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Glutamate receptor-driven gene expression in learning

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Abstract. The role of protein biosynthesis and gene expression in learning has been well documented. Similarly, the role of activation of glutamate receptors in neuronal plasticity have been shown repeatedly. In our studies we found that L-glutamate, acting through different kinds of its receptors may stimulate expression of c-fos and other genes encoding components of transcription factors both in vivo and in vitro. We have also documented elevated expression of c-fos after induction of long lasting long term potentiation and various forms of behavioral training. In this paper these data are reviewed and a hypothesis, suggesting that neuronal nuclei may act as information integration device in memory formation is proposed.

Key words: L-glutamate, excitatory amino acids, NMDA, c-fos, zif/268, transcription factor, AP-1, two-way active avoidance, LTP, copulatory behavior

INTRODUCTION

In the first half of the eighties a new synthesis of biological knowledge emerged (reviewed previously in: Kaczmarek 1986, Kaczmarek and Kamińska 1989). It became clear that long term cellular responses, apparently so contradictory like stimulation of cell cycle and induction of differentiation, have in fact a lot in common. Development of molecular biology contributed the most to these findings and the discovery of protooncogenes should be particularly praised in this regard.

Protooncogenes are normal cellular counterparts of transforming genes of tumor cells. They encode different kinds of proteins sharing, however, one feature - active role in the transduction of signals between extracellular environment and intracellular milieu including transmission of information into the cell nucleus. There are protooncogenes which code for cell stimulating ligands like growth factors, their receptors, receptor-linked tyrosine kinases, GTP-binding proteins, serine/threonine kinases, transcription factors.

It was then quickly realized that probably the most fascinating long term biological response i.e. memory formation may share similar molecular mechanisms (Goelet et al. 1986, Curran and Morgan 1987, Kaczmarek and Nikolajew 1990, Sheng and Greenberg, 1990, Robertson and Dragunow 1990). Memory formation is dependent on neurotransmitters acting on specific cell surface receptors and then activating all the aforementioned intracellular machinery. As this paper focuses on gene expression in learning, the evidence for this phenomenon will be further elaborated below.

REQUIREMENT FOR GENE EXPRESSION AND PROTEIN BIOSYNTHESIS IN LEARNING

There is an expanding body of evidence that gene expression and protein biosynthesis are critical for establishing long lasting memory traces (Davis and Squire 1984, Goelet et al. 1986, Matthies 1989a,b). For example, several research groups have documented enhanced incorporation of various precursors into RNA and proteins during behavioral training (for review see: Dunn 1986), suggesting an induction of gene expression during and after learning. There have also been numerous reports showing that inhibitors of protein and RNA biosynthesis block formation of long lasting (greater than several hours) memory in vertebrates (Davis and Squire 1984, Flood et al. 1986) as well as in invertebrates (Montarolo et al. 1987). Of particular interest is the fact that usually only an injection of these inhibitors at the time of training or immediately afterward produced memory deficits. More detailed scrutiny of the timing of the action of inhibitors on the rats' learning of a brightness discrimination task revealed that there are two sensitive periods for such treatment: one just described, and another a few hours following the training (Grecksch et al. 1980).

In good agreement with these results are the observations that two waves of protein biosynthesis have been observed following aversively motivated brightness discrimination training of rats. The first occurs just after training and soluble proteins, synthesized on free polysomes, predominate in this wave and another one with membrane proteins predominating is detectable a few hours later (for review see: Matthies 1989a,b).

Also in the LTP model (see below), an increased incorporation of precursors into proteins was observed after high frequency stimulation (Loessner et al. 1987). Likewise, the requirement of new protein biosynthesis for the long lasting component (4 hours or longer) of LTP seems to be firmly established based on effects of protein synthesis inhibitors both *in vivo* (Krug et al. 1984) and *in vitro* (Stanton and Sarvey 1984, Frey et al. 1988).

L-GLUTAMATE DRIVEN ACTIVATION OF TRANSCRIPTION FACTORS

We initially decided to investigate whether L-glutamate, a putative neurotransmitter critical for

different forms of neuronal plasticity including learning, may activate expression of c-fos nuclear proto-oncogene. This gene codes for a protein (c-Fos) known to be a component of the AP-1 (activator protein 1) transcription factor (TF) (Morgan and Curran 1991a). The TFs are proteins which regulate gene expression after binding to specific regulatory sequences (He and Rosenfeld 1991, Struhl 1991). There are at least 4 different members of the "fos family" (*c-fos*, *fos-B*, *fra-1*, *fra-2*) and protein product of each of them (c-Fos, Fos-B, Fra-1, Fra-2) may interact with any of the proteins of the "jun family" (c-Jun, Jun-B, Jun-D) in order to produce dimers acting as AP-1 (Morgan and Curran 1992a).

Injection of L-glutamate just below the hippocampal formation in the rat brain induced rapid and transient accumulation of c-fos mRNA levels in the hippocampus (Kaczmarek et al. 1988). However, we then found that injection of physiological saline alone (vehicle used to dissolve L-glutamate) produced similar, albeit less pronounced effect (Kaczmarek et al. 1988).

In order to test whether L-glu may indeed elevate c-fos expression we turned to a better controlled system i.e. neurons cultured *in vitro*. Together with the group of D.F. Condorelli (Condorelli et al., in preparation) we studied the mRNA levels coding for c-fos, fos B, c-jun, jun B and zif/268 in cerebral hemispheres' neurons isolated from fetal rat brain and cultured in vitro for 7 days. Several different agonists of L-glu were used, and all of them were able to provoke accumulation of mRNAs of aforementioned genes. However, the detailed kinetics as well as levels of induction were specific for each of the gene tested. In particular, NMDA was only a weak inducer of c-jun, while very strong of c-fos and zif/268.

We then studied an ability of L-glutamate to increase levels of DNA binding activities (measured by electrophoretic mobility shift assay, Kamińska et al. 1992) of four TFs: AP-1, CREB, NFκB and AP-2 in neurons and astroglia cultured in vitro (Łukasiuk et al. 1992, Łukasiuk et al., in preparation). A high level of specificity of L-glu-driven TFs induction was found as only AP-1 was stimulated. On the other hand, AP-2 was observed to be activated by phorbol esters (TPA) in neurons but not glia, while NFκB DNA binding was enhanced by TPA in glia but not in neurons. No significant differences in the pattern of TFs DNA binding activities were detected among various neuronal cultures: fetal cerebral hemispheres, fetal hippocampus and postnatal granule cells of the cerebellum.

It is important to note that the DNA-binding activity does not necessarily reflect the level of the TF or its functional activity. Certain TFs like CREB are known to be present in the nucleus before stimulation and their functional activity is dependent on protein phosphorylation (Montminy et al. 1990), which, apparently, does not influence the DNA binding activity (Dash et al. 1991). Other TFs like NFκB are present in the cytoplasm anchored by specific inhibitory factors and cell activation results in translocation of the functionally active TF to the nucleus (Baeuerle 1991). Another mode of activation characterizes AP-1, which has protein components that are, during induction process, first to be synthesized and then interact, forming a mature TF (Morgan and Curran 1991). Therefore, only for some but not all TFs, DNA binding activity is a measure of the TF level.

It should be stressed that in glia elevated expression of c-fos and other aforementioned genes encoding TFs as well as AP-1 itself is inducible by different L-glu agonists with a notable exception of NMDA (Condorelli et al. 1989, Condorelli et al., in press).

In conclusion, one can say that L-glutamate activates expression of genes encoding transcription factors as well as transcription factors themselves (see above and also: Szekely et al. 1987, Murphy et al. 1991a,b, Vaccarino et al. 1992, and for review: Morgan and Curran 1991a,b). This suggests the way L-glu may provoke long term neuronal responses. Several different L-glu receptors are differentially involved in activation of expression of different genes encoding TFs.

NMDA RECEPTOR IN LONG TERM MEMORY FORMATION

Obviously, the main reason for our interest in L-glu driven gene activation is the role of L-glu and particularly its NMDA receptor in learning processes. While the literature on this subject is abundant (Morris et al. 1984, Cotman and Iversen 1987, Collingridge and Singer 1990) our own work has provided additional evidence documenting that NMDA receptor plays a critical role in long but not short term memory formation. This conclusion was based on passive avoidance studies with two antagonists of the NMDA receptor, namely, dextrorphan and MK-801 (Sierocińska et al. 1991).

C-FOS EXPRESSION IN LONG TERM POTENTIATION

One promising direction for delineating the mechanisms serving functional brain plasticity and memory formation involves the study of synaptic long-term potentiation (LTP), which refers to the persistent increase of synaptic efficacy following brief, high frequency stimulation of monosynaptic pathways particularly in the hippocampus (Bliss and Lomo 1973).

We found, together with the group of H.Matthies, that specially designed stimulation of rat perforant path in freely moving rats increases c-fos mRNA levels in both pre- (entorhinal cortex) and post-synaptic (hippocampus) cells (Nikolaev et al. 1991). The stimulation session consisted of twenty 75 ms groups of high frequency current separated by 5 s and repeated 4 times at 15 min intervals. This stimulation pattern was selected because it leads to LTP reproducibly lasting longer than a few hours. Only under such conditions studying gene expression seems to be reasonable. Moreover, we assumed that transient nature of c-fos activation well known from other systems, as well as its inhibitory effect on its own expression, required a single stimulation session leading to a full long lasting response.

The rationale for the experimental paradigm we employed has to be stressed as there are controversies about c-fos inducibility in LTP formation. In general, one can say that when single session was used to develop long lasting LTP in freely moving rats, c-fos activation was observed. On the other hand, weaker stimuli, particularly in anesthetized animals, failed to provoke c-fos expression. However, they apparently failed to induce long lasting LTP as well. This issue is further discussed in: Kaczmarek 1992.

GENE EXPRESSION EVOKED BY BEHAVIORAL TRAINING

Our initial experiments with behavioral training and c-fos expression, performed with H.Matthies and his collaborators, were based on a single session of two-way active avoidance with compound conditioned stimulus (CS) consisting of darkness and tone. We found that c-fos mRNA levels were several-fold increased in the hippocampi after such training (Kaczmarek and Nikolajew 1990, Nikolaev et al. 1992a). Next, these results were reproduced after similar, though slightly modified, training with darkness as CS, as well as they were extended, showing both c-fos and zif/268 mRNA elevation in the hippocampus and visual cortex (Nikolaev et al. 1992a). In the meantime, similar experiments with brightness discrimination training paradigm confirmed the basic finding of c-fos activation and raised important questions about proper controls. It was found that both regular excitatory training as well as pseudoconditioning provoked c-fos expression (Tischmeyer et al. 1990). However, it has to be noted that pseudoconditioning should be also regarded as a training procedure leading to learning of lack of CS-US association.

This question of proper controls remains as yet not fully resolved, since there is probably no real control for learning. The fairest one could get is probably to train animals up to a plateau level of performance, and then to test them in a "no-learning" situation when only the execution of already learnt behavior occurs.

In our studies we found that the performance of a well learnt task did not by itself lead to *c-fos* and

zif/268 induction (Nikolaev et al. 1992a). On the other hand, the novel performance-elevating stimuli (Zieliński et al. 1991) were still able to activate gene expression. For instance, in our experiments rats were exposed to daily training session of two way active avoidance (50 trials per session) with darkness as the CS. We observed that while the first training session evoked clear increase of *c-fos* mRNA levels, the 9th session (at the plateau level of performance) was without any effect on *c-fos* expression. However, when the rats on the 9th day of training were exposed to a compound CS, consisting of darkness and a wide-band noise, the *c-fos* mRNA was found to accumulate in the sensory cortex (including the auditory cortex), preferentially to other brain areas (Nikolaev et al. 1992b). This c-fos activation was concomitant to increase of performance level.

Comparison of the *c-fos* expression levels observed in the hippocampus and sensory cortex after single versus multiple training sessions revealed interesting differences (Fig. 1). Single training session with darkness as CS led to much higher *c-fos* levels in the hippocampus than in the cortex. Then, as mentioned above, a session at the plateau level of performance was not efficient inducer of c-fos mRNA in neither brain structure. However, addition of auditory stimulus to produce compound CS increased c-fos expression, but only in the sensory cortex area. This suggests that mechanisms involved in c-fos activation in both experimental situations in different brain structures are somewhat different.

Elevated expression of *c-fos* following behavioral training is not limited to the aversive conditioning only. We have reported that learning of

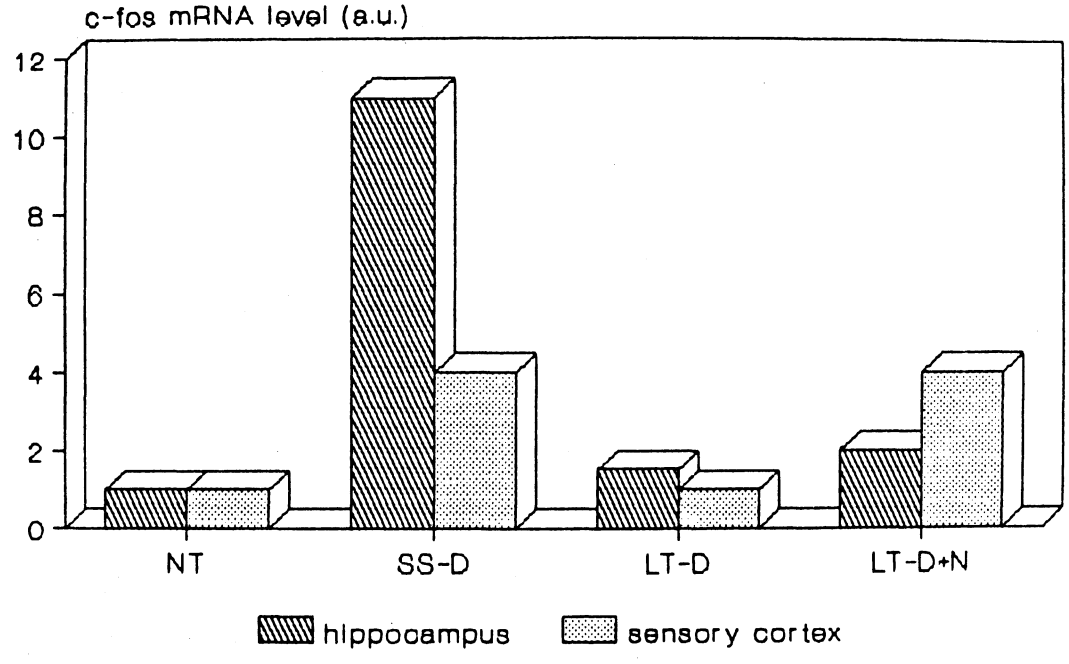

Fig. 1. Accumulation of c-fos mRNA in rat brain after two-way active avoidance training. NT, non-trained (control) rats; SS-D, material taken from brains of rats one hour after single session of the training with darkness as conditioned stimulus, CS; LT-D, material taken from animals 1 h after 9th training session with darkness as CS; LT-D+N, material taken from animals 1 hr after 9th training session with darkness and wide-band noise as compound CS. The c-fos mRNA levels are expressed in arbitrary units and are derived from densitometrically scanned northern blots after normalization to control (non-trained animals) levels. Material (separately hippocampi and sensory cortices) pooled from three animals were used for each experimental point. Performance levels (percentage of avoidance reactions) for each group was: NT - 0; SS-D - 45; LT-D - 82; LT-D+N - 98. For further details see refs.: Nikolaev et al. 1992a,b.

copulatory behavior in male rats correlates with c-fos mRNA accumulation in the sensory cortex (Bialy et al. 1992). Recently (Bialy et al., in preparation), we have found that this *c-fos* induction is dependent on activation of NMDA receptor, since 0.1 mg/kg of MK 801 completely abolished the effect. At the same time the L-glu antagonist blocked the shortening of the ejaculation latencies. Interestingly, however, it did not preclude a dramatic shortening of the intromission latencies. Both latency measures were used as indicators of copulatory performance.

AP-1 DNA BINDING ACTIVITY IN DEVELOPING RAT CORTEX

In our recent studies (Kamińska et al. 1992) we investigated the AP-1 DNA binding activity in postnatally developing rat cortex with the focus on the barrel field of the somatosensory cortex (cortical representation of vibrissae). Two main forms of plasticity could be observed in this area. Removal of a vibrissa during the first postnatal week leads to a disappearance of its cortical barrel. Then, starting from the third postnatal week removal of a vibrissa provokes functional plasticity detected for example with 2-deoxyglucose method (Kossut 1992). We have found that no AP-1 could be detected in the barrel cortex until the 21 days postnatally. Then, the AP-1 was still observed at 6 weeks, albeit at lower level. This effect was specific in the sense that a different pattern of expression was noted for AP-1 in the visual cortex and another TF (CREB) displayed a different pattern of expression in the barrel cortex. These results further suggest that there is a coincidence of AP-1 expression and certain forms of neuronal plasticity.

Summarizing the experiments on neuronal plasticity-related c-fos and AP-1 expression one can say that learning-related processes, including LTP, aversive and appetitive conditioning as well as developmental cortical plasticity coincide with *c-fos* and AP-1 activation (see above and also: Worley et al. 1990, 1991, Anokhin and Rose 1991, Anokhin et al. 1991, Rose 1991, Brennan et al. 1992, McCormack et al. 1992, Kaczorowski 1993, Kaczmarek 1993, in press). At least in one example - learning of copulatory behavior, the elevated expression of c-fos is dependent upon activation of NMDA receptor, known to be critical for long term memory formation. However, no functional significance of this coincidence has been so far provided. In particular, it is not clear to which components of learning processes (e.g. processing of sensory, emotion, motivation, attention, award, etc. information or their associations) elevated expression of TFs and their genes could be ascribed.

AP-1 IN HIPPOCAMPI FROM AGED RATS

Finally, I would like to mention our experiments on AP-1 DNA binding activity in brains of aged rats (Kamińska and Kaczmarek 1993, in press). These studies were provoked by i. findings that c-fos expression and AP-1 are somewhat altered in senescent cells in culture (Seshadri and Campisi 1990, Riabowol et al. 1992); ii. defect of AP-1 expression in lymphocytes collected from aged mice and stimulated in vitro to proliferate (Sikora et al. 1992); iii. well known aging-related learning abnormalities (Rapp and Amaral 1992); iv. aforementioned correlations between learning-related phenomena and *c-fos* and AP-1 expression. We have found that, contrary to our expectations, pentylenetetrazole (PTZ) was able to elevate AP-1 DNA binding activity even more robustly in hippocampi from aged than young animals (Kamińska and Kaczmarek 1993, in press).

These studies are worthwile to mention as an example of lack of association between learning processes and level of *c-fos* induction. In the aforementioned case one could expect that aging-related learning deficits may coincide with decreased inducibility of AP-1, however, the observed effect was quite opposite. Therefore, these results raise a caution in interpreting the data. Nevertheless, a plethora of results relating *c-fos* and AP-1 patterns of expression to neuronal plasticity is overwhelming. This situation calls for some heuristic hypothesis summarizing available information and

proposing new directions of research. Below an attempt to develop such a hypothesis is being presented.

NEURON'S NUCLEUS AS AN INTEGRATING DEVICE - A HYPOTHESIS ON MOLECULAR MECHANISMS OF LONG LASTING MEMORY FORMATION

It is widely accepted that memory is a feature of neuronal networks. The cellular and molecular bases for memory storage are located at the synapses, and memory formation is based on an enhancement of synaptic efficacy. It is not, however, clear where memory formation based on integration of information, takes place. The following hypothesis suggests that the nucleus of the neuron should be considered as an important component of the memory locus.

It has to be noted that this view is restricted only to certain form (phase) of memory, i.e., its long lasting component. On the basis of pharmacological, behavioral and other manipulations, one can distinguish at least three main forms of memory (Gibbs and Ng 1977, Matthies 1989a,b, McGaugh 1989). There is memory lasting up to minutes, memory lasting up to hours, and long lasting memory. The argument presented below will be limited only to the last kind. Its lower time-limit defined as a few hours is based on experiments with inhibitors of protein and RNA biosynthesis (see above). Similarly, experiments on memory consolidation and modulation showed that multiple neurotransmitter systems are involved over a few hours period in consolidating a memory trace (for review see: Gold and Zornetzer 1983, McGaugh 1989).

Obviously, the same mechanisms that are involved in the formation of shorter memories may also be operating as temporary support and/or intracellular information transduction systems for longer memories. Therefore, e.g. phosphorylation of preexisting proteins (Schwartz and Greenberg 1987), which may be responsible for the formation of memories lasting up to hours may be also critical for the formation of longer memories, e.g. marking the synapse destined to receive newly synthesized proteins as well as being involved in membrane-nucleus signal transduction (see below).

Based on the experimental evidence and theoretical considerations, a hypothesis of molecular mechanisms of long term memory formation is proposed. According to this hypothesis, the main information-integrating device is the nucleus of a neuron. Obviously, the information is processed by multiple neurons at the same time, but our discussion will be limited to only one neuron. During and after behavioral training the neuron receives several

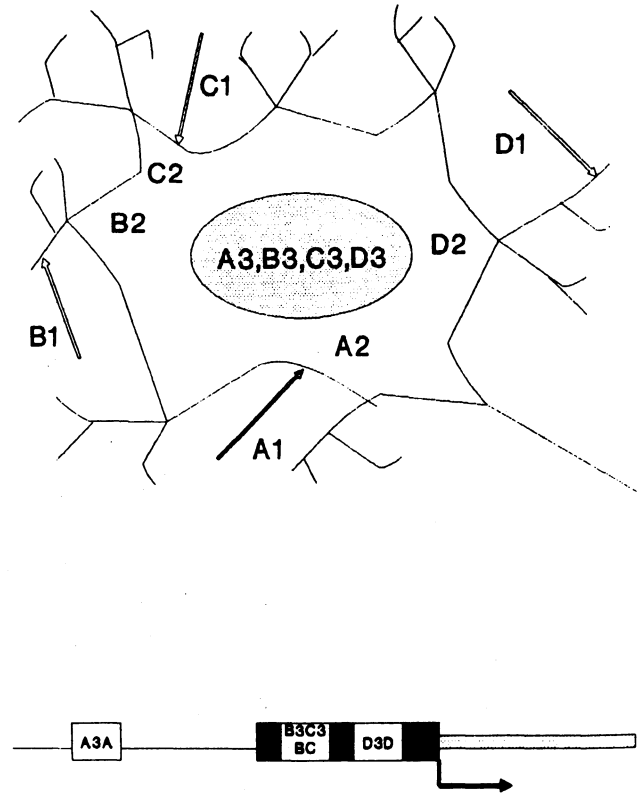

Fig. 2. Schematic illustration of information processing at the neuronal level. Top: A1, B1, C1, D1 - neurotransmitters activating neuron as a consequence of a behavioral experience; A2, B2, C2, D2, second messengers produced after activation of neurotransmitters' receptors; A3, B3, C3, D3, transcription factors accumulated in the cell nucleus as a result of second messenger activity. Bottom: Interactions between regulatory sequences and transcription factors results in expression of effector learning- related genes.

information inputs carried on by neurotransmitters (A1, B1, C1 and D1 on the Fig. 2, top) carrying various sensory as well as motivational, emotional, attentional, etc. inputs. After activation of specific receptors, second messengers (A2, B2, C2 and D2 on the Fig. 2, top) are stimulating specific protein kinases and they activate specific transcription factors (A3, B3, C3 and D3 on the Fig. 2 top and bottom). Some of them are just phosphorylated (like CREB), while others require elevated gene expression (like AP-1, ZIF/268, see above). This hypothesis assumes that, at least to some extent, transcription factors are induced independently and specifically by each receptor-derived signal. Next, the regulatory proteins interact with regulatory sequences (A, B, C and D on the Fig. 2, bottom) of "effector" learning-related genes. Only if a set of regulatory factors is simultaneously available, the effector gene can be turned on (Fig. 2, bottom), and its protein produced. Then, such a protein is directed to certain synapses which are to be strengthened. The nature of those effector proteins is obviously unknown, but one can expect that they exert their effects on synaptic efficacy. Effector genes could code for membrane (synaptic) glycoproteins (Matthies 1989a,b, Rose 1989). The expression of many different genes can be, in this way, turned on in a coordinated fashion.

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