawal component of the reflex, despite the fact that gill motor neurons have been used for the cellular analyses.

Subsequently, differential conditioning of the siphon withdrawal reflex was reported (Carew et al., 1983). Tail shock was paired 15 times with either mantle- or siphon-stimulation (the CS^+) and specifically unpaired with stimulation of the other site (the CS^-). Significantly longer siphon withdrawal times were observed in response to the CS^+ than to the CS^- 30 minutes after training.

Hawkins et al. (1983) reported a cellular analogue of this differential conditioning in the isolated CNS, with the tail attached. Intracellular depolarization of two different sensory neurons was used as the CS^+ and the CS^- and shocks to the tail or to the pedal nerves (which innervate the tail) as the UCS. The CR that was measured in these preparations was the EPSP amplitude in either a siphon motor neuron, or in the gill motor neuron L7. The CS^+ neuron was found to evoke significantly larger EPSPs in the motor neuron after training.

Hawkins et al. (1983) reported a broadening of the action potential in the paired sensory neuron and suggested that it was mediated by a decreased K^+ conductance. In addition, intracellular stimulation of the motor neuron, in place of the UCS, was not sufficient to cause conditioning in this preparation, and hyperpolarization of the motor

neuron does not interfere with synaptic enhancement. Although these latter observations indicate that motor neuron activity is not sufficient or necessary for conditioning of the EPSP in response to the sensory neuron action potential, this does not eliminate a possible role for such activity in behavioral conditioning. The authors compared the summed pre-test and post-test data from the behavioral training and the cellular analogue. A correlation between conditioning of the siphon-withdrawal duration and of the motor neuron EPSP was demonstrated. Neither simultaneous training of the EPSP and the behavioral response nor an enhancement of EPSPs in brains dissected from previously conditioned animals have yet been reported. It is possible that other changes in the nervous system are required to take place before the behavioral learnt response is expressed.

Complementary to the findings of Hawkins et al., Walters and Byrne (1983) have demonstrated a cellular analogue of differential conditioning at the synapse between the tail sensory and motor neurons. Surprisingly, these authors used a semi-intact 'split-foot' preparation in this study but no simultaneous monitoring of behavioral changes was reported. There has been a preliminary report of differential conditioning of the tail withdrawal reflex itself (Ingram and Walters, 1984). In this case mantle shock was used as the UCS, in contrast to the tail shock used for the cellular conditioning, and tests were carried out 24 hours after training instead of the 10 minute interval allowed before testing the EPSPs.

Cellular analogues of associative learning have therefore been reported by these groups and a detailed molecular basis, involving an "activity-dependent amplification of presynaptic facilitation" has been

proposed to account for the behavioral conditioning (Hawkins et al., 1983, Kandel et al., 1983). 5HT can be substituted for the UCS and will increase the spike duration of the paired sensory neuron and cause a significant increase in cAMP activity within the cell (Occorr et al., 1985). There have been no attempts to demonstrate 5HT as a successful UCS for conditioning (or sensitization) of the gill or siphon withdrawal reflex.

The changes occuring within the sensory neuron during associative learning are considered to be an amplification of those underlying sensitization: Activity in the sensory neuron just prior to the arrival of the facilitatory input brings about a higher level of intracellular Ca^{2+} which may somehow interact with the 5HT-sensitive adenylate cyclase to cause an even greater increase in the intracellular level of cAMP (Kandel et al., 1983, Hawkins et al., 1983).

Hawkins and Kandel (1984) have suggested that this simple model for associative learning also forms the basis for higher forms of learning such as blocking, second-order conditioning and the effect of contingency; although only the latter of these has actually been demonstrated behaviorally in Aplysia (Hawkins et al., 1986).

In vitro associative conditioning of the GWR to a different sensory CS (light) was reported by Lukowiak and Sahley (1981). This preparation provides a means of simultaneously monitoring cellular and behavioral responses and therefore determining the existence of a causal relationship. More recently, simultaneous conditioning of the GWR and gill motor neuron EPSP was reported after 40 paired trials of a siphon tap (CS) and a train of taps to the gill (UCS) (Lukowiak, 1986). In

this study, the number of action potentials evoked by the CS in the gill motor neurons L7 and LDG1 was found to reach an asymptote after approximately 15 trials, at which point no further increase in response was observed. In contrast, the GWR continued to increase its response to the CS throughout the 40 trials. This training procedure did not prove successful in all of the preparations, and 'non-learners' were discarded (Lukowiak, personal communication).

Lukowiak's (1986) report serves to demonstrate further that the present model of Kandel and his co-workers may not be sufficient to explain the behavioral learning. Whether the proposed mechanism is necessary for acquisition or expression of the learned response has also not been determined. 'Off-field' sensory neurons, that do not receive input from the CS, display associative-dependent synaptic enhancement (Lukowiak, 1986), suggesting that activity in the sensory neuron is not necessary for at least the cellular analogue of learning.

The model for the molecular and cellular basis of associative learning in Aplysia californica may be one of the most detailed and concise of those offered so far. In contrast to the Hermissenda model, increased cellular responses are proposed to account for increased behavioral responses and the evidence that has built up over the years has largely complemented that found previously, rather than contradicted it. A causal relationship between the changes at the central sensory-motor neuron synapses and modulation of the withdrawal reflex, however, has not yet been established. Also, the effectiveness of this learning paradigm for inducing behavioral, and cellular, changes has not been determined.

As discussed earlier, research on Pleurobranchea, and many vertebrate systems, has suggested that many sites may be involved in the acquisition and expression of a learned response; such sites may operate quite independently, or they may interact to bring about the observed behavioral modification. Similarly, multiple sites may be involved in the mediation of associative learning in Aplysia.

The experiments discussed in this thesis were designed to determine whether classical conditioning and sensitization of the GWR, utilizing stimulus parameters very similar to those used by Kandel and his associates, can be demonstrated in an in vitro Aplysia preparation. To determine whether cellular changes could be induced in this preparation, and whether they correlated with behavioral changes, gill motor neuron responses were monitored simultaneously with the behavioral responses. The question of whether the cellular changes are necessary or sufficient for the behavioral changes was addressed. Changes occurring efferent to the motor neuron soma as a result of associative and non-associative conditioning, were also monitored. In addition, the morphologies of those motor neurons relevant to this study were characterized.

MATERIALS AND METHODS

I. The In Vitro Preparation

a. The Gross Dissection

Aplysia californica were purchased from Sea Life supply (Sand City, California) or from Pacific Biomarine Inc. (Venice, California). Animals were kept in a 1200 litre, gravel filtered tank filled with artificial seawater (Instant Ocean, Aquarium Systems), at a pH of 7.9, a specific gravity of 1.023-1.025 and a temperature of 15-16°C. Dried red seaweed (MRA marine products) was fed to the animals once weekly. Food-satiated Aplysia were not used for physiological experiments as they show suppressed gill behavior (Lukowiak, 1980).

Animals used for the conditioning experiments weighed between 75 and 200g, while those used for the morphological identification of the motor neurons weighed between 10 and 15g, (smaller neurons were preferred for the latter study to facilitate staining).

Four minutes prior to dissection animals were anaesthetised with isotonic (0.33M) $MgCl_2$. An amount approximately equal to 33% of the animal's body volume was injected into the haemocoel; this completely sedated the animals within 2-3 minutes.

The animal was initially pinned ventral side down to a cork board, with one pin through the tip of its tail and another through its head, rostral to the buccal mass and head ganglia (Figure 1a). The parapodia were pinned out to either side exposing the mantle cavity. The animal was periodically rinsed with seawater to wash away any ink and opaline

FIGURE 1

The two photographs show the initial stages of the dissection.

(a) The Aplysia was pinned to the board, ventral side down. The head (H) and tail (T) were pinned down, then the parapodia (P) were pulled apart and pinned down to expose the mantle cavity (M).

(b) The skin and body wall were cut from just rostral to the mantle shelf as far as the buccal mass. The crop (C) and gizzard (Gz) were then carefully removed. The mantle and attached gill (G) and siphon (S) could then be freed.

A

B

secretions and to keep the tissues moist. An incision was made just rostral to the mantle shelf and the skin and body wall were cut with scissors rostrally to the buccal mass. The mantle and attached gill, siphon and ctenidial organs were freed by cutting posteriorly through the skin and body wall around the left edge of the mantle (preserving the pericardial sac) and around the siphon base. At this point the hepatopancreas was removed from the left side of the animal by cutting the thin connective tissue around it. Both posterior and anterior ends of the digestive tract were then cut and the entire system was pulled away, carefully avoiding spillage of gut contents onto the animal (Figure 1b). The remaining body wall connections on the right side were cut, without damage to the underlying abdominal ganglion (AG). The mantle organs could now be lifted by the mantle shelf.

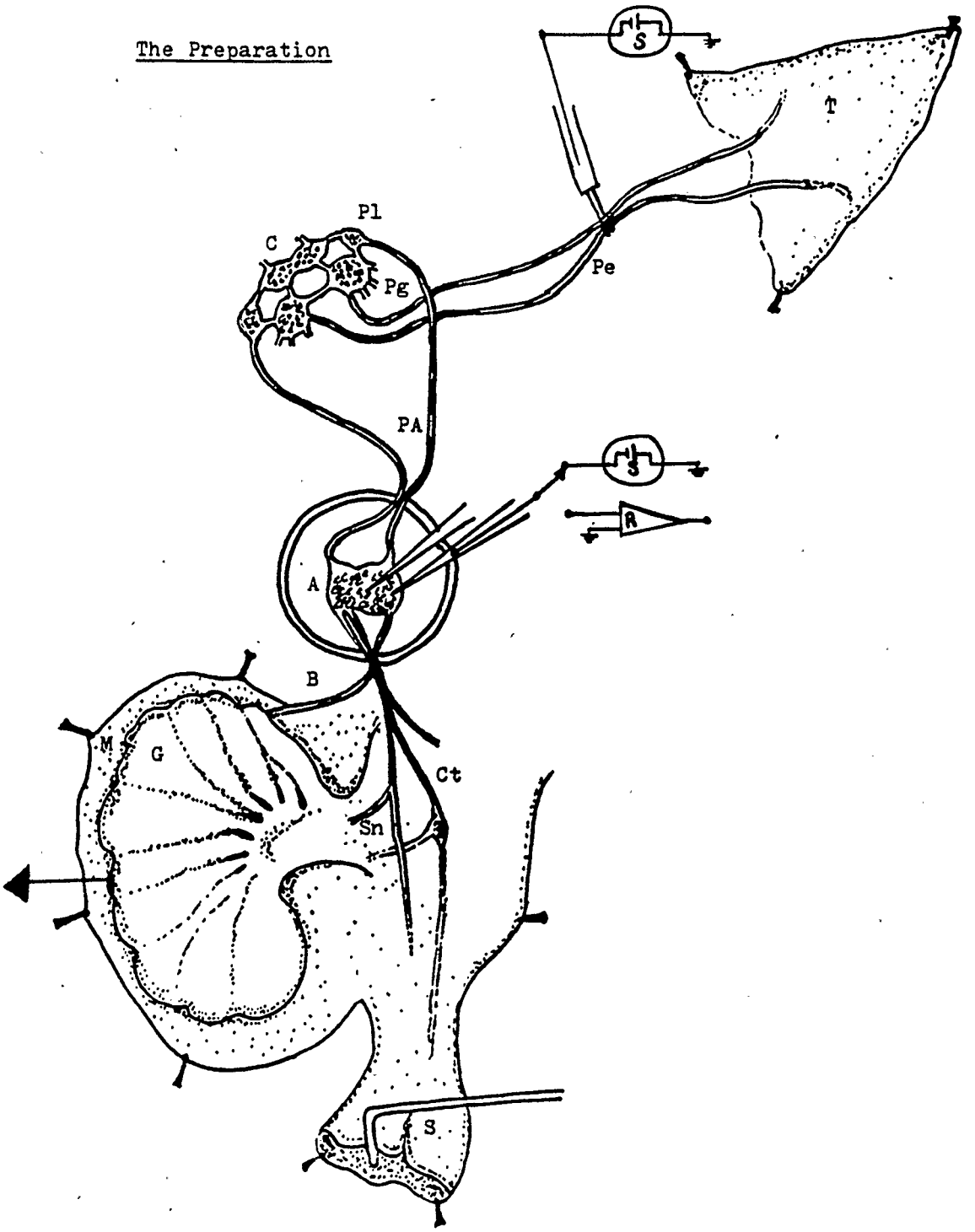
For anatomical experiments, the pleuroabdominal connectives and anterior aorta of preparations were cut just posterior to the position of the head ganglia, and the mantle organs with the attached AG were transferred to a seawater-filled experimental chamber.

The preparation used for the conditioning experiments was, however, more complicated (see Figure 2). The pleuroabdominal connectives were not cut; rather, the pleural, pedal and cerebral ganglia were gently freed from the surrounding tissue by cutting all of the surrounding nerves. Only the connectives to the AG and the two posterior pedal nerves (also referred to as the P9 nerves by Jahan-Pavar and Fredman, 1978) that exit the pedal ganglia and run down the ventral body wall to innervate the tail (i.e., the posterior part of the foot) were left intact. The pedal nerves were then carefully lifted and all of the

FIGURE 2

The preparation consisted of the siphon (S), gill (G), tail (T), abdominal ganglion (A), pleural ganglia (Pg) and cerebral ganglia (C). The gill and siphon were innervated by the branchial (B), ctenidial (Ct) and siphon (Sn) nerves from the abdominal ganglia. The tail was innervated by the pedal (Pe) nerves from the pedal ganglia. The head ganglia were connected to the abdominal ganglia via the pleuroabdominal connective nerves (PA). The gill was connected to a tension transducer by surgical thread secured to a single gill pinnule. Tactile stimuli were delivered to the siphon by a mechanical tapper. The abdominal ganglia were isolated in a chamber sealed with vaseline, the nerves passed underneath the chamber via a small vaseline filled notch. Intracellular recordings were made from motor neurons in the abdominal ganglia.

The Preparation



connectives attaching them to the body wall were cut. The tail was left attached to the pedal nerves and separated from the rest of the body by a transverse cut at the level of the posterior insertion of the parapodia. The mantle organs with the attached complete nervous system (buccal ganglia excepted), pedal nerves and tail were then transferred to the experimental dish and submerged in seawater.

b. Preparation for Conditioning Experiments

The preparation was pinned, dorsal-side-down to the clear Sylgard (Dow Corning Corp.) coated base of a lucite chamber. Pins were inserted through the body wall near the heart and close to the siphon; insect pins were used to secure the mantle shelf and to keep the siphon flat. The heart, anterior aorta, spermatheca and opaline gland were removed. In order to allow the AG to be pulled out from the organs the vulvar and spermathecal nerves were cut; the branchial, siphon and ctenidial nerves were left intact.

The AG was pinned, dorsal surface up, through the connective sheath surrounding the nerves. The head ganglia were pinned out in a similar manner to a separate platform and three pins were used to secure the tail, one on either side and one at the posterior end. A small plastic chamber (sealed with vaseline at the base) was gently placed around the AG, isolating it from the rest of the preparation. This enabled a lower fluid level to be maintained over the ganglion, facilitating both visualization of its neurons and application of sucrose containing sea water for desheathing.

The AG was left for fifteen minutes under a hypertonic sucrose/seawater solution (2M sucrose was diluted 1:1 with artificial

seawater (ASW); adapted from the procedure of Connor, 1979) before desheathing. (The sucrose osmotically brings about a slight shrinkage of the cells away from the connective sheath and facilitates removal of the sheath). Angled spring scissors were used to cut a single rostral - caudal incision along the midline, enabling fine forceps to be slid under the flap of sheath over the left dorsal surface of the ganglion, and the scissors used to remove the entire flap of sheath over this hemiganglion. The sucrose solution was immediately removed and the ganglion washed 4-5 times in ASW (Table 1).

A fine surgical thread (5-0) was connected to a single gill pinnule at one end, and to a force transducer (Narco myograph F60) at the other; the thread was allowed enough slack to avoid stretching the pinnule. Gill withdrawals could then be reliably recorded and measured on analogue (Tektronix, 5113) and digital storage (Nicolet, 2090-3) oscilloscopes and a four channel chart recorder (Gould 2400) simultaneously with the cell responses. The gill was not damaged by this procedure and the muscle did not fatigue (spontaneous movements remained constant throughout the training period).

A "tapper" was used to apply tactile stimulation to the siphon (the conditioned stimulus or CS). This consisted of a plastic wire, 1mm in diameter, which was connected to a solenoid. The duration and amplitude of the voltage applied across the solenoid coil determined the force applied to the siphon (see Peretz and Lukowiak, 1975).

Bipolar silver hook electrodes were used to deliver shocks (the unconditioned stimulus or UCS) to the pedal nerves. The electrodes were hooked underneath both nerves, whenever the nerves were to be stimulated

TABLE 1

COMPOSITION OF THE ARTIFICIAL SEAWATER

	Stock Solution (mM)	Final Concentration (mM)
$NaCl_2$	2560	425
KCl	2560	10
$CaCl_2$	341	10
$MgCl_2$	341	22
$MgSO_4$	512	2.5
Hepes		55
Ph	7.8	

they were lifted out of the seawater, in which they normally remained submerged, with the use of an X-Y-Z manipulator.

Preparations were left to rest for at least 45 mins following surgery before any of the cells were impaled.

II. Electrophysiology

The preparation was stabilized on an air table and trans-illuminated for viewing under a dissecting microscope. Single-barreled micropipettes (3M KCl filled) of 10-20 MΩ resistance were positioned with Leitz micromanipulators. A Getting M-5 or Dagan 8700 Cell Explorer electrometer containing a Wheatstone bridge allowed simultaneous current injection and recording through the electrode. An agar bridge coupled the seawater bath to a separate beaker of seawater containing a calomel reference electrode (Fisher Scientific). The signal from the amplifiers was displayed on the analogue and digital storage oscilloscopes and four-channel chart recorder. Data were also stored for subsequent analysis on floppy diskettes (Nashua MD1D and Sentinel 5S-12) and were reproduced with the use of an X-Y plotter (Hewlett Packard, 7470A). Original records that are presented in the text were all reproduced with this X-Y plotter. The CS and UCS were driven by the two channels of a Grass S88 stimulator and a second S88 was used to deliver single depolarizing pulses to the motor neuron(s).

One or two gill motor neurons (L7 and/or LDG1) were impaled and monitored in each preparation. Neurons L7 and LDG1 were identified using the criteria of Koester and Kandel (1977). Because the gill was still attached to the ganglion via the intact nerves, identification of the cells was based on the correlation of their activity with gill

behavior, as well as their position in the ganglion and their pattern of activity. The positions of cells within the ganglion can vary, as can their patterns of activity to a certain extent; the movement that is elicited when a gill motor neuron is depolarized, however, is unique to that neuron and is the best type of positive identification available when trying to locate these particular cells.

Neuron L7 is positioned at the left edge of the dorsal surface of the ganglion and is larger than the other cells around it. It receives bursts of inhibitory post-synaptic potentials (IPSPs) during spontaneous gill withdrawals and causes a spreading of gill pinnules at their bases and a closure of the gill halves (anti-flaring) when spiking is induced with depolarizing current. LDG1 is usually positioned directly medial to the large L7, just left of the rostral-caudal midline of the ganglion. This cell receives vigorous excitatory and inhibitory synaptic input and shows rapid bursts of activity during spontaneous gill withdrawals. Injection of sufficient depolarizing current to induce a series of action potentials in the cell brings about a rostral rotation of the gill and bunching of the pinnules. Both neurons are highly effective at moving the gill requiring only a few spikes to bring about a large and immediate contraction. Only cells satisfying all of these criteria were used in this study.

Responses of cells were measured precisely using the digital oscilloscope. Neurons were hyperpolarized, with about 5nA of current, throughout the conditioning sessions to reduce spontaneous activity and to enable measurements of the peak amplitudes of the evoked excitatory post synaptic potential (EPSP). In certain cases where cells could not be prevented from spiking in response to the CS, a pre-training test

response of zero action potentials was considered as a 100% response, one action potential on a later trial would then be considered as a 200% response, and so on.

III. Associative and Nonassociative Conditioning

a. The Conditioned Stimulus

A "tapper" stimulus was used of about 600 mg intensity and 50 msecs duration. This was just sufficient to cause an EPSP in the gill motor neuron(s) and often a small withdrawal of the gill. Once committed to an area of the siphon, the same patch of siphon skin was used as the stimulation site throughout the training period and tests.

b. The Unconditioned stimulus

The intensity of shocks applied to the pedal nerves (a 3sec, 10Hz train of 3 msec pulses) was sufficient to cause a brisk burst of action potentials in the motor neuron(s) and a large gill withdrawal. If habituation to this UCS occurred over the course of the experiment, the voltage was increased slightly.

c. Conditioning Protocol

The classical conditioning protocol that was used is illustrated in Fig. 3a. The UCS was specifically paired to the CS and delivered 500 msecs after the CS; this is considered the optimum interstimulus interval (ISI) for classical conditioning of the Aplysia defensive reflex (Kandel et al, 1983). The intertrial interval (ITI) was 5 minutes and each animal received 10 training trials. Gill withdrawal amplitude and cell EPSP amplitude in response to the CS were

simultaneously monitored throughout the ten training trials, and also at CS only test trials 5 mins prior to training and 30 mins after training. The preparation was left to rest between the 10th training trial and the test 30 mins later.

d. Sensitization Protocol

The CS and UCS were specifically unpaired in a control group of animals. As illustrated in Fig. 3b, the UCS was delivered 2.5 mins after the CS; the ITI was again 5 mins. These animals also received 10 trials and tests 5 mins prior to training, and 30 mins post training. Gill and cell responses were simultaneously monitored.

e. Habituation Protocol

To ensure that habituation would occur to the CS in the absence of the UCS, a third group of animals were presented with a pre-test, followed by 10 trials of CS only with an ITI of 5 mins and a final test at 30 mins after the last training trial.

IV. Depolarization of the Motor Neurons

The ability of the motor neuron(s) to move the gill was also monitored before and after training in some of the animals in both the conditioning and sensitization groups (see Fig. 3c).

Twenty minutes before the pre-test a 2-second depolarizing pulse of current was injected into the motor neuron. This was of a sufficient voltage to induce a steady train of between 10 and 20 action potentials.

FIGURE 3

CLASSICAL CONDITIONING: Experimental animals received paired presentations of the CS (siphon tap) followed 500msecs later by the UCS (pedal nerve stimulation).

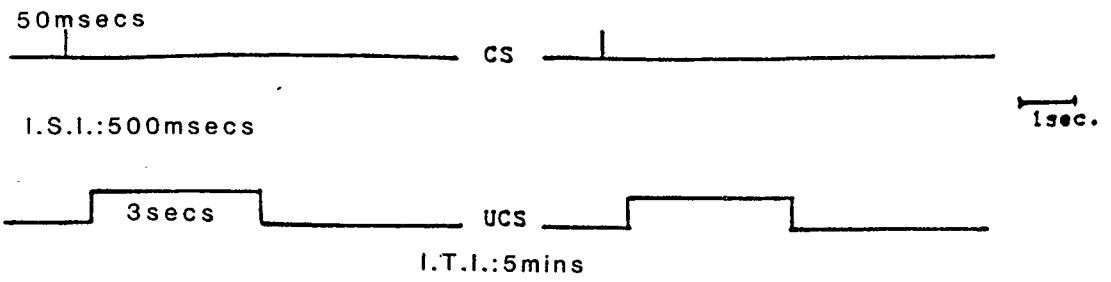
SENSITIZATION: Control animals received specifically unpaired presentations of the CS, followed 2.5 mins later by the UCS.

Both groups received 10 training trials with an intertrial interval (I.T.I.) of 5 minutes.

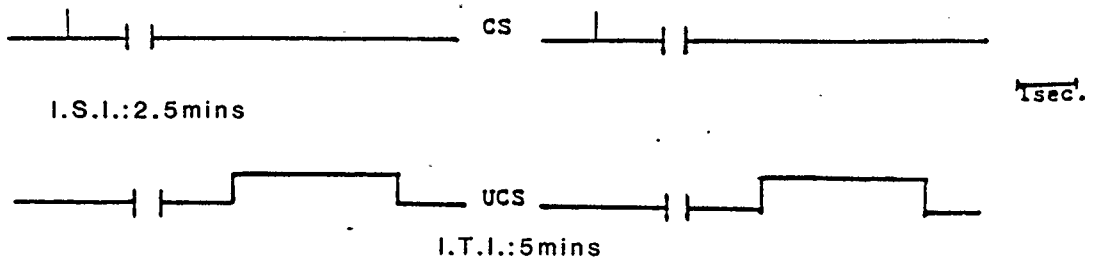
The response to the CS was tested before training (T(0)) and 30 minutes after training (T(30)).

MOTOR NEURON TEST: Before training, sufficient depolarizing current was injected into the gill motor neuron to induce a burst of action potentials. The same number of action potentials were elicited after training. The resulting gill withdrawal reflexes were measured.

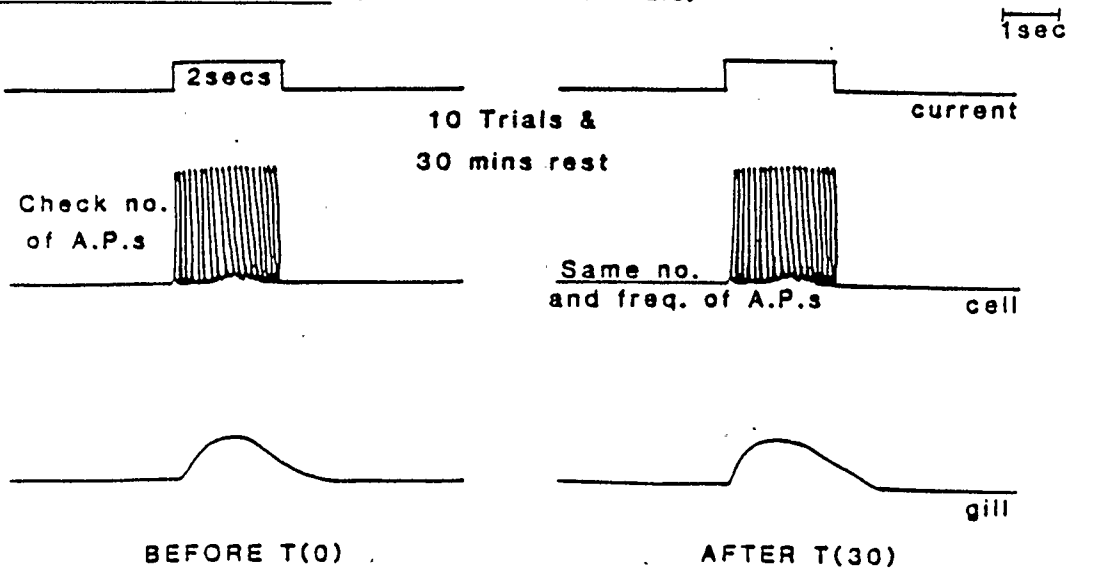
CLASSICAL CONDITIONING (n-22)



SENSITIZATION (n=11)



MOTOR NEURON TEST (24 cells/19 animals)



The gill withdrawal amplitude in response to this depolarization was recorded. An identical test was performed 5 minutes after the last CS-test, ensuring that the number and frequency of firing was identical to the original test.

V. Statistics

The comparison between sensitization-induced changes and classical conditioning-induced changes in CS-elicited gill and cell responses was made with the non-parametric Mann-Whitney U-test.

The respective distributions of outcomes following sensitization or classical conditioning were analyzed by means of a one-way chi-squared test.

All estimates for correlation were determined by the Pearsons Product Moment correlation coefficient (2-tailed).

The paired T-test (one-tailed) was utilized to contrast the pretest (T(0)) and post-training (T(30)) cell and gill responses to the CS. This same test was also used to analyze differences between gill withdrawals induced by motor neuron depolarization before and after training.

The 2-tailed independent Student's T-test was employed to compare original withdrawals elicited by LDG1 depolarization to those elicited by L7 depolarization and also to compare those changes that occurred, following training, in the withdrawals generated by LDG1 to those by L7.

The two-way chi-squared test was used to test for any association between the outcomes of training and the change in motor neuron elicited gill withdrawals.

Two populations of data were assumed to be different if $p < 0.05$.

VI. Morphological Characterization of the Motor Neurons

The preparation consisting of the mantle, siphon, gill and AG was transferred to a petri dish ($2cm^3$) and pinned dorsal side down to the clear Sylgard as in the physiology experiments. The AG was pinned out tightly on a small platform, but was not desheathed; the sheath in these small animals was easy to penetrate with the electrodes. The preparation was constantly perfused with ASW (Table 1) and epi-illuminated. Glass electrodes filled with 0.75M KCl (resistances of 10 to 20 MOhms) were used to initially identify the neurons by the criteria discussed earlier. This electrode was then removed and replaced with a Lucifer Yellow CH (LY) dye-filled electrode; the cells were therefore identified behaviorally and physiologically before introducing the LY electrode (only those cells satisfying all of the criteria for L7 and LDG1 were filled with dye).

The tip of the LY electrode had been backfilled with 3% LY, the rest was then filled with 0.1% $LiCl_2$ (Stewart 1978). Lucifer Yellow is in solution as a negative ion, therefore the dye was iontophoresed into the cell with 8nA of negative direct current; which was applied to the electrode for 1 to 2 hours. Diffusion of LY into the cell could be observed with the aid of a blue filter (BG-12, Schott) placed over the light source.

After injection, the mantle organs were removed by cutting the branchial, ctenidial and siphon nerves a short distance away from the AG. The ganglia were immediately placed in fixative (4% formaldehyde and 5% sucrose in ASW) and left at 4°C for 40 hours. Preparations were dehydrated by placing them for 15 mins in each of 70%, 80%, 90% and then

twice in 100% ethanol before clearing in methyl salycilate and wholemounted.

These LY preparations were visualised using the excitation filter (BP 436/8) and fluorescent emission filters (LP 500 and KP 600) of a Zeiss Universal epi-fluorescence microscope. Photographs were taken on Kodak Ektachrome 400ASA (color slide) and Tri-X (print) films through a permanently attached camera (Zeiss M35); exposure was determined by a Zeiss MC63 exposure meter.

RESULTS

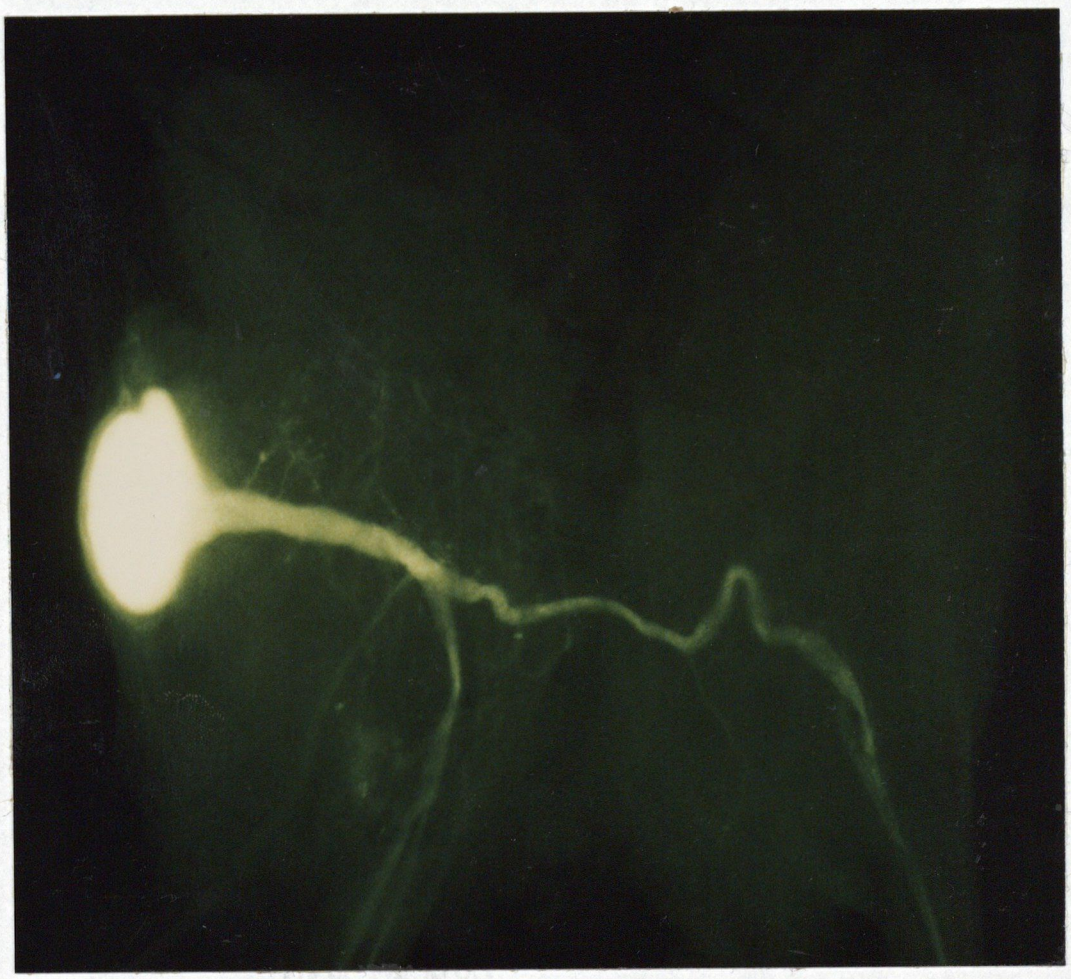
I. Morphology of the Motor Neurons

Optimum Lucifer Yellow fills were obtained by placing the ganglia in fixative immediately after filling with the dye. A previous report (Kaczmarek et al, 1979) in which Lucifer Yellow was injected into Aplysia bag cells, reported that the most successful fills were obtained after injecting the cells for a similar time period (30-120 mins.) and with a similar amount of current (2-20 nA), but with a delay of about 4 hours between injection and onset of fixation. A similar incubation interval, during which the ganglia were left in seawater prior to fixing was attempted, however this procedure was found to leave the cells with a washed out appearance and no visible axons.

The cells in Figures 4 to 6 were all from animals weighing between 10 and 15 grams. The only other report where the morphology of L7 has been examined by means of intracellular dye injection was that of Winlow and Kandel (1976), using cobalt chloride; the animals used in this latter study were larger (40-130 grams). The diameters of the L7 somas recorded in Winlow's study were, however, of a similar diameter to that presented in Figure 4. In addition, the ganglia were isolated from the mantle organs and the rest of the animal in their study, and the motor neurons were identified by topographical and physiological criteria alone.

FIGURE 4

The dorsal surface of the abdominal ganglia showing the gill motor neuron L7 injected with Lucifer Yellow. Axons can be seen in the branchial (bottom right), ctenidial (bottom middle) and siphon (bottom left) nerves. The axon in the ctenidial nerve forks into both the genital and pericardial branches. No axons were observed in either of the connectives (top right and left). There is extensive branching of the cell's dendrites in the left side of the ganglion. Magnification: 100x.



Cell L7 was found to send axons into each of the Branchial, Siphon and Ctenidial nerves and showed extensive dendritic branching within the left dorsal side of the abdominal ganglion (see Figure 4). The axon in the Ctenidial nerve sends branches into both the Pericardial and Genital nerves. As in Figure 4, the cell body of L7 is typically very large (150µm. x 240µm. in this case). This large cell body size, together with its position at the edge of the left dorsal surface of the ganglion, enables easy visual identification of L7 through the light microscope. When depolarized, the cell in Figure 4 elicited a large, reproducible gill withdrawal which was typical of that previously described for L7 (Kupfermann et al., 1974); a contraction of the whole gill and a closing together of the dorsal and ventral halves of the gill (antiflaring).

An example of an LDG1 cell that has been filled with Lucifer Yellow is shown in Figure 5. The axon can be seen to send branches into the Branchial nerve and into the Genital branch of the Ctenidial nerve. The dendritic branching of LDG1 is more localized around the area of the cell body than that of L7, but appears to be equally as dense. The cell body in Figure 5 measures 155µm. x 95µm. and lies just left of the caudal-ventral midline on the dorsal surface of the ganglion. There has been no previous report demonstrating a morphological identification of the branching pattern of LDG1 with intracellular dyes. The axon routes have, however, been previously identified by electrophysiological criteria (Kupfermann et al., 1974) and agree with the morphology revealed by the Lucifer Yellow filled cell shown in Figure 5. Depolarization of this cell also caused a large gill movement, in this

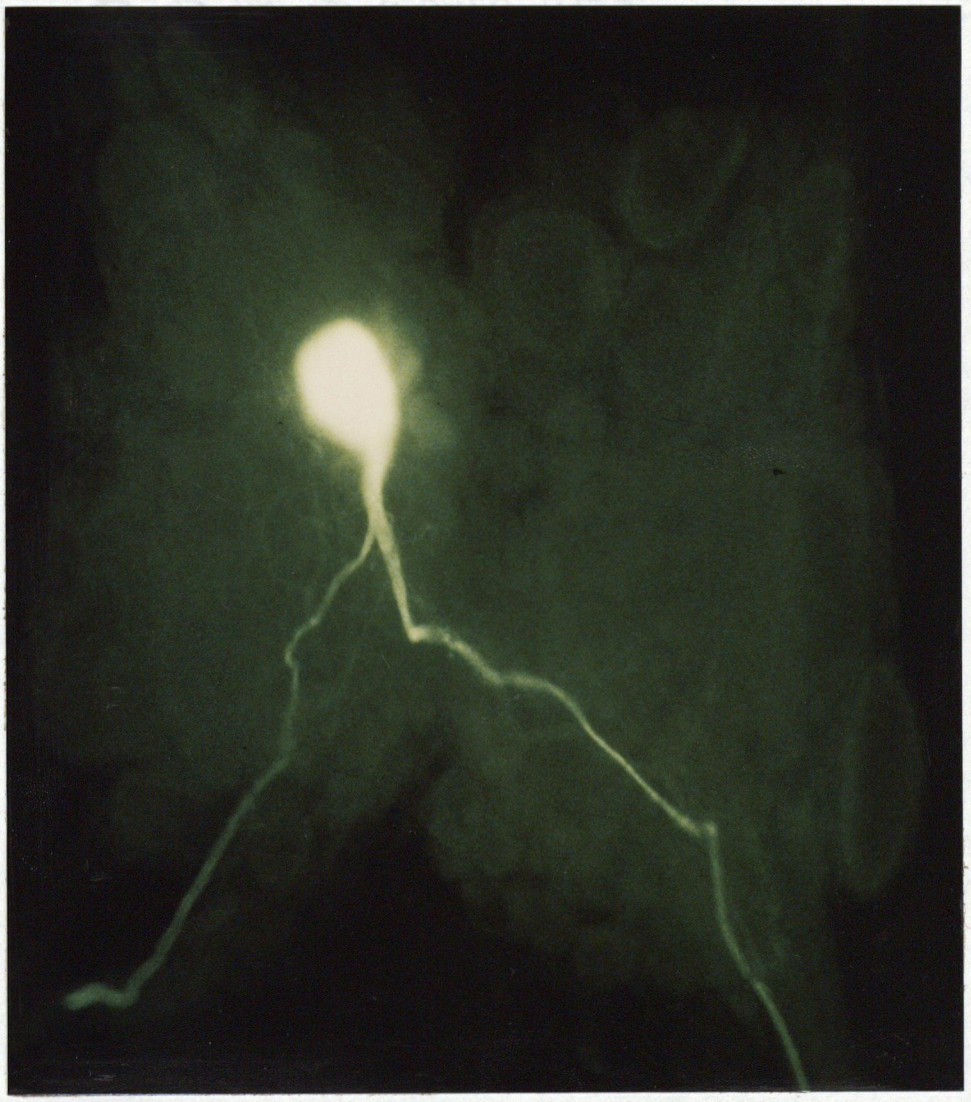
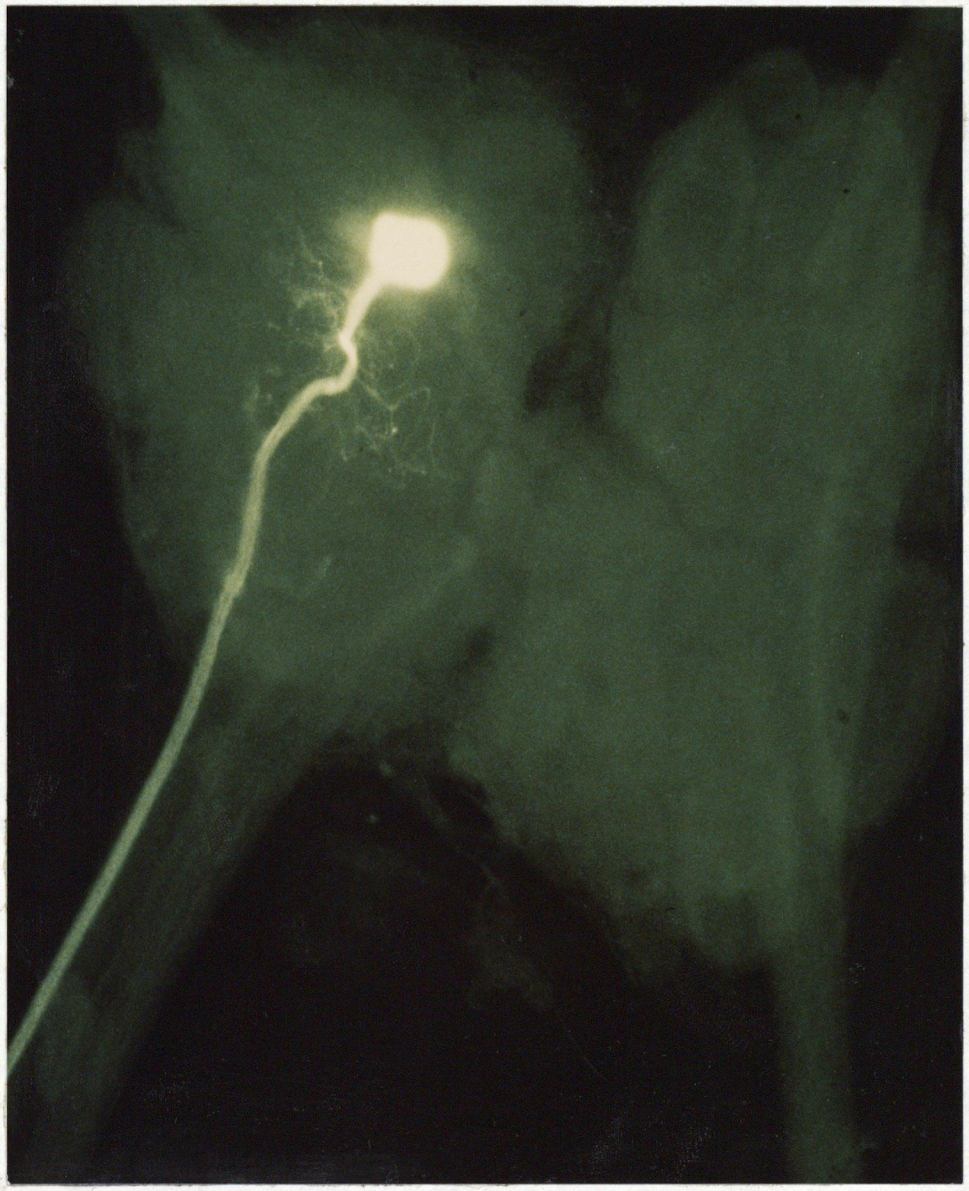


FIGURE 5

The dorsal surface of the abdominal ganglia showing the gill motor neuron LDG1 injected with Lucifer Yellow. The single axon branches into the branchial nerve (bottom right) and into the genital branch of the ctenidial nerve (bottom left). There is localized dendritic branching around the cell body. Magnification: 100x.

FIGURE 6

The dorsal surface of the abdominal ganglia showing the gill motor neuron L9 injected with Lucifer Yellow. The filled axon is seen to exit the ganglion via the siphon nerve. Magnification: 100x.



case the gill rotated and the individual pinnules drew together and displayed antiflaring. This type of withdrawal is similar to that described for LDG1 by Kupfermann et al. (1974).

In addition, a third motor neuron, which did not display the properties of LDG1 and L7, was filled with Lucifer Yellow (Figure 6). This cell is also located in the left dorsal half of the abdominal ganglion and its cell body dimensions are 90µm. x 100µm.. A small gill movement was produced by depolarizing the neuron; together with its position in the ganglion, this suggests that the cell is probably the gill motor neuron, L9 (Kupfermann et al., 1974). Only one axon can be seen to exit the ganglion from this cell's soma, and it leaves via the Siphon nerve. Although there has been no previous report of L9 having been filled with an intracellular dye, Lukowiak (1977) reported that this cell's axon innervates the gill via a small branch of the siphon nerve. The observed morphology in Figure 6, therefore, confirms this previous report. Dye-filled dendritic arborizations can also be detected within the left side of the ganglion.

II. Classical Conditioning and Sensitization

Certain problems were encountered over the course of the conditioning experiments. A major setback was that a regular absence was encountered of any cells within the abdominal ganglion that would drive the gill when depolarized. Although such cells could not elicit a gill withdrawal response, the gill muscle itself was still healthy as these preparations showed regular, large spontaneous gill movements, as does the intact animal. On several occasions many hours were spent penetrating every available cell body on the dorsal surface of the

ganglion with electrodes, only to find that none were capable of eliciting a gill withdrawal. Cells that otherwise displayed all of the characteristics of LDG1 or L7, but did not elicit a gill withdrawal when depolarized, are not included in this report.

Occasionally the preparation displayed rapid habituation to the UCS (depolarization of the pedal nerves) and sometimes only the gill and not the motor neurons, or just the cells but not the gill, would respond to the UCS. The preparations, therefore, appeared to be far more complex than originally suspected. Only animals that showed consistent gill and cell responses to the UCS were analyzed further.

Despite hyperpolarization of the motor neurons, cells would sometimes respond with action potentials to even the most gentle CS (siphon tap). In these cases the number of action potentials, rather than the EPSP amplitude was recorded as an indication of the cell's response magnitude.

Only preparations showing large, non-decrementing spontaneous gill withdrawals throughout the duration of the experiment were considered to be healthy and were included in this study.

In total, 22 animals fulfilled all of the above criteria in the classical conditioning (CC) group and 11 animals in the sensitization (sens) group. In addition, in 6 of the 22 classically conditioned animals, both motor neurons (L7 and LDG1) were monitored simultaneously. In the remaining 16 CC animals and 11 sens animals, either L7 or LDG1 was monitored.

a. Facilitation Occurs After Paired Presentations of the CS and UCS

The mean T(0) and T(30) values of all the cells and gills are presented in Figure 7. In the classically conditioned group (lower figure), there was a significant increase in the cell response to the CS following training (T_{21}=3.14, $p< 0.005$). Although the gill response to the CS had increased after classical conditioning, however, the difference in the behavioral response before and after training was not significant (T_{21}=1.55, $p> 0.05$). The lack of significant behavioral change following classical conditioning training appears to be due to the large variability in the T(30) gill amplitude values, because the actual mean increase in the gill response amplitude is greater than that shown by the motor neuron responses. No significant difference was found between the T(0) and T(30) responses of the cells (T_{10}=0.129, $p> 0.05$) or the gills (T_{10}=0.13, $p> 0.05$) in the sens group (upper figure).

In these in vitro preparations then, the motor neurons of the CC group showed a significant increase in their response to the CS as a result of paired presentation of the CS and UCS (classical conditioning). However, a significant behavioral change (gill withdrawal amplitude) as a result of such associative conditioning was not evident. Neither the motor neurons nor the gills displayed a significant increase in response to the CS as a result of specifically unpaired presentation of the CS and UCS (sensitization training).

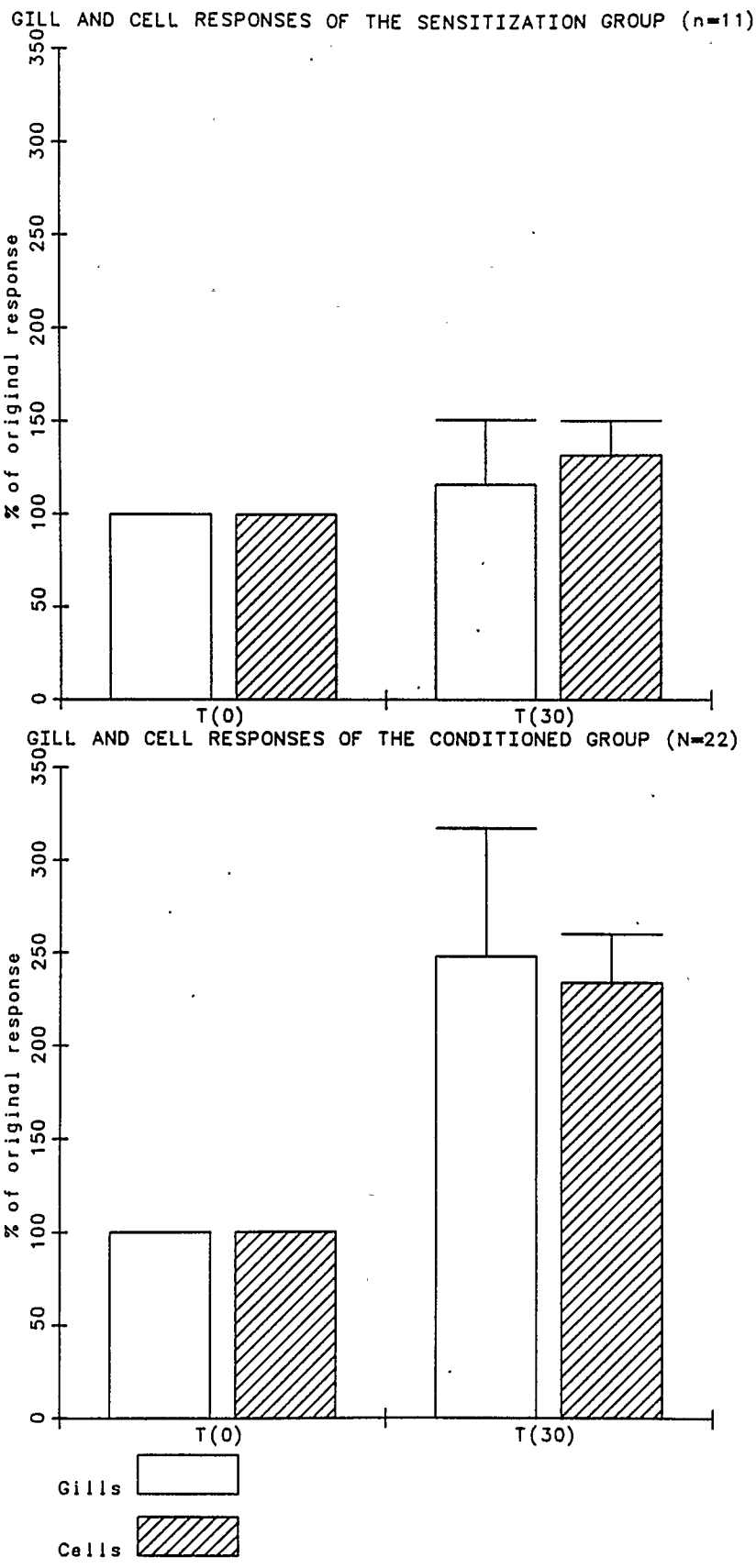
Using the isolated Aplysia nervous system, Hawkins et al., (1983) showed that, when an intracellularly induced action potential in the sensory neuron (the CS) was paired with pedal nerve stimulation (the UCS), the EPSP in the motor neuron became enhanced. As this data was

FIGURE 7

Mean responses to the CS before (T(0)) and after (T(30)) training. All amplitudes were normalized to the response at T(0).

There was no significant change in the gill withdrawal amplitude or the motor neuron EPSP amplitude after 10 unpaired trials (upper figure). The mean response of the gills in the sensitization group at T(30) was 116% and that of the cells was 133%.

After 10 paired trials (lower figure) the gill response to the CS was 248%. This increase was not, however, statistically significant due to the large amount of variability between individuals. The cell response, on the other hand, was significantly increased (to 234%) after classical conditioning training. (Error bars; +/- S.E.M.).


GILL AND CELL RESPONSES OF THE SENSITIZATION GROUP (n=11)
% of original response
0
50
100
150
200
250
300
350
T(0)
T(30)
GILL AND CELL RESPONSES OF THE CONDITIONED GROUP (N=22)
% of original response
0
50
100
150
200
250
300
350
T(0)
T(30)
Gills
Cells

found to correlate with behavioral changes observed by Carew et al., (1983) in the intact animal, it is of interest to compare the data presented here with that of these two previous reports. To facilitate its comparison to the data of Hawkins et al. and Carew et al., the data from Figure 7 are presented differently in Figure 8. In this figure the cell responses and behavioral responses are displayed on separate graphs and the effects of sensitization and classical conditioning training are compared on the same graph. In agreement with the findings of Hawkins and his collaborators, a significantly greater percentage change between T(0) and T(30) was found in the CS-elicited response amplitude of the CC group as compared to that of the sens group ($U_{22,11}=61$, $p< 0.05$). However, in contrast to the results reported in the in vivo preparation by Carew et al. (1983), such a significant difference did not exist between the percentage change in amplitude of the CC group behavioral response (gill withdrawal amplitude) and the percentage change in amplitude of the sens group behavioral response ($U_{22,11}=91$, $p> 0.05$).

A final analysis of the data was made focusing only on those cells and gills that showed an increased T(30) response (see Figure 9). It is now clear that both the behavioral and the cellular mean response is larger in the classically conditioned group than in the sensitization control group. Yet again, however, the increased cell responses were significantly greater in the CC group than those in the sens group ($U_{17,6}=22$, $p< 0.05$), while there was not a significant difference between the increased gill responses of the two groups ($U_{11,4}=14.5$, $p> 0.05$). The large variability between the behavioral responses of different animals was again apparent.

FIGURE 8

Mean responses to the CS before and after training with the behavioral (upper figure) and motor neuron (lower figure) responses displayed on separate graphs. The values are the same as those presented in the previous figure. Both the gill responses and the cell responses of the experimental group are enhanced when compared to equivalent responses of the control group. (Error bars; +/- S.E.M.).

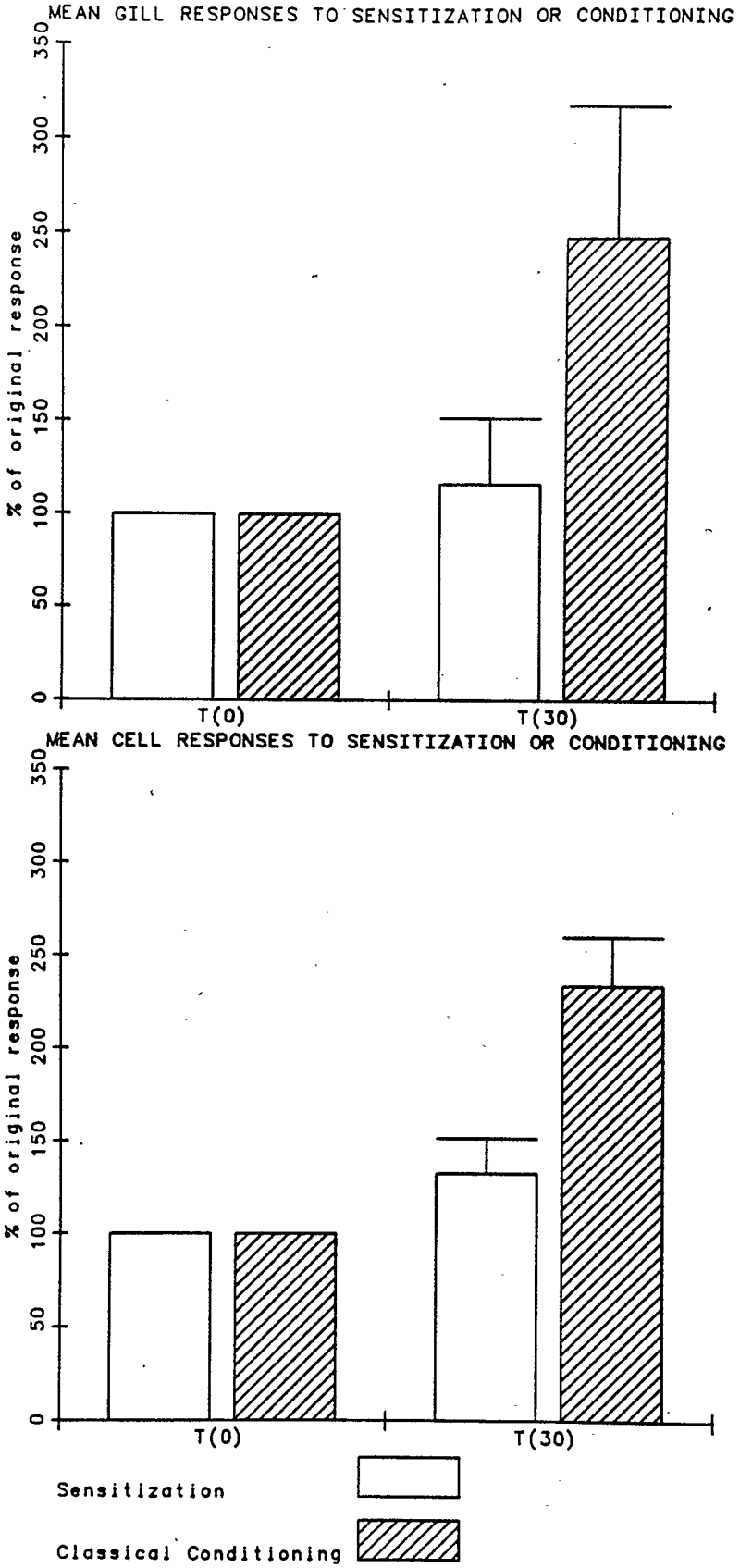
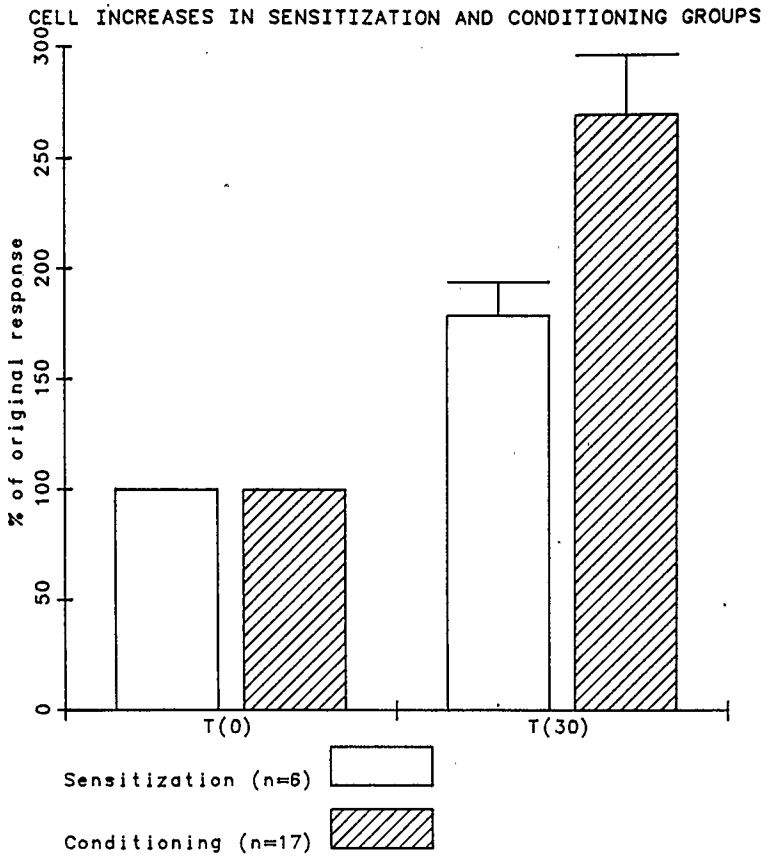
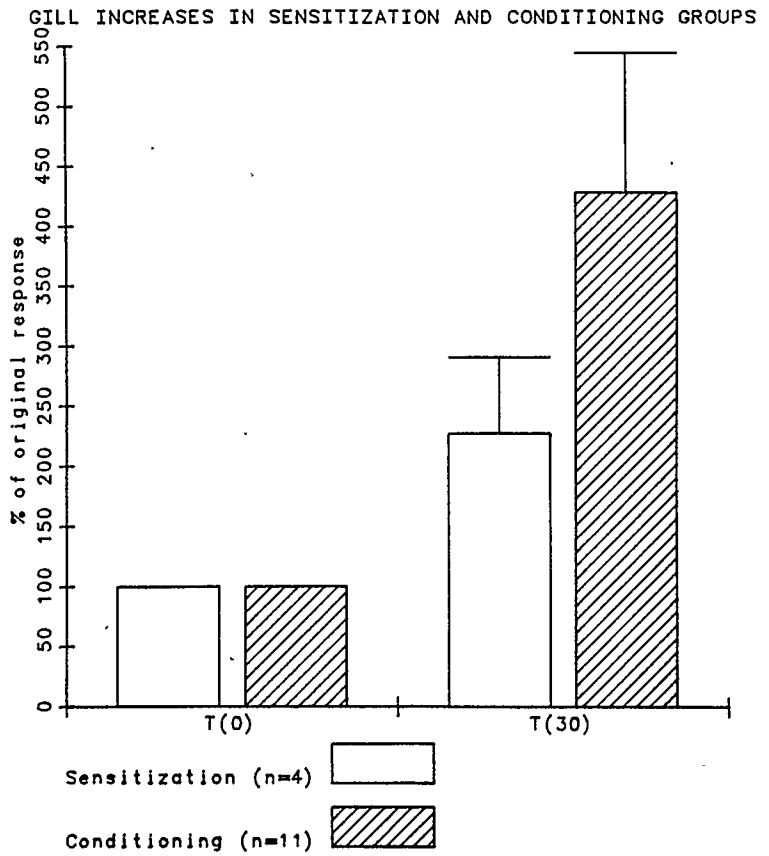

MEAN GILL RESPONSES TO SENSITIZATION OR CONDITIONING
% of original response
0
50
100
150
200
250
300
350
T(0)
T(30)
MEAN CELL RESPONSES TO SENSITIZATION OR CONDITIONING
% of original response
0
50
100
150
200
250
300
350
T(0)
T(30)
Sensitization
Classical Conditioning

FIGURE 9

Mean responses to the CS of the cells (upper figure) and gills (lower figure) whose responses were enhanced after training. All amplitudes were normalized to the response at T(0). Paired training produced a larger mean increase than unpaired training which is reflected in both the motor neuron and the behavioral responses. The mean facilitated response of the cells in the sensitization group was 180%, that of the cells in the classically conditioned group was 276%. The mean facilitated response of the gills in the sensitization was 227%, that of the gills in the classically conditioned group was 428%. (Error bars; +/-S.E.M.).


CELL INCREASES IN SENSITIZATION AND CONDITIONING GROUPS
% of original response
0
50
100
150
200
250
300
T(0)
T(30)
Sensitization (n=6)
Conditioning (n=17)


GILL INCREASES IN SENSITIZATION AND CONDITIONING GROUPS
% of original response
0
50
100
150
200
250
300
350
400
450
500
550
T(0)
T(30)
Sensitization (n=4)
Conditioning (n=11)

b. Classical Conditioning or Sensitization Training can Produce Four Different Types of Outcome

An increase in response amplitude was operationally defined as a T(30) amplitude of at least 110% of the T(0) response.

The assumptions made by Kandel and his co-workers are that changes observed in the motor neuron soma reflect those observed in the behavior itself (see introduction). Any increase or "learning" that occurs in the motor neuron's EPSP as a result of the conditioning procedure should, therefore, be apparent in the gill's response to the CS. As displayed on Table 2, of those preparations (n=22) that were subjected to a classical conditioning protocol, 45.5% showed increases in both the cell and gill responses to the CS at T(30). However, 31.8% of the CC group showed an increased cell response at T(30) in the absence of an increased behavioral response ie. the gill withdrawal response to the CS decreased or did not change as a result of training even though the EPSP in the motor neuron was facilitated. Also, a further 4.5% (one preparation) displayed an increased behavioral response without a concurrent cell increase. The remaining 18.2% of the CC group showed neither a cell increase nor a gill increase (ie. no evidence of 'learning').

In contrast to the classically conditioned group, only 27.3% of the animals in the sens group showed both increased cell and increased gill responses at T(30). The same proportion (27.3%) of sens animals displayed an increased cell response in the absence of an increased gill response. As in the CC group, only one animal (9.1% in this case) demonstrated an increased behavioral response and no increased cell response. Twice as many preparations in the sens group (36.3%) as in

TABLE 2

Summary of the responses of the gills and motor neurons to the CS at T(30). There was no correlation between the gill response and the response of the motor neuron at T(30).

BOTH: The percentage of animals that showed both cell and gill increases in response to the CS at T(30).

CELL ONLY: The number of animals that demonstrated a facilitated motor neuron response and no increase in the gill response to the CS at T(30).

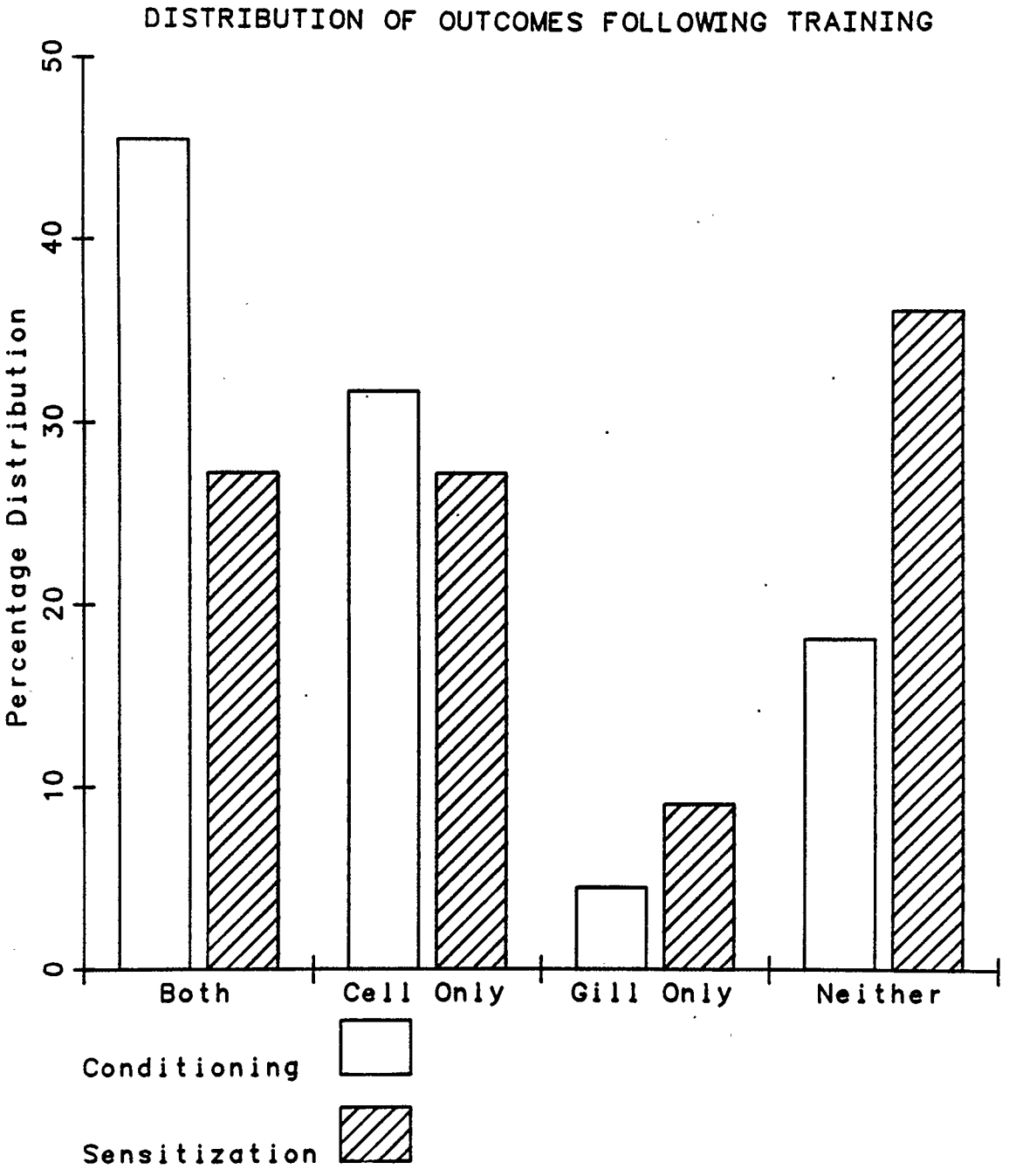
GILL ONLY: The number of animals that responded to the CS with an enhanced gill withdrawal but no facilitation of the motor neuron EPSP at T(30).

NEITHER: The number of animals that showed neither an increased gill nor an increased cell response at T(30).

	CLASSICAL CONDITIONING (n=22)	SENSITIZATION (n=11)
BOTH	45.5% (10)	27.3% (3)
CELL ONLY	31.8% (7)	27.3% (3)
GILL ONLY	4.5% (1)	9.1% (1)
NEITHER	18.2% (4)	36.3% (4)

FIGURE 10

Graphic representation of the distribution of response types after paired (conditioning) and unpaired (sensitization) training. The classically conditioned group displayed a greater tendency towards increased cell and gill responses after training than the unpaired group. Preparations in the unpaired group, on the other hand, were more likely to show neither facilitation of the cell or the gill responses.


DISTRIBUTION OF OUTCOMES FOLLOWING TRAINING
Percentage Distribution
0
10
20
30
40
50
Both
Cell Only
Gill Only
Neither
Conditioning
Sensitization

the CC group showed no change in gill and cell responses to the CS. These distributions of outcomes are illustrated in Figure 10. A chi-squared analysis was carried out to determine whether these two distributions were similar to each other, it was found that these two groups, ie. that following sensitization training and that following classical conditioning training, are not the same (X^2_3=30.58, $p < 0.01$). The CC group, therefore, displays a greater tendency towards increased cell and gill responses at T(30) than the sens group which shows more of a tendency towards neither cell nor behavioral increases after training. This affirms that the in vitro preparation can be classically conditioned and the changes observed after training are not purely due to nonassociative factors (ie. sensitization). There is an overlap between the responses of the two groups and some animals can be neither associatively or nonassociatively conditioned.

An example, from the classically conditioned group, of a preparation in which both the motor neuron and the behavioral response to the CS increased after training is presented in Figure 11. Before training, the CS elicited no gill withdrawal, and the cell responded with a small (about 14mV) EPSP. After training, however, there was an extremely large gill withdrawal in response to the CS and the motor neuron responded with several action potentials. It is also worthy of note that the depolarization elicited in the motor neuron by the CS demonstrated a longer duration after training than pre-training and that the spontaneous EPSPs in the motor neuron show a similar amplitude and frequency before and after training.

FIGURE 11

An example of a preparation from the "BOTH" response group. Recordings were taken from the gill (G) and motor neuron (MN) of a classically conditioned preparation. Before paired training the gill did not respond to the CS (arrow) and an EPSP of 14mV was detected in the motor neuron. After training a large gill withdrawal and several action potentials in the motor neuron were observed in response to the CS.

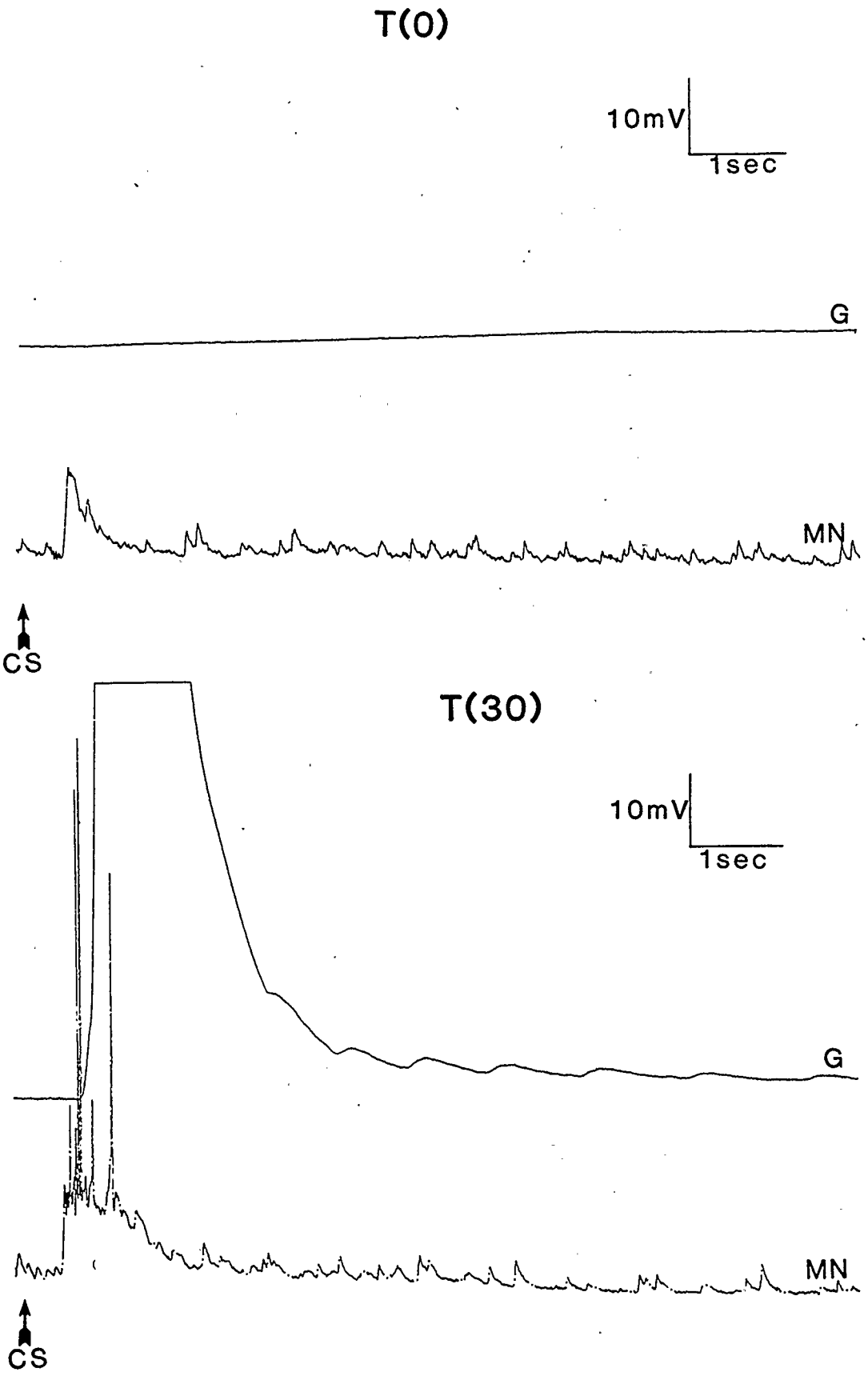

T(0)
10mV
1sec
G
MN
CS
T(30)
10mV
1sec
G
MN
CS

FIGURE 12

An example of a preparation from the "CELL ONLY" response group. This record was taken from a preparation in the experimental group. A slight gill withdrawal (G) was detected in response to the CS (arrow) before training, this response was not altered as a result of exposure to paired stimuli. The response of the motor neuron (MN), however, was greatly enhanced as a result of conditioning.

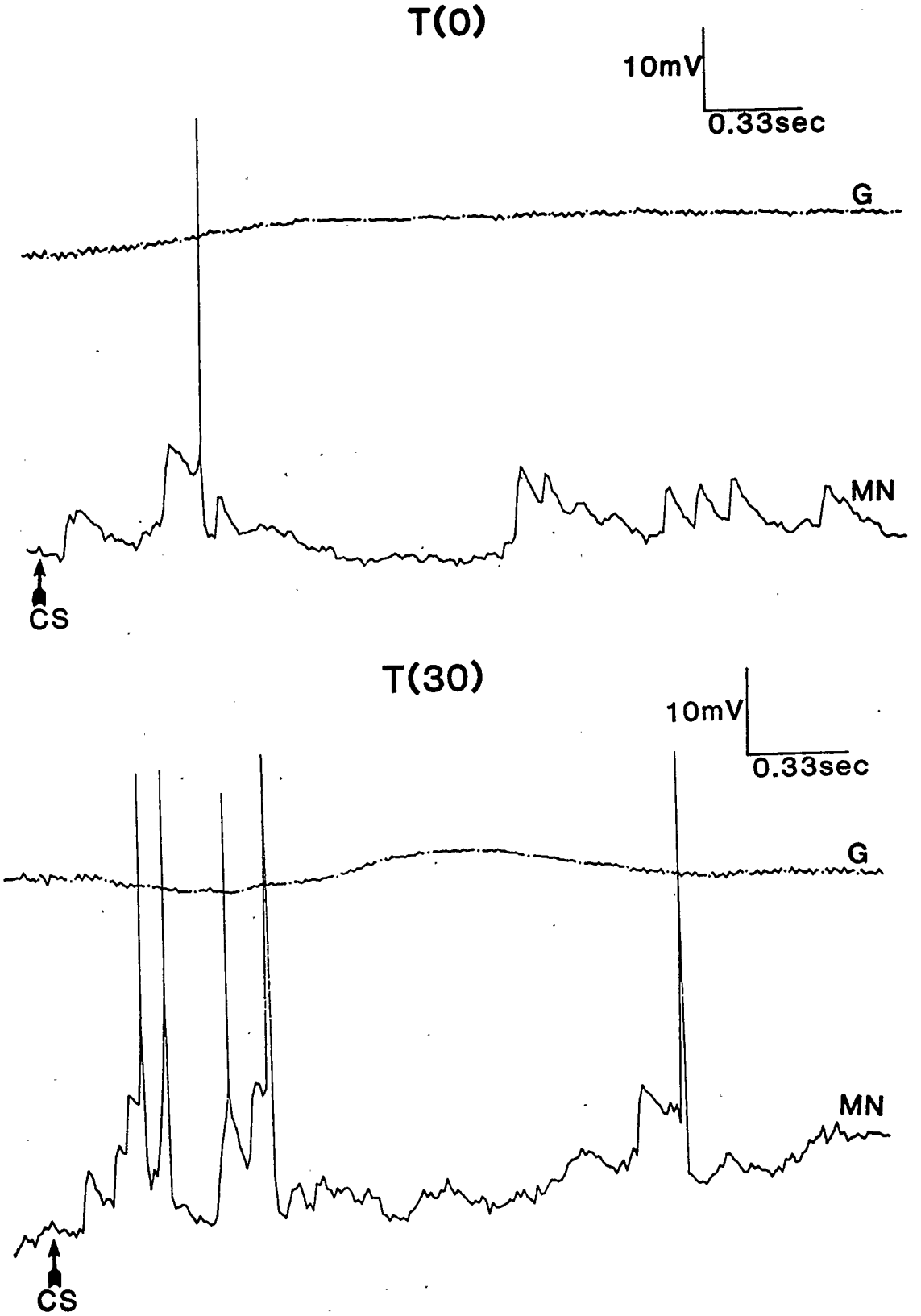

T(0)
10mV
0.33sec
G
MN
CS
T(30)
10mV
0.33sec
G
MN
CS

FIGURE 13

The cell and gill responses of the preparation from the control group that showed an enhancement of the gill response only (GILL ONLY) as a result of unpaired training. A small withdrawal of the gill (G) was observed in response to the CS (arrow) at T(0). This response was greatly facilitated at T(30). In contrast, the response of the motor neuron (MN) was not facilitated after training.

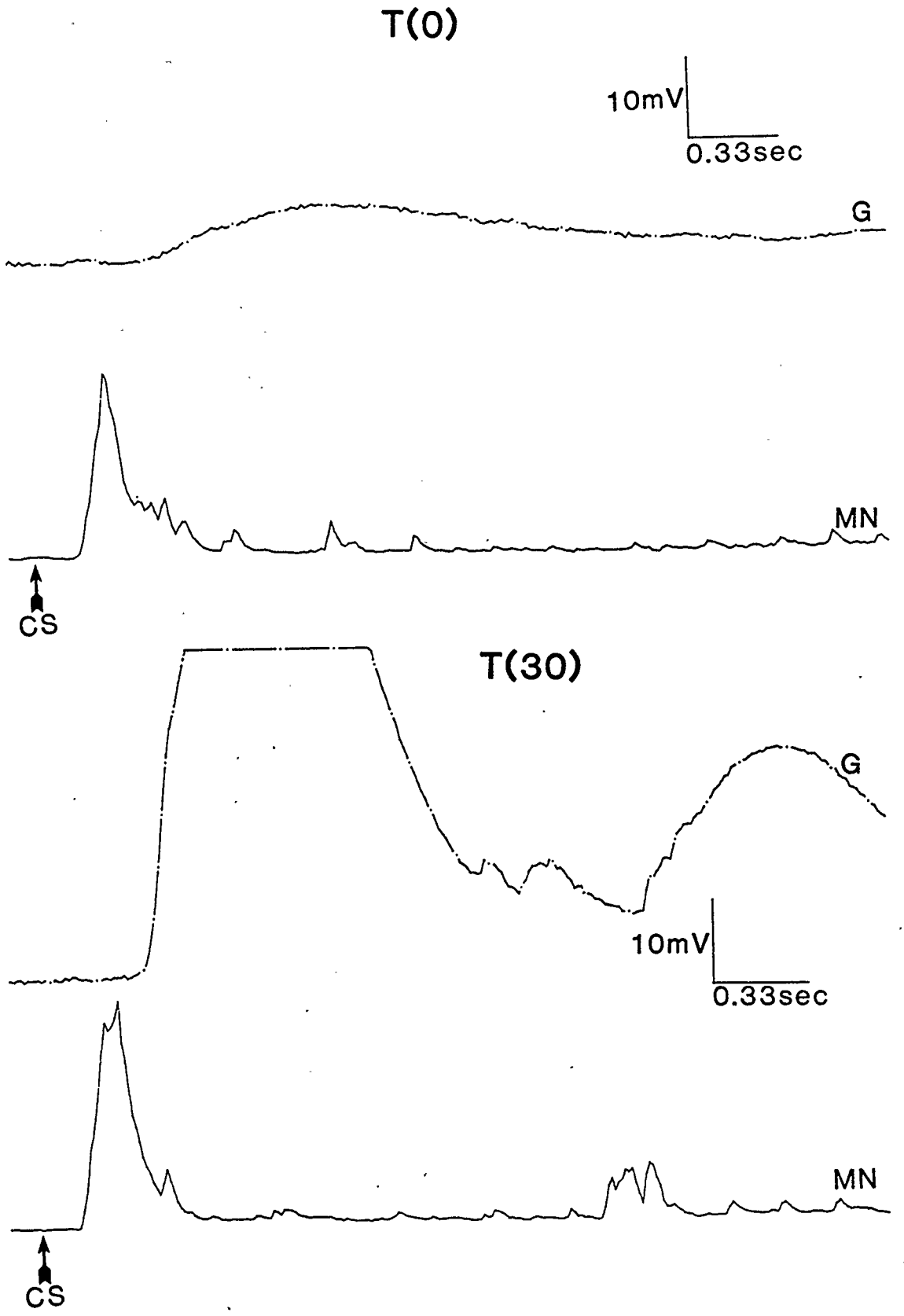
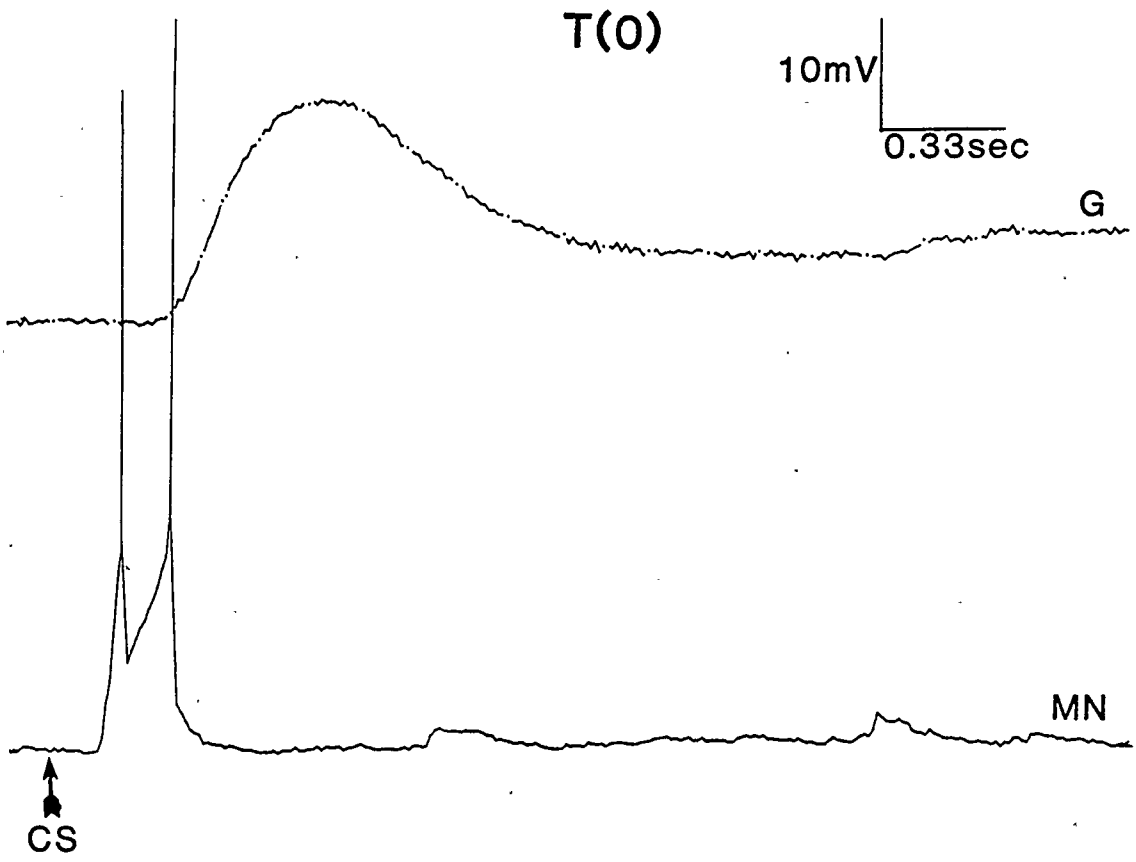

T(0)
10mV
0.33sec
G
MN
CS
T(30)
G
10mV
0.33sec
MN
CS

FIGURE 14

Recordings taken from the motor neuron and gill of a preparation from the classically conditioned group. This preparation showed neither an enhanced behavioral response or cellular response (NEITHER). The gill withdrawal response (G) to the CS (arrow) showed a very slight decrease after the paired training trials and the motor neuron response (MN) to the CS did not change.


T(0)
10mV
0.33sec
G
MN
CS

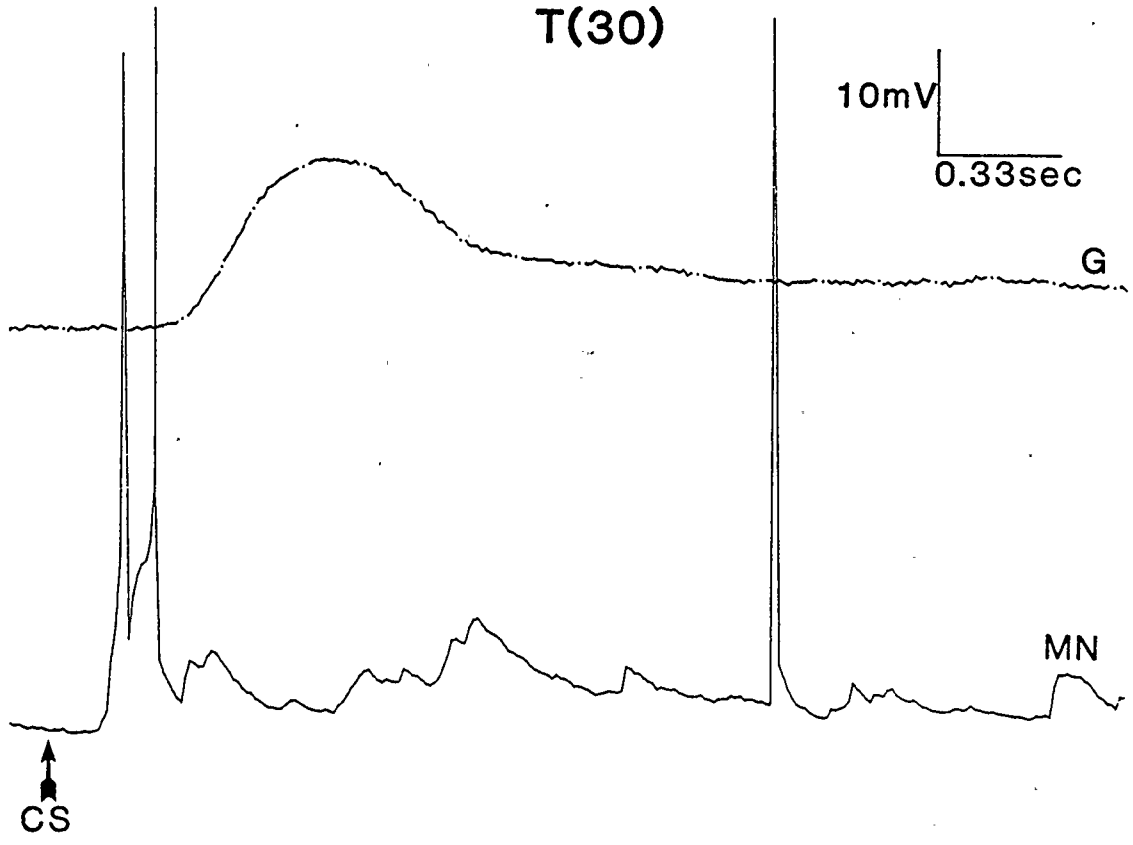

T(30)
10mV
0.33sec
G
MN
CS

Figure 12 depicts an example of a preparation, again from the classically conditioned group, that demonstrated an increased motor neuron response but no change in the gill withdrawal response. In this case, the CS elicited a small EPSP and one action potential before training, this response increased to four action potentials following training. The gill displayed a slight withdrawal to the CS before training, but this response remained the same after training.

The responses of the preparation from the sensitization group which demonstrated an enhanced gill withdrawal in the absence of an enhanced EPSP is shown in Figure 13. The gill responded to the CS with a small withdrawal before training, this response was greatly enhanced after training. The cell response on the other hand did not show an increase after training of more than 110%.

Finally, the responses of one of the preparations that showed neither an increased motor neuron or increased behavioral response is presented in Figure 14. The CS-elicited gill withdrawal decreased following training, whereas the response of the motor neuron did not change. This example was also taken from the classically conditioned group.

c. Gill and Cell Responses do not Correlate over the Course of Training

Figure 15 shows the cell and gill responses of a representative preparation from the sens group and from the CC group. The normalized response is plotted for each of the 10 training trials and for the final test (T(30)). In this particular case the non-associatively conditioned (sens) preparation was included in the 'cell only' outcome group, as the gill shows a decreased response at T(30), whereas the cell response is increased. The associatively conditioned (CC) preparation was included

in the 'both' group, as both the gill and cell responses are larger at T(30) than at T(0).

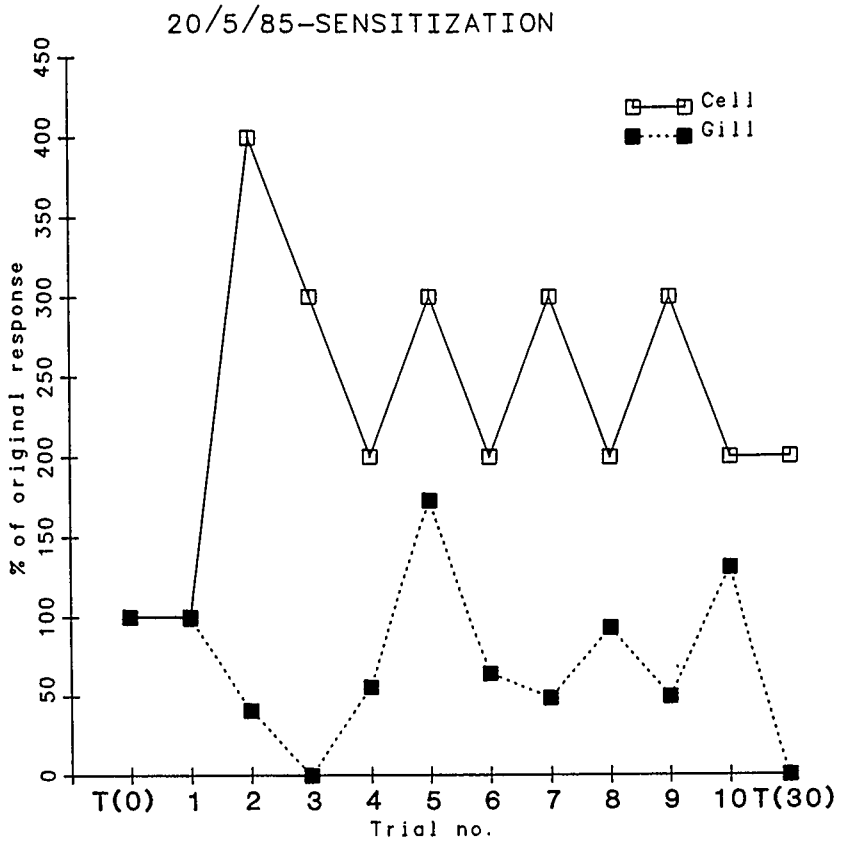
It is clear from Figure 15 that there is a large amount of variability between trials and there appears to be a distinct lack of correlation between the behavior of the motor neurons and of the gill. This is particularly evident on trials 2 and 3 of the sensitization example, the cell response is increased while the behavioral response is decreasing, and likewise on trials 4 and 5 of the CC example.

In order to determine whether any correlation exists between the responses, to the CS, of the motor neuron and the gill, a Pearson's Product Moment Correlation test was used to compare the overall mean responses of the gills and cells on each individual trial. 'Within animal' correlation was also estimated by comparing each animal's gill and cell response on T(30). No correlation was found to exist between the mean gill and the mean cell response values on each trial (r_{10}=0.53, $p > 0.05$) or between each individual animal's gill and cell responses on T(30) (r_{31}=0.32; $p > 0.05$).

The observation that the size of the EPSP measured in the motor neuron soma does not correlate with the gill withdrawal amplitude indicates that changes observed in the motor neurons of an isolated nervous system are not representative of changes in the behavior. The correlation that has been suggested to exist by Hawkins et al. (1983), therefore, does not exist when the cell responses and gill responses of the same animal are compared and the correlation is statistically tested.

FIGURE 15

CS responses of the gill and motor neuron on individual trials over the course of training. One representative animal from the control group (upper figure) and one representative preparation from the experimental group (lower figure) are presented. All amplitudes were normalized to T(0). The control animal was included in the "CELL ONLY" group, as the gill response had decreased to zero at T(30). The experimental animal was included in the "BOTH" group as both cell and gill responses were facilitated at T(30). A large amount of variability exists in the response amplitude from one trial to another. No correlation was found between the gill and cell responses of individual animals.


20/5/85-SENSITIZATION
Cell
Gill
% of original response
0
50
100
150
200
250
300
350
400
450
T(0)
1
2
3
4
5
6
7
8
9
10
T(30)
Trial no.

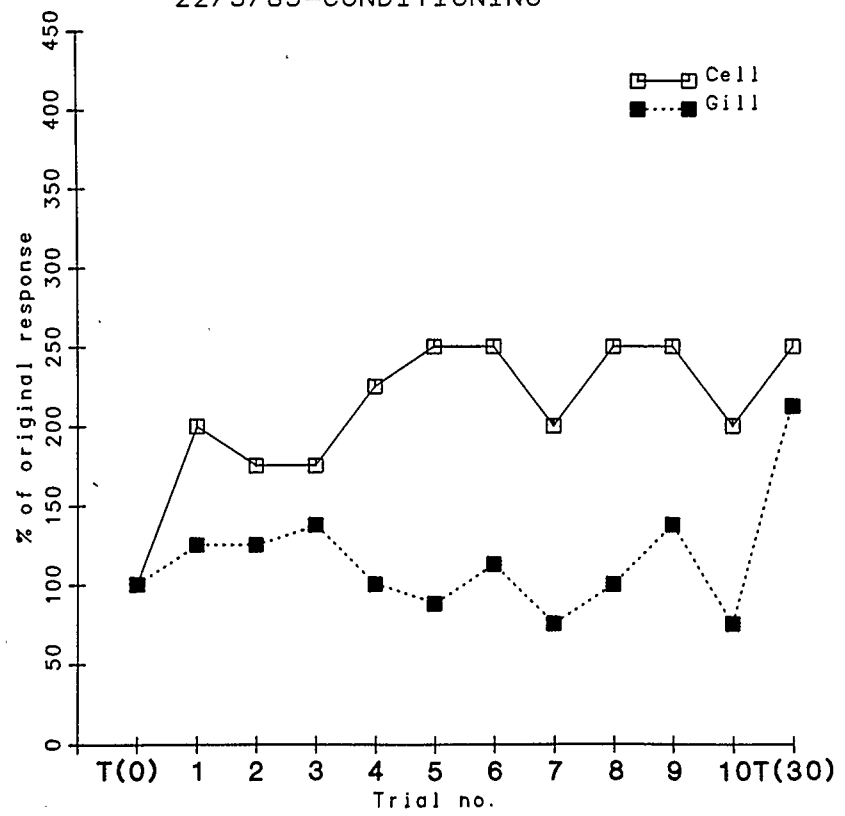

22/3/85-CONDITIONING
Cell
Gill
% of original response
0
50
100
150
200
250
300
350
400
450
T(0)
1
2
3
4
5
6
7
8
9
10
T(30)
Trial no.

FIGURE 16

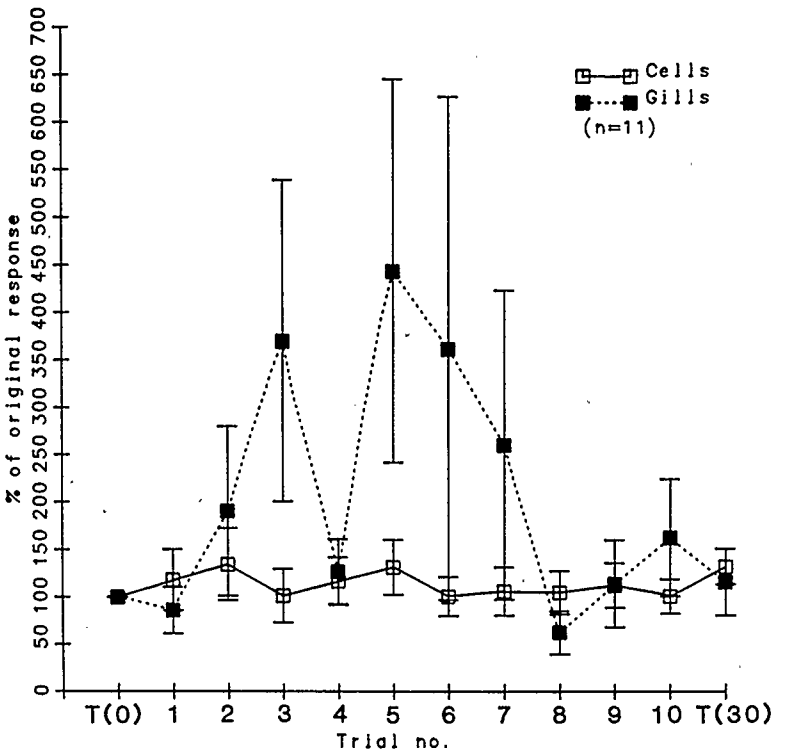
The mean gill and motor neuron responses over the course of sensitization (upper figure) and classical conditioning (lower figure). All amplitudes were normalized to T(0).

The mean cell response of the sensitization group remained stable throughout training and was slightly facilitated at T(30). The mean cell response of the experimental group gradually increased over the course of learning and was greatly enhanced at T(30). This cell response curve has the form of a typical acquisition curve.

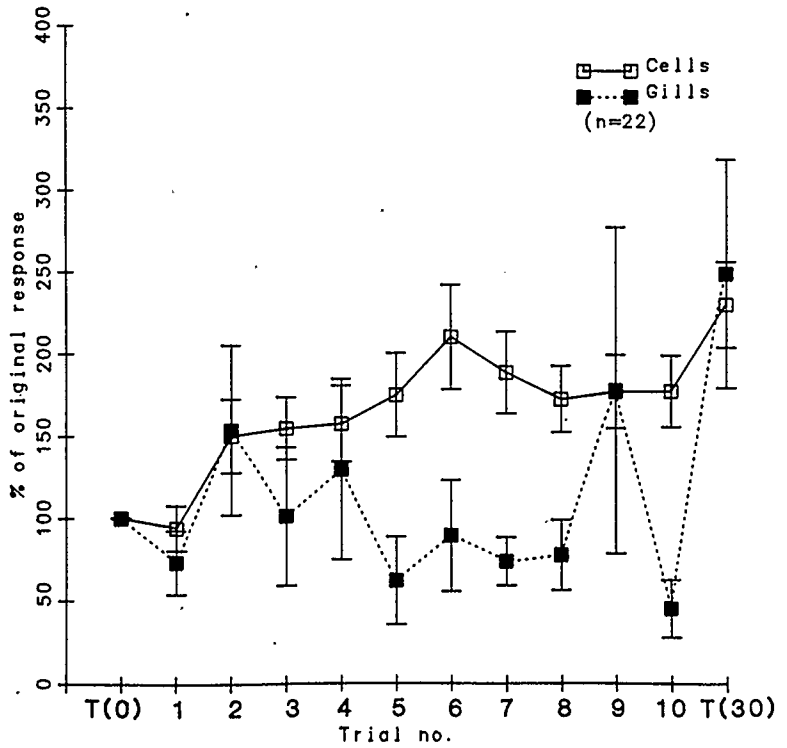
The mean gill response of the sens group was greatly enhanced early on during training, but decreased on later trials. In contrast, the mean gill response of the classically conditioned group showed only a slight enhancement early on during training, but was greatly facilitated at T(30).

There was a considerable amount of variability in the behavioral responses between animals and between trials. In contrast, the motor neuron responses show little variability between trials or between preparations. No correlation was found between the mean gill and cell responses on each trial. (Error bars; +/- S.E.M.).

MEAN RESPONSES OVER THE COURSE OF SENSITIZATION



MEAN RESPONSES OVER THE COURSE OF CONDITIONING



To examine whether any correlation exists between the responses of motor neurons within the ganglion, both LDG1 and L7 were impaled and monitored in some of the preparations. In 83% of these preparations, the cells showed a similar response at T(30). Thus, if the input to one cell increases, the input to the other cell would also be expected to increase. This suggests that the facilitation that occurs between the central sensory neurons and motor neurons occurs at all of the synapses simultaneously. The disparity observed between the gill responses and the motor neuron EPSPs represents a disparity between amplification of presynaptic facilitation in the abdominal ganglion and enhancement of the gill withdrawal reflex.

The mean gill and cell responses over the course of sensitization and conditioning are illustrated in Figure 16. The cell responses in the CC group can be seen to gradually facilitate over the course of conditioning, culminating in a mean T(30) value of about 230% that of the T(0) response. The cell responses in the sens group do not show this gradual enhancement but maintain a stable mean response of about 100% throughout the course of training and at T(30). In contrast to these cell responses, the behavioral responses show a large degree of variance between preparations (indicated in particular by the large error bars on trials 3, 5, 6 and 7 of the control group responses in Figure 16, and on trial 9 of the experimental group responses). The behavioral responses also show a large variance between trials; the gills of the CC group show no sign of the gradual response enhancement demonstrated by the cells of the same animals, and the gill responses of the sens group show a mean response value as high as 443% that of the

T(0) response value on trial 6 despite a facilitation of only 116% at T(30).

Learning curves for behavioral data, such as those in Figures 15 and 16, have not previously been presented in reports on classical conditioning of this defensive withdrawal reflex in Aplysia (eg. Carew et al., 1983). It is possible that such large variability between animals and between trials has been observed previously. When the acquisition curve for every fifth trial was plotted (Carew et al., 1981), error bars were not included, the amount of variability between animals in this previous report therefore cannot be compared to the data presented here.

III. Habituation

When habituation training was attempted a rapid and consistent decrease in the response of the cell and the gill to the CS was observed, this response decrement persisted after a 30 minute rest (ie. at T(30)). Because of the consistency of their responses, only 3 preparations were subjected to habituation training. The mean T(30) values following presentations of the CS alone are presented in Figure 17. In particular, a notable decrease in the gill response at T(30) is evident (26% of the original response). The mean cell response at T(30) is 88% of the original response. All of these preparations displayed a decreased T(30) value, behaviorally and cellularly, in response to the CS. An example of a habituated response is shown in Figure 18, where one of the three habituated preparations demonstrates the typical decreased gill withdrawal and decreased motor neuron responses.

FIGURE 17

Habituation of the gill and motor neuron response to the CS. The CS was presented alone for 10 trials with an I.T.I. of 5 minutes. After a 30 minute rest the response of the gill and the cell to the CS was recorded. All amplitudes were normalized to T(0). The behavioral response demonstrated a large decrement at T(30) to 26.5% of its response at T(0). The motor neurons showed only a small decrement in comparison, to 88.1% of their original response.

(Error bars; +/-S.E.M.).

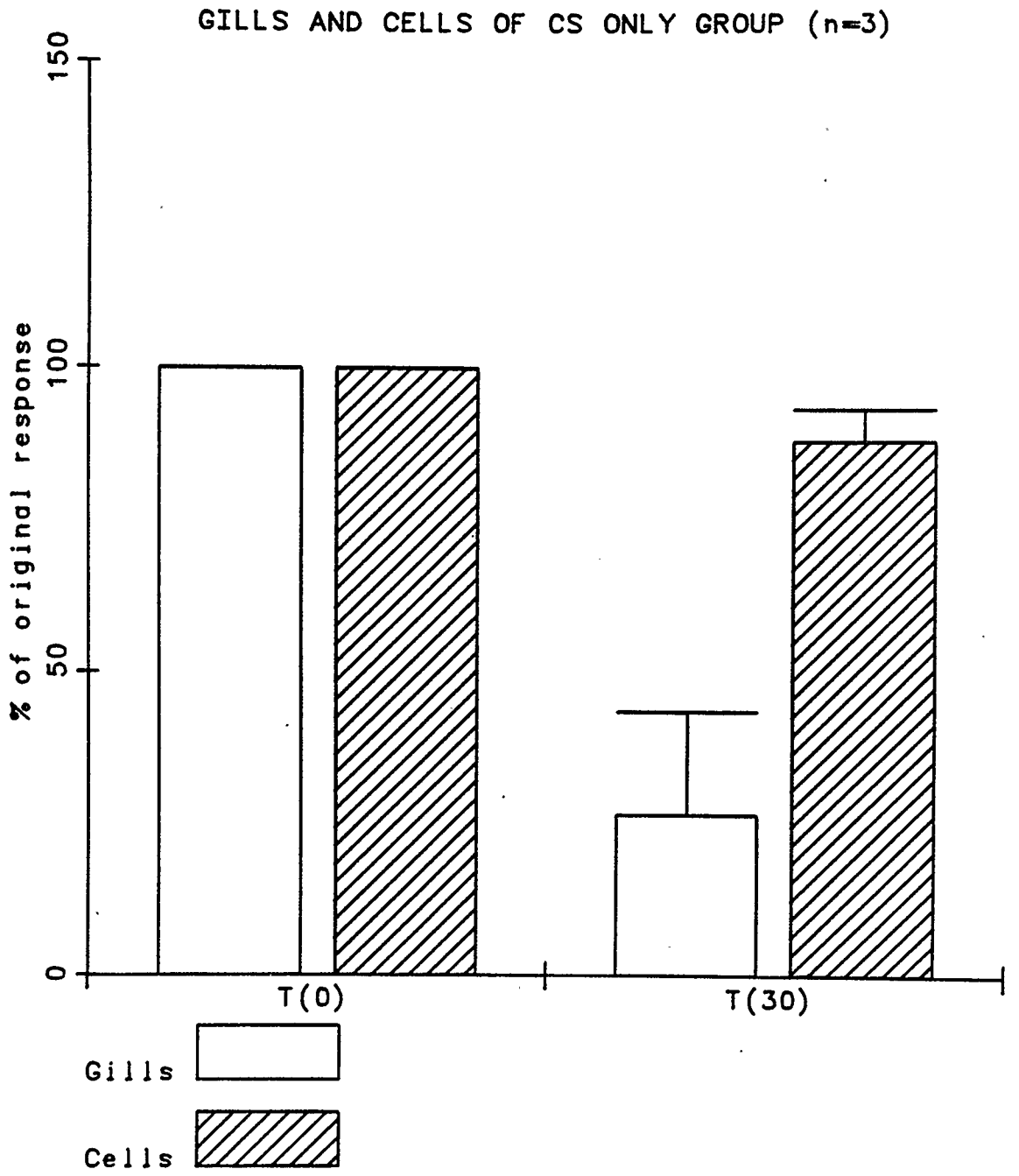
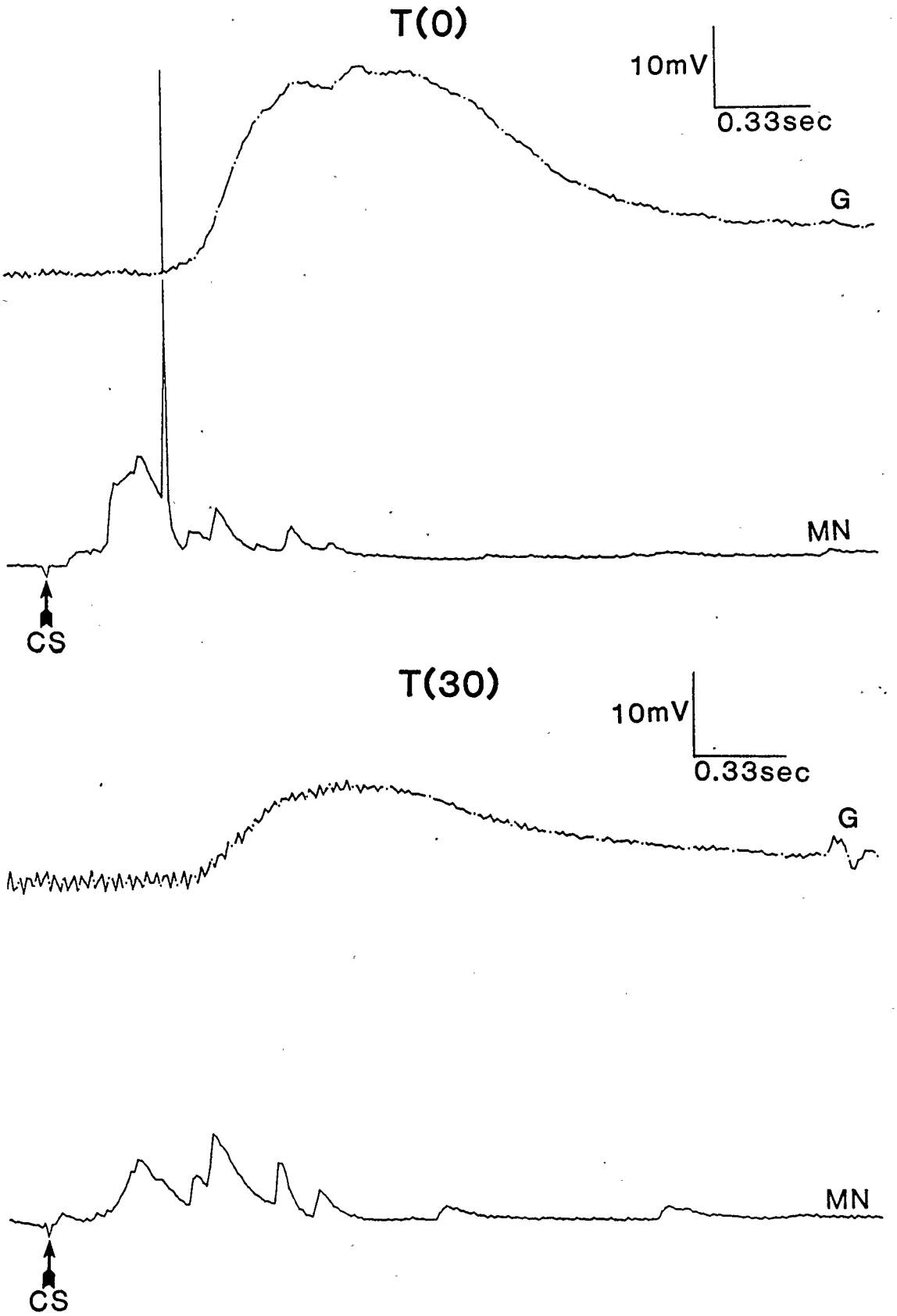

GILLS AND CELLS OF CS ONLY GROUP (n=3)
% of original response
150
100
50
0
T(0)
T(30)
Gills
Cells

FIGURE 18

Recordings from the gill (G) and motor neuron (MN) of a preparation from the CS only group. Before presentation of the habituating stimuli the CS elicited a gill withdrawal response and an EPSP followed by one action potential in the motor neuron (T(0)). After 10 trials and 30 minutes rest both the cell and the gill response had decremented (T(30)).


T(0)
10mV
0.33sec
G
MN
CS
T(30)
10mV
0.33sec
G
MN
CS

IV. Training Induced Changes in the Elicited Gill Withdrawal

In 17 cells of the CC preparations and 7 cells of the sens preparations, the electrodes remained well balanced and the same amount of depolarizing current could be passed into the motor neuron before and after training. The gill withdrawal amplitudes elicited by a set number of action potentials in the motor neuron were measured. The amplitude of withdrawal after training was calculated as a percentage of the amplitude of withdrawal generated before training. As listed in Table 3, three types of outcome were observed. A large proportion of the gills in the sens group (85.7%) showed a decrease in the amplitude of their elicited withdrawal and only 14.3% of the cells in this group elicited a larger withdrawal after training relative to pretraining. Representative data from the sens group is shown in Figure 19, where the elicited gill withdrawal has decreased to zero after training, the cell in this figure is LDG1. There is a significant decrease overall between the elicited gill withdrawals before non-associative conditioning and 30 minutes after such conditioning (T_6=2.28, $p < 0.05$). Sensitization training, then, resulted in a decrement of the motor neuron's elicited gill withdrawal response.

In the CC group, on the other hand, only 47.1% of the gills showed a decrease in their elicited withdrawal after associative conditioning, 23.5% did not change and 29.4% displayed an increased withdrawal amplitude. Overall, there was no significant change in the elicited gill withdrawal before and after associative conditioning (T_{16}=0.796, $p > 0.05$). A representative example of no change in the elicited gill

TABLE 3

Summary of the responses of the gill to depolarization of the motor neuron after paired (left) or unpaired (right) training. The amplitude of the elicited gill withdrawal was compared to that observed before training and classified into one of the three groups; decreased, no change or increased. The majority of the elicited withdrawals were decreased as a result of unpaired training. After paired training less than half of the gills responded to motor neuron depolarization with a smaller withdrawal.

	CLASSICAL CONDITIONING (n=17)	SENSITIZATION (n=7)
DECREASED ELICITED WITHDRAWAL	47.1% (8)	85.7% (6)
NO CHANGE IN ELICITED WITHDRAWAL	23.5% (4)	0.0% (0)
INCREASED ELICITED WITHDRAWAL	29.4% (5)	14.3% (1)

withdrawal before and after classical conditioning is presented in Figure 20.

All of the preparations that demonstrated an increased elicited gill withdrawal in the classically conditioned group showed a greater increase (up to 200%) than the one preparation in the sensitization group that showed an increase (114%). The increased withdrawal demonstrated by one of the classically conditioned preparations is shown in Figure 21. It appears that facilitation of L7's and LDG1's elicited gill withdrawal is occurring after classical conditioning in some cases.

The difference between the change in the elicited gill withdrawal reflex after sensitization training as compared to the classical conditioning protocol is more clearly demonstrated on the bar graph in Figure 22. Although both groups show a mean decrease in elicited withdrawal after training, the sens group shows a greater decrease overall (a mean decrease of 50% of the original response in the sens group as compared to a mean decrease of only 7.4% in the CC group).

No relationship was found between the outcome of the associative or non-associative conditioning, ie. whether a facilitated cell and/or gill response to the CS was observed at T(30), and the change in amplitude of the elicited gill movement before and after training ($X^2_6=10.6$, $p> 0.05$). Therefore, if a preparation demonstrated behavioral and/or cellular facilitation after conditioning, it was just as likely to demonstrate an increased gill withdrawal to depolarization of the motor neuron as it was to manifest a decreased withdrawal.

In addition, the elicited withdrawals by both LDG1 and L7 were found equally likely to decrease, the two motor neurons again did not differ

(T_{22}=0.16, p> 0.05). The amplitudes of gill withdrawal that each of these motor neurons generated on the original test were also not significantly different (T_{22}=0.514, p> 0.05). The number of action potentials produced by the intracellular depolarization was similar in the two different motor neurons (T_{22}=0.25, p> 0.05).

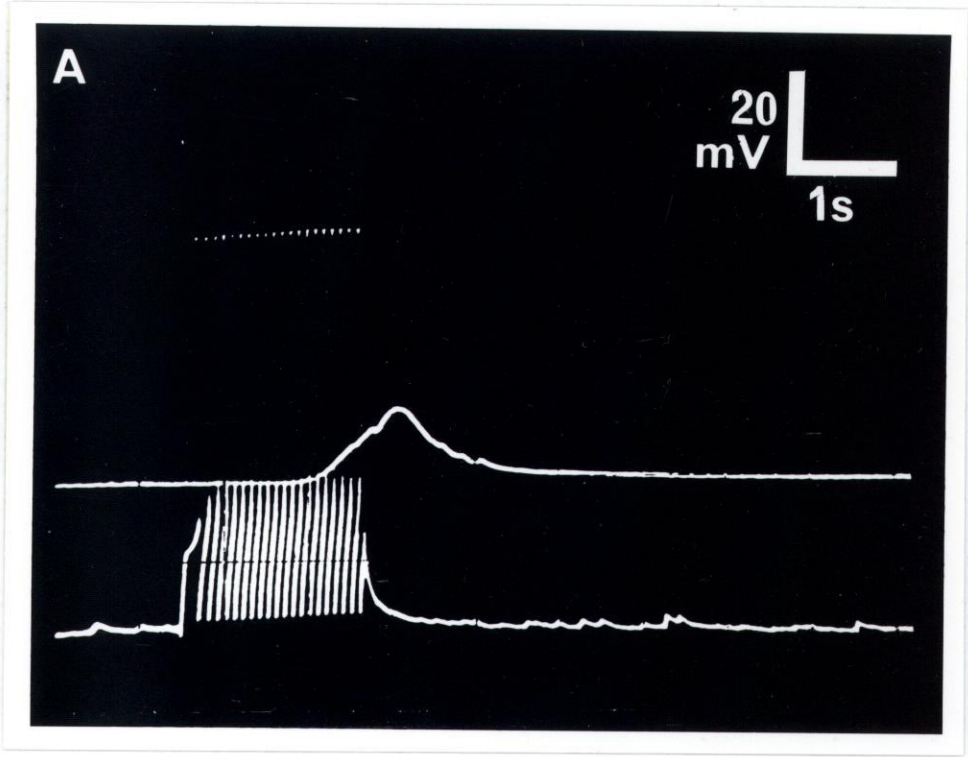
FIGURE 19

An example of a decreased elicited withdrawal.

(A) The gill withdrawal (upper trace) elicited by depolarization of the motor neuron (lower trace) before 10 unpaired training trials.

(B) The same gill and motor neuron after 10 unpaired trials and 30 minutes rest. The elicited gill withdrawal has decreased to zero.

The mean elicited gill withdrawal was significantly decreased after unpaired training.


A
20
mV
1s

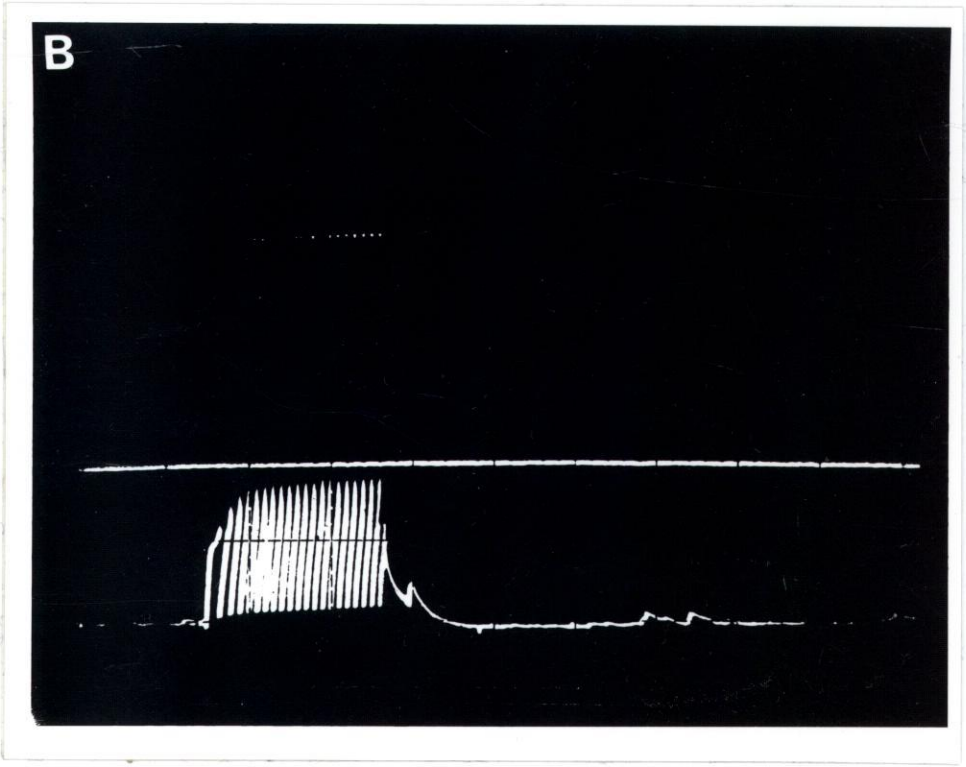

B

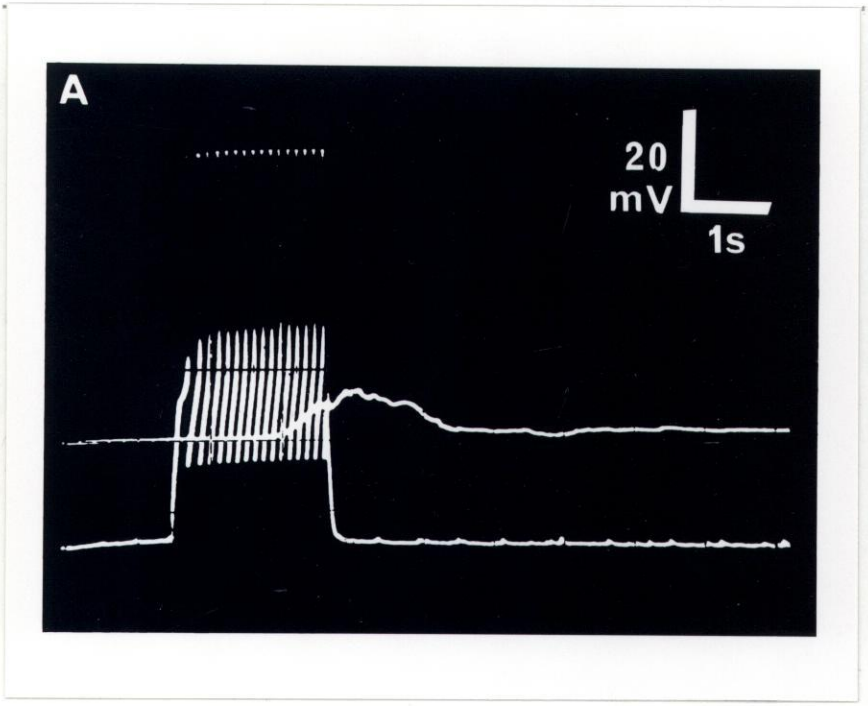
FIGURE 20

One of the four preparations from the experimental group whose elicited gill withdrawal did not change as a result of training.

(A) The gill withdrawal response (upper trace) elicited by depolarization of the motor neuron (lower trace) before 10 paired training trials.

(B) The same gill and motor neuron after 10 paired trials and 30 minutes rest. The elicited withdrawal did not change.

There was no significant change in the elicited gill withdrawal before and after associative conditioning.


A
20
mV
1s

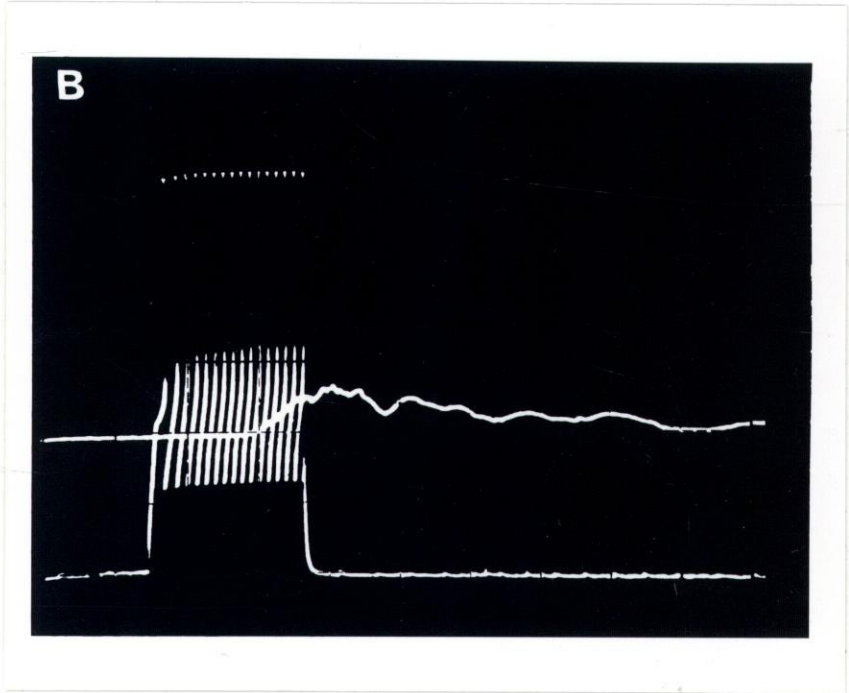
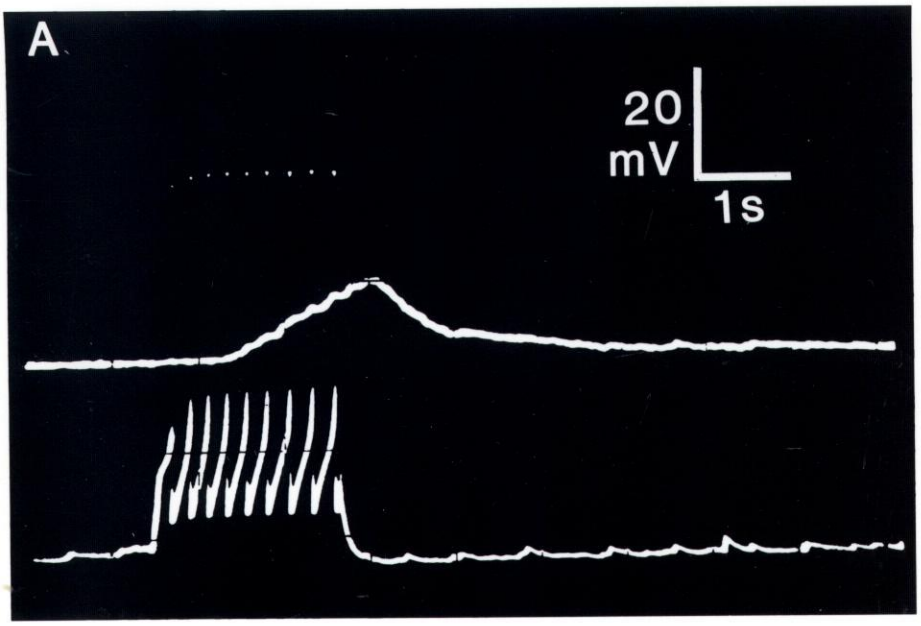

B

FIGURE 21

An example of an increased elicited withdrawal.

(A) The gill withdrawal (upper trace) elicited by depolarization of the motor neuron (lower trace) before 10 training trials.

(B) The same gill and motor neuron after 10 paired trials and 30 minutes rest. The elicited gill withdrawal was greatly enhanced as a result of associative conditioning.


A
20
mV
1s

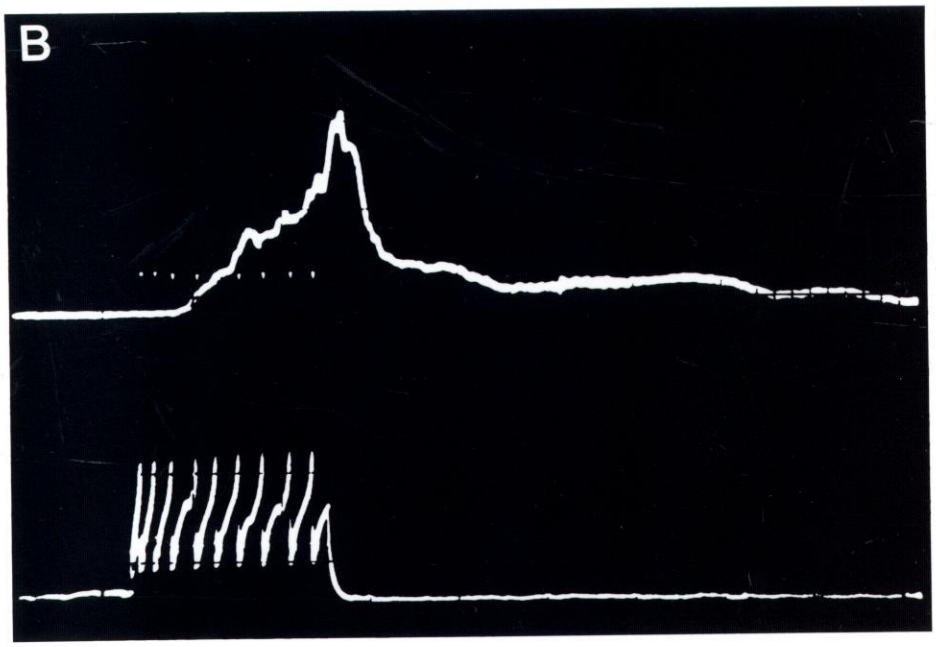
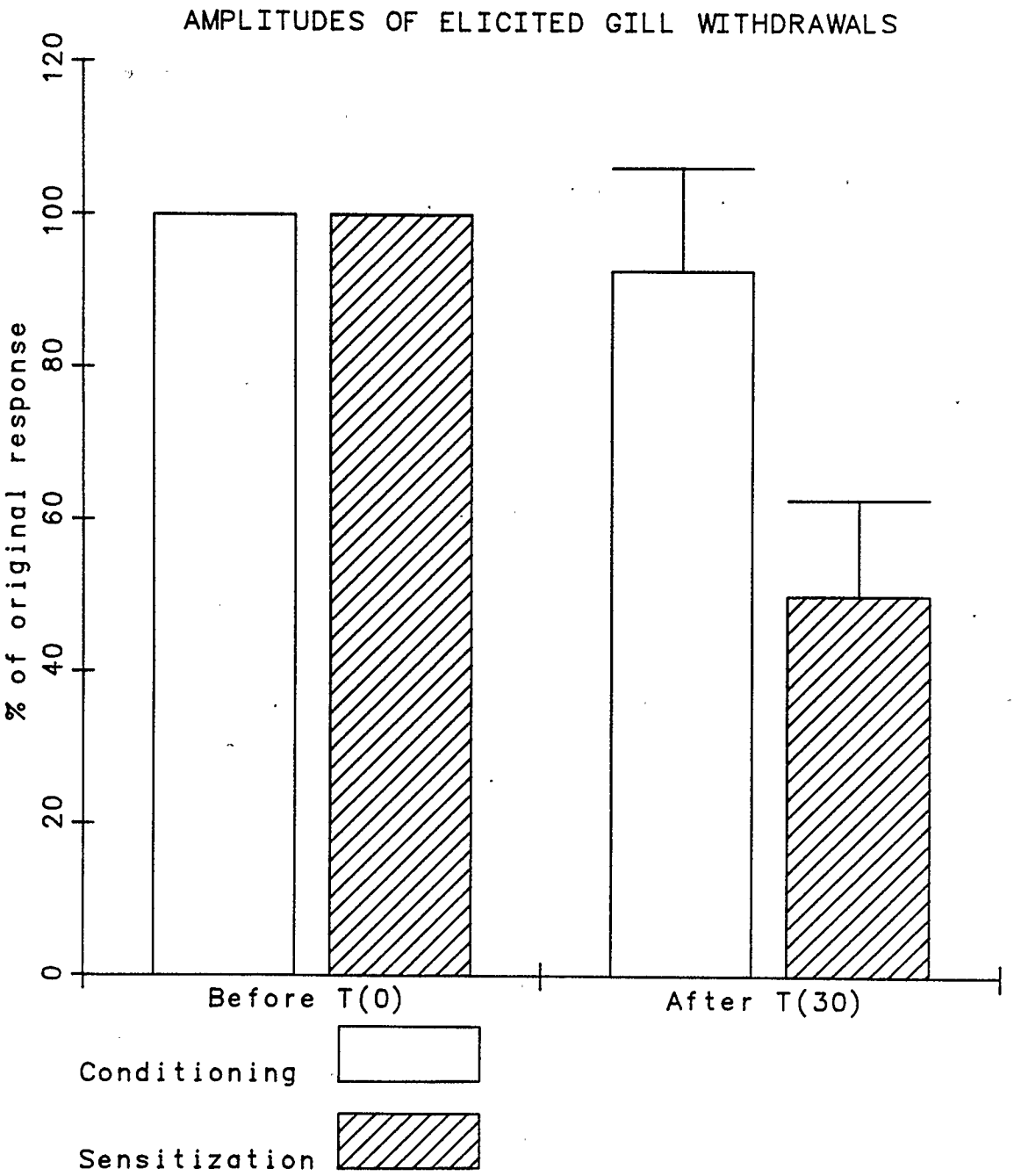

B

FIGURE 22

Mean amplitudes of the gill withdrawals elicited by direct depolarization of the gill motor neurons.

The withdrawal elicited 30 minutes after 10 training trials (After T(30)) was calculated as a percentage of that elicited before training (Before T(0)). After paired training a slight decrease to 92.6% of the original elicited gill withdrawal was observed. Unpaired training resulted in a much greater decrease, to 50% of the original elicited withdrawal.


AMPLITUDES OF ELICITED GILL WITHDRAWALS
% of original response
0
20
40
60
80
100
120
Before T(0)
After T(30)
Conditioning
Sensitization

DISCUSSION

In the course of this study the anatomy of the motor neurons L7, LDG1 and L9 has been demonstrated by intracellular fluorescent dye injection (Figures 4, 5 and 6). This is the first study in which these cells have been identified by behavioral criteria before injection of any type of dye. A previous report on the morphology of L7 (Winlow and Kandel, 1976) found some variability in its morphology between preparations. An axonal branch of L7 was found in the right connective, for example, in 2/12 preparations and one of the preparations had no axon in the branchial nerve. The neurons in this latter study were identified by their spontaneous firing patterns and position in the ganglion; it is likely that the cells were wrongly identified on occasion. It was observed during the course of the present study that cells can appear similar to L7 and LDG1 in all other ways except that they do not move the gill. Similar cells may have been positively identified and injected by Winlow and Kandel (1976). It is clear that only when behavioral criteria are included can a motor neuron be confidently identified.

As mentioned in the introduction, a causal relationship between 'activity dependent amplification of presynaptic facilitation' and classical conditioning of the Aplysia defensive withdrawal reflex has not been demonstrated. There has been considerable controversy over the relative roles that the central nervous system and peripheral nervous systems play in controlling this defensive reflex (see Mpitsos and

Lukowiak, 1985). There has also been considerable evidence for the involvement of multiple loci and mechanisms in the mediation of learning in other model systems (Lashley, 1929, Cohen and Randall, 1984, Gelperin et al., 1984). Lukowiak (1986) suggested that the cellular model of Kandel et al. (1983) may not be sufficient to explain conditioning of this defensive withdrawal reflex.

The experiments described in this report serve to directly test whether pairing specific facilitation at the sensory-motor neuron synapse (activity dependent amplification of presynaptic facilitation) is necessary and/or sufficient for the behavioral learning (conditioning of the defensive withdrawal reflex). They also test whether the enhancement (amplification of presynaptic facilitation) of these synapses that has been proposed to underlie sensitization (Klein and Kandel, 1978) is necessary and/or sufficient for sensitization of the behavioral response.

Successful classical conditioning of the motor neuron EPSP was observed after 10 paired training trials (Figures 7, 8 and 9). It is somewhat surprising that the change in the behavioral response following conditioning is not statistically significant even though the mean T(30) values depicted in Figures 7, 8 and 9 would suggest that behavioral learning has occurred. This anomaly reveals the high degree of variability between the behavioral responses of different animals. In contrast to the responses of the gill, the cellular responses show little variation. If the motor neurons were reflecting the responses of the gill equal amounts of variability would be expected. The fact that the gill behavior is more variable than that of its central neurons

suggests that other neural mechanisms must be playing a role in controlling the gill withdrawal response and its plasticity.

It has been demonstrated here, then, that an in vitro preparation of Aplysia californica can be classically conditioned when it is presented with the same stimulus parameters as those used by Carew et al. (1983) and Hawkins et al. (1983).

The type of conditioning that has been demonstrated here, and by these previous authors, is commonly referred to as "alpha-conditioning": The CS elicited a weak overt response prior to conditioning and this response increased in strength as a result of pairing. Even Pavlov's dogs sometimes salivated a little in response to novel stimuli (Pavlov, 1927). It is possible that many cases of associative learning are functionally equivalent to alpha-conditioning. The whole range of possible CS's may already be wired into the circuits responsible for the CRs and these connections may exist without the appearance of any overt behavioral characteristics. The CS used in the present study, for example, sometimes elicited a small response in the motor neuron prior to conditioning without causing a detectable gill withdrawal (see Figure 11). Most, or all, potential CS's may be prewired to be associated with most responses. The Aplysia in vitro system has also been shown capable of "true associative conditioning", with no alpha-conditioning characteristics. When a light stimulus was used as the CS (Lukowiak and Sahley, 1981) conditioning of the gill withdrawal reflex was demonstrated and the CS did not elicit an overt behavioral response prior to conditioning.

As may be predicted by the observation that 15 trials were used for training the behavior of the intact animal (Carew et al., 1983), 10

trials were not sufficient in this study to produce statistically significant behavioral learning. The data in figures 7 and 8 are presented in the same way as the equivalent data was presented in these two previous reports and the resulting graphs appear very similar. If these graphs were to be considered alone we may well reach the conclusion that changes at the sensory-motor neuron synapse reflect changes in the gill withdrawal behavior and vice versa.

When the behavioral and cellular responses of individual animals are considered (Table 2 and Figure 10) both the motor neuron and the gill are found to be capable of learning independently of the other. Such an observation has not previously been reported. The data in Table 2 and Figure 10 also reveal that the training protocol used is not 100% effective, a certain proportion of the animals in the classically conditioned group did not learn at all. Similar partial success has been reported by investigators studying other preparations such as Limax (Chang and Gelperin, 1980, Gelperin and Culligan, 1984). With some preparations only the nervous systems of successfully conditioned preparations have been investigated, increasing the chances of observing those changes that are directly related to the learned behavior (eg. Davis and Gillette, 1978). Studies using intact Aplysia and those using its isolated nervous system have not reported success rates nor whether only a selection of the animals were used in the final analysis (eg. Carew et al., 1983, Hawkins et al., 1983). An exception to this is the report of Frost et al. (1985) in which only highly sensitized Aplysia were selected for dissection after long-term sensitization training. These authors found facilitated synaptic transmission at the central sensory-motor neuron synapses of sensitized preparations. It would be

interesting to determine whether the synapses of those animals that were subjected to the sensitizing stimuli, but did not meet the authors criteria, were equally facilitated. The results of this present study would predict that these synapses may well have been facilitated in the absence of behavioral response enhancement. The successful 60% of animals that were used in the study of Frost et al. presumably displayed a large degree of variability because both behavioral and cellular data were presented as median values, with no error bars.

A follow-up experiment to this report would be to determine the percentage success rates for conditioning intact animals and isolated nervous systems. The data in Figure 10 would predict a higher success rate for conditioning of the isolated nervous system, because more cells than gills became facilitated in these semi-intact preparations.

The preparations in the classically conditioned group, and in the sensitization group, that displayed facilitated cell responses but no enhancement of behavioral responses clearly illustrate that facilitation at the central sensory-motor synapses is not sufficient for conditioning, or sensitization, of the gill withdrawal reflex.

It seems that additional changes are required for the learned or sensitized behavioral response to be manifested. These changes are mediated at a site peripheral to the motor neuron cell body because the size of the EPSP recorded in the soma is not correlated with the amplitude of the gill withdrawal reflex. If the relevant changes that mediate the learnt behavioral response occurred at a site presynaptic to the motor neuron soma an enhancement of the behavioral response would be reflected in an enhancement of the EPSP amplitude.

Two of the preparations displayed enhanced behavioral responses in the absence of enhanced EPSPs in the motor neuron (see Figures 13), this suggests that central sensory-motor neuron synapse facilitation may not be necessary for sensitization or conditioning of the gill withdrawal response. However, as such a small percentage of the preparations displayed this type of response it seems likely that central EPSP enhancement usually occurs sooner than the behavioral amplification. It is clear from the data in Table 2 that central synapse facilitation is more likely to take place within 10 trials (77% of the conditioned preparations). The behavior, on the other hand, was amplified in only 50% of the conditioned preparations within 10 trials.

In preparations where both LDG1 and L7 were monitored simultaneously, 83% of the motor neurons responded similarly after training. These two cells together control more than 75% of the gill withdrawal response (Kupfermann et al., 1974). It seems unlikely, therefore, that enhancement of the central synapses between sensory neurons and other motor neurons could mediate learning of the gill withdrawal response in those animals showing 'gill only' responses.

It is possible that the central synaptic facilitation that has been studied by Kandel and his co-workers (Camardo et al., 1984) is not directly responsible for the mediation of behavioral learning - this is suggested by the low correlation observed here between the responses of the gill and its motor neurons, and by the observation that the EPSP enhancement can be detected before the behavior is learnt. The central synapse amplification may, for example, represent a neurophysiological correlate of 'latent facilitation' in the same way that Woody has suggested cortical neuron changes may relate to eyeblink conditioning

(see introduction). Latent facilitation has recently been shown to influence conditioning of the gill withdrawal reflex in Aplysia (Lukowiak, 1986). Should these central cellular changes be responsible for such a phenomenon their facilitation would be expected to persist after extinction of the behavioral learnt response. Use of a semi-intact conditioned preparation may well, therefore, reveal the role of facilitation at the central synapses.

The in vitro preparation could also be used to investigate the role of 5HT in behavioral learning. Many of the studies carried out to investigate subcellular mechanisms of synaptic facilitation have utilized 5HT as the UCS (eg. Abrams et al., 1983, Occorr et al., 1985). 5HT has not been demonstrated as successful for conditioning of the gill or siphon withdrawal reflex and has not been paired with siphon stimulation to induce activity dependent facilitation. The semi-intact preparation provides the means for substituting 5HT application to the abdominal ganglion for pedal nerve stimulation and observing whether the cells and, in particular, the behavior can be conditioned.

Previous reports on learning in Aplysia have not presented behavioral responses on individual trials. In particular the papers of Carew et al. (1983) and Hawkins et al. (1983) are of interest as they present learning curves for conditioning of the isolated nervous system, but do not present an equivalent graph for acquisition of the withdrawal reflex itself. The two forms of learning are being directly compared and a causal relationship is being suggested (Hawkins et al., 1983) a trial by trial presentation of the behavioral data would therefore be useful for direct comparison to that of the motor neuron.

Figures 15 and 16 clearly demonstrate that the responses of the gill motor neurons are not predictive of gill behavior. In particular, the data from two individual animals (Figure 15) show that the cell and gill behavior of the same preparation do not correlate. The motor neuron responses in both groups show little variability between animals. The gill responses, on the other hand, show a large amount of variability between animals and between trials and, unlike their motor neurons, do not show the typical acquisition or control curves that would be expected. The two curves (of behavior and motor neuron responses) should be similar if "activity dependent amplification of presynaptic facilitation" at the central sensory motor neuron synapses is responsible for gill withdrawal enhancement. Clearly, the synaptic enhancement that occurs in the isolated nervous system is an event that is influenced by the training procedure and it may contribute to the behavioral conditioning, but it is not an exclusive neural equivalent of the behavioral learning.

In figure 16 an early increase is seen in the mean gill response of the control group, which later drops down to before training levels. A similar early facilitation is not observed during classical conditioning. This enhancement that is seen during sensitization is probably due to the shorter time interval between the last UCS and the next CS ie. 2.5 minutes as opposed to nearly 5 minutes in the associative protocol. The more recent exposure to the UCS in the sens group causes a facilitated response early during training. On later training trials the sens animals become habituated whereas the response of the classically conditioned group gradually increases as the 'association' or contingency is learnt. The motor neurons of the sens

animals do not undergo this early increase in response, which suggests that the effect may be peripherally mediated.

The high variability in the behavioral responses (Figure 16) - in contrast to the low variability in the cell responses - suggests, along with results that have been discussed earlier, that changes are occurring peripheral to the motor neuron cell body which influence the gill responses, but do not effect the EPSP of the motor neurons.

It is interesting that the mean gill response of the classically conditioned group increases substantially between the last training trial and the test at T(30) (see lower graph in Figure 16). This suggests that a change must occur during the 30 minutes rest after the last paired trial. This change is not reflected in the gill motor neurons, whose response is facilitated already on the last training trial and increases only slightly after the 30 minute rest. The change that mediates the increased behavioral response must, therefore, occur peripheral to the motor neuron cell body.

The in vitro preparation underwent habituation of both the cell and gill responses when presented with the CS alone (Figures 17 and 18). This would be predicted based on earlier observations (Pinsker et al., 1970, Kupfermann et al., 1970, Lukowiak, 1973). The interstimulus interval used in this study was longer than that used in these previous studies. The longer the interval between the stimuli the less pronounced and more gradual the habituation usually is (Lukowiak, 1973, Byrne, 1982). The data in Figures 17 and 18 illustrate that habituation will occur with an ISI as long as 5 minutes, and that it does not fully recover within 30 minutes of the 10th trial.

One noticeable feature of Figure 17 is that the amount of habituation demonstrated 30 minutes after training by the gill (26.5%) is much greater than that shown by the motor neurons (88.1%). A similar observation was made by Jacklet and Rine in 1977. These authors suggested that depression at the motor neuron terminals supplement the plastic properties of the central sensory-motor neuron synapses. Repeated depolarization of L7 can, in fact, lead to habituation of its elicited gill withdrawal reflex at certain intertrial intervals, impulse frequencies and trial durations (Lukowiak, 1977b).

When the motor neurons were depolarized after training the majority of the preparations in the sensitization group responded with a smaller gill withdrawal than they did before training (see Table 3). Less than half of the classically conditioned group demonstrated such a decreased elicited withdrawal. The fact that the animals in the sensitization group underwent a significant decrease in their motor neuron elicited gill withdrawals suggests that habituation of the neuromuscular junction occurred over the course of the unpaired training. This observation from the sensitization group confirms the conclusions of Jacklet and Rine (1977) and Lukowiak (1977b) that habituation can occur peripheral to the central sensory-motor neuron synapse. This is in direct contrast to the report of Carew et al. (1974) in which gill habituation was attributed entirely to central synapse depression.

It seems likely that some form of facilitation, peripheral to the motor neuron cell body is counteracting this habituation in the classically conditioned group. If facilitation was not occurring in the periphery due to the paired presentation of the CS and UCS, habituation of the elicited gill withdrawal reflex would be equally evident in both

the control and experimental groups. The motor neuron induced withdrawal did not habituate, however, in more than half of the classically conditioned preparations and 29% of them were facilitated (Figure 21).

Lukowiak and Peretz (1977) determined that functional differences exist between the peripheral terminations of LDG1 and L7. In their study repeated depolarization of LDG1 (with an ISI of 30 seconds) was found to result in enhancement of its elicited gill withdrawal reflex, in contrast to the decrement observed with a similar treatment to L7. In the present study LDG1's elicited withdrawal was found equally likely to decrease as that of L7. This suggests that with a longer interstimulus interval, ie. 5 minutes, LDG1's elicited gill withdrawal reflex is more likely to be reduced and not enhanced.

The additional or alternative site of facilitation that seems to be involved in mediation of the learnt withdrawal response has not been characterized or localized in this study. As these changes seem to occur peripheral to the motor neuron cell body the peripheral nervous system is a likely candidate. The peripheral gill ganglion, for example, is conveniently located half way along the branchial nerve between the abdominal ganglion and the gill. The cells of this ganglion have been found to move the gill when depolarized and they receive both CS input (from the siphon) and UCS input (from the pedal nerves) (Colebrook and Lukowiak, 1985). In addition, the central gill motor neurons have been observed to synapse onto some of these peripheral cells in a different species of Aplysia (Kurokawa and Kuwasawa, 1985). As these cells have already been shown capable of habituation (Peretz

and Moller, 1974), it is feasible that their responses to central neurons become facilitated or that they mediate facilitation in the periphery. Synapses within the gill ganglion or the gill could become enhanced by a similar mechanism to the central presynaptic facilitation that Hawkins et al. (1983) have described or by a different mechanism. Peretz and Moller (1974) proposed, for example, that a potentiation of hyperpolarization causes habituation of the gill ganglion cells' firing rate. Since an increase in spike activity in only one cell can facilitate the gill withdrawal amplitude (Peretz and Moller, 1974) a decrease in this hyperpolarization and hence an enhanced firing rate in these peripheral cells may well contribute to sensitization and/or classical conditioning of the gill withdrawal reflex.

A semi-intact preparation has now been developed that allows intracellular recording from gill ganglion and abdominal ganglion cells with simultaneous observation of the gill behavior; the pedal nerves and siphon are also accessible for stimulation (Colebrook and Lukowiak, 1985). Such a preparation could be utilized to determine whether changes occur within these peripheral nerve cells during learning, and furthermore whether such changes supplement central changes or are sufficient to mediate the behavioral learning alone. Peretz and Moller (1974) describe a technique for removing the gill ganglion, this may be useful for identifying the ganglion's relative contribution to conditioning of the gill withdrawal reflex.

An alternative (or additional) mediator of the conditioned gill withdrawal reflex could be one or more central neurons having a direct action in the periphery, such as the gill motor neuron L9 (see Figure 6). This motor neuron is capable of eliciting only a minor gill

withdrawal reflex that probably does not contribute to normal defensive withdrawals of the gill (Kupfermann et al., 1974). Activity in L9 can potentiate L7's ability to elicit a gill withdrawal response (Lukowiak, 1979a) and can also prevent, or reverse habituation of the gill withdrawal reflex (Lukowiak, 1979b). L9 activity does not influence the decrement of activity across the central sensory-motor neuron synapses during the presentation of habituatory siphon stimulation (Lukowiak, 1979b). Also, L7's ability to move the gill is not potentiated by L9 activity if the small branch of the siphon nerve, which carries L9's axon to the gill is severed (Lukowiak, 1979a). L9's modulation over the gill withdrawal reflex is therefore mediated peripherally, either by a direct effect on the gill muscle itself or via modulation of the terminals of central or peripheral motor neurons. The possibility that L9 activity may play an active role in associative learning of the gill withdrawal reflex could be tested by recording from L9 during training. Alternatively L9's input to the gill could be eliminated by cutting the branch of the siphon nerve that innervates the gill, or by removing L9 from the network by injecting it with a fluorescent dye and irradiating it (Miller and Selveston, 1979).

A recent report in which fluorescent dyes were used to monitor the activity of cells within the abdominal ganglion before and after sensitization has revealed that more central neurons come into play after exposure to sensitizing stimuli (London et al., 1986). Central modulators of peripheral activity, such as L9, may be included amongst these 'lit up' cells.

Both dopamine and the molluscan neuromodulator FMRFamide can potentiate gill withdrawal reflexes when perfused through the gill

(Ruben and Lukowiak, 1983, Cawthorpe and Lukowiak, pers. comm.). In addition, the peptide SCP_B can potentiate gill withdrawals that are elicited by motor neuron depolarization if it is perfused over the abdominal ganglion. The peptide may be affecting central neurons that modulate the peripheral nervous system (Lukowiak and Murphy, 1985). Such modulators could play a role in nonassociative and associative learning of the reflex. It would be of interest to determine whether perfusion of these transmitters through the gill (or over the ganglion in the case of SCP_B) during training increases the percentage of successful learners.

Dopamine and peptides are both good candidates for modulators of learning. Peptides have been implicated in mammalian learning for a number of years (eg. De Weid and Gispen, 1977) and, more specifically, SCP_A and SCP_B have recently been proposed as possible mediators of presynaptic facilitation in Aplysia (Abrams et al., 1984). Dopamine can potentiate synaptic transmission in mammalian sympathetic ganglia (long term enhancement) (Libet and Tosaka, 1970) and dopaminergic systems have been implicated in a variety of learning and memory situations (Beninger, 1983). The use of a dopamine antagonist during training would reveal whether peripheral dopamine modulation is critical for behavioral learning - this is of particular importance in light of the suggestion that L9 may be part of a dopaminergic pathway (Mpitsos and Lukowiak, 1985).

It is surprising, perhaps, that no relationship was found between the response of the gill to the CS at T(30) and the change in amplitude of

the elicited gill withdrawal reflex. This suggests that multiple mechanisms are contributing to classical conditioning of the gill withdrawal reflex and a combination of these processes mediate the learning. Neither central synapse facilitation nor the mechanism that counteracts habituation of the neuromuscular junction are sufficient or necessary for the learned behavioral response.

As with so many of the systems mentioned in the introduction many loci and mechanisms seem to be involved in classical conditioning of the Aplysia gill withdrawal reflex. No one particular locus or mechanism is likely to be necessary or sufficient. Just as the data in this thesis shows central activity dependent amplification to neither be sufficient or necessary for behavioral conditioning of the in vitro preparation, a previous report by Carew et al. (1984) has claimed that changes in the central motor neurons themselves are not necessary or sufficient for EPSP amplification. By the same token this does not mean that changes in the motor neurons or amplification at the central sensory-motor neuron synapses are not normally involved in learning of the behavior.

It is likely that higher centers and local changes combine to produce behavioral changes, in the same way that learnt reflexes are mediated by the vertebrate spinal cord. This report emphasizes that any study undertaken in the hope of revealing the contributions of various systems and mechanisms to a learnt behavior should attempt to include as much of the nervous system, and indeed the animal itself, as possible. Studies investigating the 'isolated nervous system', or parts of it, may well be of use in determining cellular and subcellular methods of modification but are of little relevance until the contribution of these isolated

changes to learning in the intact behaving organism has been established.

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