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Review

NADPH oxidase in stroke and cerebrovascular disease

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Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) was originally identified in immune cells as playing an important microbicidal role. In stroke and cerebrovascular disease, inflammation is increasingly being recognized as contributing negatively to neurological outcome, with NOX as an important source of superoxide. Several labs have now shown that blocking or deleting NOX in the experimental stroke models protects from brain ischemia. Recent work has implicated glucose as an important NOX substrate leading to reperfusion injury, and that NOX inhibition can improve the detrimental effects of hyperglycemia on stroke. NOX inhibition also appears to ameliorate complications of thrombolytic therapy by reducing blood–brain barrier disruption, edema formation, and hemorrhage. Further, NOX from circulating inflammatory cells seems to contribute more to ischemic injury more than NOX generated from endogenous brain residential cells. Several pharmacological inhibitors of NOX are now available. Thus, blocking NOX activation may prove to be a promising treatment for stroke as well as an adjunctive agent to prevent its secondary complications.

Keywords: Ischemia, Inflammation, Neuroprotection, Superoxide, NADPH oxidase

Introduction

The major therapeutic strategy for treatment of acute ischemic stroke is rapid recanalization, either by pharmacological means through thrombolytic agents, or mechanical thrombectomy.\(^1,2\) However, the time window for intervention limits these therapies to a small number of patients, and their inappropriate use can actually worsen outcome. This worsened outcome has been blamed on complications of delayed recanalization such as worsened brain edema or symptomatic brain hemorrhage, a phenomenon commonly referred to as ‘reperfusion injury’.\(^3,4\) Thus, therapies to minimize reperfusion injury might expand populations of stroke patients eligible for treatment. Reactive species, radicals derived from oxygen, or nitric oxide are thought to be major contributors to this damage. Upon reperfusion, the brain is quickly exposed to oxygenated blood, and injured mitochondria of the ischemic brain are rendered incapable of detoxifying free radicals.\(^5\) Furthermore, immune cells which infiltrate ischemic tissue or plug ischemic microvasculature can also generate reactive species through several enzyme systems.\(^5\) Recent studies have focused on the role of superoxide generating systems in immune cells and their consequences on reperfusion injury. One enzyme system is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or NOX, originally found on leukocytes, but now recognized in several types of cells in the brain. Inhibition of NOX can potentially reduce the amount of superoxide generated during reperfusion, and thus limit reperfusion injury. Such a strategy has the potential to not only treat acute ischemic stroke, but also reduce complications of recanalizing strategies by using it in combination with thrombolytics or mechanical thrombectomy devices.

NOX, classically viewed as a purely peripheral phagocytic enzyme involved in the killing of bacteria, fungi, and microbes, is now recognized as a major contributor of this reactive oxygen species (ROS) in the vast majority of central nervous system diseases. The superoxide produced by activation of the NOS enzyme is soluble in solution and can combine with other ROS to form more toxic reagents \(in vivo\). The importance of NOX in the production of ROS and subsequent neuronal cell death cannot be overstated. Several other enzymes found throughout the cell are involved in ROS generation including, xanthine oxidase, lipoxygenase, cyclooxygenases, and substrate-coupled nitric oxide synthetase, but none of these can produce the large amount of ROS observed in non-phagocytic cells in both normal and pathological conditions.\(^7\) In the last 10 years, the research into
the NOX enzyme has led to a better understanding of its role in stroke and related diseases, and the development of inhibitors has opened up the field to a potentially important therapeutic target.

**NOX**

NOX is a membrane-bound enzyme complex with components which can be found in the plasma membrane as well as in the cytoplasm. NOX was originally found in leukocytes and is a major source of ROS generation. The complex is normally latent in neutrophils and is activated to assemble in the membranes during respiratory burst. NOX is a multi-component enzyme comprising a cytoplasmic subunits (p47phox, p67phox, and p40phox and Rac2) and upon phosphorylation, these subunits can form a complex and translocate to the plasma membrane to dock with the plasma membrane subunits (p91phox, p22phox). The catalytic core of the enzyme is thought to comprise gp91phox and p22phox. Catalysis of NOX occurs in the p91phox subunit (Nox2) and is initiated by transferring of electrons from molecular oxygen through redox coupling with NADPH, FAD, and heme to produce superoxide anion \((O_2^-)\).

Superoxide can be produced in phagosomes, which contain ingested bacteria and fungi, or it can be produced outside of the cell. In a phagosome, superoxide can spontaneously form hydrogen peroxide that will undergo further reactions to generate ROS. Superoxide is capable of killing bacteria and fungi by mechanisms that are not yet fully understood, but may inactivate critical metabolic enzymes, initiate lipid peroxidation, and liberate redox active iron.

NOX activation depends on phosphorylation, especially of the p47phox subunit. While other subunits can be phosphorylated, p47phox phosphorylation appears to be the key in the membrane translocation of other subunits. Kinases known to phosphorylate p47 include several of protein kinase C isoforms (beta, delta, and zeta) as well as p38 and p21 mitogen-activated kinases and protein kinase B. Further, it appears that NOX can be regulated by the inflammatory transcription factor, nuclear factor-kappaB (NF-kappaB). NF-kappaB can induce gp91phox expression, as cells deficient in NF-kappaB’s p65 subunit express less gp91phox in response to lipopolysaccharide stimulation. Vascular ROS are produced in endothelial, adventitial, and vascular smooth muscle cells and derived primarily from NOX, a multisubunit enzyme catalyzing a superoxide anion production by the one-electron reduction of oxygen using NADPH as the electron donor: \(2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+\).

An important breakthrough came through in 1999 with the discovery of a gp91phox homolog in non-phagocytic cells, Nox1, which was originally named Mox-1. This Nox1 was first discovered in vascular smooth muscle cells. To date, six homologs of gp91phox have been identified, Nox1, Nox3–5, Duox1, and Duox2. The gp91phox is now referred to as Nox2 and all the NOX homologs are the catalytic components of the enzyme involved in the electron transport function. All NOX family members share a core structure consisting of 6-transmembrane domains (including the two heme-binding regions) and a cytoplasmic C-terminus which contains the NADPH binding region.

NOX has also been detected in the brain, but knowledge of its distribution and function in normal central nervous system tissue has been lacking. This is partly due to the lack of tools and, in particular, the lack of good specific antibodies available for any components of this enzyme. A detailed description of the expression of all the NOX isoforms is described in a recent review by Sorce and Krause. mRNA for all the subcomponents of NOX has been observed in both neurons and microglial. An immunohistochemical study in mice using polyclonal antibodies revealed all the major components of NOX (p22phox, Nox2, p47phox, p67phox, and p40phox) predominantly in the neurons of hippocampus, cortex, amygdala, striatum, and thalamus, but not in the cerebellum. Nox4 has also been identified in very specific neurons in the cortex, hippocampus, and cerebellum of mice by both in situ hybridization and immunohistochemistry. More recently, Nox5 has shown to be expressed in the cerebrum and Duox1 is highly expressed in the cerebellum. In rats, neuronal immunohistochemistry staining for Nox2 and p47phox was found predominantly in the hippocampus, cerebral cortex and cerebellum. This is in contrast to the study by Green et al. who found that in the normal rat brain, Nox2 is present only on perivascular cells, but can be highly expressed in activated microglial cells. These differences in expression patterns are probably due to different affinities, expression patterns, and staining procedures of the antibodies, as well as species differences. In addition to the neuronal and glial expression patterns of Nox, the cerebral vasculature has also been shown to express subunits of NOX as well as many of the Nox homologs. This is important because ROS are consistently involved with certain cerebral vascular diseases such as hypertension and stroke. mRNAs for Nox1, Nox2, Nox4, p22phox, and p47phox have been shown to be expressed in rat basilar arteries and Nox4 is also expressed at the protein level. In vitro studies have shown NOX expression in neurons, astrocytes, and microglia. Immunohistochemistry studies have shown that NOX subunits are widely distributed in the cortex, the hippocampus, and in the cerebellum in vivo.
**NOX involvement in cerebrovascular disease**

NOX isoforms have been described in the cerebral vasculature. Thus, cerebrovascular disease may benefit from NOX as a target in the brain, as well as in its blood vessels. NOX may also be an important target in the prevention of reperfusion injury, an increasingly observed complication of recanalizing strategies used to treat stroke in patients. Upon reperfusion, the brain is quickly exposed to oxygenated blood, and injured mitochondria of the ischemic brain are rendered incapable of detoxifying reactive species.\(^5\)

Furthermore, immune cells which infiltrate ischemic tissue or plug ischemic microvasculature can also generate ROS through several enzyme systems.\(^6\) Recent studies have focused on the role of the superoxide generating NOX systems in immune cells and their consequences on reperfusion injury. NOX can potentially reduce the amount of superoxide generated during reperfusion, and thus limit such injury. This strategy has the potential to not only treat acute ischemic stroke, but also reduce complications of recanalizing strategies by using it in combination with thrombolitics or mechanical thrombectomy devices.

There is increasing evidence that inflammation accompanying ischemic stroke accounts for some of its progression, at least acutely.\(^5,29,30\) A robust inflammatory reaction characterized by peripheral leukocyte influx into the cerebral parenchyma and activation of endogenous microglia follows focal cerebral ischemia. This leads to the generation of ROS which can then stimulate ischemic cells, even ischemic neurons, to secrete inflammatory factors. Generation of ROS by inflammatory cells occurs via several enzyme systems, but NOX is the major enzyme that generates superoxide. How NOX is activated in stroke is not entirely clear, but phosphorylation of the p47\(^{phox}\) subunit appears important. p47\(^{phox}\) phosphorylation can occur through several kinases also upregulated and activated by brain ischemia, including several protein kinase C isoforms and the p38 and p21 mitogen-activated kinases. While numerous forms of the enzyme have now been described,\(^8\) phagocytic NOX, also referred to as Nox2, is associated with immune cells.

NOX has been documented to increase in the brain after experimental stroke.\(^23\) Mice deficient in the gp91\(^{phox}\) subunit had smaller infarcts than mice with an intact enzyme in models of focal cerebral ischemia followed by reperfusion.\(^31-33\) Further, NOX appears to play a significant role in reperfusion injury, as reperfusion permits the restoration of glucose to the ischemic brain. Interestingly, reperfusion in the presence of glucose appears to increase neuronal NOX activity and NOX deficiency or inhibition prevents this (Fig. 1). Further, NOX requires glucose metabolism through the hexose monophosphate shunt to supply NADPH, and blocking this pathway with 6-aminonicotinamide prevented the detrimental effects of reperfusion. Thus, the restoration of glucose (rather than oxygen, which is traditionally thought to be a source of ROS in this setting), appears to ‘fuel’ NOX by serving as a requisite electron donor to produce damaging levels of superoxide.\(^34\) NOX also appears to be a primary source of ROS generated by NMDA receptor activation.\(^35\)

The importance of glucose and NOX raises another important question. Since many stroke victims also have diabetes mellitus, NOX may be highly activated in this population. To address this, we generated a rodent model of hyperglycemia where animals given intravenous glucose to cause acute elevations in blood levels were subjected to 90 minutes of transient middle cerebral artery occlusion. We found that compared to animals subjected to middle cerebral artery occlusion...
but maintained normoglycemic, hyperglycemic animals suffered larger infarcts and worse neurological outcome. We then developed a model of hemorrhagic transformation caused by tissue plasminogen activator (tPA) treatment. Under condition of hyperglycemia, rats had more severe hemorrhage and higher mortality. This was also associated with increased infarct size, blood–brain barrier (BBB) disruption, and superoxide production. However, these negative consequences of tPA treatment could be abrogated by treatment with a NOX inhibitor, in this case, apocynin. Our results indicate that hyperglycemia can exacerbate post-tPA brain hemorrhage, and this effect might be mediated by NOX through increased superoxide generation.36

NOX may also explain a relatively new phenomenon of ischemic ‘post-conditioning’. Post-conditioning is a phenomenon of reduced ischemic injury when interrupted reperfusion is applied using three cycles of 15-second reperfusion followed by 15-second occlusion followed by eventual reperfusion. One group found that post-conditioning group led to less superoxide generation compared to conventional reperfusion. This was correlated to decreased expression of NOX subunits gp91phox and p47phox and activated Rac1-GTP in the post-conditioning group with subsequent reduction in superoxide generation.37

The role of NOX in the inflammatory response accompanying stroke has certainly been widely studied for obvious reasons. Mice deficient in NOX’s gp91 demonstrated decreased mRNA expression of TNF-alpha, CCL2, CCL3, and inducible nitric oxide synthase compared to wild-type litter mates. IL-1beta protein expression was also reduced in gp91 knockout mice 1 and 3 days after cerebral ischemia, and this was related to reduced ischemic brain damage.38 Bradykinin, well known to participate in BBB permeability, was shown to increase after experimental stroke. This led to the release of IL-1beta which in turn activated NOX and led to BBB breakdown. Animals treated with apocynin to inhibit NOX led to decreased IL-1beta release and BBB breakdown compared to those treated with vehicle.39 Blocking NOX with apocynin decreased nitrogen species generation, inflammatory molecule release, apoptosis, and transcriptional factor degradation in rat model of experimental stroke. Rats treated with apocynin actually had reduced IkappaB degradation with subsequent reduction in IL-1beta and ICAM-1 presumably because NF-kappaB activation was suppressed. Apocynin treatment was also associated with an overall anti-apoptotic effect of preventing Bcl-2 decrease and decreased expression of Bax.40

Immune cells are known to participate in ischemic brain injury in two broad compartments: within the brain itself and the peripheral circulation. This is supported by the observations that inhibition of brain resident microglia or the inhibition or the prevention of infiltration of circulating leukocytes appears to ameliorate damage from experimental stroke. In the ischemic brain, cells that generate superoxide through NOX are largely the brain’s resident immune cell, the microglia. Another source is from circulating leukocytes that have infiltrated the ischemic brain. Nox2 is well known to exist on microglia and leukocytes. To determine which compartment contributes more to NOX-mediated brain injury, we used a bone marrow chimera model, where we transplanted bone marrow from wild-type or Nox2-deficient mice into Nox2 or wild-type hosts, respectively.41 In the resulting phenotypes, Nox2 was present only in the circulating cells in one type of chimera, and present only in the brain of the other. Following experimental stroke, we found that Nox2 deletion in the circulation or the brain led to better outcomes compared to animals with fully intact Nox2, but the effect was most pronounced when Nox2 was deleted in the circulating cells. Thus, it appears that Nox2 derived from circulating cells contributes significantly to stroke pathogenesis, and may suggest an important intravascular target which may circumvent any requirement that a pharmaceutical needs to penetrate the BBB.

Other NOX subtypes have been studied in stroke models. In a recent study by Kleinschnitz et al.,42 Nox4 was found to have an especially profound effect in experimental stroke, as Nox4-deficient, but not Nox2- or Nox1-deficient mice were found to have improved neurological outcome. A pharmacological inhibitor had a similar effect. The lack of any effect by Nox1 or Nox2 deficiency goes against other reports in the scientific literature, and the authors could not identify any specific factors to explain this discrepancy as many of their models and paradigms matched those previously published.

**NOX Inhibitors**

Several NOX inhibitors are currently available, but are generally very low in specificity and selectivity. There has not been much clinical development since many are associated with toxicities that could hinder their use in humans. Yet, safer and more specific inhibitors are under development.

The most promising NOX inhibitor is apocynin which has been mentioned above. Its chemical name is 4-hydroxy-3-methoxy-acetophenone, and it is a methoxy-substituted catechol which is derived from the root extract of *Picrorhiza kurroa*, a medicinal herb that has been used for centuries by the Chinese to treat inflammatory diseases.43 Apocynin is a commonly used NOX inhibitor with relatively low affinity (IC50: ~10 μmol/l) in neutrophils.44 It does not seem to interfere with other polymorphonuclear
neutrophil defense mechanisms, as it does not affect phagocytosis or intracellular killing.43 Apocynin inhibits the release of superoxide through NOX by blocking migration of p47phox to the membrane, thus interfering with assembly of the functional NOX complex.45 The inhibitory action of the compound is not entirely specific to NOX, however. Some of its inhibitory activity at least initially may involve myeloperoxidase (MPO) because apocynin does not inhibit NOX in cells deficient in MPO.46 MPO together with hydrogen peroxide can facilitate apocynin dimerization, and these dimers can prevent assembly of an active enzyme complex. Furthermore, agents such as zymosan that promote the release of MPO also enhance the efficacy of apocynin.47 In cells that are not rich in MPO, apocynin can reduce oxidant stress through a non-specific oxidative scavenger effect instead of NOX inhibition.48 However, besides MPO, other peroxidases, such as horseradish peroxidase, can also induce apocynin dimer formation with a consequent NOX inhibitory effect.45,49 In addition, in vivo studies showed that MPO secreted by neutrophils can be taken up by endothelial cells, in which apocynin can then be metabolized to active dimers, thus inhibiting vascular NOX.45 In line with this concept, it was observed that supplementation with thiold provided either as gluthathione or cysteine prevents the inhibitory effect of apocynin on the NOX. Apocynin dimer formation may be responsible for its delayed inhibitory property.50 and it has been suggested that this dimer is what blocks NOX activity.47

Apocynin has been studied by a few groups in brain ischemia models. From our own lab, we found that a dose of 2.5 mg/kg given parenterally just prior to reperfusion, or 1.5 hours after ischemia onset, resulted in reduced infarct volume and improved neurological outcome.51 We also found that superoxide is largely generated in neurons and some microglia/monocytes, with no generation in brain vascular endothelial cells. Apocynin markedly reduced superoxide in the brain. However, apocynin at higher doses (3.75 and 5 mg/kg) failed to show any benefit, and actually increased the severity of brain hemorrhage. Thus, this rather narrow therapeutic dose range may limit its translation to the clinical level. However, other groups have shown salutary effects of apocynin at doses as high as 50 mg/kg.31,52 In global cerebral ischemia, 5 mg/kg apocynin attenuated hippocampal injury when given prior to ischemia onset.53 In safety studies of uninjured mice, apocynin was well tolerated in single oral doses of up to 1000 mg/kg.54 Interestingly, apocynin failed to have an further beneficial effect in Nox2 deficient mice subjected to stroke, and might speak somewhat to its specificity or its dependence on NOX2.41

In addition to apocynin, there are several other inhibitors with a more limited scope as they pertain to stroke. Diphenyleneiodonium (DPI) is the most commonly used inhibitor of NADPH oxidase.55 DPI impairs NOX by flavoprotein inhibition so that NOX cannot produce superoxide. One experimental study has shown that its administration in combination with dimethylsulfoxide reduced infarct size and BBB disruption.56 However, DPI can also inhibit other flavoenzymes in vivo. Flavoenzymes play a crucial role in many metabolic pathways so this is not a therapeutically viable inhibitor, but it is valuable as a standard in vitro assay.

Another inhibitor is 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), which an irreversible serine protease inhibitor.57 AEBSF appears to have a direct effect on the plasma membrane components of NOX and interferes with the binding of the cytosolic components p47phox and p67phox. AEBSF does not interfere with the electron transport and does not scavenge the oxygen radicals. Unfortunately, AEBSF modifies many proteins by covalent attachment preferentially on tyrosine, and to a lesser extent on lysine, histidine, and the amino-terminus. AEBSF is quite stable in aqueous solution and the extent to which the protein is modified continues to increase for several days and this significantly limits its use in vivo. Tosylphenylalaninchloromethane is another irreversible serine protease inhibitor similar to AEBSF and has a similar mechanism of action.58

Other NOX inhibitors include phenylarsine oxide and gliotoxin.59 Phenylarsine oxide appears to prevent assembly of the NADPH enzyme complex via interacting with cysteine residues. However, this interaction with the cysteine groups makes this inhibitor quite non-specific as it will also interact with other enzymes and proteins.60,61 Gliotoxin is extracted from Aspergillus and it appears to inhibit the enzyme by blocking the phosphorylation of p47phox.47,62 It also seems to react with the thiol groups, thus limiting its specificity.

Honokiol is one of a group of NOX inhibitors that have been extracted from plants. Honokiol was isolated from the herb Magnolia officinalis and has been of particular interest because this compound appears to inhibit superoxide production after the respiratory burst and not before the enzyme is activated as with other inhibitors.63 Honokiol has also been shown to reduce lesion size in experimental focal cerebral ischemia followed by reperfusion,53,64 and this decrease was correlated to a reduction in neutrophil infiltration and activation, and decreased lipid peroxidation.63

The only inhibitor to date that directly interacts with a specific NOX homolog appears to be a plant derived naphthoquinone called plumbagin.65
Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural yellow pigment that comes from the roots of the black walnut plant Plumbago zeylanica. Plumbagin inhibits non-phagocytic NOX activity in HEK293 and LN229, a cell line that only express NOX4 and in a cell line transfected for Nox4.\(^\text{65}\) The regulation of Nox4 appears to be different from the other NOX homologs which require p47\(^{\text{phox}}\) and p67\(^{\text{phox}}\), and it has been observed that Nox4 alone can produce superoxide activity.\(^\text{14,66,67}\) The method by which it inhibits Nox4 is unknown but it is unlikely that it is due to cytotoxic effects as the cells were viable after 1 hour incubation with plumbagin.\(^\text{65}\) It has been shown to have significant anti-cancer activity\(^\text{68,69}\) and may work by blocking superoxide production as many cancers have been shown to produce ROS and specifically express NOX homologs.\(^\text{16,70}\) NOX4 is the dominant NOX homolog in vascular smooth muscle cells and its inhibition by plumbagin may well explain its anti-atheroscerotic effect.

The most selective NOX inhibitor to date is a chimeric peptide gp91ds-tat.\(^\text{71}\) This peptide is constructed from the sequence of gp91phox that is known to be involved in the binding of gp91phox to p47phox and can inhibit the oxygen radical production in cell-free assays (gp91 docking sequence or gp91ds). In order to deliver this peptide into the cells, the gp91ds was linked to HIV coat peptide (HIV-tat) that is known to be involved in internalization.\(^\text{71}\) This gp91ds-tat specifically binds to p47phox and prevents the formation of the NOX complex. While this is the most specific inhibitor for NOX, it cannot distinguish between the phagocytic or non-phagocytic enzyme and it has little oral bioavailability as it is a peptide.

In addition to the above inhibitors, there are NOX inhibitors that have either been specifically developed by the pharmaceutical industry or are in clinical trials.

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, is a mimic of glutathione peroxidase which also reacts with peroxyxirite, inhibits a variety of enzymes such as lipoxgenases, nitric oxide synthases, NOX, protein kinase C, and H\(^{+}/K^{+}\)-ATPase.\(^\text{72}\) Ebselen is therefore quite non-specific, but is being used at some centers for the treatment of stroke in Japan. Ebselen has shown efficacy if the treatment is started within 24 hours of the stroke.\(^\text{73,74}\) Currently, a multicenter phase 3 ebselen trial is underway. A few other inhibitors have been developed by the pharmaceutical industry including VAS2870 (Vasopharm),\(^\text{75,76}\) and S17834 (Servier).\(^\text{77}\) There is no information regarding these compounds mechanism of action, although VAS2870 seems more specific to inhibit Nox2, whereas S17834 seems more specific in inhibiting vascular Nox. Finally, as noted in a review by Miller et al.,\(^\text{78}\) angiotensin converting enzyme inhibitors, angiotensin receptor-1 antagonists, and the HMG-CoA (statins) drugs can all indirectly inhibit NOX activity.\(^\text{79,80}\) These are very effective cardiovascular drugs and it is tempting to suggest that their beneficial clinical effects may be enhanced by their ability to inhibit superoxide production \textit{in vivo}.

Conclusions

NOX is an enzyme that broadly expressed in immune cells, especially those that reside in and infiltrate into the brain parenchyma after ischemic injury. While contributing to the oxidative stress exacerbated by reperfusion, NOX appears to depend on glucose as a substrate, further emphasizing the detrimental effects of hyperglycemia in stroke. By blocking the NOX activation in experimental stroke, several preclinical studies have demonstrated improved neurological outcome, and reduced severity of brain injury. This is associated with the reduced superoxide generation and the activation of downstream activities, such as reduced apoptosis, detrimental cytokine release, and thus inflammatory responses. Further, inhibiting NOX within the intravascular space seems to also be a robust therapeutic strategy, thus circumventing the need for an inhibitor to necessarily penetrate into the brain in order to be effects. Yet, an effect and safe inhibitor has yet to make it to the clinical level.

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