Some authorities have suggested that, because of their many primitive anthropoid characteristics, one group of Fayum primates, the parapithecids, may be related to the New World platyrrhines (14). However, recent reports of the geology of the Salla basin in Bolivia (15) have shown that the earliest platyrrhines are probably from about 25 million years ago, substantially younger than the Fayum primates. Thus, the new minimum age estimate of the Jebel Qatrani formation indicates that the Fayum primates are substantially older than the first appearances of all modern anthropoid radiations, a chronology that accords well with morphological analyses that place some species at the base of the higher primate radiation.

REFERENCES AND NOTES

16. We thank the Geological Survey of Egypt for assistance and cooperation, especially B. Isawi, R. Elissa, B. el-Khushal, A. A. el-Awady Kandil, A. el-Ghaw Thabiri, Shelly, and M. Askaliy, and M. Kraus, P. Charrath, D. Donning, T. Rasmussen, P. Gingerich, R. Kay, S. Wing, and D. Krause for information and suggestions. Supported in part by BNS 8310913, BNS 8546024, and BNS 8521665, from NSF and 70869500 and 809479 from the Smithsonian Foreign Currency Program Duke Primate Center publication 415.

A Critical Period for Macromolecular Synthesis in Long-Term Heterosynaptic Facilitation in Aplysia

P. G. Montarolo,* P. Goelet, V. F. Castellucci, J. Morgan, E. R. Kandel, S. Schacher†

Both long-term and short-term sensitization of the gill and siphon withdrawal reflex in Aplysia involve facilitation of the monosynaptic connections between the sensory and motor neurons. To analyze the relationship between these two forms of synaptic facilitation at the cellular and molecular level, this monosynaptic sensibrimotor component of the gill-withdrawal reflex of Aplysia can be reconstituted in dissociated cell culture. Whereas one brief application of 1 µM serotonin produced short-term facilitation in the sensorimotor connection that lasted minutes, five applications over 1.5 hours resulted in long-term facilitation that lasted more than 24 hours. Inhibitors of protein synthesis or RNA synthesis selectively blocked long-term facilitation, but not short-term facilitation, indicating that long-term facilitation requires the expression of gene products not essential for short-term facilitation. Moreover, the inhibitors only blocked long-term facilitation when given during the serotonin applications; the inhibitors did not block the facilitation when given either before or after serotonin application. These results parallel those for behavioral performance in vertebrates and indicate that the critical time window characteristic of the requirement for macromolecular synthesis in long-term heterosynaptic facilitation is not a property of complex circuitry, but an intrinsic characteristic of specific nerve cells and synaptic connections involved in the long-term storage of information.

Memory, the retention of learned information over time, is thought to have at least two components—short-term memory lasting minutes to hours, and long-term memory lasting for days to weeks or even years (1). Behavioral studies in vertebrates suggest that long-term memory requires the synthesis of new protein, whereas short-term memory does not (2). Moreover, long-term memory is most sensitive to disruption by inhibitors of protein synthesis when the inhibitors are applied during and immediately after training (3). There is no deficit in the retention of the learned behavior when the inhibitors are applied even 1 to 2 hours after training. This time window is remarkably characteristic and has been demonstrated for memory produced by associative as well as nonassociative forms of learning and for various vertebrate species (3). Although comparable data are lacking in humans, analogous clinical studies of convulsions and head trauma indicate that human long-term memory is particularly susceptible to disruption during and soon after seizure (4).

The interpretation of earlier experiments, based on the systemic application of protein synthesis inhibitors, was limited, however, in two ways. First, the protein synthesis inhibitors often led to serious side effects such as seizure and sickness (3). Second, the studies were based on the examination of behavioral performance rather than on the analysis of the cellular correlates responsible for the neuronal plasticity underlying the altered behavior. Inhibitors of protein synthesis can affect behavioral performance not only through their action on memory processes, but also through their action on other systems that influence performance, such as attention or motivation. As a result, it has not been possible to determine whether new protein synthesis is important for memory and whether the characteristic time window for long-term memory reflects a specific requirement for macromolecular synthesis by particular neurons involved in the storage of long-term information.

To overcome these limitations, we analyzed memory processes at the cellular and molecular level, using the gill-withdrawal reflex of Aplysia. The neural circuitry of this reflex behavior has been delineated (5), and one important cellular locus has been identified in mediating short-term and long-term...
sensation (6). Both short-term sensitization, lasting minutes to hours, and long-term sensitization, lasting days, involve changes in the strength of the connections that the sensory neurons make on their central target cells, the interneurons and the motor neurons. Short-term sensitization results from the heterosynaptic facilitation of transmitter release from the terminals of the sensory neurons produced by the actions of serotonergic and other modulatory neurons activated by tail or head shock (7). Long-term sensitization also leads to an enhancement in the strength of the connections between sensory and motor neurons (6). In addition, long-term sensitization results in a growth process leading to an increase in both the number of varicosities of the sensory neurons and in the incidence of active zones for transmitter release (8).

As a first step in exploring the molecular mechanisms underlying long-term plasticity, we have further simplified the analysis of the neuronal circuit by reconstituting the mono-

Fig. 1. Long-term facilitation of sensorimotor connections with repeated applications of 5-HT is blocked in the presence of inhibitors of protein or RNA synthesis. (A) Experimental protocol. After 5 days in culture, sensory and motor cells were impaled and the motor cell was hyperpolarized 50 mV below the resting potential to permit accurate measurement of the amplitude of the EPSP. The first recording session (0 hour, dark bar), either in the absence or presence of the inhibitors, consisted of the following. Every 20 seconds, a single spike was evoked in the sensory neurons by injecting a depolarizing pulse. After the fifth action potential, 5-HT was applied in the bath to a final concentration of 1 μM and the extent of facilitation assessed with three additional evoked EPSPs (see Fig. 2). In cultures with two sensory neurons, short-term facilitation was assessed during the recording from the second cell. Four minutes later, the 5-HT was washed out by perfusion with 50% L15 and 50% artificial sea-

The cultures were then perfused for 15 minutes in drug-free medium. Actinomycin D (50 μg/ml) was added 1 hour before the first recording session, after which the cells were perfused with drug-free medium. Actinomycin D was not perfused continuously because a 1-hour exposure irreversibly blocked total RNA synthesis in Aply-

Summary of long-term facilitation induced by 5-HT and blockade by protein and RNA synthesis inhibitors. A, actinomycin D; E, emetine; AD, acti-

neurons by more than 90%. (B) Sensorimotor connections show long-term facilitation after repeated 5-HT applications. The first evoked EPSP in the motor cell L7 [top traces in (B1), (B2), and (B3), during the 0-hour recording session (left column)] is compared to the first evoked EPSP for the same connection at the 24-hour recording session (right column). When no 5-HT applications were given [control (B1)] the amplitude of the EPSP shows a slight decline. Twenty-four hours after a single 5-HT application (1X 5-HT (B2)) the EPSP is unchanged. A significant increase in the amplitude of the EPSP was obtained after five applications of 5-HT (5X 5-HT (B3)). For details on recording sessions and protocol, see Fig. 1A. Calibration bars: 10 mV, 20 msec. (C)
gether with one to two sensory neurons from the abdominal ganglion LE cluster (10) and examined the strength of the connection on day 5 (Fig. 1A, bar at 0 hour; Fig. 1B, 0-hour column). We then exposed the cultures to either a single 5-minute application of 5-HT or to five applications of 5-HT (5 minutes each) at 15-minute intervals (Fig. 1A). We measured the response to the first 5-HT application to determine the extent of short-term facilitation (Fig. 1A, arrow at 0 hour). The identical cells and connections were reexamined 24 hours later (Fig. 1A, bar at 24 hours; Fig. 1B, 24-hour column). The cultures treated with five applications of 5-HT showed a significant increase in the strength of their connections of 71 ± 31% (SEM), whereas untreated controls or cultures receiving a single application showed no significant change in their synaptic strength (Fig. 1A, B and C).

Because the behavioral expression of long-term memory in vertebrates can be blocked by interfering with protein synthesis during training (3), we next determined whether the long-term facilitation of synaptic strength in culture produced by 5-HT can be affected by inhibitors of protein or RNA synthesis present during 5-HT applications. Using the same protocol described in Fig. 1A, we first exposed the cultures to one of two different inhibitors of protein synthesis—10 μM anisomycin or 100 μM emetine (1J)—or to one of two different inhibitors of RNA synthesis—actinomycin D (50 μg/ml), an inhibitor of total RNA synthesis (12), or α-amanitin (2 μg/ml), a specific inhibitor of RNA polymerase II activity (13). The presence of either translational or transcriptional inhibitors during the applications of 5-HT blocked the enhancement of synaptic strength produced by 5-HT when retested 24 hours later: −16 ± 14% for anisomycin; −21 ± 10% for emetine; +3 ± 18% for actinomycin D; and +11 ± 9% for α-amanitin (Fig. 1C). By themselves, the inhibitors had no significant effect on synaptic strength, resting potential, or input resistance (Table 1).

Although both the protein and RNA synthesis inhibitors blocked long-term facilitation, none of these inhibitors affected short-term facilitation produced by a single application of 5-HT (Fig. 2). In the same experiments in which the inhibitors blocked long-term facilitation, short-term facilitation was normally expressed, both when the inhibitors were present or 24 hours after the inhibitors had been washed out.

We next examined the critical time course for new protein synthesis required for long-term facilitation by studying in more detail the extent of inhibition by anisomycin and the recovery of protein synthesis after drug washout. *Aplysia* neurons in culture were exposed to anisomycin for 3 hours and pulse labeled with [35S]methionine in the presence of this inhibitor or at various times after its washout (Fig. 3). When anisomycin was present, protein synthesis was inhibited by over 95%, but it recovered rapidly to control levels within 2 to 4 hours after removal of the inhibitor (Fig. 3). This suggests that transient inhibition of protein synthesis, during and immediately after the application of 5-HT, might be sufficient to block the long-term facilitation of the sensorimotor connection.

We tested this idea directly by defining...
more precisely the exact time period during which new protein synthesis is required for the expression of long-term facilitation (Fig. 4A). We applied anisomycin for a 3-hour period at various times before (12 hours) and after (0.5 hour and 4 hours) the applications of 5-HT. Whereas anisomycin blocked the facilitation when given at -1 hour [-16 ± 14% (SEM)], exposure to anisomycin at other times did not interfere with the long-term facilitation of the sensorimotor connections (Fig. 4A). There was little or no effect of anisomycin given at -12 hours (65 ± 10%), at 0.5 hour (51 ± 19%), or at 4 hours (90 ± 32%). Thus, expression of long-term facilitation requires new protein synthesis only transiently, during and immediately after 5-HT applications.

We also explored whether an additional requirement for new macromolecular synthesis might be revealed at periods other than during the 5-HT applications by exposing the cultures to inhibitors of macromolecular synthesis for the entire 22-hour period following the 5-HT applications. Cultures were exposed to anisomycin (10 μM) or the transcriptional inhibitor α-amanitin (2 μg/ml) for a 22-hour period, extending from 0.5 hour after the last 5-HT application until the onset of the recording session at 24 hours. Neither inhibitor significantly interfered with the 5-HT-induced long-term facilitation (Fig. 4B). The period of inhibition affected neither the strength of the connections nor their capacity to undergo short-term facilitation with a single application of 5-HT.

Our findings indicate that the monosynaptic component of the Aplysia gill-withdrawal reflex can show both short-term and long-term facilitation in dissociated cell culture with applications of 5-HT. As is the case with sensitizing stimuli to the tail or head, a single exposure to 5-HT produced facilitation that lasted minutes (9), repeated 5-HT exposures produced facilitation that lasted more than 24 hours. This result suggests that enhancement in the connections between sensory and motor neurons in long-term sensitized animals can be produced by repeated actions of a single modulating neurotransmitter (such as 5-HT) that is released with each sensitizing stimulus.

We do not know the intracellular signaling systems whereby repeated actions of 5-HT (or other modulatory transmitters) produce long-term enhancement of the connection. Short-term facilitation of transmitter release by sensory neurons produced by a single sensitizing stimulus is associated with an increase in cyclic adenosine monophosphate synthesis and protein phosphorylation that produces closure of a serotonin-sensitive potassium channel and altered handling of calcium (14). Long-term facilitation could work through an independent and parallel signaling system. Alternatively, the two types of systems could be in series so that some or all of the steps involved in the short-term process may also participate in the long-term process initiated by repeated 5-HT exposures. The structural changes described by Bailey and Chen (8) suggest that at least some additional steps are likely to be involved in the long-term facilitation of sensorimotor connections. The long-term change may also involve alterations in the postsynaptic motor neuron, perhaps in the density or distribution of transmitter receptor proteins (15).

As is the case with vertebrates, long-term behavioral sensitization in Aplysia also requires new protein synthesis. In a partially intact animal preparation, consisting of all the components of the behavior, long-term sensitization of gill-withdrawal was blocked when repeated tail shocks were given while the abdominal ganglion was exposed to anisomycin, whereas short-term sensitization was not affected (16). In dissociated culture, we now have been able to examine a significant component of the cellular substrate of this long-term memory for sensitization—the changes that occur in the monosynaptic component of the gill-withdrawal reflex—at the level of a single identified and behaviorally relevant synaptic connection. As is the case with the intact behavior, the long-term facilitation of this connection shows a dependence on protein and RNA synthesis that parallels the memory. By contrast, both the short-term memory and the short-term facilitation are not dependent on new macromolecular synthesis. This result is consistent with the findings of studies on invertebrates such as Aplysia, Hermisenda, and Drosophila that short-term memory is
Table 1. Effects of inhibitors on physiological properties of sensory and motor cells. The values (mean ± SEM) were measured on cells as in Fig. 1A. Drug treatments alone had no significant effect on initial amplitudes of the excitatory postsynaptic potential (EPSP) evoked in L7. Percent change in EPSP amplitude was measured as in Fig. 1C. The drugs had no significant effect on sensory or motor cell resting potentials (V_m) and L7 input resistance (R_m).

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>n</th>
<th>Connections</th>
<th>EPSP amplitude</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8</td>
<td>13</td>
<td>-14 ± 12</td>
<td>- ± 6</td>
</tr>
<tr>
<td>5x 5-HT</td>
<td>9</td>
<td>16</td>
<td>+71 ± 31</td>
<td>+ ± 4</td>
</tr>
<tr>
<td>Anisomycin (10 μM)</td>
<td>12</td>
<td>18</td>
<td>+12 ± 19</td>
<td>+ ± 6</td>
</tr>
<tr>
<td>Emeitine (100 μM)</td>
<td>4</td>
<td>5</td>
<td>-19 ± 14</td>
<td>- ± 6</td>
</tr>
<tr>
<td>Actinomycin D (50 μg/ml)</td>
<td>5</td>
<td>9</td>
<td>+2 ± 11</td>
<td>+ ± 6</td>
</tr>
<tr>
<td>α-Amanitin (2 μg/ml)</td>
<td>6</td>
<td>7</td>
<td>-6 ± 21</td>
<td>- ± 6</td>
</tr>
</tbody>
</table>

Moreover, our findings indicate that the narrow time window of 1 to 3 hours during which protein and messenger RNA (mRNA) must be synthesized to express the function of long-term facilitation of senso-motor connections in vitro—a feature characteristic of long-term behavioral memory for many forms of learning in vertebrates—is not a systems property but reflects a fundamental property in the storage of information by specific neurons and their connections. At this storage site, long-term facilitation differs from short-term facilitation in requiring the synthesis of gene products not required for short-term facilitation. Thus, whereas the proteins and mRNA's critical for short-term facilitation already exist and turn over slowly (17), certain proteins and mRNA's necessary for long-term facilitation must either be induced or, if constitutively expressed, are only transiently accessible to modification (18).

Although our data indicate that the synthesis of gene products is necessary for long-term facilitation, we do not know the nature of the gene products or how their expression affects the phenotypic properties of the sensory or motor cells. We also do not know how the synthesis of these gene products is regulated. Exposures to 5-HT could lead, via the appropriate activation of second messenger systems within sensory neurons (19), to long-lived modifications of constitutively expressed gene products, or to direct induction of effector genes (20), resulting in synaptic enhancement. However, the time window for macromolecular synthesis in long-term facilitation resembles that found in certain development processes (21) and in the initiation of lordosis behavior in rats by steroid hormones (22), both of which appear to involve sequential gene activation. In these cases an external signal (such as transmitter or hormone) triggers the transient expression of early regulatory genes that have products that are thought to regulate the expression of effector genes responsible for the persistent phenotypic changes observed. The long-term facilitation of sensorimotor connections may thus involve a sequential induction mechanism. The second messenger might first activate early regulatory genes, which act on later effector genes to bring about maintained changes in the synthesis of gene products responsible for synaptic enhancement (18).

Evoked patterns of the neural circuit of the gill-withdrawal reflex in culture has allowed replication of a component of the long-term change in two identified cells and their connections, and has isolated a single transmitter as the extracellular signal necessary for inducing this long-term change. In addition, it has been possible to constrain the critical expression of gene products underlying this long-term change to a specific 1- to 3-hour time period. These simplifications should facilitate the search for the macromolecules required for the retention of long-term behavioral sensitizer- in Aplysia and allow study of the mechanisms regulating the synthesis of these macromolecules. Application of these methods for studying long-term changes in other systems might elucidate the general nature of these mechanisms.

REFERENCES AND NOTES


1. 5 DECEMBER 1986 REPORTS 1253
For all groups, 128 cultures were examined before pathologic diagnosis (9), it occurred to me that skeletal muscle and erythrocyte membranes share the same genetic lactate transporter (or a common subunit), which is deficient in this patient. This defect may be a common cause of elevated serum creatine kinase levels, as seen in the patient described here and of unexplained episodes of rhabdomyolysis and myoglobinuria.

Lactate Transporter Defect: A New Disease of Muscle

WILLIAM N. FISHEBEIN

New methods were used to identify the abnormality in a patient who showed evidence of neuromuscular dysfunction on extensive clinical examination. The methods revealed that the lactate content of the patient's skeletal muscle does not decline normally after exercise and that his red cells are defective in lactate transport. These results suggest that skeletal muscle and erythrocyte membranes share the same genetic lactate transporter (or a common subunit), which is deficient in this patient. This defect may be a common cause of elevated serum creatine kinase levels, as seen in the patient described here and of unexplained episodes of rhabdomyolysis and myoglobinuria.

TREATED MUSCLE HAS A GREATER DYNAMIC RANGE THAN ANY OTHER TISSUE. Upon strenuous exercise its oxidative demands may increase a hundredfold, and its glycolytic rate, a thousandfold (1-3). Moreover, it is the inherent paradox of the contractile machinery that attainment of maximal force by the recruitment of all fiber groups, with the greatest demand for oxidative energy provision, simultaneously produces the greatest blockade of capillary blood flow and hence the most anaerobic state. It is particularly for this tissue that the distinction between conditioning and disease becomes blurred, since poor conditioning may produce more functional compromise in muscle than many diseases produce in other target organs. Muscle is thus a prime candidate for functional or metabolic diseases, that is, enzyme or carrier defects that limit maximal performance, but may be unaccompanied by anatomic pathology. Examples of such defects have thus far been limited to enzyme deficiencies, such as those involved in glycolysis and glycogenolysis (4-6) and myoadenylate deaminase (7). I have previously referred to some of these enzymes as "perquisitory" catalysts, because, although not essential for muscular activity, they provide important perquisites for maximal performance (8). One may anticipate, therefore, that defects in such catalysts may produce "diseases of healthy people."

Since most patients whose major complaint is of muscle pain or weakness are never identified with a specific disorder or pathologic diagnosis (9), it occurred to me that a logical candidate for the pathogenesis of some of these cases would be the lactate transporter of the cell membrane. This transporter has been known for about a dozen years. It was first demonstrated in bacterial cells, then later studied in detail in mammalian tumor cells (10) and human red blood cells (11). It is now clear that it is present in most animal cells and tissues that produce lactate above their utilization level, which include erythrocytes and skeletal muscle.

Often referred to as the organic anion or substituted monocarboxylate transporter because of its generic carrier properties (12), this transporter's main function is to export lactate, the major organic acid that accumulates intracellularly. Although, to my knowledge, there have been no definitive studies on its isolation and characterization, the transporter seems to be a membrane protein that cotransports a lactate anion and a proton externally (OH⁻ uptake has not been excluded), without a requirement for adenosine triphosphate (ATP), to reduce and prevent intracellular metabolic acidosis (10-12). The carrier must contain one or more essential sulfhydryl groups, since micromolar concentrations of organic mercurial compounds inhibit the transporter, and permit one to conclude that it is responsible for about 90% of lactate efflux; the remainder is carried by diffusion and by the chloride/carbonate exchanger (13). The transport rate is sensitive to the pH gradient across the cell membrane, and increases markedly as the intracellular pH falls with accumulation of lactic acid (10-12).

The human red blood cell, which has no aerobic energy-yielding pathway, is almost completely dependent upon glycolysis for its energy stores, and therefore upon rapid lactate export. Lactate efflux is even more critical for muscle, which produces more lactate than it uses under all conditions, even at rest. Although aerobic electron transport is a much more efficient energy producer, glycolysis is much faster, and muscle metabolism is designed to maintain an adequate supply of pyruvate (and hence lactate) for all oxidative capabilities. If the excess lactic acid is not exported rapidly, the developing acidosis will render inefficient both the contractile apparatus and the energy-yielding pathways.

Over the past year we have developed a clinical assay for the human erythrocyte lactate transporter and an ischemic exercise test suitable for evaluating muscle lactate transport. These have been applied to normal subjects and to patients with unexplained muscle dysfunction. I report here the first case of what may prove to be a common muscle disease.

The patient was a 26-year-old military drill instructor in superb physical condition. He had had three brief episodes of severe, diffuse anterior chest pain over the past 5

Fig. 1. Rise and decline of plasma lactate in nine control subjects and the affected patient (dotted line) after 2 minutes of ischemic forearm exercise. All cases show a peak lactate immediately after exercise, and all, except the patient, show a considerable decline during 6 minutes of reperfusion.

Biochemistry Division, Armed Forces Institute of Pathology, Washington, DC 20306-6000.