Epigenetic learning in non-neural organisms

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Learning involves a usually adaptive response to an input (an external stimulus or the organism's own behaviour) in which the input-response relation is memorized; some physical traces of the relation persist and can later be the basis of a more effective response. Using toy models we show that this characterization applies not only to the paradigmatic case of neural learning, but also to cellular responses that are based on epigenetic mechanisms of cell memory. The models suggest that the research agenda of epigenetics needs to be expanded.

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1. Learning and cell memory

1.1 *Learning requires memory*

Learning has been one of the central processes studied by psychologists, neurobiologists, and cognitive scientists, and its definition and normal usage are rooted in these disciplines. Seen from a general biological perspective, learning is a special type of adaptive plasticity, which involves memory. Memory, however, is necessary but not sufficient for learning. For example, if as a result of an environmental induction there is a persistent change in the behaviour and the internal state of an organism, even when the original stimulus that induced the behaviour and the internal state is long gone, we may speak about this persistence as memory. This notion of memory also applies to cells, and this is indeed the sense in which biologists speak about "cell memory". Nevertheless, we would not say that mere persistence of past activities means that the cell has learnt. Learning implies both latency and recall.

We therefore say that simple forms of learning occurred when:

(i) One or more inputs (e.g. external sensory stimuli or the organism's own behaviours) start a reaction that leads to a behavioural response.

(ii) The input-response relations are memorized. By "memorized" we mean that some physical traces of the reaction persist. The organism is no longer in its initial (pre-input) state, but when the input has gone it does not go on exhibiting the behavioural response. It is the threshold for responding to the input that has been changed as a result of the past response.

(iii) The memorized relations can be recalled upon later exposure to one or more of the inputs. The response appears more readily or with less exposure to these inputs.

Many different types of learning that are in line with this characterization have been formulated in the fields of psychology and neurobiology. The simplest types of learning in neural organisms entail modifications – by inputs and outputs – in the efficiency or strength of existing (reflex) connections between neurons. A neural memory trace can be seen as the result of a temporary pattern of activity (firings) in a neural network, leading to changes in synaptic weights, which persist in the absence of the firing pattern and the behavioural response. Recall can be seen as the initiation of firing activity in the network in which these synaptic weights were stored, a firing that leads to the behavioural response. Crucially, the persistent synaptic pattern of

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modified weights does not lead to overt behavioural action in the absence of the input. Hence, the notion of memory in neurobiology and psychology directly implies latency and learning. However, the characterization of learning given above applies not only to learning in neural organisms, but also to learning in the immune system and in sophisticated machines like robots. This definition is also appropriate for some responses of unicellular organisms, and possibly also of non-neural multicellular organisms such as some plants, fungi, sponges, and slime moulds. The study of learning in such organisms requires that a clear distinction between the notions of memory and learning is made, and that cell memory mechanisms are characterized. We propose a new general framework for studying learning in unicellular and non-neural organisms, which is based on what has been discovered about epigenetic control mechanisms.

1.2 *Epigenetics, cell memory and cell heredity mechanisms*

Our focus in this paper is on the epigenetic control mechanisms that underlie cell heredity. Since the notions of epigenetics, epigenetic memory, and epigenetic inheritance are used in an overlapping and inconsistent manner in the literature, we shall define them as they are used in this paper.

Epigenetics explores the regulatory mechanisms that can lead to inducible persistent, developmental effects: to the establishment of variant cellular states that are transmitted across cell divisions, or that are dynamically maintained for a long time in non-dividing cells. At higher levels of biological organization, epigenetic mechanisms generate the context-dependent self-sustaining interactions between groups of cells that lead to physiological and morphological plasticity and persistence. The mechanisms underlying cellular and organismal dynamic persistence are referred to as *epigenetic control mechanisms*, or *epigenetic control systems*. Usually changes in DNA sequence are not involved, but in some cases, for example in the mammalian immune system and in ciliate development, epigenetic control mechanisms do generate regulated alterations in DNA.

The notion of cell memory is important in studies of cell biology and differentiation (Holliday 1994; Jablonka and Lamb 1995). In complex multicellular organisms, cells become increasingly more specialized. Most differentiated cells do not divide, yet they dynamically retain their characteristics over long time (for example, nerve cells). Dividing, determined cells also retain their characteristics, as do all stems cells and some differentiated cells, such as β pancreatic cells (Dor *et al.* 2004). *Cell memory* therefore refers to the retention of functional or structural states in both dividing and non-dividing cells in the absence of the conditions that originally induced these states. For example, in non-dividing nerve cells the effect of maternal licking during a sensitive period leads to a persistent change in the activity of the glucocorticoid receptor gene in the neural cells in the hippocampus (Weaver *et al.* 2004). In determined dividing stem cells, the functional and structural state of the cell persists through cell divisions (Gilbert 2006).

The relation between cell memory and cell heredity is very simple. *Cell heredity* (or *epigenetic cellular inheritance*) occurs when variations that are not the result of DNA differences or persistent inducing signals in the cell's environment are transmitted from mother cell to daughter cell. Hence, cell heredity = cell memory mechanisms + cell division. In addition to cell heredity during development, there are many examples showing that epigenetic variations can be transmitted between generations of unicellular and multicellular individuals (Jablonka and Raz 2008).

Cell memory can be based on a very simple kind of material continuity: when the amount and stability of the induced regulatory gene product is very high, the gene product may go on performing its role even when the stimulus is gone, as long as its dilution following cell division leaves its concentration above the threshold that is required for its activity. Such transcriptional memory is, however, short-term, and does not require any special mechanism (for an example, *see* Zacharioudakis *et al.* 2007). For memory to be more persistent, autocatalysis is necessary. It is important to note that according to our definition, cell memory is a *system property*, not a property that can be applied to a particular macromolecule (for a review of a molecular reductive approach *see* Morange 2006), and therefore a search for "memory molecules" which does not start from the description of the relevant system dynamics is doomed to fail.

The autocatalytic mechanisms underlying cell memory and cell heredity are called *epigenetic inheritance systems* (EISs). Jablonka and Lamb (2005, 2007a, b) distinguished four types of epigenetic control mechanisms, all based on autocatalysis, that lead to epigenetic inheritance (the transmission from mother cell to daughter cell of variations that are not the result of DNA differences) and cell memory (the persistence in non-dividing cells of variations that are not the result of DNA differences):

(i) *Self-sustaining feedback loops:* When, as a result of induction, the product of a gene acts as a regulator that directly or indirectly maintains the gene's own activity, the persistence of this activity in non-dividing cells qualifies as cell memory, and when the transmission of these products during cell division results in the same states of gene activity being reconstructed in daughter cells, it qualifies as cell heredity. Such positive feedback may lead to alternative and heritable cell phenotypes.

(ii) *Structural inheritance:* In both dividing and non-dividing cells, pre-existing three-dimensional

structures can act as templates for the production of similar structures, and lead to their persistence over time. When, as a result of induction, alternative self-templating structures are formed, the variants persist and breed true. This type of spatial templating includes a wide spectrum of mechanisms, including prion-based inheritance in fungi (Wickner *et al.* 2004; Shorter and Lindquist 2005), the inheritance of cortical structures in ciliates (Grimes and Aufderheide 1991), and the reconstruction of what Cavalier-Smith (2004) calls "genetic membranes".

(iii) *Chromatin marking:* Chromatin marks are the proteins and small chemical groups attached to DNA which influence gene activity. Different chromatin marks can be generated as a result of changing developmental conditions, and relicts of chromosome marks can dynamically persist over time, and may segregate with the DNA strands during replication, nucleating the reconstruction of similar marks in daughter cells. Chromatin marks include modifiable histone and non-histone proteins that are non-covalently bound to DNA, as well as small methyl groups that are covalently bound directly to the DNA. Chromatin marks can have a range of stabilities, from transient to very persistent.

(iv) *RNA-mediated inheritance:* This mechanism is based on transcriptional states that are actively maintained through interactions between small, transmissible, RNA molecules and the mRNAs or the DNA/chromatin regions with which they pair (Bernstein and Allis 2005; Matzke and Birchler 2005). New patterns of interactions can be induced and persist over time in non-dividing cells, and can also be transmitted between cell- and organism-generations through an RNA-replication system and/or via the interaction of small RNAs with chromatin, which leads to heritable modifications of chromatin marks (through DNA methylation or histone modifications). RNA-DNA and RNA-RNA pairing interactions can lead not only to functional silencing, but also to targeted gene deletions and gene amplifications (Mochizuki and Gorovsky 2004).

Developmentally regulated and persistent changes in gene activity can also result from developmentally induced alterations at the level of DNA: for example, particular developmental stimuli may give rise to amplification, deletions or rearrangements of genes, based on the chromatin and RNA-mediated EISs, which may qualify as "memorized" developmental responses (Meyer and Chalker 2007; Nowacki *et al.* 2008). Although developmental alterations in DNA structure do occur (and can be readily accommodated by our toy models), we are focusing on epigenetic mechanisms of cell memory and cell heredity; these epigenetic mechanisms seem central to the processes of physiological and cellular adaptation during development, and are among the most intensely studied processes in modern biology. Since epigenetic variations can also be transmitted between individuals and generations, they play a role in heredity and evolution. Different taxa differ in the kind of epigenetic mechanism employed for between-generation inheritance: in unicellular organisms self-sustaining loops and prions are commonly employed in addition to chromatin marking, while in multicellular organisms, between-generation transmission through gametes is based on chromatin and RNA-mediated EISs (Jablonka and Raz 2008). Here we argue that epigenetic control mechanisms may be the mechanisms underlying cell *learning*, a topic that has received relatively scant attention from biologists. The simple memory and learning systems that we describe below can be instantiated through any of the four epigenetic control mechanisms, but we focus on the chromatin marking epigenetic mechanism for two reasons. First, chromatin marking is involved in many cases of stable epigenetic inheritance in all taxa (Allis *et al.* 2007; Jablonka and Raz 2008). Second, storing cell memory in patterns of chromatin marks is a general mechanism of cell memory and cell heredity. Although specific regulatory interactions are necessary to trigger and alter the activity patterns of specific genes, *memorizing* these specific activity patterns through chromatin marking (e.g. DNA methylation, histone modifications) is a very general mechanism that can be applied to any pattern of gene activity. Of course, cell memory can also be based on self-sustaining metabolic reactions (*see* for example Balaban *et al.* 2004; Tagkopoulos *et al.* 2008), on small replicating RNAs, or on three-dimensional templating, but these require additional and more constraining assumptions about the specificity of the regulatory interactions involved in memorizing. Because of the complementary generality and specificity of the chromatin marking memory mechanism, it is possible to construct very simple toy models that highlight central features of memory and learning in cells. In such models, memory span (the persistence of epigenetic marks as measured in time units) depends on the kinetics of induction and decay of the epigenetic marks.

2. From memory to recall

2.1 *Toy models of cell memory and heredity*

On the basis of our understanding of EISs, we present toy models of cell memory and learning. Our toy models are general schemes that describe input-output relations

at the transcriptional level. Following induction, changes in the chromatin structure of genes (represented as + or – marks) may persist or decay. The + or – marks in the models represent methyl groups, histone modifications, or DNA binding proteins, all of which are known to serve as both regulatory and memory elements in cells (Allis *et al.* 2007). We start by presenting five toy models that show the phenotypic effects of cellular memory/heredity under simple, biologically plausible assumptions (figure 1). We then present four more models showing how simple learning can take place in such systems (figures 2, 3).

2.1a *Constant memory and output:* In this paradigmatic case of epigenetic inheritance and cell memory, shown in figure 1a, the mark persists or is inherited between generations. The input is an inducer that leads to a change in the state of chromatin of the gene, resulting in the gene's activation and a phenotypic, behavioural output. As long as the mark is maintained, so is the output. Once established, the mark is maintained or inherited between generations with a certain, more or less constant, error rate. A classical example of such cell memory is the stable transmission of the inactive (or active) state of the X chromosome in female mammals (Heard 2005), and there are many known cases of locally induced and enduring patterns of gene activity associated with persistent chromatin changes (Allis *et al.* 2007). Intergenerational inheritance of chromatin marks, especially DNA methylation, has also been described in plants and animals (*see* for example Cubas *et al.* 1999; Anway *et al.* 2005, 2006; reviewed by Jablonka and Raz 2008). When the environment in which offspring develop does not match the environment in which their parents have developed and acquired their persistent phenotype and epigenetic marks, it may lead to pathologies. Gluckman and Hanson (2005) and Gluckman *et al.* (2007) argue that such mismatch is a cause of common metabolic diseases such as diabetes. On the positive side, a "memorized", ongoing defensive response to an insult may protect the organism or the cell against this insult upon a second application, and may even partially (and immediately) protect the cell against more extreme insults of the same type. Constant memory and constant output can also enhance the sensitivity of cells to a previously encountered stimulus. This seems to be the case with the increased affinity of *Tetrahymena* to serotonin following an initial exposure to this hormone; the organisms are able to respond to a thousand-fold lower concentration of the hormone following an initial exposure to a higher concentration. An epigenetic memory based on DNA methylation probably underlies the remarkably stable transmission of enhanced sensitivity to serotonin (Csaba and Kovacs 1990,1995; Kohidai *et al.* 1990; Csaba 2008).

2.1b *Memory with decay:* Marks are established, but, in the absence of the stimulus, over time or with cell divisions they are gradually erased, and the magnitude of the phenotypic response correspondingly diminishes (figure 1b). The lingering modifications (dauermodifications), found in *Paramecium* following induction of new phenotypes by various physical and chemical treatments (Jollos 1921), may be a good example of such linear memory decay. Lingering modifications may also be important in development, functioning as part of an internal molecular clock (if, for example, a certain number of modifications is removed with every cell division, or every unit of time).

2.1c *Decay with a threshold:* The behavioural phenotypic response disappears when the mark decays and the traces fall below the threshold value (figure 1c). Such responses are probably very common. It is known that in some cases of transgenerational epigenetic inheritance, especially in mammals, epigenetic memory is often fairly short, lasting only two or three generations (Jablonka and Raz 2008). The stability of developmental stages may also often be short, depending on the signals received by determined cells during differentiation. In many such cases it is likely that this is due to memory decay with a threshold. It seems that if an input is not repeatedly applied, the modifications of the mark will fade and a state of no response will be reached.

2.1d *Memory with delayed output (priming):* An input brings about a change in the patterns of marks which *does not* lead to an immediate phenotypic response; the later phenotypic response, however, depends on the already pre-established marks. The response occurs at a later developmental stage, when a second, different input enhances the mark and leads to the corresponding response (figure 1d). Many types of developmental changes, e. g. some stages in the transition determination → differentiation, may involve such memory mechanisms. Vernalization, an exposure to chilling that "prepares" the plant for flowering following a second signal (a change in day length) months later, is another example (Sung and Amasino 2004). Developmental deprivation, the absence of the initial input, leads to a later absence of the phenotypic response despite the presence of the second input; in other words, the organism has been deprived of a crucial early maturational input. Such is the case with maternal licking in rats: the amount of maternal licking received by offspring during a sensitive period establishes an internal primed state that, at a later stage of development (which depends on various hormonal inputs), leads to characteristic responses (Meaney 2001; Weaver *et al.* 2004, 2005); deprivation of a normal amount of early licking fails to establish a mark on a crucial gene associated with the neuro-hormonal system and leads to the development of easily stressed rats. Many persistent physiological states in adults are the effect of

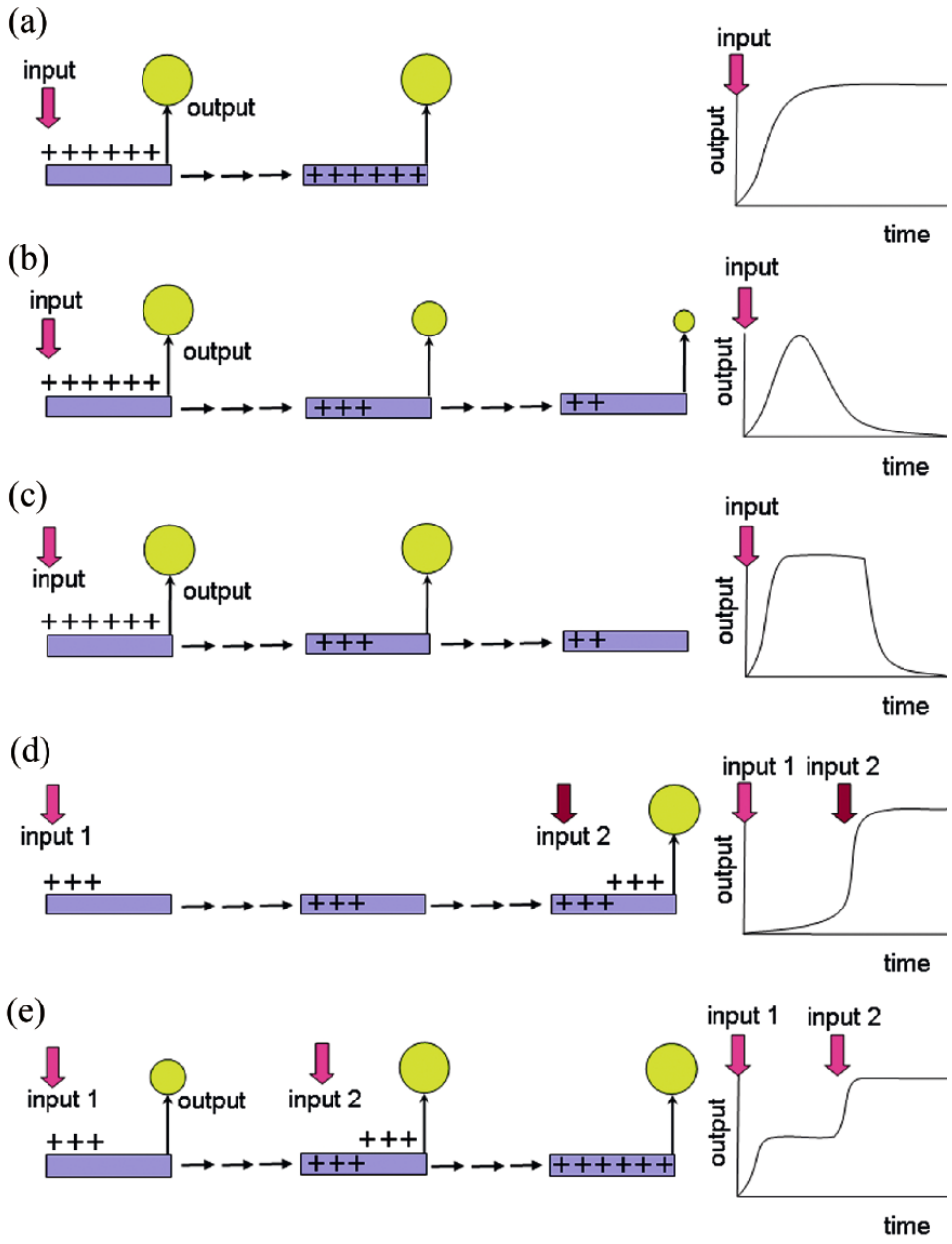

Figure 1. Five types of cell memory. Rectangles denote genes, and plus signs represent activation-related chromatin modifications; when these modifications are memorized, they are placed within the gene. Arrows between states indicate either cell generations or time units within a single generation. (**a**) Constant memory and output; (**b**) memory with decay; (**c**) decay with threshold; (**d**) memory with delayed output (priming); (**e**) memory with cumulative marking and assimilation.

maturational inputs, and many late onset chronic diseases may be the effects of deprivation early in development.

2.1e *Memory with cumulative marking and assimilation:* Inputs are applied continuously, the mark is enhanced, and the phenotypic response gradually increases; when a threshold is reached, the mark becomes stable and persists in the absence of inputs, and the phenotypic response is also persistently manifest (figure 1e). Some examples of good memory (as depicted in figure 1a) may be the result of such a cumulative process of mark enhancement. The study by Allen *et al.* (1990) of the hereditary stabilization of the effects of a transgene in a pure line of mice seems to belong to this category. Upon repeated transmission of the transgene through the mother and selection for low expression, an inserted transgene became progressively more methylated

until it became fully methylated and silent, and was stably transmitted in this state, even following the introduction of low-methylation modifiers from another strain.

2.2 *Simple learning*

Ideas about simple learning are based on the paradigmatic cases of neural learning. The simplest types of neural learning involve modifications – by inputs and outputs – in the efficiency or strength of existing (reflex) connections between neurons. Habituation and sensitization, the two basic types of simple non-associative learning, are such reflex modifications. We start by describing these elementary types of neural learning and then apply their basic features to learning in cells.

Habituation is defined as a decrease in the magnitude of a behavioural response to an iterative stimulus (Eisenstein *et al.* 2001). Habituation enables the organism to ignore irrelevant stimuli, thereby minimizing energy waste. The neural circuit underlying the behavioural response involves a sensory neuron, which is connected to a postsynaptic motor neuron (or another effector cell) via a synapse. In principle, habituation is implemented through a decrease in the strength of this synapse upon repetitive stimulation of the presynaptic neuron. In practice, the neural circuit that implements habituation is more complex, involving additional excitatory and inhibitory interneurons. Depending on the number of repetitive stimuli and the pattern of stimulation, habituation may be short-term, lasting from seconds to minutes, or long-term, lasting from minutes to weeks. Conceptually, one may view habituation as a process in which iterative inputs to a sensor connected to an effector, lead to negative feedback from the effector to the sensor.

Simple sensitization – the behavioural mirror image of habituation – involves an increase in the magnitude of a behavioural response to a stimulus, or the lowering of the response threshold upon repeated stimulations of the same type. In a two-cell circuit that exhibits simple sensitization, following repeated stimulation, the synaptic weight of the synapse connecting the presynatic and postsynaptic cells is increased. Thus, simple sensitization may abstractly be viewed as a behavioural process in which iterative inputs to a sensory element in a network, connected to an effector, lead to positive feedback from the effector the sensor. Like habituation, sensitization may be short-term or long-term.

Sensitization can take complex forms, and a specific unlearnt ("innate") response may be affected by the general excitatory state of the animal, and by the state of other (interacting) reflex pathways, which can modify the response pattern (Razran 1971; Dyal and Corning 1973). Thus, in *associative sensitization*, input to one sensory neuron, A, elicits a response from the motor neuron, while input to a second sensory neuron, B, does not; however, repeated pairing of an input to A with an input to B, in whatever order, leads to strengthening the synaptic connection between B and the postsynaptic neuron. As a result, input to B does now elicit a response from the motor neuron, and thus an association has formed: pairing the two inputs sensitizes the response. (Note that following this training, input to A is not needed in order to elicit a response from the effector following stimulation of B.)

Yet another form of non-associative learning is *pseudo-conditioning*, in which the application of an *unpaired* stimulus (itself inadequate for eliciting the specific response) sensitizes the reaction, with the result that the animal reacts to the original eliciting stimulus more readily. In this type of learning, a sensory neuron, A, elicits a specific response from the effector, and a second sensory neuron, B, does not; however, B has connections to many neurons, including A, and when it is stimulated, it enhances activity in all of these. As a result, inputs to A coupled with inputs to B lead to stronger output from the effector, and the result may even seem like conditioning: with no activity in B, mild stimulation of A will not elicit any response from the effector, but with activation of B, a threshold is reached and a specific response is elicited by the effector.

2.3 *Toy models of simple cell learning*

On the basis of the toy models of cell memory and heredity, and the simple cases of neural learning we have discussed, we suggest 4 toy models of non-associative (figure 2) and associative (figure 3) learning in cells.

2.3a *Sensitization: decay with threshold and recall:* This is the simplest type of learning, and we describe two cases. In the first case (figure 2a, case 1), following the stimulus, the gene is marked and there is a behavioural output; in the absence of the input the mark decays but a partial mark persists, and when the input is introduced again additional sites are marked and the output increases. The second case (figure 2a, case 2) is a simple modification of decay with a threshold (depicted in figure 1c): when a second input of the same type as the first input is applied, the threshold is lowered, so the size of the second input required to elicit the reaction is smaller, or the response is faster, because a partial mark is already present.

2.3b *Habituation: inhibitory modifications and recall:* An input to the gene brings about an output that acts as a negative regulator of the gene, leading to inhibitory epigenetic marking (figure 2b). As a result, upon recurring stimulation of the same type that activates the gene, the output is smaller because of the memorized inhibitory marking. Note that habituation of this type is more complex than sensitization in that it requires that the output negatively regulates the gene

(imposes a "negative" mark). Such feedback from output to the gene is possible (and plausible), but is not necessary for sensitization.

2.3c *Pseudo-conditioning sensitization linked to a generally amplifying input:* As illustrated in figure 2c**,** an input to the gene leads to an output and to the marking of the gene, with traces persisting; another, general and non-specific weak input (star-like structure) cannot lead to an output from the gene; however, when the gene is activated by input 1 and marked, the affinity of the marked gene for the general activator increases, and it can now elicit the output even in the absence of the normal input to gene 1.

2.3d *Associative activation:* Associative activation (figure 3) requires a relation between two genes to form a metabolic circuit: gene 1 is activated by input 1, leading to an output (circle), and gene 1 is also partially marked by input 1; gene 2 is activated by another input and generates an output (square). Once gene 1 is marked by input 1, the output from gene 2 serves as input to it. Hence gene 1 can be induced and generate its characteristic output upon induction of gene 2.

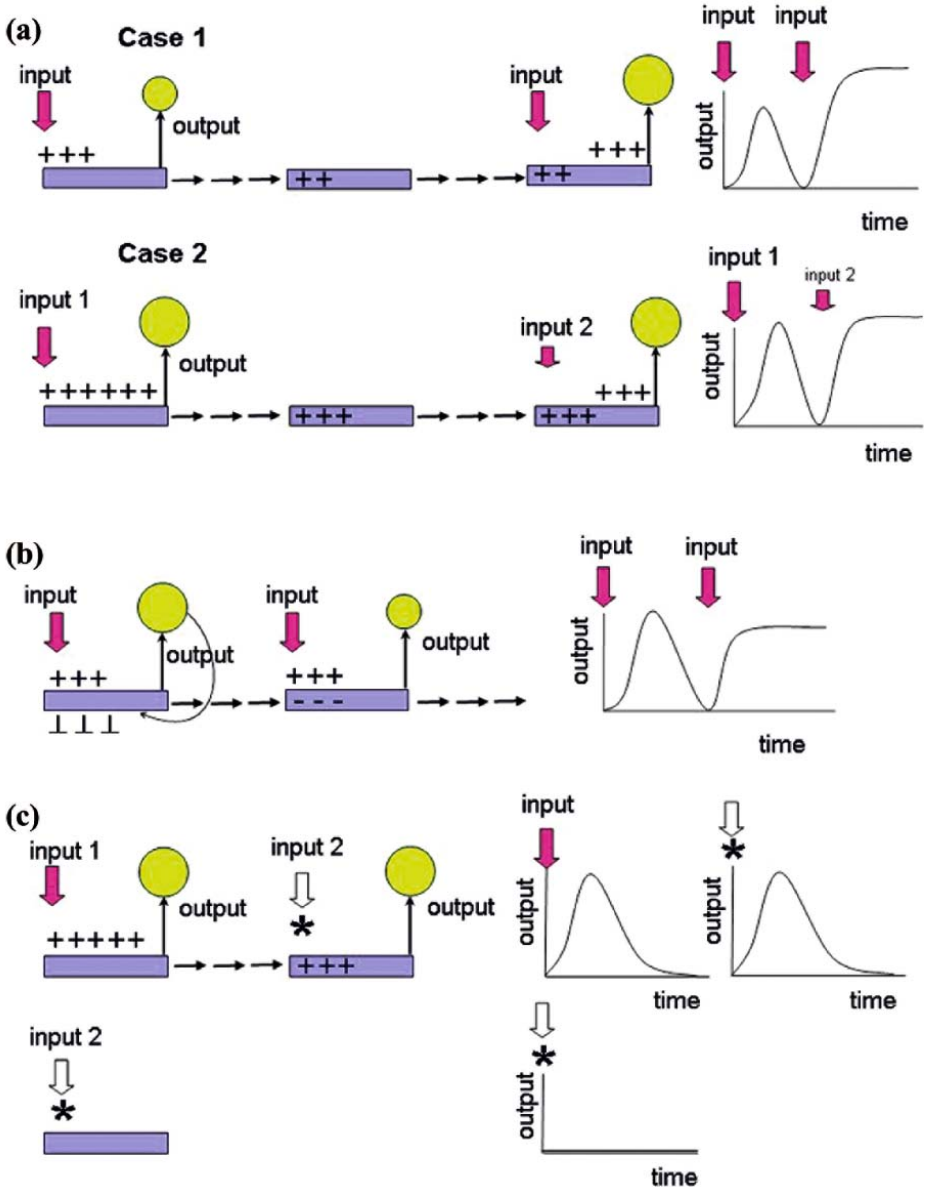

Figure 2. Simple cell learning. Rectangles represent genes; plus or minus signs represent activation or suppression-related chromatin modifications; memorized modifications are placed within the gene. (**a**) Sensitization**;** (**b**) habituation; (**c**) pseudo-conditioning.

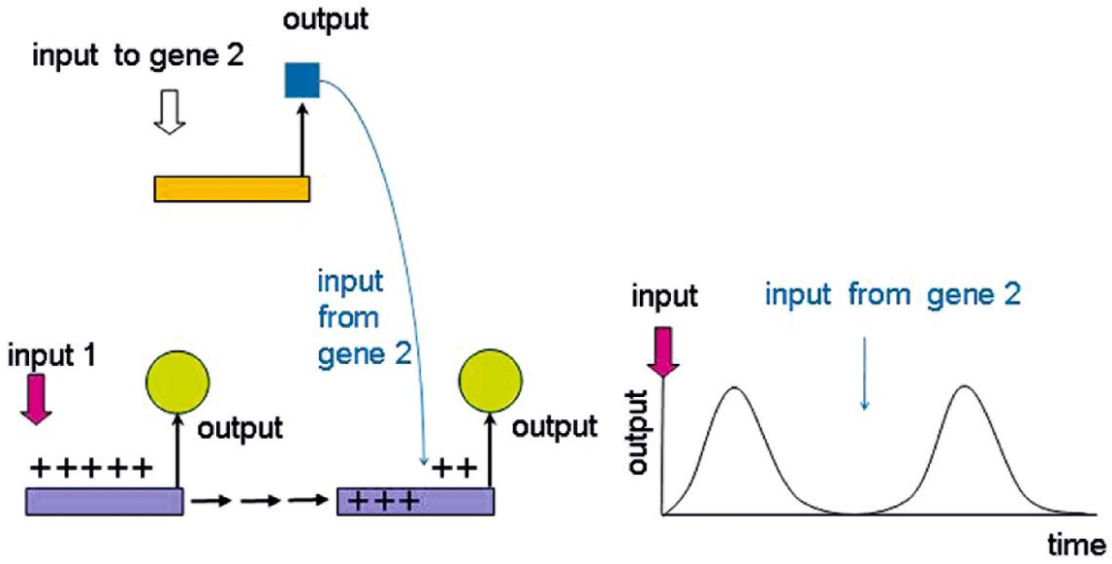

Figure 3. Associative cell learning.

The last two toy models (2.3c and 2.3d) show how as a result of pairing of a non-inducing input with an inducing one, the former can elicit a response from the gene.

Our discussion of memory, priming, and learning suggests a straightforward way of distinguishing between learning and developmental maturation processes: while learning requires latency and recall, maturation requires latency but not recall (there is no facilitated response to a recurring input). It is interesting that maturational processes can be more complex and require more numerous interactions among regulators than simple learning. There is therefore no simple correlation between the complexity of response and learning. It is also clear that maturational processes may be involved at different stages of learning, for recall may be delayed and require additional, non-identical, inputs.

Based on our approach to memory and learning in cells, we suggest two new concepts – "epigenetic recall" and "epigenetic engram" – that may be useful for the general study and discussion of cell learning (*see* Jablonka and Raz 2008). Both “recall” and “engram”, a term originally coined by Semon in 1904 (Schacter 2001), are used in a sense similar to that in psychology.

Epigenetic engram – a cellular structure or activity that acts as a memory trace, and is a remnant or specific modification of an originally induced epigenetic mark or structure; such a memory trace may persist for a long time in non-dividing cells, and it may be transmitted during cell division (mitosis and/or meiosis in eukaryotes); it facilitates the reconstruction of the original phenotypic response upon subsequent induction in the next generation of cells or organisms. Since an engram can be seen as an internalized trace of past activity which "stands for” past input–response relations, it can be considered as a “representation” that is the result of the effects of a past input on the system.

Epigenetic recall – the facilitated reconstruction of a previously induced phenotypic response, based on persistent epigenetic engrams.

We would like to stress again that although our models are based on the chromatin marking EIS, the other EISs too can lead to the formation of engrams and bring about recall.

3. Expanding the epigenetics research program

Although epigenetic memory in non-dividing cells, in dividing cells, and across generations of organisms is a very intensely researched topic, the kinetics of memory decay, the maximal “memory span” of a mark, the relation between the nature and extent of the mark and gene expression, have not been systemically investigated. Although some information of this type is available for specific systems, as the examples that we have given show, these topics are not routinely investigated as part of the research program of epigenetics. Gluckman and Hanson (2005) suggested that marks in one generation that are faithfully inherited might lead to a non-matching (yet predictable) effect in the subsequent generation, if the environments of parent and progeny are drastically different. These considerations have led to medically important insights. It would be interesting to see how deprivation, which results in the absence of (or in abnormal) memorized marks, can affect the development of offspring and how deprivation can be compensated for.

The role of memory in "preparing" the organism for more extreme conditions than those experienced by the parent (or experienced by the same organisms at a previous stage) is also of interest.

There are at present only a few known cases of what might be seen as epigenetic learning in non-neural, single-celled or multicellular organisms. However, the language of learning and intelligence is often used (e.g. Trewavas 2003) when organisms exhibit memory or show plastic open-ended behaviour, such as chemotactic movement in bacteria or in roots. Exploration and selective stabilization mechanisms, which often underlie such behaviour, occur at the cellular, physiological, behavioural and social levels, and are all based on a similar principle – the generation of a large set of local variations from which only a small subset is eventually stabilized and manifested. Which particular output is realized depends on the initial conditions and the number of possible points around which development can be stably organized (these points are referred to as attractors). There are many such processes in biology (*see* Kirschner and Gerhardt 2005; Ginsburg and Jablonka 2007). Although these kinds of processes can lead to flexible new adaptive responses, the responses may be defined as learnt ones only if they are coupled to memory mechanisms, and only if partial memory traces, which facilitate a future response to the recurring input, occur. The distinction between a learnt response and a memorized constant response is central to our definition of learning. A systematic search, guided by simple yet plausible models and plausible molecular mechanisms, should uncover and distinguish between cases of learning and cases of a constant response that seem like learning.

For example, a case that looks like sensitization, but is in fact a manifestation of the effects of constant response in a changing environment, is seen in *Escherichia coli.* Growth of the bacteria under inorganic phosphate (P_i) limitation induces the synthesis of many proteins. These proteins scavenge traces of P_i or phosphorylated compounds from the extracellular medium. The expression of the genes encoding these proteins is controlled by a two-component regulatory system consisting of the sensor PhoR and the transcriptional activator PhoB. The regulatory genes *phoB* and *phoR* form an operon, which is subject to autoamplification, so that signal transfer through the PhoB-PhoR system stimulates its own expression. Since the regulatory proteins are quite stable, upon exposure of the cell to inducing conditions, previously induced cells (with high concentrations of the regulatory proteins) respond more rapidly than cells with no recent induction history (Hoffer *et al.* 2001). Memory resides in the autoamplification dynamics (through positive feedback) coupled with the stability of the proteins within the cells. There is no latency and no recall in this system: the previously induced response (having the scavenging proteins) simply persists, and the recurring stimulus (limitation of P_i) does not alter it. However, the functional effect of the constant response is only unraveled when P_i is limiting: in these conditions a more rapid response is an inevitable consequence of the persistence of the previously induced scavenging proteins.

Learning in single celled organisms has been investigated mainly in ciliates, and there are several reliable reports documenting non-associative learning in *Stentor* and *Paramecium* (Wood 1992). Thus, for example, Wood (1988a, b) showed that repetitive mechanical stimulation of *Stentor* leads to habituation of the contraction response; it seems that the basis of the habituation in this case is a (post-transcriptional) change in the voltage-dependent mechanoreceptor channels. This is a case of genuine learning, because the response (contraction) that followed the first input (mechanical stimulation) disappeared after the initial input was gone, but memory traces of the reaction leading to the response remained, resulting in facilitated (in this case reduced) responses following the application of additional inputs of the same type. Another example of habituation in *Stentor*, decreased upward-swimming upon repeated exposure to conditions eliciting this response, has also been documented (Hinkle and Wood 1994). Similar cases have been reported in *Paramecium*, and in this single-celled organism there have also been many attempts to demonstrate associative learning. Most of these attempts, carried out decades ago, seem to be controversial, but recent evidence suggests that *Paramecium* can learn to associate between light and electrical stimulations (Armus *et al.* 2006).

At the molecular level, changes in the mechanoreceptor channels in *Stentor* are similar to the changes occurring during short-term habituation in *Aplysia*, in that no transcription or protein synthesis is required. In unicellular organisms, the effector and sensory components are, of course, part of the same cell, while in multicellular neuronal organisms the two components reside in different, sometimes very distant, cells. Nevertheless, in both cases the molecular machinery underlying the learning phenomena are basically the same. In long-term habituation, however, such as that found in *Aplysia*, protein synthesis is required (Hawkins *et al.* 2006). The epigenetic mechanisms we have discussed may be involved in establishing such long-term memory and learning. For example, DNA methylation changes may underlie the stabilization of a gene expression pattern that leads to ongoing and stimulus-independent synthesis of a chemoreceptor protein. We are not aware of any studies that have looked for sensitization and habituation in cell lineages within a multicellular organism during development.

Although our toy models and discussion are focused on cell learning, learning may also occur in multicellular organisms that do not have a nervous system. In such organisms, the problem of intercellular coordination

arises, so the mechanisms may be more complex or different from those in single cells. What would need to be memorized is not just the state of single cells, but rather *patterns of interactions/communication between cells.* The central question then is how these interaction patterns are instantiated, and how communication patterns can be remembered. It is plausible that changes in the three-dimensional conformation of molecular structures (e.g. receptors for paracrine factors secreted by one cell type and received by neighbouring cells) that connect cells in an organ (e.g. a flower of a carnivorous plant) may be involved; the same mechanisms as those associated with the maintenance of form through growth (Ettinger and Doljanski 1992) may operate to preserve traces of previously induced temporary changes in morphological features. Memory will then be instantiated as partially-altered, three-dimensional, intercellular conformation patterns that would lead, upon repeated stimulation, to the more ready formation of previously induced responses. For example, a receptor protein that binds a hormone or a paracrine factor could be involved in such memory, if the receptor alters its conformation so that it acquires prion-like properties, as suggested by Si *et al.* (2003) for self-sustaining changes at the synapse. DNA methylation and histone acetylation are known to be involved in some cases of long-term memory in the nervous system (Levenson and Sweatt 2005; Miller and Sweatt 2007; Gräff and Mansuy 2008). It is plausible that the epigenetic learing mechanisms that we described will be found in nerve cells following firing and wiring, and this will then forge an interesting link between epigenetic learning and neural learning. However, the mechanisms underlying epigenetic memory and epigenetic learning may also be involved in maintaining connectivity paterns among non-neural cells. Epigenetic changes may lead to the production of altered patterns of connectivity if these genes code for receptors or for enzymes involved in the synthesis of paracrine factors or hormones. Self-sustaining physiological intercellular loops based on localized signalling patterns (through diffusible signals) are also likely to be involved, with close-to-threshold concentrations of signalling molecules being the memory traces.

As in unicellular organisms, non-neural multicellular organisms exhibit complex adaptive behaviours that may seem like learning. An example is the condition-dependent movement of cellular slime mould *Physarum polycephalum*, which looks like sensitization but, according to our criteria for learning, is not. The mould, which belongs to the phylum Amoebozoa, moves in humid warm conditions at a rate of about one centimeter per hour, but when the temperature and humidity drop it decreases its rate of movement. When three exposures to dry air lasting for 10 min were given to the mould at regular intervals (e.g. every 30 min), the mould slowed down when a fourth pulse of dry air was due, even if none was actually applied. Expectation gradually faded away if no dry period recurred, but applying a single dry pulse about 6 h later commonly led to another anticipatory slowing, which was in step with the earlier rhythm (Saigusa *et al.* 2008). The team that studied this behaviour developed a model based on the coupling and reorganization of oscillators. In our terms, the behaviour can be described as a case of constant memory with the activity of the activated ("wound") oscillator persisting over time, and eliciting the response without any need for a new external input.

Learning via sensitization and habituation (and possibly their modulations) can, however, be expected in multicellular organisms that live in a complex, yet more or less recurring conditions. Plants (especially those able to move), fungi, sponges (especially their motile larvae), slime moulds, and possibly also *Trichoplax* (a primitive non-neural multicellular creature that crawls on the ocean floor) and *Volvox* (motile multicellular algae) are all likely to anticipate and learn. However, the evidence for learning in non-neural multicellular organisms is scant, and it seems that few relevant experiments have been done to investigate this issue. We found no evidence for learning in *Volvox*, although there is a possibility of memory, since the phototactic threshold (the minimum light intensity required to get any phototactic response) was reported to rise by more than three orders of magnitude after spheroids that had been kept in the dark for a few hours were exposed to direct sunlight for a few moments (Kirk 1998). We are not aware of any experiments on memory or learning in sponge larvae or in *Trichoplax*.

Plants store information about past experience and this affects the way they respond to present inputs. Abramson and his colleagues (2002) showed that differential responses of *Philodendrum* plants to light depended on their previous experience, but no learning (according to our definition) occurred. There are, however, reports that suggest that habituation may occur in plants. The legume *Mimosa pudica* responds to touch: when touched, its compound leaves fold-up. If leaves are repeatedly prodded by the same kind of stimulus, they eventually stop folding upon touch. Applewhite (1975) reviewed data showing that the extent of habituation can be modulated; for example, *Mimosa* leaves can be conditioned to distinguish the touch of wet droplets from dry poking objects, retaining their sensitivity to one type of touch while becoming habituated to the second. Habituation in the carnivorous plant *Drosera* (Sundew) has also been reported (Applewhite 1975). The basis of this habituated behaviour may be persistence at the level of receptors, but since the response (the folding behaviour) decays in the absence of the input, and the extent of the response decreases upon repeated stimulation, this is a case of true habituation. It would be very interesting to study the molecular basis of these responses, and elucidate the mechanisms of memory involved.

A study on induced defenses against predators in wild radish (*Raphanus raphanistrum)* showed that induction (exposure to a predator) in the parental generation made the offspring better adapted to the predators than those of un-induced parents. Agrawal and his colleagues (1999) suggested that the persistent effect might be either a direct, maternally-induced effect (in which case it would qualify as a case of constant transgenerational memory), or the result of more rapid induction of plant defenses in the offspring of damaged mothers. If the latter proves to be the case, it will represent a case of sensitization, with the epigenetic recall underlain by as yet uncharacterized epigenetic engrams.

The few cases of actual and possible learning that we have surveyed are all different and for many of them the underlying biochemical mechanisms are unclear. We know of no evidence for mechanisms like those illustrated in our toys models, but we predict that such mechanisms exist and underlie different types of non-neural learning in unicellular and multicellular organisms. We suggest that unicellular organisms, especially actively moving protists, and non-neural organisms with the ability to adaptively and rapidly alter their location and morphology (for example, *Volvox*, sponge larvae and "sensitive" plants) may be good candidates for such study. Evolutionary considerations can give us clues to the function of such systems and to the conditions in which they may be favourable, and hence point to the kind of biological systems in which they may be found.

4. Evolutionary implications

Memory and learning are clearly of potential advantage to organisms that live in fluctuating but recurring environments: when inputs are likely to recur and the adaptive developmental response to these inputs is very costly, it is beneficial to reduce the cost by memorizing. We must assume, of course, that the cost of having memory systems is not too high and its formation does not require any unlikely mechanism. These are reasonable assumptions, since the memory mechanisms are part of already existing epigenetic control systems, which have obvious adaptive benefits. Only small modulations of these are necessary to turn them into memory systems.

The advantage of remembering is clear for organisms that live in conditions that persist, but not for very long. In an environment that persists for a very long time, a constitutive response is expected. On the other hand, a response to accidental and transient, non-recurring changes needs to be forgotten. Memorizing should be favoured in conditions that recur: when the environment changes every few ontogenetic time units, or every few generations. For example, epigenetic inheritance is likely to be favoured in environments that fluctuate at an intermediate rate – that last for more than one generation, but not for very many (Lachmann and Jablonka 1996; Balaban *et al.* 2004; Lewis 2007; Rando and Verstrepen 2007). It may be particularly important for microorganisms that live in environments that are neither very rapidly changing (where readily reversible responses that depend on the stimulus are advantageous), nor very slowly changing (where practically irreversible mutational changes are beneficial). The "memory span" that evolves will be proportional to the rate of fluctuation as measured in generations. In general, efficient epigenetic inheritance in intermediate length fluctuating environments is likely to evolve (i) if the parental (or past) environment carries reliable information about the offspring's (or future) environment (Jablonka and Lamb 1995); (ii) when the response to induction is lengthy and incurs a high cost (Lachmann and Jablonka 1996). The advantage of correctly anticipating environmental conditions may be particularly great if the anticipatory response increases protection against more extreme adverse conditions, or if it increases the sensitivity of the organism and enables it to detect rare, low-concentration useful factors.

We suggest that true learning, epigenetic sensitization and habituation will often be selectively superior to persistent developmental memory and to epigenetic inheritance, because the cost of a memorized response that is no longer adequate (which occurs when memory is perfect) is reduced, and the cost of development-from-scratch (which occurs when reset is complete and full induction is required) is also reduced. The transmission of epigenetic engrams that lead to an inducer-requiring yet facilitated response may therefore often be an optimal compromise. The danger of a tyrannical (no longer adequate) memory is avoided, and the expensive need for developing-from-scratch (when there is delay in responding) that comes with too thorough "forgetting" is also avoided. Shorter-term forgetting is much better than both not forgetting at all, and total amnesia.

It is of interest that repeated stimulations, rather than a single stimulus, often elicit habituation or sensitization in neural organisms and in *Mimosa* and *Drosera*. This makes functional (and hence evolutionary) sense, since the only events that are worth remembering are recurring ones. Rare events need not be remembered, and enduring events lead to enduring stimuli and hence remembering is superfluous. It is not difficult to envisage how repeated stimulation may operate within the framework of the toy models we have suggested. Repeated stimulation may lead to cumulative marking if it adds (positive or negative) modifications to the gene, but as long as a critical level is not reached, there is no adaptive output. Only when the critical level of modification is reached does the gene produce the output and the mark persists (i.e. there is memory). This is similar to priming, but in this case priming occurs through the effect of the same stimulus. Another, more realistic case, which takes decay into consideration, is also easy to envisage, as illustrated in figure 4. Assume that there are 5 sites that can be marked

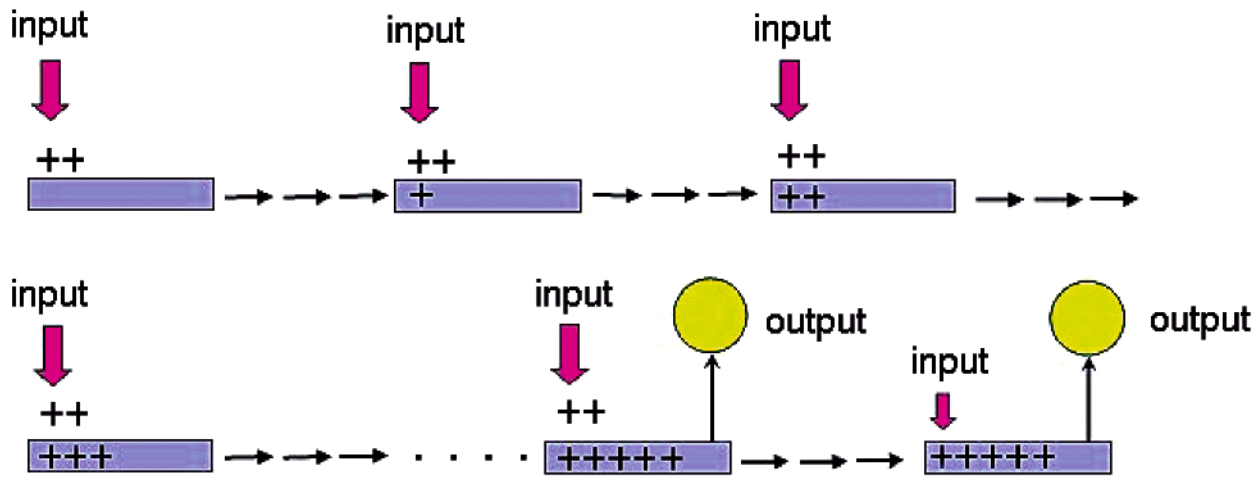

Figure 4. Training: multiple-input dependent response. Note that output occurs only following repetitive inductions and cumulative markings (5+).

and that persist, and that whenever there is a stimulus, 2 sites are marked (positively or negatively), and when the stimulus is over one site remains marked. As long as fewer than five sites are marked, there is no change in the output of the gene. Eventually, following recurrent stimulations, the mark will be "saturated" and elicit the adaptive response. Further repeated stimulation may be needed for sensitization (as shown in figure 4) or for habituation. Such dependence on recurring stimuli ensures that the memorized response adequately predicts future enviornemtnal conditions.

An additive marking mechanism may also operate at the receptor level, if we assume that the conformation of the receptor undergoes small, partially persistent, changes upon each stimulation, and a change in behaviour occurs only when enough partial changes have accumulated. In other words, a new threshold is reached only following recurrent stimuli. The dependence of cumulative marking on repetitive inputs rather than on one continuous input may be based on reversible interactions between the marking enzyme and the marked sequence, which requires release and re-loading of the marking enzyme, with release (and hence re-loading and remarking) depending on the absence of the inducing input.

Associative sensitization and pseudo-sensitization will be selected in an environment in which the conditioned normal (primary) inducing stimulus and the secondary dependent stimulus are usually, but not always, coupled, yet the benefit of a sensitized response is significantly greater than that of a superfluous response. For example, if tissue damage is often, but not always, associated with a change in salinity, it may be advantageous if a change in the salinity input alone induces a defensive response (for similar reasoning *see* Tagkopoulos *et al.* 2008). An occasional superfluous defensive response is not too costly, whereas a needed defensive response is always life saving.

5. Conclusions

We presented simple toy models of memory and learning in single cells. Since the molecular mechanisms that may underlie memory and learning are well characterized, we suggested that modulations in the conditions in which these mechanisms operate, and modulations in the dynamics of memory formation and decay can lead to quite complex plastic adaptive responses that may enhance reproductive success. The simplicity of the models we presented, their biological plausibility and their evolutionary logic suggest that learning in cells and in non-neural organisms may be common, and that experiments exploring the dynamics of memory formation and decay may be fruitful.

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