The Roles of MAPK Cascades in Synaptic Plasticity and Memory in *Aplysia*: Facilitatory Effects and Inhibitory Constraints

Shiv K. Sharma and Thomas J. Carew

Department of Neurobiology and Behavior, Center for the Neurobiology of Learning and Memory, University of California, Irvine, California 92697, USA

Synaptic plasticity is thought to contribute to memory formation. Serotonin-induced facilitation of sensory-motor (SN-MN) synapses in *Aplysia* is an extensively studied cellular analog of memory for sensitization. Serotonin, a modulatory neurotransmitter, is released in the CNS during sensitization training, and induces three temporally and mechanistically distinct phases of SN-MN synaptic facilitation. The role of protein kinase A and protein kinase C in SN-MN synaptic facilitation is well documented. Recently, it has become clear that mitogen-activated protein kinase (MAPK) cascades also play a critical role in SN-MN plasticity. Here, we summarize the roles of MAPK cascades in synaptic plasticity and memory for sensitization in *Aplysia*.

MAPK Pathways

Protein phosphorylation is one of the most common posttranslational modifications that cells use to both respond to extracellular stimuli and to regulate the functions of proteins. Members of one family of protein kinases, the mitogen-activated protein kinases (MAPKs), play important roles in various cellular processes. Initially, the role of MAPK pathways was primarily examined with respect to growth factor signaling and stress responses (Segal and Greenberg 1996; Ichijo 1999; Zhang and Liu 2002). However, recently it has become increasingly clear that these pathways play important roles in the adult brain and regulate various forms of synaptic plasticity and memory in several different systems, ranging from invertebrates to mammals. Three different families of MAPKs have been well characterized, the extracellular signal-regulated kinase (ERK), p38-MAPK, and JNK-MAPK (Johnson and Lapadat 2002). The basic module of MAPK cascades consists of three kinases that act in a sequential manner, MAP kinase kinase kinase (MAPKKK) → MAP kinase (MAPKK) → MAPK (Johnson and Lapadat 2002). MAPKKKs are serine/threonine kinases that, when activated, phosphorylate and activate the next kinase in the chain, the MAPK. MAPKKs are dual-specificity kinases that phosphorylate both threonine and tyrosine residues on MAPKs and activate them. MAPKs are serine/threonine kinases that, when activated, phosphorylate a large number of substrate proteins in the cell, including transcription factors. Several members within each MAPK family are known (Widmann et al. 1999).

The best-characterized MAPK pathway with respect to synaptic plasticity and memory is the ERK pathway. JNK-MAPK and p38-MAPK have been most extensively studied for their role in...
stress responses. Recently, however, their role in synaptic plasticity and memory also has begun to be elucidated (Bolshakov et al. 2000; Zhen et al. 2001; Alonso et al. 2003; Bevilaqua et al. 2003; Guan et al. 2003). Several excellent reviews on the role of ERK pathway in vertebrate synaptic plasticity and memory have been recently published (Grewal et al. 1999; Sweatt 2001; Thielts and Klann 2001; Adams and Sweatt 2002; Thomas and Huganir 2004). Thus, we will limit this review to the role of MAPK pathways in Aplysia synaptic plasticity and memory. Currently, the role of the JNK-MAPK pathway has not been examined in Aplysia. Hence, we will limit our discussion to the roles of ERK and p38-MAPK pathways.

Role of ERK in SN-MN Synaptic Facilitation in Aplysia

As mentioned earlier, it is now well established that STF of SN-MN synapses induced by SHT depends upon covalent modification of pre-existing proteins, whereas ITF requires new protein synthesis, but no transcription, and LTF requires both translation as well as transcription. The requirement for new RNA synthesis for LTF has led to the identification of several transcription factors that play critical roles in the formation of LTF. These include both activating and inhibitory isoforms of cAMP response element binding (CREB) protein (Bartsch et al. 1995, 1998), the cCAAT enhancer-binding protein (C/EBP; Alberini et al. 1994), and Activating Factor (ApAF; Bartsch et al. 2000). The formation of LTF requires not only the activation of CREB1, the activating isoform of CREB, but also down-regulation of CREB2 activity, the inhibitory isoform of CREB (Abel et al. 1998). CREB1 is phosphorylated by protein kinase A (PKA). However, CREB2 lacks a consensus PKA phosphorylation site, but it contains phosphorylation sites for ERK (Bartsch et al. 1995). In addition, C/EBP contains an ERK phosphorylation site. These observations led Martin et al. (1997a) to examine whether ERK might play a role in the formation of LTF of SN-MN synaptic facilitation. Martin and colleagues examined the role of ERK in cultured SN-MN synapses using pharmacological as well as antibody tools. They found that five pulses of SHT that induce LTF of SN-MN synapses led to translocation of ERK to the nucleus of presynaptic SN cells. A single pulse of SHT, which induces only STF of SN-MN synapses, did not lead to any significant nuclear translocation of ERK. Furthermore, ERK translocation was specific to the presynaptic SN cells; no translocation occurred in the postsynaptic MNs. Because treatment with SHT leads to the elevation of cAMP levels inside the cells, the next question was to examine whether the agents that elevate cAMP levels would also translocate ERK to the nucleus. To explore this question, Martin et al. (1997a) increased intracellular cAMP levels in two ways, first, by using forskolin, an activator of adenylate cyclase, and second, by using Sp-cAMPS, a cell-permeable analog of cAMP that is also resistant to phosphodiesterase action. Similar to the observations with five pulses of SHT, application of forskolin or Sp-cAMPS led to the translocation of ERK in the presynaptic SN nucleus. Consistent with the findings using SHT, elevation in intracellular levels of cAMP had no effect on nuclear ERK levels in the postsynaptic MNs. Having established that ERK was in the right place (i.e., the nucleus) to regulate transcription factor(s), they next examined whether ERK activity was required for SN-MN synaptic facilitation. They used a pharmacological inhibitor (PD098059) that inhibits MEK, the upstream kinase that dually phosphorylates and activates ERK, as well as specific ERK antibodies, and found that application of the MEK inhibitor or injection of the ERK antibody in the SNs blocked LTF of SN-MN synapses. Consistent with the observation that no significant translocation of ERK to the SN nucleus occurs with a single pulse of SHT, the STF induced by SHT was unaffected either by MEK inhibitor treatment or ERK antibody injection.

The role of ERK in LTF of SN-MN synapses in the intact CNS has been examined by Purcell et al. (2003). They found that pharmacological blockade of tyrosine kinase activity blocked the induction of LTF, and conversely, functionally enhancing endogenous tyrosine kinase activity by inhibiting tyrosine phosphatases facilitated the induction of LTF, enabling a single pulse of SHT to induce LTF. Consistent with a requirement of ERK activity for the induction of LTF in cultured SN-MN synapses (Martin et al. 1997a), Purcell et al. (2003) found that the "gain of function" observed by tyrosine phosphatase inhibition coupled with one pulse of SHT was critically dependent on ERK activation. Furthermore, similar to the observations with tyrosine phosphatase inhibitor, human recombinant BDNF also facilitated the induction of LTF in an ERK-dependent manner (Purcell et al. 2003). These studies suggest that growth factor-mediated signaling, acting through ERK, may be important for the induction of LTF of SN-MN synapses.

LTF of SN-MN synapses is associated with growth of new synaptic connections, and down-regulation of cell adhesion molecules is thought to contribute to this process (Bailey and Kandel 1999). For example, Mayford et al. (1993) showed that SHT decreases the expression of ApCAM, a cell adhesion molecule, at the surface membranes of the SNs. In subsequent studies, Bailey et al. (1997) found that only the transmembrane form of ApCAM was internalized after treatment with SHT, and that phosphorylation of ApCAM by ERK was important for internalization of ApCAM. They examined this issue in two ways; first, they mutated the threonine residues in the ERK consensus phosphorylation sites and showed that this modification in ApCAM prevented its down-regulation and internalization by SHT. Second, they examined whether inhibition of MEK had any effect on internalization of ApCAM. They found that the MEK inhibitor blocked internalization of ApCAM. These studies suggest that ERK could contribute to growth of new synaptic connections by down-regulating ApCAM.

In another study, Zhang et al. (1997) showed that transforming growth factor β (TGFβ) induces LTF of the SN-MN synapses in Aplysia. Further examination showed that the effect of TGFβ on SHT-induced LTF was not additive, suggesting that TGFβ is part of the cascade initiated by SHT that is involved in LTF formation. Consistent with this possibility, an inhibitor of TGFβ signaling blocked SHT-induced LTF. Although the signaling mechanisms of TGFβ are not yet known in Aplysia, ERK is an attractive candidate target, as TGFβ-mediated rescue of SN-MN synaptic depression is dependent on ERK activity (Chin et al. 2002). In addition, ERK plays a role in regulating SHT-induced phosphorylation and dispersion of synapsin in the SNs, and thus may modulate the synaptic vesicle trafficking accompanying synaptic plasticity (Angers et al. 2002).

The role of ERK in the induction of ITF at the SN-MN synapses in the CNS has been examined by Sharma et al. (2003a). They took advantage of the fact that tail-nerve shock induces the release of SHT in the CNS (Marinesco and Carew 2002), and thus substituted tail-nerve shock for SHT application, thereby using a stimulus closer to the tail shock used to induce behavioral sensitization of tail-elicted siphon withdrawal reflex (Sutton et al. 2001). Sharma and colleagues found that five shocks to the tail nerve failed to induce ITF when ERK activation in the CNS was inhibited using the MEK inhibitor (U0126). In contrast, the same stimulus induced ITF when the CNS was treated with the inactive analog of the MEK inhibitor (U0124).

From the studies described above, it is clear that long-lasting SN-MN synaptic facilitation, both in the SN-MN culture system as well as in the CNS, is critically dependent upon ERK activity, and that ERK is specifically recruited for the induction of ITF and LTF, but not for STF. In addition, ERK seems to...
play important roles in both cytoplasmic as well as nuclear signaling.

**Role of ERK in Memory for Sensitization**

Given that ERK is necessary for the induction of synaptic facilitation in the intermediate-term and long-term temporal domains, this leads directly to the issue of the role of ERK in memory formation in comparable time domains. The requirement of ERK activity in the formation of memory for sensitization of tail-elicted siphon withdrawal reflex was examined by Sharma et al. (2003a). They found that STM, which is induced by a single tail shock, was independent of ERK activity, as it was unaffected when ERK activation was inhibited using MEK inhibitor. On the other hand, ITM that is induced by five tail shocks was critically dependent upon ERK activity. An interesting finding in that study was that only the induction, but not the expression of ITM, required ERK activity, suggesting that once memory has been induced, ERK-independent mechanisms are involved in maintaining it in the intermediate-term time domain. Consistent with a critical requirement of ERK in LTF (Martin et al. 1997a; Purcell et al. 2003), LTM induced by five tail shocks also required ERK activity (Sharma et al. 2003a). In addition, LTM induced by tyrosine phosphatase inhibition coupled with two tail shocks (that normally induce only STM; Sutton et al. 2002) also required ERK activity (Purcell et al. 2003).

**ERK Activation by SHT**

The cDNA clone for *Aplysia* ERK was isolated by Michael et al. (1998), who also showed that five spaced pulses of SHT that induce LTF of SN-MN synapses also induce ERK activation in the cultured SNs, whereas a single pulse that induces only STF had no significant effect. In subsequent studies, the activation of ERK in the intact CNS was examined by Sharma and colleagues (Sharma et al. 2003a; see also, Purcell et al. 2003). They found that five spaced pulses of SHT induced a sustained (−3 h) activation of ERK in the SNs of the pleural ganglia. Furthermore, five spaced tail shocks (the stimulus used to induce ITM and LTM) to the intact animal also induced a temporal profile of ERK activation that was similar to the activation profile with five spaced pulses of SHT. A sustained activation of ERK has also been observed with repeated spaced stimuli in hippocampal neurons (Wu et al. 2001). As mentioned earlier, Sharma et al. (2003a) found that the expression of ITM did not require ERK activity. What then is the significance of a sustained ERK activation? The answer to this question is not known at present. It is possible that sustained ERK activity is required for the development of LTM. Examination of LTM with delayed application of MEK inhibitor after training would directly address this possibility.

Despite a significant progress in linking ERK activation to the regulation of long-lasting SN-MN synaptic plasticity and memory, the molecular pathways that link SHT receptors to ERK activation are not fully understood. In vertebrate systems, many signaling pathways, including receptor tyrosine kinases, PKA, and PKC, can regulate ERK activation (Grewal et al. 1999; Sweatt 2001). In *Aplysia*, forskolin has been shown to activate ERK (Michael et al. 1998) and translocate it to the nucleus (Martin et al. 1997a), suggesting that CAMP, perhaps acting through PKA could mediate SHT-induced ERK activation. The generation of cAMP by SHT could activate ERK in at least two ways as follows: (1) through the Rap-B-Raf pathway (Vossler et al. 1997), and (2) through cAMP regulation of guanine nucleotide exchange factors (GEFs; de Rooij et al. 1998; Kawasaki et al. 1998). However, a recent study (Dyer et al. 2003) concluded that neither cAMP nor PKC (another second messenger activated by SHT) play a significant role in SHT-induced ERK activation. Another recent study by Purcell et al. (2003) has suggested that a growth factor (possibly BDNF, or more likely a similar growth factor) may play an important role in ERK activation and contribute to long-lasting SN-MN synaptic facilitation. However, the important question of whether a BDNF-like growth factor is released by the neuronal cells (e.g., SNs or MNs) in *Aplysia* remains to be investigated. Supportive evidence that a growth factor is released upon treatment with SHT comes from studies by Kandel and colleagues (Giustetto et al. 1999) who showed that SHT-induced LTF of SN-MN synapses is blocked by TrkB-Fc receptor bodies (which will sequester the released growth factor and thus prevent its action on the cell). Along these lines, it would be interesting to examine whether the TrkB-Fc receptor bodies that block SHT-induced LTF would also block ERK activation.

**Role of p38-MAPK in SN-MN Synaptic Facilitation**

Thus far, we have discussed the role of ERK pathway in SN-MN synaptic plasticity and memory. Do other MAPK pathways have any role in these forms of plasticity? Recently, the role of another MAPK family member, p38-MAPK, was examined in SN-MN synaptic facilitation (Guan et al. 2003). Guan and colleagues isolated the cDNA for *Aplysia* p38-MAPK and found that it belongs to the α-β family of p38-MAPK in vertebrates. They examined the role of p38-MAPK in LTF of cultured SN-MN synapses in two ways. First, they blocked the p38-MAPK activity using a pharmacological inhibitor. This manipulation reduced the threshold for the induction of LTF; a single pulse of SHT that normally induces only STF, led to the induction of LTF when p38-MAPK was inhibited. Second, they blocked p38-MAPK activity using specific antibodies and showed that it had a similar effect as the pharmacological inhibitor. These results suggest that p38-MAPK acts as an inhibitory constraint in the induction of LTF. Consistent with this, SHT inhibited p38-MAPK activation, and injection of phosphorylated (and thus activated) p38-MAPK into the SNs blocked the induction of LTF by five pulses of SHT. It is currently not known whether the gain of function observed with p38-MAPK inhibition in conjunction with a single pulse of SHT requires ERK activity, as it was blocked by inhibitor of MEK. These studies show first, that calcineurin acts as yet another inhibitory constraint on the induction of long-lasting memory in *Aplysia*, and second, at least one pathway regulated by calcineurin is the ERK-signaling cascade. Thus, p38-MAPK and calcineurin join the members of “memory suppressor” genes (Abel et al. 1998) involved in facilitation and memory in *Aplysia*.

**Role of p38-MAPK in SN-MN Synaptic Depression**

In *Aplysia*, whereas SHT produces SN-MN synaptic facilitation, the effects of a neuropeptide, FMRFa (Phe-Met-Arg-Phe-NH2) are the opposite; FMRFa induces depression of SN-MN synapses (Pi-
omelli et al. 1987). Similar to SHT-induced facilitation, FMRFa induces short-term depression (STD) as well as long-term depression (LTD) of SN-MN synapses (Montarolo et al. 1988; Guan et al. 2002). STD produced by FMRFa relies on the release and metabolism of arachidonic acid (Piomelli et al. 1987), whereas LTD requires translation and transcription (Montarolo et al. 1988; Guan et al. 2002). The role of p38-MAPK pathway in SN-MN synaptic depression has recently been examined by Guan et al. (2003). They found that a pharmacological inhibitor of p38-MAPK blocked STD induced by FMRFa. In addition, five pulses of FMRFa failed to induce LTD in the presence of the p38-MAPK inhibitor, or when p38-MAPK antibody was injected in the SNs. Furthermore, injection of phosphorylated (and thus active) p38-MAPK in the SNs reduced the threshold for LTD induction and enabled a single pulse of FMRFa (that normally induces only STD) to induce LTD. Consistent with a role of p38-MAPK in FMRFa-induced synaptic depression, FMRFa induced the activation of p38-MAPK in the pleural ganglia.

How does FMRFa, acting through p38-MAPK, induce depression of SN-MN synapses? p38-MAPK activity is required for the release of arachidonic acid by FMRFa (Guar et al. 2003). Thus, STD seems to be mediated by FMRFa-induced activation of p38-MAPK, which then regulates the release of arachidonic acid. The role of p38-MAPK in LTD is less clear. As mentioned earlier, LTD requires new gene expression and protein synthesis. Guan and colleagues (Guan et al. 2002, 2003) found that blocking CREB2 function using specific antibodies blocked LTD induction by five pulses of FMRFa, and that CREB2 is a substrate of p38-MAPK. Thus, one possibility is that activation of CREB2 via p38-MAPK regulates the expression of genes that are necessary for the induction of LTD.

Molecular Targets of MAPKs

Because ITF and ITM require translation but not transcription, the role of ERK in ITF and ITM may be to regulate translation of pre-existing mRNAs. It has been reported in vertebrates that BDNF-induced protein synthesis requires ERK activity, as it is inhibited by the MEK inhibitor (Takei et al. 2001). In addition, recently, it has been shown that ERK regulates protein synthesis in the hippocampal cells (Kelleher et al. 2004). As far as the role of ERK in LTF and LTM is concerned, in addition to regulating protein synthesis, ERK could also contribute to the synthesis of new mRNAs by regulating the activity of transcription factors. This seems plausible, as ERK can phosphorylate CREB2 and C/EBP (Michael et al. 1998). Phosphorylation of CREB2 by ERK has been hypothesized to relieve the CREB2-mediated repression on CREB1 activity (Michael et al. 1998). In addition, C/EBP is an immediate early gene induced by SHT that is essential for the induction of LTF (Alberini et al. 1994) and is thought to contribute to the induction of late-response genes necessary for LTD formation. Phosphorylation of C/EBP by ERK has been shown to promote its DNA-binding activity (Yamamoto et al. 1999).

In addition to CREB2 and C/EBP, there are two other transcription factors that could potentially be regulated by ERK and could contribute to LTD and LTM formation. One is Elk1, and the other is CREB1 itself. Elk1 is a substrate of ERK that has been shown to be activated after LTP induction and learning (Berman et al. 1998; Cammarota et al. 2000; Davis et al. 2000). ERK could regulate the activity of CREB1 through p90rsk, the intermediary kinase between ERK and CREB (Xing et al. 1996; Impey et al. 1998). CREB-mediated CRE-dependent gene expression is critical for the establishment of LTD (Dash et al. 1990). Moreover, phosphorylation of CREB-binding protein (CBP) by ERK (Alt-Si-Ali et al. 1999) could regulate its histone acetyltransferase activity that may be important for chromatin restructuring needed for the induction of genes during LTF formation. The regulation of these proteins by ERK remains to be examined in Aplysia.

Studies of synaptic plasticity using bifurcated synapses in culture, in which one SN makes synapses with two MNs (Martin et al. 1997b), have provided considerable insights into the mechanisms of integration of events at different synapses. If five pulses of SHT (to induce LTF) are applied to one synapse, and five pulses of FMRFa (to induce LTD) are applied to the other synapse, who wins? The answer is that the induction of LTF by SHT is blocked (Guan et al. 2002). This effect has been explained, at least in part, by p38-MAPK-mediated phosphorylation of CREB2, its recruitment to, and displacement of CREB1, from C/EBP promoter (Guan et al. 2002, 2003). This results in the reduced expression of C/EBP that is required for LTD formation. As mentioned earlier, it has also been suggested that ERK-mediated phosphorylation of CREB2 may relieve the repression caused by CREB2 on CREB1-mediated transcription. How does phosphorylation of the same protein (CREB2) by two different MAPKs (ERK and p38-MAPK) mediate different effects? Although the phosphorylation sites in CREB2 for ERK and p38-MAPK are not known, it is possible that the two kinases phosphorylate different amino acid residues in CREB2, and bring about differential change, such that when CREB2 is phosphorylated by ERK, the repression on CREB1-mediated transcription is relieved, whereas when CREB2 is phosphorylated by p38-MAPK, its affinity for C/EBP promoter increases, resulting in CREB1 displacement and reduced expression of C/EBP. Alternatively, the outcome of CREB2 phosphorylation by ERK and p38-MAPK could depend upon complex interactions with different proteins. Although still speculative, some intriguing possibilities for MAPK-mediated signals are emerging.

Conclusions and Perspectives

It is clear from the studies described above that ERK, as well as p38-MAPK pathways play important regulatory roles in synaptic plasticity. Figure 1. A schematic diagram for the role of ERK in synaptic facilitation and memory for sensitization in Aplysia. SHT, released in response to tail shocks, induces ERK activation. Activated ERK could regulate protein synthesis and contribute to ITF and ITM induction. In addition to regulating protein synthesis, ERK could regulate transcription factors, which then contribute to the transcription of genes required for LTD and LTM. ERK could also contribute to LTD and LTM formation by phosphorylating, and thus promoting internalization of ApCAM, which leads to facilitation of synaptic growth. The p38-MAPK may indirectly inhibit ERK, and its inhibition by SHT could potentially facilitate ERK activation. The regulation of different steps is discussed in more detail in the text.
plasticity and memory formation. The ERK pathway seems to be recruited specifically for long-lasting synaptic plasticity and memory, as its inhibition has no effect either on STF induced by SHT or STM induced by tail shock. It is also evident that there are several inhibitory constraints in the induction of long-lasting plasticity and memory. A schematic diagram for the role of ERK in synaptic facilitation and memory is shown in Figure 1. A major challenge now is to identify the mechanisms of regulation of the signaling molecules in response to SHT treatment and sensitizing stimuli. For example, it is not known whether SHT-induced ERK activation follows the classical growth factor-receptor tyrosine kinase-Ras-Raf1 pathway that is well documented in vertebrates. If so, is a growth factor released in response to SHT treatment, and what is its identity? As mentioned earlier, the observations in the SN-MN cultures by Kandel and colleagues that sequestering a released growth factor using the TrkB-Fc receptor bodies blocked SHT-induced SN-MN synaptic facilitation suggest that a BDNF-like growth factor may be released in response to SHT treatment. Along this line, it would be interesting to examine whether the TrkB-Fc receptor bodies block SHT-induced ERK activation. Also, as several different SHT receptors have now been characterized in Aplysia (Barbas et al. 2003; Cohen et al. 2003), it will be important to identify the receptor(s) that links SHT to ERK activation. Future studies would also examine the interaction between the p38-MAPK and ERK pathways in the formation of long-lasting synaptic plasticity and memory. Finally, in order to fully appreciate the role played by these regulatory molecules, it will be important to identify the genes whose transcription, and the mRNAs whose translation is under the regulatory control of these important signaling molecules.

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