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Forum Review

Sources and Targets of Reactive Oxygen Species in Synaptic Plasticity and Memory

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ABSTRACT

Increasing evidence suggests that reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, act as necessary signaling molecules in processes underlying cognition. Moreover, ROS have been shown to be necessary in molecular process underlying signal transduction, synaptic plasticity, and memory formation. Research from several laboratories suggests that NADPH oxidase is an important source of superoxide in the brain. Evidence is presented here to show that ROS are in fact important signaling molecules involved in synaptic plasticity and memory formation. Moreover, evidence that the NADPH oxidase complex is a key regulator of ROS generation in synaptic plasticity and memory formation is discussed. Understanding redox signaling in the brain, including the sources and molecular targets of ROS, are important for a full understanding of the signaling pathways that underlie synaptic plasticity and memory. Knowledge of ROS function in the brain also is critical for understanding aging and neurodegenerative diseases of the brain given that several of these disorders, including Alzheimer's disease and Parkinson disease, may be exacerbated by the unregulated generation of ROS. *Antioxid. Redox Signal.* 9, 233–244 .

INTRODUCTION

OVER THE LAST SEVERAL YEARS, several studies have indicated that reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H_2O_2), are important signaling molecules underlying mammalian learning and memory. Previously, ROS had been described as a class of destructive molecules that hinder neuronal function and have been implicated in degenerative processes underlying Alzheimer's and Parkinson diseases (1, 70, 104, 124), as well as processes thought to underlie the general aging-associated decline of cognitive function (15, 35, 100). However, recent work has shown that ROS are required for normal cognitive function at cellular and behavioral levels of analyses. Specifically, ROS have been shown to be required for a form of synaptic plasticity called long-term potentiation (LTP), learning and memory, and for biochemical signal transduction cascades that are believed to underlie LTP and memory formation. Thus, the source of ROS responsible for these brain functions and how these small highly reactive molecules are regulated are important questions that this review will attempt to address.

Positive correlations have been made between LTP and learning. One such correlation is that signal transduction cascades that are activated during LTP have been shown to be required for memory formation. For instance, inhibition of NMDA receptors can block LTP and can interfere with certain types of memory formation (112). Furthermore, deficiencies in memory formation are observed in mice with gene specific mutations in loci coding for signal transduction elements that are critical for LTP (7, 12, 72, 81). Many of these signal transduction elements, such as small messenger molecules and protein kinases, become active once NMDA receptors are activated, thereby permitting the influx of Ca^{2+} into the postsynaptic terminal. Thus, NMDA receptor-dependent LTP in acute hippocampal slices has been used as an *in vitro* model to study synaptic plasticity and the cellular mechanisms underlying memory formation.

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In the hippocampus, high-frequency stimulation (HFS) of CA3 Schaffer collaterals can induce LTP at synapses in area CA1. HFS induces the activation of postsynaptic NMDA receptors, resulting in the influx of Ca^{2+} into the postsynaptic terminal. The transient rise in Ca^{2+} results in the production of small messenger molecules, such as cyclic adenosine monophosphate (cAMP), nitric oxide, arachidonic acid, and ROS (106). These small messenger molecules in turn activate kinases such as protein kinase (PKC) and extracellular signal-regulated kinase (ERK) and inhibit phosphatases such as calcineurin (protein phosphatase 2B or PP2B) (107, 117). Many of these signaling molecules and enzymes are involved in the induction and expression of LTP, as well as memory formation (107, 117, 121). In addition to these well-studied signaling pathways, NMDA receptor activation has been shown to produce ROS (31), which also are required for LTP and hippocampus-dependent memory (56, 67, 71, 111). Until very recently, the source of ROS required for LTP and memory formation was unclear.

NADPH oxidase has been shown in multiple cell types to generate superoxide as a response to specific extracellular and intracellular stimuli (76, 91) . The best known of these responses is the phagocytic oxidative burst (76). This burst generates large amounts of superoxide rapidly, transiently, and in a well-controlled manner. This pool of superoxide is used to break down phagocytosed material and as a signal to initiate signal transduction cascades underlying the bactericidal response (76). Furthermore, in several cell types, NADPH oxidase production of superoxide has been implicated in signaling required for transcriptional activation and cell proliferation (38, 59, 96, 98, 102).

In this review we will briefly discuss the evidence that ROS are important signaling molecules underlying LTP and memory, including known biochemical targets of ROS signaling in the brain. We also will discuss some of the hypothesized sources of ROS, focusing on recent evidence pointing to the role that NADPH oxidase plays in the generation of ROS that are required for NMDA receptor-dependent signal transduction, synaptic plasticity, and memory formation.

ROS ARE CRITICAL SIGNALING MOLECULES IN SYNAPTIC PLASTICITY

Synaptic plasticity is the physiological process that is thought to underlie learning and memory at the cellular level. One form of plasticity that has been commonly studied is LTP. Many of the molecular processes underlying LTP also are required for learning and memory (67, 103). Thus, LTP has become a popular model to study the mechanisms that underlie learning and memory behavior.

Investigation into the role of ROS in LTP has revealed an interesting, yet complex role for oxidative species such as superoxide and H_2O_2 in the molecular processes that lead to changes in synaptic strength. The role of ROS is specific to the identity of the oxidative molecule involved (*i.e.*, superoxide or H_2O_2) (43, 56) and the concentration of ROS during the process (41, 42, 56).

The use of transgenic animals and pharmacological approaches to block the activity of ROS has been useful in identifying the necessity of these signaling molecules in synaptic plasticity and memory formation. LTP studies in the rodent hippocampus have revealed that scavenging superoxide blocks LTP induced with high-frequency stimulation (HFS–LTP) (48, 51), suggesting that superoxide is required for HFS–LTP. On the other hand, overproduction of H_2O_2 as a byproduct of superoxide dismutase (SOD) activity also was shown to inhibit LTP (29). To better understand the specific role of ROS, investigators have also applied exogenous sources of either superoxide or H_2O_2 to various *in vitro* systems and have observed interesting effects on cellular plasticity.

Xanthine-xanthine oxidase (X/XO) is often used to generate superoxide *in vitro* (55). Exogenous application of X/XO to hippocampal slices resulted in a short-term depression of synaptic transmission that eventually potentiated in an LTP-like manner that was inhibited by SOD (55). The role of H_2O_2 has also been investigated using the application of exogenous H_2O_2 to study the effect of this ROS on the molecular mechanisms underlying cellular plasticity. Kamsler and colleagues have shown that H_2O_2 could either potentiate or depress HFS–LTP in a concentration-dependent manner (41). Also, low concentrations of H_2O_2 potentiate or depress intracellular Ca^{2+} levels (63, 123).

Many of the studies implicating ROS involvement in the molecular mechanisms underlying synaptic plasticity and memory investigate a narrow range of superoxide or H_2O_2 concentrations. However, very interesting results were obtained when a range of ROS concentrations were tested for their effects on synaptic plasticity (41, 42, 55, 56). For example, high concentrations of superoxide or H_2O_2 resulted in the depression of excitatory postsynaptic field potentials (fEPSPs) measured in hippocampal area CA1, whereas lower concentrations resulted in a potentiation of the fEPSP (41, 55).

The role of ROS in aging-related changes in synaptic plasticity and cognitive performance is an interesting and thriving area of research; we refer the reader to the review of Hu *et al*. presented in this forum. However, it should be mentioned that during aging there seems to be a shift in the role of ROS in synaptic plasticity and memory that may be dependent on the changes during aging that result from the accumulation of oxidative damage or changes in oxidant regulation via enzymatic antioxidants (100).

ROS ARE REQUIRED FOR LEARNING AND MEMORY

SOD mutant mice have been critical models for determining the role of ROS in learning and memory. SOD scavenges superoxide and dismutates the molecule to H_2O_2, which can then be rapidly degraded by catalase. There are three SOD isozymes: cytoplasmic-Cu/Zn-SOD (SOD-1), manganese-containing mitochondrial SOD (SOD-2 or MnSOD), and extracellular-SOD (EC-SOD), which also is a Cu/Zn-containing SOD. Mice that either overexpress SOD or express a dysfunctional SOD have been analyzed using various behavioral paradigms with surprising results.

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The earliest evidence that ROS are required for learning and memory came from mice that overexpress SOD-1. Behavioral analyses of the SOD-1 overexpressing mice indicated that the mice displayed altered behavior in open field analysis, and more interestingly, showed decreased escape latencies in the hippocampus- and NMDA receptor-dependent Morris water maze paradigm as compared to wild-type mice (29, 66, 112). These data were the first to suggest that scavenging of superoxide impairs learning and memory. Consistent with a requirement of superoxide in learning and memory, Levin *et al.* showed that mice that either overexpress EC-SOD or are genetically deficient for the EC-SOD gene had impaired performance in the win-shift 8-arm radial maze (60), also a hippocampus-dependent task (80). Further analysis of the EC-SOD overexpressing mice revealed that these mice were impaired in hippocampus-dependent contextual fear conditioning (85, 86), but were normal for cue-dependent fear conditioning (35, 85, 111).

Hippocampus-dependent learning and memory formation has been a useful model in determining the role of ROS in cognition; however, behavioral analysis of SOD overexpressing mice has revealed potential roles for ROS in cognitive performance associated with other brain regions as well. EC-SOD overexpressing mice displayed alterations in performance on the 8-arm radial maze task that was dependent on motivational state induced by food restriction (60, 62). Under low motivational state, EC-SOD overexpressing mice showed impairments in learning; however, under a high motivational state EC-SOD overexpressing mice were able to learn, though at a slower rate, and express long-term memory (62).

Taken as a whole, the data generated from the analysis of SOD mutant mice suggest that ROS are critically involved in the molecular processes involved in cognition, particularly processes underlying learning and memory formation.

ROS ARE CRITICAL SIGNALING MOLECULES THAT MODULATE SIGNALING PATHWAYS INVOLVED IN LTP AND MEMORY

The molecular mechanisms underlying synaptic plasticity and memory have been intensely studied (107). Investigations of signal transduction cascades involved in synaptic plasticity have revealed a wide range of molecular players, including protein kinases (52), phosphatases (52, 95), transcription factors (40), translation factors (50), GTPases (116), and other Ca^{2+}-dependent enzymes (88, 121). Interestingly, ROS have been shown to be important modulators of many of these pathways (43), including some evidence of direct modification of these signaling enzymes by ROS. As mentioned earlier, NMDA receptor-dependent signaling is one critical pathway that is thought to underlie synaptic plasticity and long-term memory formation that has been shown to contain several signaling molecules that are directly affected by ROS.

Glutamatergic synaptic transmission results in the activation of AMPA receptors and voltage-dependent calcium channels during fast synaptic transmission. Given the appropriate pattern of stimulation, NMDA receptors can become activated, which results in a transient spike in postsynaptic calcium levels that leads to the activation of numerous signal transduction cascades. Among the signaling events that occur following NMDA receptor activation are the activation of various protein kinases, including calcium/calmodulin-dependent protein kinase II (CaMKII) (121), protein kinase C (PKC) (49, 51), and extracellular signal-regulated kinase (ERK) (7, 24, 25), as well as the inhibition of protein phosphatases such as calcineurin and protein phosphatase 1 (6, 68, 79, 118). NMDA receptor stimulation also has been shown to result in the production of ROS (13). Following ROS generation, several target proteins become oxidatively modulated. For example, NMDA receptor activation was shown to result in the oxidation of neurogranin (NG) in a time- and dose-dependent manner that is sensitive to NMDA receptor antagonists (64). Neurogranin is a postsynaptic PKC substrate that binds to and sequesters calmodulin (CaM) (64); either oxidation or phosphorylation (36, 49) of neurogranin promotes the release of CaM, thereby promoting Ca^{2+}/CaM signaling via the activation of CaMKII. This signaling module is likely to trigger the induction of LTP (121). NMDA receptor-mediated oxidation of neurogranin occurs within 3–5 min of stimulation and quickly returns to basal oxidation levels (64) and H_2O_2 can directly cause the oxidation of NG (64). In addition, the generation of superoxide by X/XO increases the phosphorylation of neurogranin by the activation of autonomous PKC (49). ROS also can directly oxidize and activate PKC (will be discussed below). Taken together, these reports suggest that neurogranin is an important target of NMDA receptor-induced ROS production, and when either oxidized or phosphorylated by PKC, promotes LTP and possibly long-term memory formation (64, 72, 119, 120).

Another signaling enzyme that is an important modulator of LTP and memory formation is PKC. LTP-inducing stimulation resulted in the activation of PKC that is NMDA receptor-dependent (51, 55, 109). Interestingly, the exogenous application of SOD and catalase inhibited not only LTP, but also the LTP-induced activation of PKC (51, 55), suggesting that superoxide and H_2O_2 are necessary for PKC activation. In addition, direct application of the superoxide-generating system X/XO to hippocampal slices induced not only an LTP-like potentiation, but also the persistent activation of PKC (54). SOD, but not catalase, was shown to block the X/XO-induced activation of PKC (54, 55). Furthermore, the PKC inhibitor bisindomaleimide blocked X/XO-induced LTP and HFS-induced LTP (55), suggesting a common pathway involving PKC for both HFS-LTP and X/XO-induced LTP. The mechanism of PKC activation via oxidation seems to be mediated by direct modification of the zinc finger region of the kinase; $ZnCl_2$ blocked the X/XO-induced activation of PKC, whereas the zinc chelator (TPEN) activated PKC (54). Furthermore, X/XO stimulated the release of zinc from PKC (54). Interestingly, peroxynitrite, a strong oxidant that is generated via the reaction of superoxide and NO (92), also can modulate PKC activity in a concentration-dependent manner. Low concentrations of peroxynitrite activated PKC, whereas high concentrations of peroxynitrite inhibited PKC (53). At all concentrations tested, peroxynitrite increased the nitration of PKC (53). Although the activation of

PKC by peroxynitrite was inhibited by reducing agents, peroxynitrite-induced inhibition of PKC activity was resistant to reducing agents (53). It remains to be determined whether peroxynitrite-induced modulation of PKC occurs during LTP.

Activation of NMDA receptors, either pharmacologically or electrically with LTP-inducing HFS, activated ERK (47, 65, 99). Consistent with the importance of ROS involvement in NMDA receptor-dependent signaling, superoxide and to a lesser extent H_2O_2, were required for NMDA receptor-dependent activation of ERK (47). In this study, NMDA receptor-dependent activation of ERK in acute hippocampal slices was blocked by exogenously added SOD, SOD mimetics, and catalase. Furthermore, it was shown that application of either X/XO or H_2O_2 to hippocampal slices resulted in the activation of ERK, which was inhibited by the general antioxidant *N*-acetyl-cysteine (44). Thus, the activation of ERK during NMDA receptor-dependent LTP and long-term memory may require ROS.

Protein phosphatases also have been shown to be important modulators of synaptic plasticity (117) that are regulated by ROS. For example calcineurin (protein-phosphatase 2B; PP2B) is thought to suppress LTP and long-term memory formation by opposing the effect of LTP-inducing kinases such as CaMKII and PKC (117). Calcineurin is highly sensitive to redox modification by ROS (75). For example, basal calcineurin activity was reduced by either X/XO or H_2O_2, but was enhanced by the addition of SOD. Furthermore, strong oxidizing agents inhibited calcineurin, whereas reducing agents enhanced calcineurin activity, possibly through direct oxidative modification of calcineurin protein (75). Consistent with the idea that a redox-sensitive calcineurin might play a role in LTP, FK506 (a calcineurin inhibitor) blocked H_2O_2-mediated enhancement of LTP and restored LTP in slices treated with concentrations of H_2O_2 that normally inhibited LTP (41). Interestingly, aged wild-type mice showed increased phosphatase activity that was similar to the levels of phosphatase activity observed in young wild-type mice that had been treated with exogenously applied H_2O_2. Thus, modulation of calcineurin by ROS may promote the expression of LTP following NMDA receptor activation.

Another potential target of ROS-mediated signal transduction during hippocampal synaptic plasticity and memory is the redox-sensitive transcription factor NF-κB. Several lines of evidence suggest that LTP and long-term memory may involve redox dependent activation of NF-κB. It has been observed that NF-κB becomes activated following LTP induction (4, 27) and that NF-κB can be activated by redox-mediated signaling (30, 32, 40). Furthermore, NF-κB has been implicated in the formation of long-term memory in nonmammalian organisms (26, 122) as well as in the consolidation of fear memories in rodents (122). Taken together, these findings suggest that NF-κB may be an important target of redox mediated signaling during synaptic plasticity and normal cognitive function. However, further studies are required to directly demonstrate that ROS-dependent activation NF-κB occurs during synaptic plasticity and memory.

Regulation of intracellular Ca^{2+} is an important function that controls synaptic plasticity and memory (88). ROS-mediated signaling could modulate intracellular Ca^{2+} via the oxidative modification of Ca^{2+} channels, thereby altering the Ca^{2+} response during plasticity-inducing stimuli. As mentioned previously, ROS can directly modulate voltage-dependent Ca^{2+} channels (63); however, there is also evidence that oxidative regulation of ryanodine receptors could be involved in redox-mediated modulation of intracellular Ca^{2+}. Ryanodine receptors are very sensitive to redox modulation, which results in alterations in channel function (34). This is intriguing because mutant mice that lack one form of the ryanodine receptor, RyR3, have been shown to express alterations in LTP and spatial memory (9, 28). Thus, ROS-mediated alteration of ryanodine receptor function may be an important step during the molecular events involved in the modulation of intracellular Ca^{2+} that underlies LTP and memory.

It seems clear that ROS are important modulators of signal transduction cascades that underlie synaptic plasticity and memory formation. Further work to identify other targets of ROS signaling will be important to better understand the significance of redox signaling in the brain. Another important goal will be to identify the sources of ROS that are involved in modulating in these redox-sensitive signaling pathways. Uncontrolled ROS production would quickly be come unhealthy for the neural tissue producing it; thus a mechanism that yields a potent, yet controlled source of ROS should be identifiable. There are several candidate sources of ROS that meet these criteria that will be discussed in the next section.

POTENTIAL SOURCES OF ROS INVOLVED IN LTP AND MEMORY

Although ROS have been shown to be critical signaling molecules that are required in the molecular events that underlie synaptic plasticity and memory formation, the source of ROS during these events has yet to be determined. Several sources have been hypothesized and evidence showing the plausibility of ROS generation from these sources has been provided; however, experiments determining the distinct physiological significance of these potential sources of ROS have been elusive. Among the hypothesized sources of ROS are mitochondria, monoamine oxidase, cyclooxygenase, nitric oxide synthase, and NADPH oxidase. Each of these potential sources is known to generate ROS under pathophysiological conditions, but the physiological role of the ROS produced by these sources has not been determined. Experiments designed to determine the physiological significance of each of these sources of ROS, especially with respect to a role in synaptic plasticity and memory formation will be an important goal for understanding the role of ROS signaling in the brain.

Mitochondria

Mitochondria produce superoxide as a metabolic byproduct of the electron transport chain and oxidative phosphorylation. Typically, mitochondrial production of superoxide is studied in models of oxidative stress, apoptosis, and neurodegeneration; however, there is evidence that suggest that mitochondria may be a source of ROS that is stimulated by appropriate physiological stimuli. For instance, elevating Ca^{2+} and Na^{+} is sufficient to produce free radicals from isolated rat mi-

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tochondria (23). Also, NMDA receptor activation via glutamate application to cultured forebrain neurons induced a localized generation of ROS that was blocked by MK-801 (94). Interestingly, glutamate application caused intracellular pH to decrease in a Ca^{2+}-dependent manner (94), which suggests that NOS could play a role in superoxide generation as well (33). NMDA receptor stimulation also was shown to result in the production of superoxide that occluded superoxide production induced by the uncoupling of protein transport with FCCP (13) The authors of this study suggested that this was evidence for mitochondrial production of superoxide; however, these data do not rule out the possibility that FCCP affects proton uncoupling on NADPH oxidase (2). Dugan *et al*. showed that NMDA-induced dihydrorhodamine (DHR) oxidation may be caused by ROS generated by mitochondria because the oxidation was inhibited by mitochondrial complex I (rotenone) and III (antimycin A) inhibitors (22). Interestingly, activation of NMDA receptors through the application of glutamate seemed to be necessary; however, FCCP treatment in the presence of MK-801 and NBQX was sufficient to cause the oxidation of DHR (22). Although these reports suggest that the mitochondrial electron transport chain may be a potent source of ROS during increased Ca^{2+} signaling, none of these reports distinguish the effects of high Ca^{2+} and ROS generation during the induction of synaptic plasticity from those underlying neurotoxicity and apoptotic signaling. Moreover, mice that overexpress SOD-2, which should scavenge superoxide produced by mitochondria, exhibit normal hippocampal LTP and memory (Hu and Klann, unpublished observations). Finally, there is much evidence suggesting that mitochondrial production of ROS is tightly regulated and that increased ROS production by the mitochondria quickly produces oxidative disease states, including the induction of apoptosis and neurodegeneration. Thus, at this time, the evidence suggests that mitochondria are not a likely source of ROS signaling during synaptic plasticity and memory.

Monoamine oxidase and cyclooxygenase

Monoamine oxidase and cyclooxygenase also are potential sources, albeit indirectly, of ROS that could be involved in synaptic plasticity and memory. In cerebellar granule cells a cyclooxygenase inhibitor (indomethican) and a monoamine oxidase inhibitor (nialamide) blocked NMDA- and kainic acid (KA)-induced ROS production, which suggests that the oxidative-metabolic activity of each of these enzymes may be responsible for the generation of ROS (14). Rotenone did not block either NMDA or KA-induced ROS production, which suggests that mitochondria are not the source of ROS as assessed in this system (14). In addition, the phorbol ester phorbol-12-myristate-13-acetate (PMA), which is a potent stimulator of NADPH oxidase, stimulated ROS production in a mechanism that was different than NMDA- or KA-induced ROS production because indomethican, nialamide, and rotenone were unable to block PMA-stimulated ROS production (14). These data indicate that there may be as many as three different sources of ROS in cerebellar granule cells. Further investigation into the potential role of monoamine oxidase in ROS generation during synaptic plasticity may lead to important insights regarding psychiatric disease, given the known effects of monoamine oxidase inhibitors on psychiatric disorders such as major depression (83).

Nitric oxide synthase

Nitric oxide synthase is well known for its role in generating nitric oxide (NO) gas, which has been observed to be a critical signaling molecule involved in synaptic plasticity and memory (97, 125). However, NOS may also be an important source of ROS for the molecular events required for LTP and memory formation. For example, purified NOS can generate superoxide that is dependent on Ca^{2+} and calmodulin (90). Interestingly, L-NAME, but not L-NMMA, could block the superoxide generation by NOS (90). In addition, it was shown that NOS could generate H_2O_2 under a variety of conditions (33). Specifically, low L-arginine and low pH each were shown to independently promote the generation of H_2O_2 production by purified NOS (33). On the other hand, H_4-biopterin inhibited H_2O_2 and promoted NO production by NOS (33). Importantly, only certain NOS inhibitors inhibited NO and H_2O_2 formation. For instance, L-NNA, L-NAME, and L-NMMA all inhibited NO formation; however, only L-NNA and L-NAME also blocked H_2O_2 formation by NOS, and L-NMMA failed to block H_2O_2 formation (33, 89). Further evidence for NOS generation of superoxide has come from experiments using cultures of primary cerebellar granule neurons, where glutamate receptor stimulation induced NOS-dependent production of superoxide if the cultures were pretreated with arginase (18).

NOS contains two enzymatic domains, one that generates NO and another that contains NADPH oxidase activity. NOS expression was shown to result in increased ERK activation that was inhibited by co-transfection of SOD (115), which suggested a role for either superoxide or H_2O_2 in NOS-mediated ERK activation. Interestingly, a mutation in NOS that rendered the NO synthase portion of the enzyme incompetent, yet retained NADPH oxidase activity, had no effect on ERK activation. In contrast, deletion of the NADPH-binding region of NOS blocked NOS-dependent ERK activation (115). These results suggest that NOS may generate superoxide that is critical for signal transduction cascades that is separable from NO generation.

Experiments that use various pharmacological agents to inhibit NOS activity often fail to discriminate between the specific roles that each of these domains play. For instance, L-NAME, which is often proposed to inhibit NO generation by NOS, is actually a very good inhibitor of NOS-mediated NADPH oxidation and superoxide generation (89). In addition, L-NMMA was shown to have little to no effect on the detection of the superoxide-dependent formation of DMPO-OOH, a superoxide-dependent electron spin-adduct, via neuronal NOS (89) suggesting that this pharmacological agent is good at dissecting the dual enzymatic function of NOS. Furthermore, diphenylene iodonium (DPI) is often used as an NADPH oxidase inhibitor (77); however, DPI also was shown to inhibit the effect of L-arginine inhibition of NOS-mediated superoxide formation (89), likely via interaction with the NADPH oxidase domain of the NOS enzyme. Unfortunately, many of the experiments that implicate generation of NO by NOS in synaptic plasticity use pharmacologi-

cal inhibitors of NOS that fail to distinguish the enzymatic generation of NO from NOS from the generation of superoxide by NOS. There is substantial evidence that NO is an important signaling molecule during synaptic plasticity (125), but the potential role of superoxide generated by NOS is relatively unexplored.

Taken together, these reports suggest the possibility that NADPH oxidation by NOS could be an important source of ROS that is independent of NO generation during synaptic plasticity and memory formation. However, there is another enzyme complex whose primary enzymatic activity is the oxidation of NADPH and concomitant production of superoxide. The role of NADPH oxidase in the generation of superoxide will be the focus of the remainder of this review.

NADPH oxidase

The NADPH oxidase complex is a plausible source of ROS in synaptic plasticity and memory that previously has been considered primarily as a generator of superoxide in non-neuronal cells, including immune system cells (21, 76), endothelial cells (37), and glia (21, 124). NADPH oxidase has been shown to be well regulated, such that a burst of superoxide could be generated in response to particular stimuli, which could subsequently be turned off; thus, generation of ROS via the NADPH oxidase system is rapid, well controlled, and specific to particular signaling events (91). Interestingly, several of the activators and effectors of the NADPH oxidase complex also have been implicated in signal transduction mechanisms that underlie synaptic plasticity and memory formation. Moreover, recent evidence has been provided that directly support a role of NADPH oxidase-dependent superoxide generation during brain function, which may explain why human patients with mutations in genes encoding subunits in the NADPH oxidase complex display mild cognitive deficits (82).

The structure and regulation of the NADPH oxidase have been well studied and extensively reviewed elsewhere (105). Briefly, the NADPH oxidase complex consists of five subunits, three cytosolic (p67^{phox}, p47^{phox}, and rac), and two membrane-spanning (gp91^{phox} and p22^{phox}). The membrane-spanning components exist as a heterodimer; gp91^{phox} is the catalytic subunit that is responsible for the transfer of electrons between NADPH to molecular oxygen, as well as the H^+ conductance that has been associated with this process. The regulation of NADPH oxidase activity is mediated through complex interactions between the cytosolic and membrane-associated components. Translocation of p67^{phox} and GTP-bound active Rac to the membrane are essential for the activation of gp91^{phox}-mediated electron transfer. p47^{phox}, once phosphorylated, acts as an "organizer" of the complex and mediates correct positioning and association of the p67^{phox} "activator" subunit to the gp91^{phox} and p22^{phox} heterodimer. Thus, translocation of all the cytosolic subunits in response to specific stimuli is required for the full activation of the NADPH oxidase complex. A model of NADPH oxidase is shown in Fig.1.

Interestingly, many of the signaling agents involved in LTP and memory formation also regulate NADPH oxidase activ-

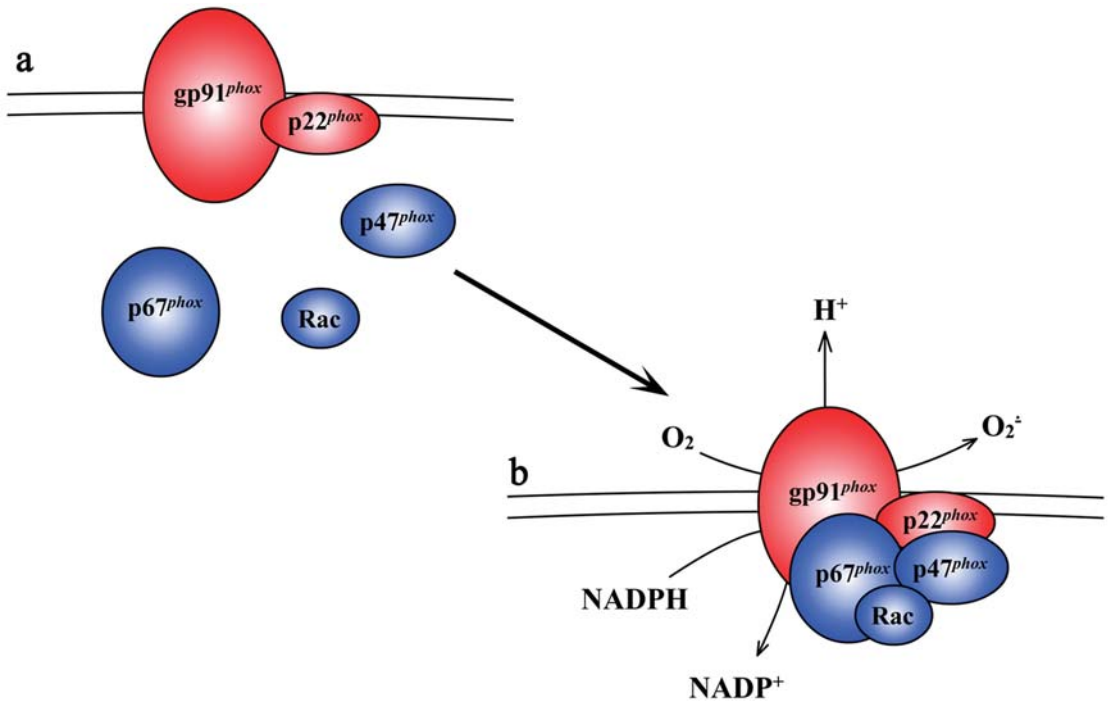

FIG. 1. Subunit composition and activation of the NADPH oxidase complex. (**A**) The NADPH oxidase complex consists of two membrane bound subunits (gp91^{phox} and p22^{phox}) and three cytosolic subunits (p67^{phox}, rac, and p47^{phox}), which upon activation translocate and associate with the membrane bound subunits (**B**). Upon activation, the NADPH oxidase complex transfers electrons from NADPH substrate to molecular oxygen, thus producing superoxide. During this process NADPH oxidase also pumps protons across the membrane. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

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ity. The transcription-dependent regulation of NADPH oxidase subunits implicates transcription factor components known to be important in the regulation of LTP and memory formation. Specifically, treatment of murine monocytic cell with lipopolysacharride (LPS) and interferon-γ (IFN-γ) resulted in the increased expression of gp91^{phox} mRNA and protein that was dependent on NF-κB (5), which has been shown to be an important transcription factor involved in LTP and memory (4, 27, 122). More near term signaling events also have implicated a potential role for NADPH oxidase in the generation of ROS-mediated signaling in neurons.

The phorbol ester PMA is a widely used compound that induces NADPH oxidase activation via the phosphorylation of p47^{phox} through activated PKC (19). In cerebellar granule cells, PMA stimulated ROS production (14) and in hippocampal slices, PMA treatment led to a LTP-like potentiation that is dependent on PKC activation (55). Furthermore, phospholipase A_2 (PLA_2)-dependent genesis of arachidonic acid also was shown to induce the activation of NADPH oxidase in intact neutrophils (69). Consistent with the possibility that this type of signaling might be involved in synaptic plasticity, hippocampal slices treated with arachidonic acid during a brief train of tetanization resulted in an LTP-like potentiation (78). Moreover, NMDA applied to cultured cerebellar granule cells led to the generation of superoxide that was mimicked by the application of arachidonic acid and inhibited by mepacrine, a PLA_2 inhibitor (58). In addition, NMDA- and glutamate-induced oxidation of dichlorofluorescin (DCF) in cerebellar granule cells could be blocked via PLA_2 inhibition (31). The PI3 kinase-Akt pathway is another kinase signaling pathway that plays a critical role in synaptic plasticity (45, 74, 114) and has been shown to be an important activator of NADPH oxidase in non-neuronal cells (16, 102).

Not only have upstream activators of NADPH oxidase been shown to be important regulators of plasticity and memory formation, but downstream effectors of NADPH oxidase-generated ROS in non-neuronal cells also parallel important signaling pathways involved in synaptic plasticity and memory. For instance, NADPH oxidase generated ROS have been shown in T-cells to regulate phosphorylation and activation of the ERK signal transduction pathway (38), which is a critical signaling pathway in LTP and memory formation. The evidence above is consistent with the hypothesis that NAPDPH oxidase is an important source of ROS in signal transduction pathways in the brain. More direct evidence recently has been provided that implicates NADPH oxidase in normal brain function.

NADPH OXIDASE EXPRESSION IN THE BRAIN

Consistent with an important function for the NADPH oxidase complex in the central nervous system, all components of the complex, including the various homologs of specific subunits are expressed in various regions throughout the brain (10, 17, 73, 93, 101, 108, 113). Serrano *et al* has shown that mouse hippocampi are immunoreactive for gp91, p47, p67, p40, and p22^{phox} proteins (73, 101) and that p47^{phox} and gp91^{phox} immunoreactivity was observed in pyramidal neurons in area CA1 (101). Furthermore, the NADPH oxidase subunits gp91^{phox} and p67^{phox} also have been found in synaptosomal fractions prepared from the whole brain and the hippocampus (108), suggesting a localized distribution that is consistent with a role for NADPH oxidase in synaptic plasticity. Interestingly, gp91^{phox} and p47^{phox} proteins also were found in several other areas of the brain including the cortex, habenula, paraventricular thalamic nucleus, anterior and posterior basolateral nucleus, basomedial nucleus of the amygdala, and striatum (101).

As discussed earlier, gp91^{phox} is the catalytic subunit of NADPH oxidase that is responsible for the transfer of electrons between NADPH to molecular oxygen. Recently, several homologs of gp91^{phox} have been described, some of which also are expressed in the brain. In addition to gp91^{phox} (also referred to as NOX-2), NOX-4 (17, 113), and NOX-5 (17) have been shown by rt-PCR to be expressed in the adult brain and NOX-4 was detected using *in situ* hybridization in the mouse cortex, cerebellum, and pyramidal cells of hippocampus (113). Furthermore, NOX-3 was shown to be highly expressed in the inner ear by rt-PCR and by *in situ* hybridization (10). p47^{phox} and p67^{phox} and their respective homologs also were detected in the brain using rt-PCR analysis (10) and by Northern blot analysis (73). Rao *et al.* also showed that NADPH oxidase is expressed and functional in lens epithelium (93). Thus, there are likely to be multiple homologs of gp91^{phox}. Whether the other NOX proteins are regulated in a similar manner to gp91^{phox} and whether they are critical for ROS signaling in the brain remains to be determined.

The expression pattern of NADPH oxidase suggests that it may be involved in ROS-dependent signaling throughout the brain. Consistent with this notion, it was shown in cultured hippocampal neurons that PMA could stimulate the redistribution of the cytosolic subunits of the NADPH oxidase complex to the membrane (108). Also in hippocampal slices, PMA induced the generation of superoxide that was inhibited by either DPI or AEBSF (108), two pharmacological inhibitors of the NADPH oxidase complex (20, 77). Furthermore, stimulation of the cellular prion protein (PrPc) was shown in a number of neuronal and non-neuronal cell lines to lead to the activation of NADPH oxidase, which induced the activation of the MEK-ERK pathway in an NADPH oxidase- and ROS-dependent manner (96). Thus, a functional NADPH oxidase is expressed in the brain, suggesting that this superoxide-generating complex may be involved in signaling, synaptic plasticity, and memory.

NADPH OXIDASE-MEDIATED SIGNALING IN THE BRAIN

NMDA receptor-dependent activation of ERK is well known to be involved in various forms of synaptic plasticity and memory (88, 106, 107). Consistent with a role for NADPH oxidase in mediating this type of signaling, DPI, an NADPH oxidase inhibitor, was shown to inhibit NMDA receptor-mediated ERK activation (47). Moreover, mice that

lacked the p47^{phox} subunit (39) also lacked the NMDA receptor-dependent activation of ERK (47). These findings are consistent with previous reports that have implicated NADPH oxidase activity with MEK-ERK signal transduction in non-neuronal cells (38, 96). However, it was unclear from these studies whether the response to NMDA receptor activation was one typical of synaptic plasticity or one typical of neurotoxicity. However, a recent series of studies with NADPH oxidase mutant mice indicate that this enzyme is indeed critical for synaptic plasticity.

A ROLE FOR NADPH OXIDASE IN HIPPOCAMPAL SYNAPTIC PLASTICITY AND MEMORY

Recent studies with pharmacological inhibitors of NADPH oxidase as well as studies with mutant mice that are genetically-deficient for either gp91^{phox} (87) or p47^{phox} (39) indicate that NADPH oxidase is involved in LTP. Two pharmacological inhibitors of the NADPH oxidase complex, DPI and apocycnin (77, 84), blocked early-phase LTP (E-LTP) and mutant mice that lacked either the gp91^{phox} or p47^{phox} subunits also expressed deficient E-LTP. Interestingly slices from gp91^{phox} KO mice and slices from wild-type mice treated with DPI also expressed deficient post-tetanic potentiation (PTP),which is a form of NMDA receptor-independent short-term plasticity (46). Other forms of presynaptic plasticity were normal in both p47^{phox} KO and gp91^{phox} KO mice (46). Thus, NADPH oxidase appears to be required for E-LTP.

Behavioral studies with the NADPH oxidase mutant mice indicate that superoxide produced by this enzyme may have an important role in hippocampus-dependent learning and memory. Consistent with the idea that NADPH oxidase-generated superoxide is necessary for learning and memory, it was shown that gp91^{phox} knockout mice displayed mild deficits in the Morris water maze, and that p47^{phox} knockout mice displayed deficits in the contextual fear conditioning (46). Interestingly, these mice also showed differences in the accelerating rotating rod apparatus and in the open field analysis, suggesting that areas other than the hippocampus may be affected in these mutant animals (46). Thus, in addition to its role in hippocampal LTP, NADPH oxidase appears to play a role in several types of hippocampus-dependent memory.

CONCLUSIONS AND FUTURE DIRECTIONS

Here we have discussed evidence that ROS, including superoxide and H_2O_2, are important signaling molecules in a variety of neuronal and non-neuronal systems. Importantly, ROS have been shown to be critical signaling molecules underlying fundamental cognitive functions including learning and memory (Fig. 2). This is atypical of previous views that placed ROS in a class of oxidatively destructive molecules that when produced led to toxic processes underlying cellular degeneration and apoptosis (8). We also have presented evidence that NADPH oxidase is likely an important source of ROS in the brain. This is evidenced by the fact that NADPH oxidase has been shown to be required for biochemical signal transduction cascades, synaptic plasticity, and cognitive behaviors involved in the formation and expression of memory. The relatively small amount of research aimed at determining the role of NADPH oxidase in brain function already has uncovered interesting results that warrant further investigation.

NADPH oxidase was shown to be required for hippocampus-dependent learning and memory, as well as for normal performance in behavioral paradigms that require other brain regions. This is consistent with the observation that ROS-dependent learning and memory has a motivational component (61) and that subunits of the NADPH oxidase complex are expressed in brain regions other than the hippocampus, including the cortex, cerebellum, striatum, and amygdala (101). Future research into the role that NADPH oxidase plays in cognitive function should address the role of ROS signaling in these other brain regions as well.

Interestingly, there are several homologs of the main catalytic subunit gp91^{phox} that have been shown to be expressed in various regions of the brain, which include the cortex and

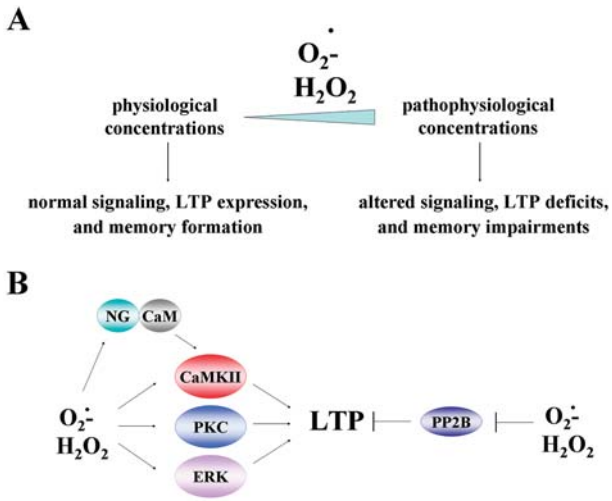

FIG. 2. Schematic depicting the role of ROS in the molecular mechanisms underlying cognition. (**A**) Low concentrations of ROS are required for signaling, synaptic plasticity, and memory formation; however, as the concentration of ROS increases, their function switches from a signaling molecule to an inhibitory or even toxic molecule. (**B**) *On the left*: superoxide and H_2O_2 activate protein kinase C (PKC) and extracellular-signal regulated kinase (ERK) and oxidize neurogranin (NG), which then releases calmodulin, resulting in the activation of calcium/calmodulin-dependent protein kinase II (CaMKII). *On the right*: superoxide and H_2O_2 also inhibit calcineurin (a.k.a. protein phosphatase 2B, PP2B). Activation of PKC, ERK, and CaMKII promote LTP, whereas the activity of PP2B tends to block LTP; thus activation of PKC, ERK, and CaMKII, along with the inhibition PP2B are all plausible, redox-sensitive, mechanisms by which ROS could promote synaptic plasticity in a concentration-dependent and cellular signaling state-dependent manner. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

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the hippocampus. These other NADPH oxidase subunit homologs may be important for plasticity and cognition. Interestingly, it is known that each of these homologs require different upstream signals for oxidase activation. For example, NOX-5 contains EF-hand regions that respond directly to Ca^{2+} influx (11). Determining the role that the various NADPH oxidase subunit homologs play in synaptic plasticity and memory should be an important goal for future investigations.

Not only is it important to determine the role of NADPH oxidase in generating ROS involved in plasticity and cognition, but it will be equally important to determine the role of other sources of ROS in these processes. We have mentioned several other potential sources of ROS that have been implicated in signal transduction and synaptic plasticity. Future research should address the distinct roles each of these sources of ROS play in mediating the molecular signaling underlying synaptic plasticity and memory.

An important issue that has been addressed only sparingly is the identity of the relevant targets of ROS signaling during synaptic plasticity and memory. As summarized in Fig. 2, several studies point directly to activation and inactivation of various kinases and phosphatases (44, 52, 54, 55, 109, 110). Although there have been few reports in neuronal systems, direct oxidative modification of ion channels, including voltage-gated Ca^{2+} channels and the ryanodine receptor have been shown to regulate the levels of intracellular Ca^{2+} (28, 63). Also shown to be an important signaling target of ROS are redox-sensitive transcription factors such as NF-kB (40). The elucidation of all of the targets of ROS signaling, in addition to the sources of ROS responsible for redox regulation of these targets, will be critical in understanding the roles that these highly reactive molecules play in normal cognitive function, and importantly, how perturbation of these signaling systems could lead to alterations in cognition, including neurodegenerative conditions mediated by oxidative stress.

We have argued that ROS signaling plays an important and necessary role in synaptic plasticity and memory formation. Moreover, NADPH oxidase is likely to be one of the important sources of ROS mediating these effects. Two key features make NADPH oxidase an attractive candidate for an ROS source in these physiological processes. First, the enzymatic complex generates large amounts of superoxide quickly, and second, can do so in a well-regulated manner. Dysfunction in either of these aspects could lead to neuronal dysfunction, as well as potential cognitive problems. Recent work indicates that not generating enough superoxide via NADPH oxidase leads to deficient synaptic plasticity and cognitive function in mice (46). Interestingly, human patients with mutations that render NADPH oxidase inactive also may express mild cognitive deficits (82). One can imagine that if NADPH oxidase regulatory mechanisms were altered, especially the mechanisms responsible for shutting down superoxide production, the well-known destructive role that ROS are known for in the brain could be fulfilled. Exuberant ROS generation could quickly lead to oxidative stress and subsequent neuronal dysfunction and damage. A fuller understanding of ROS signaling may lead to a better understanding of the degenerative mechanisms that underlie disorders such as Parkinson disease (57) and Alzheimer's disease (1) that may be in part caused by aberrant ROS generation. Thus, understanding the regulatory mechanisms that underlie NADPH oxidase-mediated signaling in the brain, as well as the regulation of other potential sources of ROS, should be an imperative for future research.

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ABBREVIATIONS

Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; CaM, Ca^{2+}-calmodulin; CaMKII, CaM-dependent kinase II; DCF, dichlorofluorescin; DHR, dihydrorhodamine; DPI, diphenylene iodonium; EC-SOD, extracellular superoxide dismutase; E-LTP, early phase long-term potentiation; ERK, extracellular-signal regulated kinase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; fEPSPs, field-excitatory postsynaptic potentials; H_2O_2, hydrogen peroxide; HFS, high frequency stimulation; IFN-γ, interferon-gamma; KA, kainic acid; L-NAME, l-N(G)-nitroarginine methyl ester; L-NMMA, N-G-monomethyl-L-arginine acetate; L-NNA, *N*-L-nitroarginine; LPS, lippopolysacharride; LTP, long-term potentiation; NG, neurogranin; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; PLA_2, phospholipase A2; PMA, phorbol 12-myristate 13-acetate; PP2B, protein phosphatase 2B or calcineurin; PrPc, cellular prion protein; PTP, post-tetanic potentiation; ROS, reactive oxygen species; SOD, superoxide dismutase; TPEN, *N*,*N*,*N*′,*N*′,-tetrakis(2-pyridylmethyl) ethylenediamine; X/XO, xanthine/xanthine oxidase.

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